CELLULASES FROM EXTREMELY THERMOPHILIC ANAEROBIC BACTERIA: A COMPARISON OF SEVERAL NEW CELLULOLYTIC ISOLATES AND THE PARTIAL PURIFICATION AND CHARACTERISATION OF COMPONENTS OF THE CELLULASE COMPLEX FROM ONE ISOLATE

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ABSTRACT

Forty-seven thermal pool sites in the central volcanic plateau of the North Island of New Zealand were sampled and enrichment cultures on crystalline cellulose screened for growth and cellulase activity. Eight anaerobic cellulolytic cultures resulted. Cellulolytic isolates were obtained from five of these cultures. All were obligate anaerobic non-sporing rods, staining Gram-negative. Most grew well at 75°C but none grew at 80°C. CMCase activity from some isolates exhibited exceptional thermal stability, with half-lives at 85°C in excess of 10 hours. Isolates from two sources, designated TP8 and TP10, were capable of completely hydrolysing crystalline cellulose and accumulated reducing sugar and avicelase activity in the growth medium. Levels of CMCase and avicelase in our most active culture supernatants were between 10 and 35% of those of *Clostridium thermocellum* grown on the same medium (but at 60°C). All produced acetic acid and ethanol in varying proportions, with a consequent tendency to decrease the pH of the growth medium. Cellobiose at 0.2%(w/v) repressed production of CMCase activity by most of the isolates by about 50%, but led to a slightly increased CMCase production by two of them.

One of the isolates capable of completely hydrolysing crystalline cellulose (TP8.T6.3.3.1) was subjected to repeated subculture, reselection and reisolation to ensure its purity. The cell-free cellulase complex of this organism was found to produce partial solubilisation of a range of natural cellulosics, and also contained ß-glucosidase activity.

Ammonium sulphate precipation and adsorption onto crystalline cellulose were the best of the methods tested for concentrating the low levels of cellulolytic activity in the culture supernatant of TP8.T6.3.3.1. Adsorption onto cellulose was much the cheapest alternative and also doubled as a preliminary fractionation and purification step.

Three fractions, separated on the basis of their affinities for crystalline cellulose, were found to interact synergistically in

hydrolysing crystalline cellulose when recombined.

Conventional ion exchange and gel permeation column chromatography served only to subdivide the TP8.T6.3.3.1 cellulase complex into subcomplexes. These were fractionated further using preparative SDS-PAGE and IEF. The cellulose-binding component of the cellulase complex was found to contain an unexpectedly large number of active components (20 being a conservative estimate). Several of these component proteins exhibited clear differences in relative reactivities towards a range of cellulosic substrates. When rerun on SDS-PAGE, almost all produced single protein bands with mobilities indicative of molecular weights covering the range 20,000 to 140,000. Isoelectric points were all in the range pH 4.5-5.0. However, narrow range isoelectric focussing (pH 4.5-5.0) produced further subdivision of these single bands. The bands produced by isoelectric focussing (7-10 per SDS-PAGE band) each bore a share of the endoglucanase activity. We were unable to individually characterize the component activities at this level, and it was not clear whether these multiple components were artefacts of the isoelectric focussing, distinct products of multiple genes, modified products of a relatively small number of genes or microheterogeneity due to ageing of the proteins.

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I also wish to thank Professor Roy Daniel for his guidance throughout, Professor Hugh Morgan and Yvonne Casey for their advice regarding the microbiological aspects, Rex Julian for his expert photography and Dianne Bovill and Lesley Howey for the majority of the word-processing.

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LIST OF ABBREVIATIONS

BSA	Bovine serum albumin
CMC	Carboxymethyl cellulose
DNSA	Dinitrosalicylic acid
GLC	Gas/liquid chromatography
HPLC	High pressure liquid chromatography
IEF	Isoelectric focussing
MOPS	Morpholinopropane sulphonic acid
PABAH	para-hydroxybenzoic acid hydrazide
PAGE	Polyacrylamide gel electrophoresis
pI	Isoelectric pH
p-NPG	para-nitrophenol ß-1,4 glucopyranoside
Rf	Migration relative to the solvent front
SDS	Sodium dodecyl sulphate
TEMED	N,N,N′,N′-tetramethylethlyenediamine
TNP-CMC	Trinitrophenol carboxymethyl cellulose

All other abbreviations used are in common usage and in keeping with international convention.

CHAPTER ONE

INTRODUCTION

1.1 PREAMBLE

It has become traditional for any review of cellulase research to commence with a reminder that cellulose is the most abundant biopolymer on earth. Being continually replenished by such large amounts each year, it surely must become the major source of liquid fuels and chemical feedstocks once all the exploitable fossil resources have been exhausted.

Such a progression is somewhat akin to pointing out that there is a vast supply of hydrogen in the centre of the sun and that all we need do is tap it. The question of the availability of cellulose is of central importance. The majority of the world's waste cellulose is simply burnt or decomposes naturally, and this situation appears likely to persist. Economic and political forces may one day combine to make the production of potential fuels such as ethanol from cellulosic crops an attractive alternative to their current uses, but this remains speculative. The funding of research into making liquid fuels from cellulose has ebbed and flowed with international oil price fluctuations. In the short term, at least, other applications of cellulase research may be more significant, as will be discussed later (see section 1.6).

From a purely academic standpoint, study of naturally occurring cellulase systems presents an interesting challenge insofar as they generally have been found to be multiple enzyme systems. Questions are raised regarding the need for such apparently complex systems, and purification of the individual component enzymes presents a technical challenge.

Study of the action of cellulases must be approached using somewhat unconventional techniques. Whereas most text-book enzyme theory is based on reactions in solutions, the cellulase-cellulose system involves an interface between an enzyme in solution and an insoluble, heterogenous substrate surface. Besides making it difficult to derive

an adequate kinetic model of insoluble cellulose hydrolysis by cellulase (Lee (S.E.) *et al.*, 1978, Lee (Y-H) *et al.*, 1980, Ladisch *et al.*, 1981, Lee and Fan, 1983, Lee (S.B.) *et al.*, 1983) this situation leads us to confront such fundamental concepts as the existence of "hydrogen bondase" enzymes, synergisms between enzymes, and the involvement of lectins in directing the binding of certain cellulases to native cellulose.

Indeed the whole subject of the mechanism of cellulolytic hydrolysis of native cellulose is currently unresolved. This is hardly surprising in view of our present high level of uncertainty regarding the structure of the substrate.

1.2 NATIVE CELLULOSE: STRUCTURE AND NATURAL PROTECTION

Cellulose is a linear glucose polymer composed of anhydroglucose units coupled to each other by ß-1,4-glucosidic bonds. Two hydrogen bonds support each glucosidic bond in maintaining the rigidity of the cellulose molecule (Gardner and Blackwell, 1974). Since adjacent glucose molecules alternate by 180° in orientation, the basic repeating unit is cellobiose. Native cellulose molecules found in cotton and wood are reported to contain about 15,000 and 10,000 glucose residues respectively (Sjostrom, 1981), which implies molecular lengths of 5-8µm.

Coupling of adjacent cellulose molecules by hydrogen bonds and van der Waal's forces results in their parallel alignment to produce a crystallite structure. This structure has been studied by X-ray diffraction analysis, by methods based on the absorption of polarized infrared radiation and most recently by ^{13}C nuclear magnetic resonance spectroscopy. Until the publication of the ^{13}C-NMR results (Atalla and van der Hart, 1984) there was general agreement about the structure of the unit cell of the crystallite of native cellulose. The favoured model was that of Meyer and Misch (1937) in which all the cellulose chains are oriented in the same direction, lying parallel and bonded in one plane by van der Waals forces alone (Sihtola and Neimo, 1975). This is clearly depicted diagrammatically by Sjostrom, (1981). The model contains only one type of unit cell, containing four glucose residues.

However, recent data from ^{13}C-NMR spectroscopy (Atalla and van der Hart, 1984) are consistent with the existence of two distinct crystalline forms in native cellulose. One form is dominant in bacterial and algal celluloses whereas the other form is dominant in celluloses from higher plants. This confirmed an earlier report that *Acetobacter* and *Valonia* celluloses are structurally different from

higher plant celluloses (Marrinan and Mann, 1956). It is estimated that *Acetobacter* cellulose is composed of 60 to 70% of one crystalline form whereas cotton is approximately 60 to 70% the other form (Atalla and van der Hart, 1984). These authors do not propose any new molecular models incorporating these two types of crystalline structure. They demonstrate clearly that neither form is identical with cellulose II (a crystalline form found in regenerated cellulose, produced by dissolving cellulose in phosphoric acid and precipitating in water). This cellulose II structure, comprising antiparallel chains and rendered thermodynamically more stable than cellulose I by hydrogen bonding in all three planes (Kolpak *et al.*, 1978) has not been found to occur naturally. Two other crystalline forms of cellulose are recognised, but again these are artificial, being produced by treatments involving anhydrous ethanolamine (form III) or high temperatures (form IV).

There is some disagreement in the literature as to how the cellulose molecules are organized into the fibres visible in native cellulose . Various theories are described by Cowling (1975) and Tong (1980). The most common theory is that groups of cellulose molecules, arrayed in crystalline structures over 85% of their length, and in more amorphous configurations over the remaining 15%, comprise the basic structural units, termed elementary fibrils (Sihtola and Neimo, 1975, Tsao *et al.*, 1978). These in turn are attached side by side to each other, probably by hydrogen bonding, to form microfibrils with transverse dimensions of 5-30nm. In plants, the microfibrils are then laid down in various arrangements during the cell wall synthesis. The microfibrils are distinct entities in that few cellulose molecules, if any, cross over between them (Cowling, 1975). The orientations of microfibrils in the primary and secondary cell walls have been extensively discussed (Roelofsen, 1965). The microfibrils may associate, controlled by means unknown, to form bundles, sheets, rings, or helices which normally have a skeletal role and can later be extracted as fibres from many different parts of plants.

With the exception of the cotton seed hair, which is 89% cellulose (Cowling, 1975), most plant cellulose is not encountered in nature in a pure and readily accessible form. Rather, the microfibrils of both primary and secondary cell walls are embedded in a continuous matrix of non-cellulosic polysaccharides termed hemicelluloses. In wood, these are mainly heteropolymers of glucose, xylose, galactose, mannose and arabinose, with a degree of polymerization seldom exceeding 200. These

have a supportive function in that they act as packing around the much longer and more rigid microfibrils, but are relatively easily hydrolyzed. In secondary cell walls much greater rigidity is conferred by lignin which also encrusts the cellulose. Lignin is a complex three-dimensional polymer (Freudenberg, 1965) which is extremely resistant to hydrolysis and serves to protect the skeletal cellulose within by forming a physical barrier around it. The lignin and cellulose form a mutually inter-penetrating system of high molecular weight polymers. Further protection of the cellulose can be conferred by the many specific cellulase inhibitors, mainly phenols, produced by a wide variety of plants (Mandels and Reese, 1965).

Even though native cellulose is normally well-protected by the matrix in which it is embedded, the basic close-knit structure of native cellulose itself tends to prevent enzymatic hydrolysis. Rowland (1975) showed that only 42% of the hydroxyl groups of native cotton fibre were accessible to D_2O (M.W. 20) and that molecules above M.W. 3000, which includes virtually all enzymes, would be excluded to the outermost surfaces of the cotton fibre. A comparison of the dimensions of a range of cellulase molecules with the diameters of capillary voids in wood and cotton fibres showed that only the gross capillaries such as cell lumina, pit apertures and pit membrane pores were large enough to accommodate the majority of the cellulases (Cowling, 1975). Even after removal of lignin and hemicellulose and being fully water-swollen, the cell wall capillaries, i.e. spaces between microfibrils and amongst the cellulose molecules in the amorphous regions, were only just large enough to accommodate the smallest cellulases.

1.3 CELLULASE TERMINOLOGY DEFINED

1.3.1 Endoglucanases

Endo-ß-(1-4)-glucanases (or 1,4-ß-D-glucan 4-glucanohydrolases, EC 3.2.1.4) are characterised by their random hydrolysis of ß-(1-4) glucosidic linkages, although the degree of randomness may vary amongst the several different endoglucanases which are normally produced by a single organism (Wood and McCrae, 1979). Acting on soluble cellulose derivatives, their random cleavage causes a rapid decrease in chain length, and hence viscosity, relative to the release of reducing end groups. When acting on cellodextrins, the rate of hydrolysis increases with the degree of polymerization within the limits of substrate solubility, with cellobiose and cellotriose being the major final products (Reese,

1977). The fact that little monomer is produced reflects a degree of non-randomness in that the terminal linkages are less affected than the internal linkages. The anomeric linkage of the product is not inverted by the hydrolysis.

1.3.2 Exoglucanases

Exo-ß-(1-4)-glucanases (or 1,4-ß-D-glucan cellobiohydrolases, EC 3.2.1.91) cleave cellobiose units from the non-reducing ends of cellulose molecules. Some examples have been purified to the point where they produced no detectable reduction in CMC viscosity although some reducing sugar production from CMC was still possible, (Wood, 1975). Presumably endwise hydrolysis would advance until a substituted glucose residue was encountered.

1.3.3 Exoglucosidases

Exo-ß-(1-4)-glucosidases (or 1,4-ß-D-glucan glucohydrolases, EC 3.2.1.74) cleave glucose units successively from the non-reducing end of the glucan. They can be distinguished from ß-glucosidases by their "preference" for substrates of longer chain length and by the inversion of their products (Reese *et al.*, 1968).

1.3.4 ß-glucosidase

ß-glucosidases (or ß-D-glucoside glucohydrolase, E.C.3.2.1.21) hydrolyse cellobiose and other very short chain ß-1,4-oligoglucosides up to cellohexaose to form glucose (Reese *et al.*, 1968). The term "cellobiase" is a misleading pseudonym since it suggests complete specificity for cellobiose. Most are in fact active on a range of ß-dimers of glucose (Lee and Fan, 1980). Unlike exoglucosidases, the rate of hydrolysis decreases markedly as the degree of polymerization of the substrate increases, and the beta configuration is retained by the product.

1.3.5 CMCase

The capacity to hydrolyse carboxymethyl cellulase (CMCase) should not be considered synonymous with endoglucanase activity, since other classes of enzyme can also be involved, particularly in the release of reducing sugars (Wood, 1975) and further discussed in Section 3.1.1).

1.3.6 Avicelase

Similarly, the capacity to hydrolyse Avicel, which is microcrystalline wood alpha-cellulose (i.e. material resistant to hydrolysis by 17% w/v NaOH) is not indicative of the action of any one class of enzyme alone (see Section 3.2).

1.3.7 Cellulase Complex

This normally refers to the complete repertoire of enzymes involved in cellulose hydrolysis. Often they are in some physical association. ß-glucosidases are usually excluded since they are not normally found in any close physical association with the cellulases. A "complete" cellulase complex is the group of enzymes involved in the hydrolysis of crystalline cellulose. On occasions the term "cellulase" is applied to individual components of the complex.

1.4 THE MECHANISM OF ENZYMATIC HYDROLYSIS OF CRYSTALLINE CELLULOSE

Virtually all of our current understanding of the mechanisms of cellulase action is based on studies of fungal systems, and its relevance to bacterial cellulases is a matter of conjecture. Reviews of the evidence accumulated since concerted study of various fungal systems began in the 1950's are provided by Reese and Mandels, (1971), Nisizawa (1973), Wood, (1975), Wood and McCrae, (1979), and Lee and Fan (1980).

Controversy still surrounds the precise sequence of action of the various types of enzymes which have so far been implicated in the hydrolysis of crystalline cellulose. In fact even the number of types of enzymes recognised as being involved is in dispute. Fig. 1.1 is a diagrammatic representation which best accommodates accumulated experimental data to date.

The question of which enzyme acts first on crystalline cellulose has been difficult to resolve. Because of the difficulties in identifying the individual actions of the various component enzymes of a cellulase complex, most groups have attempted to purify individual components before studying their actions. This approach presents two major problems. Firstly, in almost all cases these purified components alone have very little activity towards crystalline cellulose. Secondly, the likely synergistic effects of minor contamination by other enzymes may drastically affect the conclusions reached. The extreme precautions which must be taken to ensure homogeneity of cellulases are demonstrated by Sprey and Lambert (1983).

Despite these difficulties, Wood and McCrae (1979) draw largely on studies of the effects of purified endoglucanases on the cotton fibre, namely its fragmentation into short fibres, an increase in the capacity to absorb alkali and a loss in tensile strength, to conclude that endoglucanases are the initiators of the attack. Electron microscope observations by White (1982) revealed that a purified endoglucanase was

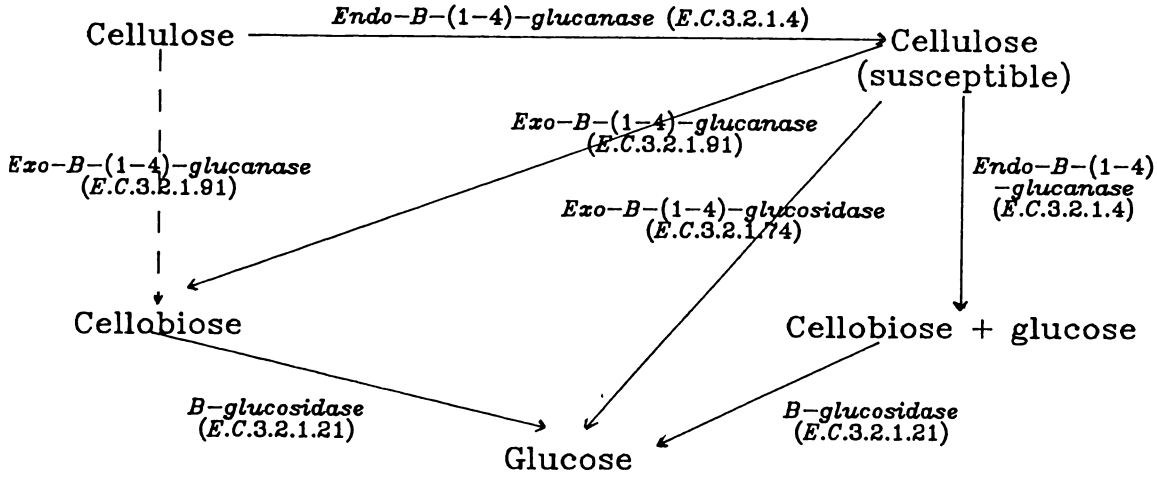


Figure 1.1 Mode of action of fungal cellulase on cellulose. (A modification of Ryu and Mandels, 1980)

capable of producing ribbon-splaying in sheets of cellulose produced by the bacterium *Acetobacter xylinum*, whereas a glucan cellobiohydrolase had no effect when acting alone.

Without first attempting to isolate the cellulase components, Klesov and Grigorash (1980) claim to have demonstrated by a study of the kinetics of the hydrolysis of cotton lint that endoglucanase is the component acting first. This conclusion was based on findings from seven cellulase complexes from fungi of three different genera, for which it was found that the steady-state rate of glucose formation was proportional to the content of endoglucanase in the cellulase preparations.

It might be argued that the above information, particularly that relied upon by Wood and McCrae in formulating their "endoglucanase first" hypothesis, does not really pertain to hydrolysis of the crystalline portions of cellulose on a molecular scale. The fact which is often overlooked is that crystallinity in native cellulose exists at several structural levels and the bonding forces and spatial constraints which must be overcome to disrupt the crystallinity at each level will be different. Swelling, splaying of microfibrils, and fragmentation

into short fibres may well be necessary preliminaries but do not constitute evidence that the hydrolysis of the most recalcitrant crystalline groupings of cellulose molecules within the elementary fibrils involves initial attack by endoglucanases. The studies of Klesov and Grigorash (1980) were also run under conditions in which the extent of cotton hydrolysis was low (3-7%) and the crystalline structures at the molecular level may have remained intact.

Chang *et al.* (1981) object to the "endoglucanase first" concept on the basis of studies at the molecular level. They collected together molecular weight and crystallinity data for various crystalline cellulose substrates measured before and after their hydrolysis by a range of fungal cellulase complexes to the extent of producing from 7% to 48% weight loss. The residual cellulose molecules had molecular weights which were only slightly reduced from the original samples and crystallinity remained unchanged. They interpreted these results as indicating that the mode of hydrolysis was not by random scission into the interior of cellulose elementary fibrils as might be expected of an endoglucanase - initiated attack followed by exoglucanase and exoglucosidase hydrolysis. Instead they proposed a quantum mode of degradation in which, once attacked by an enzyme, a cellulose molecule is degraded directly down to cellobiose and glucose before the enzyme(s) detach and attack another molecule.

The mechanism of lysozyme action, which also involves cleavage of ß-1,4-glucosidic bonds, is comparatively well studied. Although lysozyme is inactive towards cellulose (Piszkiewicz and Bruice, 1969), it has been suggested that cellulase catalysis may follow the same mechanistic pathway as hen egg-white lysozyme, which involves a carboxyl group acting as a general acid, protonating the leaving groups (Shepherd, 1981). This idea has been supported by a recent determination of the N-terminal amino acid sequence of an endo-ß-1,4-glucanase from the cellulase complex of the white-rot fungus *Schizophyllum commune*. The sequence from Glu-33 to Tyr-51 was homologous with the active site sequence of hen egg-white lysozyme, including the lysozyme catalytic residues (Glu-35, Asp-52) and the substrate binding residue Asn-44 (Yaguchi *et al.*, 1983).

1.4.1 The importance of synergism

Synergistic action between endoglucanases and exoglucanases (cellobiohydrolases) has frequently been shown to play a major role in the hydrolysis of crystalline cellulose. Perhaps the clearest demonstrations of such synergism are those of Selby and Maitland, (1967) and Wood, (1969). In these examples neither class of enzyme had significant activity towards crystalline cellulose, whereas acting in combination they were very much more effective. There is however an accumulating body of evidence demonstrating that substantial, even complete hydrolysis of crystalline cellulose can be due to action by one class of enzyme alone (e.g. Halliwell and Griffin, 1973, Berghem *et al.*, 1976, Kanda *et al.*, 1976a, Tong, 1980, Chanzy *et al.*, 1983, Nummi *et al.*, 1983). However, these cases serve merely to demonstrate that synergism is not vital for crystalline cellulose hydrolysis and do not preclude its possible involvement in the action of the unfractionated complexes.

In contrast, there was no evidence of any such synergism between an exoglucanase and two endoglucanases produced by *Thermoascus aurantiacus* (Tong, 1980, Shepherd *et al.*, 1981). These authors concluded that hydrolysis of cellulose by this particular fungus "involves a multienzymatic process whereby degradation of cellulosic materials of any complexity can be carried out independently by at least one of the cellulase components".

For bacteria, the only published demonstration of synergism being involved in crystalline cellulose hydrolysis has been by Creuzet *et al.*, (1983) studying the thermophilic anaerobe *Clostridium stercorarium*. This synergism involved cellobiohydrolase, endoglucanase and ß-glucosidase activities. Although the effects of the synergism were not as dramatic as for some fungal systems, the purity of the components must be doubted given the one-step separation employed. Cross-contamination would have reduced the degree of synergism observed.

Cellobiohydrolase production by bacteria appears rare, the only published examples being in *Cellvibrio gilvus* (reclassified as *Cellulomonas gilvus*) (Storvick and King, 1960), *Ruminococcus flavefaciens* (Pettipher and Latham, 1979), *Cellulomonas uda* (Nakamura and Kitamura, 1983) and *Clostridium stercorarium* (Creuzet *et al.*, 1983). Storvick and King (1960) in fact concluded that endwise cleavage of cellobiose units was the sole mode of attack by

each of the four components which they separated, but conceded that some other factor must have been involved in the growing cultures since their purified enzymes all had very limited activity towards native cellulose. Wood and Wilson (1984) reached a similar conclusion regarding the cellulolytic system of *Ruminococcus albus*. They demonstrated synergism between the *R. albus* endoglucanase complex and a purified exoglucanase from *Trichoderma koningii* in the hydrolysis of Avicel. This suggested that the vital factor missing from their cell-free *Ruminococcus* cellulase complex could have been an exoglucanase.

1.4.2 Affinity Factors

It is a fairly common observation amongst bacteria that the ability to grow on native cellulose is not necessarily associated with the ability to obtain isolated cell-free preparations capable of hydrolysis of this same substrate (Beguin and Eisen, 1978), although cellulose hydrolysis must obviously be extracellular. It has been suggested (von Hofsten, 1975) that the bacterial cell surface might be important in aligning cellulolytic enzymes, possibly in a specific sequence, so as to facilitate the disruption of crystalline cellulose. The presence of cellulose in most cellulase-producing cultures could also account for the lack of an active cellulase preparation derived from the supernatant if an important component remains bound to the cellulose.

An alternative explanation involves specific physical associations of enzymes, possibly involving a noncellulolytic "affinity factor", which are easily disrupted but difficult to reform. *Ruminococcus albus* is reported to require an affinity factor in addition to a hydrolytic factor for maximal hydrolysis of crystalline cellulose (Leatherwood, 1969). However Wood *et al.* (1982) found no evidence of such a factor in their studies of *R. albus*. An affinity substance is definitely involved in the cellulolytic system of *Clostridium thermocellum* (Ljungdahl (*et al.*, 1983). Few characteristics of the substance were published apart from its colour (yellow), its virtual insolubility in water or buffer solutions and its sensitivity to oxidation in air. Endoglucanases could be removed from binding to the affinity factor by washing with water, but the affinity factor was more firmly bound to cellulose and required an acetone wash to solubilize it. A "cellulose-binding-factor" has also been identified as being

produced by *C. thermocellum*, being largely bound to the cell surface in early culture growth but being released in water soluble form later (Bayer *et al.*, 1983). It bound strongly to cellulose but was released by triethylamine. This solubility in water and buffers distinguishes it from the yellow affinity substance of Ljungdahl *et al.* (1983).

1.4.3 Cellulase complexes as discrete functional units

Lamed *et al.*, (1983) found the cellulose-binding-factor of *C. thermocellum* to comprise a discrete, multisubunit complex (M.W. about 2 million) which exhibits multiple cellulase activities in addition to the property of cellulose binding, and hence is not only responsible for adherence of the cells to cellulose but also constitutes a major part of the cellulolytic apparatus. The majority of the 14 or so polypeptides in the complex were shown to be cellulolytic but were not separated for individual characterisation. The largest component (M.W. 210,000) was not cellulolytic, but was clearly important in causing adherence of the complex to the cell surface. Several complementary lines of evidence were presented which suggested that the isolated complex serves as a discrete structural and functional unit, with a complicated quaternary structure. The subunits seem to be arranged in a defined supramolecular fashion designed for highly efficient cellulose degradation.

Close physical association between members of an individual cellulase complex may well be a widespread phenomenon. Wood and McCrae, (1979) measured numerous cross-synergisms between an endoglucanase of one species of fungus and an exoglucanase of another. They found considerable variation in the degrees of cooperation between the endo- and exoglucanases of different species, and concluded that for maximum effectiveness these two types of enzyme might have to function in close physical association. In a separate experiment Wood ad McCrae (1979) investigated the synergistic effects of four purified endoglucanases, all obtained from *Trichoderma koningii*, when combined with cellobiohydrolase from the same organism. These endoglucanases all produced different degrees of synergism with the cellobiohydrolase, and only when all four were recombined with the latter was the activity of the unfractionated complex restored. Clearly the possibility of a multi-subunit complex acting as a

physical entity even in *Trichoderma* must be considered.

Sprey and Lambert (1983) present evidence that inadequately robust dissociating conditions have led many groups to mistake purified complexes for purified enzymes. They claimed that even SDS-PAGE did not split a cellulase complex which they isolated by preparative IEF from *T. reesei*. However IEF in urea, following pretreatment of the complex with urea and octylglucoside (a detergent), split the complex into six proteins which included a CMCase, a xylanase and two ß-glucosidases.

Given that a cellulose crystal is stabilized by hydrogen bonding and van der Waal's forces, Wood and McCrae (1979) conclude that it would be beneficial for initial cleavage of a glucosidic bond by an endoglucanase to be immediately followed by cellobiohydrolase action to remove a cellobiose unit, thus preventing the glucosidic linkage from reforming, but they don't see the need for a discrete functional complex of the two enzymes. Rather, they suggest that cellobiohydrolases might be already positioned along the crystalline sections of the cellulose, awaiting the approach of an endoglucanase. This would explain the frequently observed greater affinity of cellobiohydrolases for crystalline cellulose while retaining the concept of an initial attack on crystalline sections being made by endoglucanases. However, as discussed earlier, there is very little evidence to support random endo-acting cleavage at the molecular level of crystalline cellulose hydrolysis and considerable evidence to the contrary (Chang *et al.*, 1981).

1.4.4 Other factors

1.4.4.1 The role of exoglucosidases The importance of exoglucosidases (E.C.3.2.1.74) in cellulose hydrolysis has been grossly underestimated, according to Klesov and Grigorash (1980). This is probably because of difficulties in reliably distinguishing exoglucosidase action from the combined effects of other components of the complex. Klesov and Grigorash (1980), through a study of the kinetics of the enzymatic hydrolysis of cotton by eight cellulase complexes from three fungal genera, concluded that exoglucosidase plays a predominant role as compared to the combined action of cellobiohydrolase and cellobiase in the formation of glucose, but is not involved in the initial attack of the crystalline

substrate. By an alternative approach, in which other types of cellulolytic activity were selectively inactivated by ultrasonic treatment, Klesov and Churilova (1980) demonstrated that 82% of the glucose formed by the fungus *Geotrichum candidum* acting on CMC could be attributed to exoglucosidase action. This work has prompted little comment by groups outside Russia. Wood and McCrae (1979) speculated that a component of the *Penicillium funiculosum* complex might have been an exoglucosidase, and found it unable to act synergistically with endoglucosidases. Li *et al.*, (1965) and King and Vessel (1969) isolated a component fitting the exoglucosidases definition from Trichoderma reesei. No exoglucosidases have yet been found in the bacterial cellulase systems.

1.4.4.2. Phosphorylases Until the demonstration of cellobiose phosphorylase in *Clostridium thermocellum* (Sih *et al.*, 1957), hydrolases were the only enzymes known to be involved in cellulose degradation. The characterization and methods of production of *C. thermocellum* cellobiose phosphorylase have been described by Alexander (1961, 1968, 1972a). Cellobiose is phosphorylated to produce glucose 1-phosphate plus glucose. Subsequently cellobiose phosphorylase has also been found in several other cellulolytic bacteria (Ayers, 1958, Hulcher and King, 1958, Sato and Takahashi, 1967, Sasaki *et al.*, 1983).

Another enzyme, cellodextrin phosphorylase, which produces glucose-1-phosphate and cellobiose from cellodextrins, also occurs in *C. thermocellum* (Sheth and Alexander, 1969, Alexander, 1972b). Phosphorylation conserves some of the energy of the glucosidic bond broken. Phosphorylases are intracellular enzymes and unable to function in the absence of ATP, so will not complicate studies of cell-free supernatants. They may however play a significant role in the *in vivo* metabolism of soluble cellodextrins and cellobiose (Avgerinos and Wang, 1980), and possibly also function in the synthetic direction (Sheth and Alexander, 1969, Wang, 1978).

1.4.4.3 An enzymatic oxidative mechanism Eriksson *et al.* (1974) proposed an enzymatic oxidative mechanism as being important in the fungal degradation of cellulose. White rot fungi have been found to produce an extracellular enzyme which catalyzes the oxidation of cellobiose with simultaneous reduction of quinones, effectively coupling lignin and cellulose degradation (Westermark and Eriksson, 1974a,b). It appears most unlikely that these oxidative systems could function in anaerobic bacteria.

1.4.5 Cellulase multiplicity

1.4.5.1 Occurrence and possible explanations The multiplicity of cellulase components which is exhibited by virtually all cellulolytic organisms is of fundamental interest because of its implications with regard to our understanding of the mechanism of cellulose hydrolysis and the regulation of cellulase biosynthesis. Evidence for the existence of several cellulolytic components in extracellular culture filtrates of various fungi has long been available (Jermyn, 1952, Reese and Levinson, 1952, Gilligan and Reese, 1954). Some of the observed multiplicity was within the then-recognized classes of cellulolytic activity. Since then, several well-documented examples of such intra-class multiplicity have been published. At least eleven endoglucanases have been reported for *Penicillium citrinum* (Olutiola, 1976), five endoglucanases for *Sporotrichum pulverulentum* (Eriksson and Pettersson, 1975), and at least eight different enzymes with some form of cellulolytic activity have been shown to be produced by *C. thermocellum* (Lamed *et al.*, 1983).

Trichoderma reesei is the best studied organism as regards cellulase multiplicity and its causes, but there are still conflicting opinions about the origins and true extent of the multiplicity (Labudova and Farkas, 1983). It seems unlikely that a microorganism, with a limited gene pool, would carry genes specific for each of the many forms of cellulase which it produces, thus making them true isoenzymes. More acceptable is the concept that post-translational modifications may be responsible, with differences in carbohydrate content (Wood, 1975) or modifications by limited proteolysis (Nakayama *et al.*, 1976, Gritzali and Brown, 1979,

Langsford *et al.*, 1984) being currently favoured explanations. Recent amino acid sequencing of a cellobiohydrolase from *T. reesei* (Fagerstam *et al.*, 1984) did not reveal any heterogeneity, despite no attempt having been made to first separate the various subforms of the enzyme, and the variable glycosylation theory appears to be gaining ground. The multiple forms of cellulases produced by *Thermomonospora* were found to be produced simultaneously rather than sequentially (Hagerdal *et al.*, 1978, 1979), so proteolytic modification seemed an unlikely explanation of the multiplicity.

Most fungal cellulases appear to be glycoproteins (Wood, 1975). Less is known about bacterial cellulases, and it is unusual for procaryotes to glycosylate proteins. However, glycosylation of proteins may be more common amongst thermophilic procaryotes. An extracellular protease produced by an extremely thermophilic *Thermus*-like organism contained 13% carbohydrate (Cowan and Daniel, 1982a). Two endoglucanases purified from *C. thermocellum* have been shown to contain roughly 10% carbohydrate per unit weight of protein (Ait *et al.*, 1979a, Petre *et al.*, 1981). *Pseudomonas fluorescens* (Yamane *et al.*, 1970) and *Cellulomonas* species (Beguin and Eisen, 1978, Langsford *et al.*, 1984) also secrete glycosylated cellulases. It is particularly difficult to ascertain the true content of carbohydrate in cellulases since most cling tenaciously to cellulose and could conceivably carry some through several purification steps.

1.4.5.2 The implications of cellulase multiplicity Whatever the causes of cellulase multiplicity, it is clear that enzyme substrate specificities are affected, which may offer an organism an advantage over competitors for the same substrate. For example, *Trichoderma* species exhibit remarkable adaptability in their cellulase synthesis. Rautela and King (1968) found that cell free extracts of *T. reesei* cellulase from cultures grown on any one of the three water-stable crystalline forms of cellulose exhibited lower activation energy in attacking that particular crystal lattice form used in culture growth than in attacking the other cellulose crystal lattice forms. It appeared that the organism was able to adapt its synthesis of the various cellulase components to

specifically suit each form of cellulose. The mechanism which it uses to detect a certain crystal structure and to relay this information to the sites of enzyme synthesis is an intriguing mystery. Wood and McCrae (1979) presented evidence, using *T. koningii*, that it is the endoglucanase components which become most specifically "adapted" to the growth substrate. The wide range of structures involved in native celluloses may well account for a large part of the multiplicity of cellulases.

Cellulase multiplicity has been largely ignored in the formulation of cellulose hydrolysis mechanisms. Certain complications arise. For instance, synergism between two physically distinct cellobiohydrolases of *T. reesei* has been demonstrated by Fagerstam and Pettersson, (1980). This "exo-exo" type of synergism challenges the most widely favoured mechanism of cellulose hydrolysis, i.e. that of Wood and McCrae (1979). Pertinent to this is the observation of van Tilbeurgh *et al.* (1982) that one of these two "cellobiohydrolases" (CBHI) did not produce the reaction products expected of an exo-acting cellobiohydrolase when acting on a range of 4-methylumbelliferyl ß-D-glucosides derived from cellodextrins.

Synergisms between three endoglucanases have also been demonstrated (Kanda *et al.*, 1976b). It is widely accepted that multiple forms of endoglucanase can exhibit differing relative viscosity-reducing and saccharifying activities (Okada *et al.*, 1968, Wood and McCrae, 1979).

Endoglucanase isoenzymes from several different fungi were grouped by Rabinovich *et al.* (1983) into three basic groups differing in affinity for cellulose by three orders of magnitude. The first group comprised endoglucanases which were very effectively adsorbed onto crystalline cellulose. These were only produced by fungi capable of growth on crystalline cellulose, and not by fungi which could only utilize amorphous cellulose. The second group, with an affinity for cellulose 50-100 times lower, produced greater hydrolysis of amorphous cellulose than did the first group, but had no action on crystalline cellulose. It was suggested that this group play a major role in cleaving external

amorphous regions in cellulose. A third group of endoglucanases were very poorly adsorbed on cellulose, and were suggested as having a role in hydrolysing soluble intermediate products of cellulose hydrolysis. The above, supported by later work by this same group (Chernoglazov *et al.*, 1983), in which evidence of the purity of the endoglucanases is included, and by earlier findings of Yamane *et al.* (1970), Kanda *et al.* (1976a, b) and Beguin and Eisen, (1978) with different organisms, clearly requires that the concept of two or three distinctly different types of endoglucanase with differing types of activity should be incorporated into any proposed mechanism for cellulose hydrolysis. To discover their respective roles, a suggestion by White (1982) of a system employing antibodies specific to each enzyme and which are labelled so as to allow them to be distinguished under the electron microscope might be helpful.

In order to understand regulation of cellulase synthesis and function we must also consider individually the multiple forms of each type of cellulase. Churilova *et al.* (1980) demonstrate this in the case of *T. koningii*. Two purified endoglucanases were oppositely affected by cellobiose, which had a weak net inhibitory effect on the unfractionated cellulase complex as a whole.

Published discussions of the multiplicity of cellulases (Wood, 1975, Gong and Tsao, 1979) largely ignore the likely adaptive significance of such systems. One might speculate that they constitute a multi-pronged means of evading plant chemical defense mechanisms, since stable cellulase inhibitors are known to be widespread amongst plants (Mandels and Reese, 1965, Avgerinos and Wang, 1980). Some plant cell walls, when damaged, release proteins which inhibit specifically the wall-degrading enzymes secreted by microbes (Albersheim and Anderson, 1971, McNeil *et al.*, 1984). It has also been suggested that different isoenzymes of cellulases might be specific for different chain lengths of substrate (Gilligan and Reese, 1954, Hash and King, 1958, Reese *et al.*. 1959).

Lectin-type binding between cellulase and natural cellulosics has been proposed (Sprey and Lambert, 1983). Multiple cellulase forms, particularly those shown to differ

only in carbohydrate content, might confer on an organism the capacity to utilize this type of binding on a variety of naturally encountered cellulosics. The activation energy required to initiate cellulose hydrolysis may be reduced if the enzyme binding is directed to particularly susceptible sites by a lectin interaction. Plant cell wall cellulosic structures may contain recognition factors which facilitate cellulose breakdown by the plant's own intracellular cellulases in certain situations, such as in the formation of abscission zones, perforation of cell plates, fusion of tracheids, senescing and softening of fruits (Byrne *et al.*, 1975) and in actively growing tissue, in which cellulase is thought to be necessary to "loosen" the restrictive wall framework for cell expansion to occur (Fan and Maclachlan, 1967). It would seem most energetically efficient for saprophytes and pathogens to produce cellulases capable of mimicking the plant cellulases in interaction with these proposed recognition factors on the cellulose. Assuming no universal recognition system prevails throughout the plant kingdom, the production of multiple forms of cellulase might be explained, at least in part, as an adaptation allowing the most efficient hydrolysis of a wide range of cellulosics. To avoid wasteful overproduction of enzymes inappropriate to a particular type of cellulosic substrate, some mechanisms for substrate-specific independent induction of each enzyme form would be required, such as that demonstrated to exist in *T. reesei* by Rautela and King (1968) and described earlier in this section.

1.4.6 Impediments to the elucidation of the mechanism of enzymatic cellulose hydrolysis

The absence from the literature of an established and proven mechanism for the enzymatic hydrolysis of cellulose is due to several factors:

(i) Incomplete separation of components of most cellulase complexes studied, with insufficient attention being paid to activity differences between multiple forms of each type of cellulase.

(ii) Inadequate methods for determining the activities of individual components of the cellulase complex when still a

part of that complex.

(iii) Inadequate understanding of cellulose structure and its possible source-dependent variation, particularly with respect to naturally occurring cellulosic forms.

(iv) The wide variety of cellulase systems, substrates and hydrolysis conditions employed by the various research groups.

(v) Insufficient emphasis being placed on kinetic analysis of the enzymatic hydrolysis of cellulose and a lack of recognition of the possibility that the rate-limiting step may change during the course of the hydrolysis. It must be conceded however that the heterogeneity of native crystalline cellulose makes this approach very difficult.

1.5 MICROBIAL SOURCES OF CELLULASES

1.5.1 Fungal Sources

Production of extracellular cellulolytic enzymes is widespread amongst fungi, but in the search for particularly good sources relatively few fungal species have been found capable of producing complete solubilization of native cellulose (reviewed by Mandels 1975, 1982, Saddler, 1982, Enari, 1983).

Most fungal cellulases are stable over the pH range 3-8, active over 3.5-7.0 and have optima between pH 4.0 and 5.5 (Tong, 1980). The lowest reported optimum is pH 2.5, from *Aspergillus niger* (Ikeda *et al.*, 1973).

One species in particular, *Trichoderma reesei*, has given rise to several mutants which show clearly superior rates of solubilization of native cellulose (Montenecourt and Eveleigh, 1979). The differences between the best mutants and their parent wild strains appear to be only quantitative, with the properties of the complex, including relative proportions of endo- and exoglucanases, being unchanged (Mandels, 1982). All are catabolite repressed by glucose and cellobiose (Mandels, 1982). Cellobiose accumulation as an end-product of cellulose hydrolysis can also substantially inhibit further cellulase activity (Sternberg *et al.*, 1977). Therefore *Trichoderma* cellulase preparations are often supplemented with ß-glucosidase from other fungi, e.g. *Aspergillus*, to achieve complete breakdown of the cellulose to glucose, which is less inhibitory than cellobiose.

Trichoderma cellulases are commercially produced in several

countries as crude cell-free preparations containing other carbohydrases; xylanases, mannanases etc. Advantages of *Trichoderma* as a source of cellulase are:

(i) that it produces a complete cellulase containing all the required components for total hydrolysis of crystalline cellulose

(ii) that a very high proportion of the extracellular protein (approaching 100% according to Mandels, 1982) is cellulase

(iii) that it is relatively resistant to chemical inhibitors (Mandels and Reese, 1965).

Disadvantages are:

(i) an inability to attack lignin

(ii) low specific activities of its component enzymes (*T. reesei* will excrete cellulases at a concentration of 1.5-2.0 mg protein.ml^{-1} in order to hydrolyse cellulose to a level of 7.5 mg.ml^{-1}. (Sternberg, 1975)).

(iii) low levels of ß-glucosidase

(iv) thermal instability, having a half-life at 60°C of less than 10 minutes (Ng and Zeikus, 1981a).

White rot fungi appear to have considerable potential industrially since they enzymatically degrade all wood components, including lignin (Eriksson, 1982, Palmer and Evans, 1983). Mutants of the thermotolerant white rot fungus *Sporotrichum pulverulentum* have recently been produced which possess filter paper degrading activity equivalent to one of the best strains of *T. reesei*, but have six times the ß-glucosidase activity and, of course, the capacity to degrade lignin (Eriksson and Johnsrud, 1983). Thermal stability of the complex does not appear to be as good as that of *T. reesei* cellulase however.

1.5.1.1 Fungal producers of thermostable cellulases. Thermophilic fungi, capable of growth at temperatures of up to 55-60°C (Tansey and Brock, 1972), are sources of more thermostable cellulases, and work has been reported on cellulases from *Humicola insolens* (Hayashida and Yoshioka, 1980a,b), *Sporotrichum thermophile* (Canevascini *et al.*, 1979, 1983, Coutts and Smith, 1976, Margaritis and Creese, 1981), *Talaromyces emersonii* (Folan and Coughlan, 1978, 1981, McHale and Coughlan, 1980, 1981a, b, 1982), *Thermoascus aurantiacus* (Tong, 1980, Tong *et al.*, 1980, Shepherd *et al.*, 1981),

Chaetomium thermophile var. dissitum (Eriksen, 1974, Eriksen and Goksoyr, 1976, 1977) and *Thielavia terrestris* (= *Allescheria terrestris*) (Skinner and Tokuyama, 1978).

Direct comparison of thermostabilities of cellulases from the various organisms is not possible due to the lack of consensus regarding the test conditions, but despite this problem, it is clear that the most thermostable cellulase complex so far reported comes from *Thielavia terrestris* (Skinner and Tokuyama, 1978). The filter-paper hydrolysing activity of its cell-free culture filtrate has a half life at 100°C of about 50 minutes, provided filter paper is present during this 100°C treatment, and 30% of the original activity remains after 120 minutes. Strangely however, the authors claim that the system exhibits "optimum" cellulolytic activity at between 60°C and 70°C. At such temperatures, at pH 5.0, the system hydrolyses cotton to the extent of 20% within 24 hours. *Trichoderma* culture filtrates can completely degrade cotton fibres in 24 hours (Halliwell, 1965). *Th. terrestris* exhibits optimum growth between 40°C to 50°C.

Stability over a wide range of pH is also a sought-after attribute, particularly stability to highly alkaline pHs which are often employed in pretreatment of natural cellulosics (Mandels *et al.*, 1974, Brown, 1983). *Humicola insolens* YH-8 perhaps offers the best combination amongst the fungi of reasonable cellulase thermostability and a very wide pH stability range (3-11 for CMCase, 3.5-9.5 for avicelase). Attached carbohydrates were shown to have a major role in producing these stabilities, but did not exert a gross influence on specific activity, hydrolysis products, hydrolysis curves or adsorbability onto Avicel (Hayashida and Yoshioka, 1980).

The highest maximum temperature for growth among these thermophilic cellulolytic fungi appears to be 60-62°C, exhibited by *Chaetomium thermophilae* (Eriksen, 1974).

1.5.2 Bacterial Sources

Cellulolytic bacteria cover a wide range of taxonomic groups. Most plant pathogens and saprophytes possess some cellulolytic capacity, but the cellulase systems of only a few species have been studied. In many cases bacteria which can hydrolyse crystalline

cellulose do not appear to give rise to a "complete" extracellular cellulase complex. Some possible explanations have already been discussed (Section 1.4.2). In addition it is known that species or strains of cellulolytic bacteria occur naturally in close, complementary relationships, the rumen providing an excellent example (Hungate, 1947, Wood *et al.*, 1982). Further, there are several known examples of a very close stable natural association with a specialist fermenting bacterium (e.g. Bellamy, 1979, Brandon, 1979, Peck and Odum, 1981, Khan and Murray, 1982). In the absence of the symbiont, end-product accumulation may rapidly prevent the functioning of the cellulase complex of an isolate. Such natural symbioses may also partly explain why cellulolytic bacteria are considered to be difficult to isolate (Hungate, 1950, McBee, 1950 - see Section 1.7).

Mesophilic bacteria (i.e. with growth optima up to 40°C) which have been found capable of substantial hydrolysis of crystalline cellulose are listed in Table 1.1.

1.5.2.1 Thermophilic cellulolytic bacteria. Thermophilic cellulolytic bacteria (i.e. those which display growth temperature optima in the range 45°C-65°C) come from four groups; the actinomycetes, the sporocytophagas, the bacilli and the clostridiae.

(i) Actinomycetes A considerable amount of interest has been shown recently in the cellulolytic capacity of species of *Thermomonospora*, aerobes that grow at 55-60°C (Hagerdal *et al.*, 1978, 1979a, b, 1980, Stutzenberger, 1979, Ferchak *et al.*, 1980, Ferchak and Pye, 1983). Maximum levels of extracellular CMCase and avicelase were achieved after only 18-24 hours of growth, and were the highest reported for culture supernatants of any of the bacterial genera (90μmoles.min^{-1}.ml^{-1} and 0.28μmoles.min^{-1}.ml^{-1} respectively) (Hagerdal *et al.*, 1979). Hyperproducing mutants have subsequently been produced (Meyer and Humphrey, 1982), and recently a catabolite repression-resistant mutant of *Th. curvata* has been produced which is capable of cellulase production in the presence of glucose, with 10mM glucose stimulating both growth and cellulase secretion (Fennington *et al.*, 1984).

TABLE 1.1 Mesophilic bacteria reported to produce significant hydrolysis of crystalline cellulose.

Ruminococcus flavefaciens	Pettipher and Latham(1979)
Ruminococcus albus	Wood et al. (1982), Taya et al. (1983)
Bacteroides succinogenes	Wood et al. (1982), Taya et al. (1983)
Other *Bacteroides* sp.	Miyoshi(1978), Guiliano et al. (1983).
Pseudomonas fluorescens var. *cellulosa*	Yamane et al. (1970), Yoshikawa et al. (1974), Suzuki et al. (1975), Hwang and Suzuki (1976).
Pseudomonas fulvus (=*Cellvibrio fulvus*)	Berg et al. (1972), von Hofsten and Berg (1972)
Cellulomonas gilvus (=*Cellvibrio gilvus*)	Hulcher and King (1958), Storvick and King (1960), Breuil and Kushner (1976).
Cellulomonas fimi	Whittle et al. (1982).
Cellulomonas uda	Nakamura and Kitamura (1983)
Other *Cellulomonas* sp.	Stewart and Leatherwood (1976), Beguin et al. (1977), Beguin and Eisen (1978), Choi et al. (1978), Hagget et al. (1979), Rodriguez and Enriquez (1980), Choudhury et al. (1980a, b, 1981), Peiris et al. (1982).
Acetivibrio cellulolyticus	Patel et al (1980), Khan (1980), Khan et al. (1980), Saddler et al. (1980), Saddler and Khan (1980), Breuil and Patel (1981), Patel and MacKenzie (1982).
Bacillus sp.	Tewari and Chahal (1977), Sashihara et al. (1984).
Clostridium cellobioparum	Hungate (1944)
Clostridium papyrosolvens	Madden et al. (1982)
Other *Clostridium* sp.	Petitdemange et al. (1983) Leschine and Canale-Parola (1983)
Cytophaga sp.	Chang and Thayer (1977)
Sporocytophaga myxococcoides	Osmundsvag and Goksoyr (1975), Vance et al. (1980).

Thermostability of the cellulase complex is considerably better than that of *Trichoderma reesei* (half lives at 60°C in excess of 30 hours and less than 10 minutes respectively) (Hagerdal *et al.*, 1979, Zeikus *et al.*, 1981).

Specific activities of the *Thermomonospora* endo- and exoglucanases are reported to be comparable to those of *Trichoderma reesei* (Ferchak *etal.*, 1980), with extracellular protein levels reaching $1.7mg.ml^{-1}$ in the stationary phase.

The pH range for stability of CMCase and avicelase components of the complex was 6 - 7.3, with optimal activity around pHs 6 and 7 respectively. The ß-glucosidase activity had a sharp pH-activity profile, being optimal at 6.6 but totally inactive at pH7. The stability of the ß-glucosidase was relatively poor, with a half-life of about 1 hour at 60°C (Hagerdal *et al*, 1979).

A group at Cornell University has successfully achieved expression of *Thermomonospora* endoglucanase cloned into *E. coli* (Collmer and Wilson, 1983).

(ii) Sporocytophaga A thermophilic anaerobic *Sporocytophaga* species (a gliding bacterium) was found by Bellamy, (1979). It had a growth range of 45-65°C, with a 55-60°C optimum, at an optimum pH of 7.5-8.0, and was capable of hydrolysing filter paper. Bellamy was not able to isolate the cellulolytic *Sporocytophaga* from a non-cellulolytic *Bacillus*, but the major products of the co-culture were ethanol and acetic acid in a 1:1 ratio. The stability of the enzyme system was not described.

(iii) Bacillus A thermophilic, cellulolytic, acidophilic, aerobic *Bacillus* species has been isolated and found capable of growth in the range 46-70°C, (optimum 65°C) and at pH 2.0-5.0 (optimum 3.5-4.0). An enzyme was purified from this organism which was active towards CMC, cellulose floc and xylan. It had a half-life at 80°C of 15 minutes and an optimum pH of 4.0, was stable in the pH range 2-6 and was not affected by 1mM concentrations of HgCl or $AgNO_3$ (Uchino and Nakane 1981).

All *Bacillus* species, whether mesophilic or not, possess

a certain inherent degree of thermostability (Zeikus, 1979). Some alkalophilic *Bacillus* species which grew well in pH 10-11 at 37°C produced CMCase active over the pH range 4.4-12.8, with near optimal activity across pHs 5-11, and were claimed to be stable at 75°C for 10 minutes at least (Sashihara *et al.*, 1984, Horikoshi *et al.*, 1984). Two of the CMCase genes of an alkalophilic *Bacillus* sp. have been cloned successfully into *E. coli*, which expressed the enzymes constitutively, but with extracellular release not exceeding 14% (Sashihara *et al.*, 1984).

(iv) Clostridia Thermophilic, anaerobic, cellulolytic clostridia are the most widely studied thermophilic cellulolytic bacteria. Only two species in this category are currently recognized, *Clostridium thermocellum* (McBee, 1950, 1954) and *C. stercorarium* (Madden, 1983). Both are Gram negative spore-formers. *C. thermocellulaseum* (Enebo, 1951) which has been lost, and *Clostridium* strain M7 (Lee and Blackburn, 1975) were probably not further distinct species in this group, but merely strains of *C. thermocellum* since the distinguishing features were mainly based around the range of substrates fermented. The reliability of such a basis for distinguishing between strains is questionable. For example, a *C. thermocellum* culture which is unable to utilize glucose can be adapted to do so, provided yeast extract is supplied, through the induction of glucokinase (Patni and Alexander, 1971a). Fructose and mannitol were also only metabolized once the appropriate PEP-phospho-transferase systems had been induced (Patni and Alexander, 1971b). It is possible that cultures can be similarly adapted to utilize pentoses, starch and other carbohydrates which serve at present to distinguish *C. thermocellum* from *C. thermocellulaseum* and strain M7. Such adaption might also explain apparent contradictions in the literature regarding the capacity of *C. thermocellum* to grow on xylose (McBee, 1948, 1950, 1954 vs. Ng *et al.*, 1977 and Garcia-Martinez *et al.*, 1980). Alternatively, differences in utilizable substrates might also be attributable to contamination by the ubiquitous *C. thermohydrosulfuricum* or *C. thermosaccharolyticum*. The former possesses extremely

thermotolerant spores, making it difficult to eradicate by autoclaving, so it may well have been present in uninoculated media assumed to be sterile (Hyun *et al.*, 1983). Once these fermenters become established in a coculture with *C. thermocellum*, reisolation of the latter can be difficult (Zeikus *et al.*, 1983, Saddler and Chan, 1984).

C. thermocellum has a growth optimum of 60-64°C and a maximum of 68°C (McBee, 1954, Johnson *et al.*, 1981). Its cellulase is active and stable in the absence of substrate from 37°C to 65°C, but rapidly loses activity at 80°C. The CMCase activity was less thermostable than the avicelase, each losing 87% and 15% of its activity respectively after 5 hours at 70°C in the absence of substrate (Johnson *et al.*, 1982). The ß-glucosidase was least stable, losing 40% of its activity after 7 hours at 60°C (Ait *et al.*, 1979a). Optimum pH values for the avicelase and CMCase activities are still disputed. Lee and Blackburn, (1975) found both to have optima at pH 6.5, whereas Ng *et al.* (1977) claimed an optimum of 5.4 for avicelase and 5.2 for CMCase, while Johnson *et al.* (1982) found the optimum pH for avicelase to be 5.7 and that for CMCase to be pH 6.1, with a rapid decline in activity below pH 5.4 or above pH 6.2.

There is also controversy over whether or not *C. thermocellum* cellulase is inhibited by its end-products, cellobiose and glucose. The evidence for and against is reviewed by Duong *et al.*, (1983). The various opposing conclusions were all reached using different substrates and assay techniques which may well have a bearing, as might the use of different strains of the bacterium. Avgerinos *et al.* (1981) reported producing a mutant strain from which the cellulases were neither inhibited nor repressed by cellobiose or glucose.

Highly ethanol-resistant strains have been isolated which can degrade cellulose in the presence of up to 8% ethanol (although even 5% ethanol in the medium inhibited growth by 50%) (Gomez, 1980).

Direct comparison of the extracellular cellulases of *C. thermocellum* and *T. reesei* (Ng and Zeikus, 1981a) revealed a higher endoglucanase/exoglucanase ratio in *C. thermocellum*

and a predominance of long-chain rather than short-chain oligosaccharides as initial hydrolysis products from microcrystalline cellulose. The *C. thermocellum* cellulase system was more sensitive to inhibition by certain metal ions, including Ag and Hg at low concentrations which suggested the involvement of sulphydryl groups. The *T. reesei* hypercellulolytic strain QM9414 used in the comparison produced extracellular cellulase with higher total and specific activities towards various cellulosics than that of *C. thermocellum* LQR1 (Ng and Zeikus 1981a).

A component of the system which is required for full activity towards crystalline cellulose is oxygen-sensitive (Johnson *et al.*, 1982). This sensitivity may be restricted to a yellow cellulose-affinity substance recently discovered (Ljungdahl *et al.*, 1983).

The cellulases of *C. thermocellum* are constitutive (Park and Ryu, 1983). Further characteristics of the *C. thermocellum* cellulase complex are reviewed by Duong *et al.* (1983).

Two genes coding for endoglucanases have been successfully cloned into *E. coli*, which expresses them intracellularly. The two genes were found to share no homology and were not contiguous on the *C. thermocellum* chromosome (Cornet *et al.*, 1983a,b). The DNA sequence for one of these genes (*celA*) has been determined (Beguin *et al.*, 1985).

The other recognized clostridial cellulolytic thermophile, *C. stercorarium*, was only recently isolated (Madden, 1983). It differs from *C. thermocellum* in being unpigmented, in having a higher ratio of endo- to exoglucanase activity and in being able to ferment xylose. Cellobiose inhibition of a purified endoglucanase is marked (50% inhibition by 1mM cellobiose) and 1mM Hg^{2+} and thiol reagents also inhibit, as does 1mM SDS (Creuzet and Frixon 1983).

C. stercorarium has a growth optimum of 65°C near pH 7.3 and growth at 60°C and 70°C is about 60% of that obtained at 65°C (Madden, 1983).

Growth occurs more slowly on cellulose (generation time of 16h) than for *C. thermocellum* (generation time of 7h),

probably due to the lower production of exoglucanase by *C. stercorarium* (Madden, 1983).

An endoglucanase which has been purified from *C. stercorarium* appears to have the best thermostability of any purified cellulase component in the literature, (i.e. a half-life of 1 hour at 85°C) (Creuzet and Frixon, 1983). This is second only to the stability of the crude filter paper-solubilizing activity of *Thielavia terrestris*, with a half life of 50 minutes at 100°C (Skinner and Tokuyama, 1978). The only report in the literature of cellulolytic bacteria capable of growth at 75°C and beyond is a preliminary account of some cellulolytic enrichments capable of fermenting cellulose at pHs as extreme as 2 and 8.6 and at temperatures up to 84°C (Ljungdahl *et al.*, 1981).

Weimer *et al.* (1984) have found seven thermophilic anaerobic bacteria which ferment xylan but not cellulose. Some grow up to 75°C. They deserve mention since they were Gram negative but not spore-forming. They appeared not to fit into any established genus, although they resembled *Thermoanaerobium brockii*, *Thermobacteroides acetoethylicus* and *Thermoanaerobacter ethanolicus* in various ways. It is possible that this new group might include some cellulolytic members also capable of growth at 75°C.

1.5.3 Advantages of bacteria as sources of cellulase

(i) They appear to be simpler to genetically engineer than the genetically more complex eucaryote fungal systems.

(ii) They less frequently produce antibiotics or substances toxic to humans and animals than do fungi.

(iii) They grow more rapidly than fungi, and some species will grow at higher temperatures than the 60-62°C limit for fungal growth.

(iv) Protease production appears to be less common amongst cellulolytic bacteria than fungi, (Tansey and Brock, 1972), so bacteria might be expected to produce more stable cellulase activity in culture supernatants.

1.5.4 Disadvantages of bacteria as sources of cellulase

(i) Currently the yields of cellulase activity per ml of culture are generally much lower than for the best fungal systems.

(ii) Many do not produce "complete" cellulase complexes which are extracellular. Cell-bound enzymes or other "affinity factors" are frequently involved in hydrolysis of crystalline cellulose by bacteria.

(iii) Many require anaerobic growth conditions, making them difficult to isolate and handle.

1.6 CELLULASE APPLICATIONS

Current and potential applications of cellulases fall into two main classes:

(i) direct use of enzymes in food processing, animal feed preparations, pollution control and pharmaceutical processes, and

(ii) production of sugars as intermediates in the fermentation of cellulose to a variety of more valuable end-products. The major uses and potential uses recognized to date are listed in Table 1.2.

Table 1.2 Current and potential applications of cellulases (Coughlan and Folan 1979)

Food processing	Pharmaceuticals
Improving digestibility of foods	Digestive aids
Producing unicellular vegetables	Elimination of unwanted fibres
Extraction of flavours, oils, juices, proteins from fruit and vegetables	Pollution control
Removing soyabean seed coat	Clearing solid wastes in municipal dumps
Recovery of agar from seaweed	Digestion of excreta in septic tanks and drains
Treating rice grains for sake production	Fermentation
Improving solubility of raw materials in brewing	Providing substrate to convert to methane, ethanol, glycerol, citric, and lactic acid, vitamins, antibiotics, single cell protein
Isolation of potato starch	
Animal feeds	
Improving digestibility of usual forage	
Processing wood and wood by products as feed	

Application in many of these areas is currently restricted by economic considerations. Firstly there is the cost of lignocellulose pretreatment, since no systems are currently capable of hydrolysing the

cellulose ~~in native cellulose~~ in native lignocellulose at economic rates. Secondly, the expense of cellulase production must be considered, particularly since most available cellulolytic enzyme systems have relatively low specific activities (about 100 times lower than comparable amylolytic enzymes (Montenecourt *et al.*, 1981)). Cellulase recycling alternatives such as readsorption onto fresh substrate (Castanon and Wilke, 1980), immobilization onto a solid matrix (Woodward and Zachry, 1982) and the use of ultrafiltration membrane reactors (Ohlson *et al.*, 1983) appear to be economic necessities. Clearly a means of producing relatively cheap cellulase with improved stability and specific activity compared with that of *Trichoderma reesei*, the main commercially exploited cellulase to date, would be of value for applications requiring cell-free cellulase preparations.

In other applications which involve the use of growing cellulolytic cultures, the yield of cellulase activity and specific growth requirements assume greater significance, and may take precidence over such factors as high stability and specific activity of the cellulase. Although genetic manipulation should eventually result in improved cellulase yields from organisms producing cellulases with the highest specific activities and stabilities, there will always be applications which demand a particular class or organism, e.g. aerobe, anaerobe, acidophile, alkalophile, and no one "super-bug" will best satisfy all requirements.

1.6.1 Advantages conferred by the use of thermophilic anaerobic cellulolytic bacteria in industrial fermentation processes

There are considerable process advantages in obtaining a fermentation system operable at temperatures of 70-80°C. The following is a summary of the advantages listed by Wiegel, (1980).

(i) The cell yield of thermophilic anaerobes tends to be low compared to mesophiles, which is an advantage in solvent/fuel directed fermentations in which cell material is essentially a waste product.

(ii) Thermophiles exhibit high catabolic activities at temperatures optimal for growth (Wiegel, 1980), allowing rapid fermentation and high efficiency.

(iii) Oxygen solubility is decreased by the use of high temperatures, simplifying establishment and maintenance of anaerobic conditions.

(iv) Viscosity of the medium decreases at elevated temperatures,

requiring less energy for stirring, and allowing faster diffusion rates.

(v) Recovery of ethanol (boiling point 78°C) could be made a continuous process by application of a mild vacuum or an anoxic gas stream.

(vi) Cooling should not be necessary whereas it is often required to sustain exothermic cellulose fermentation by mesophiles. Once established, a thermophilic fermentation may become self-sustaining with little or no requirement for external heat input.

(vii) Sterility of the substrate is of reduced importance since no fungal and very few bacterial species will be able to grow in the fermentor at 70-80°C. However contamination by *Bacillus stearothermophilus* and *Clostridium thermohydrosulfuricum*, which are widespread and grow rapidly at 70°C, may require some countermeasure.

1.6.2 Proposed systems using thermophilic and extremely thermophilic bacteria for industrial ethanol production

A high percentage of known anaerobic thermophiles and extreme thermophiles produce ethanol from sugars as their main product, possibly because it is volatile and thus less likely to accumulate to toxic levels in the medium than alternative products.. The diversity of thermophiles which hydrolyse cellulose, a favoured raw material, is much more limited, and to date only *Thermomonospora* species and *C. thermocellum* have to our knowledge been employed in the thermophilic pilot schemes. The former does not produce ethanol and so has been teamed up with a sugar-fermenting organism such as *C. thermohydrosulfuricum* (Nolan *et al.*, 1981). Simultaneous coculture of aerobic *Thermomonospora* and the anaerobic clostridial species is of course impossible. Due to its mycelial growth habit, which would assist in cell harvesting, and to a relatively high cell mass production, *Thermomonospora* single cell protein could be a valuable by-product (Humphrey *et al.*, 1977, Meyer and Humphrey, 1982). Most attention has been paid however to systems involving *C. thermocellum*, and several groups that were previously exploring the industrial potential of cellulose saccharification by *T. reesei* have switched their attention to *C. thermocellum* - based systems. Although *C. thermocellum* can produce ethanol when acting alone, cocultures involving a

specialist fermenter allow much higher yields of ethanol and the utilization of pentoses (Wiegel, 1982), e.g. with *C. thermosaccharolyticum* (Saddler and Chan, 1983, Wang *et al.* 1983), *C. thermohydrosulfuricum* (Zeikus *et al.*, 1983), *Thermoanaerobacter ethanolicus* (Ljungdahl and Wiegel, 1981). The latter organism appears to be ideal for co-cultures with thermophilic cellulose-degrading bacteria since it exhibits optimal growth and ethanol production between pH 5.8 and 8.5, has a high ethanol tolerance (8% w/v) and will grow at temperatures as high as 78°C with little effect of temperature on the ethanol yield (up to 1.9 mole ethanol per mole of glucose or pentose) (Wiegel, 1982).

A French group (Petitdemange *et al.*, 1983) maintains that their butyric acid-producing mesophilic coculture of *Clostridium* strain H 10 and *C. acetobutylicum* is also a promising approach to efficiently obtaining useful chemicals from cellulosic materials. They claim higher rates of cellulose utilization than those obtained by groups employing thermophilic cocultures. Nonetheless, recognition of the many merits of thermophilic fermentation processes (Section 1.6.1) must surely dictate their use when economic and political forces eventually allow the commercial production of fuel and chemical feedstocks from lignocellulosics. Perhaps the genetic engineers will one day be able to clone cellulase genes into one of the extremely thermophilic fermenting bacteria, thus obviating the need for cocultures.

1.7 ISOLATION OF CELLULOLYTIC BACTERIA IN PURE CULTURE

The difficulties of isolating cellulolytic thermophilic anaerobes have long been recognized. McBee (1950) provides an amusing review of the early unsuccessful attempts at isolation of such organisms, dating back to MacFadyen and Blaxall (1894). The first cultures of thermophilic cellulose fermenting bacteria of demonstrable purity were those of McBee (1948), obtained through the use of techniques developed by Hungate (1944). McBee's success may be attributed to the stringent criteria of culture purity which he adopted, namely: (i) The strain must arise from a single, well-isolated colony in the cellulose agar (ii) Upon dilution into cellobiose agar it must yield only one colony type (iii) A single isolated colony from a high dilution of the cellobiose agar must be transferred to a second cellobiose agar dilution series with similar results. (iv) All the colonies in the highest dilution showing growth in the second cellobiose agar series must be

macroscopically similar and yield only cellulose-digesting colonies when transferred into cellulose agar.

There are several explanations for the exceptional difficulties encountered in the isolation of cellulolytic anaerobes. Firstly, the cellulolytic organism may not survive in the absence of a symbiont, unless certain nutrients, growth factors or enzymes are added to the growth medium or certain metabolic products are removed. Brandon (1979) was able to develop stable mixed cultures containing anaerobic cellulolytic bacteria but found the components of the mixed cultures lost viability in monoculture when grown on cellulose. Viability could not be restored by supplementing the medium with filtered culture supernatant from growing mixed cultures and only actual remixing of the organisms was sufficient to allow growth on cellulose. Removal of metabolic products is thus probably an essential part of the symbiosis. Alternatively, essential cellulases may have been removed from Brandon's filtered mixed-culture supernatants due to adsorption on the cellulose filter paper. Only very few micro-organisms, mostly aerobic fungi, produce a complete cellulase complex (Enari, 1983) and according to von Hofsten *et al.* (1971), the "true cellulolytic activity" which allows a culture to hydrolyse crystalline cellulose may often be due to a syntrophism.

A very close physical association may exist between two species of bacteria. Khan and Murray, (1982), in attempting to isolate a particular cellulolytic mesophilic anaerobe, found that discrete colonies obtained on agar slants gave rise inevitably to two distinctly different bacteria. If the requirement for cellulase production was removed by addition of yeast extract, cellulolytic activity was lost permanently and a pure culture of the non-cellulolytic bacterium could be produced. However, the cellulolytic partner could not be isolated in pure culture. Electron micrographs revealed a close physical association of the two types of cell in co-cultures. Such growth of two anaerobes as a single colony is not unique, with several other examples from the literature being listed by Khan and Murray, (1982).

Isolation of extremely thermophilic cellulolytic anaerobes presents further problems stemming from their high optimum growth temperatures. The traditional methods of streaking agar surfaces of plates or roll-tubes had been found inappropriate by other members of our group. Plated agar would dry out during incubations at 70-75°C lasting several days and roll-tubes, such as those of Hungate (1950), rapidly collected

a film of liquid on the agar surface which resulted in colony cross-contamination. Also, the thin layer of agar in the roll-tube was prone to collapse due to a softening of the agar.

Production of sterile media is vital to any bacterial isolation programme, especially if dilution series play an integral part in the scheme. Some literature examples of remarkably heat-stable spores are given by Hyun *et al.* (1983), who state that over the past years their laboratory cultures have been "routinely contaminated, despite cautious use of rigorous aseptic culture techniques". This problem has resulted in their supplying other labs unknowingly with *Clostridium thermocellum* cultures that were contaminated by pentose-fermenting species. They, in turn, have found spore-forming contaminants in cultures of several supposedly non-spore-forming ethanol-producing thermophilic bacteria supplied by other investigators and major culture collections.

Standard autoclaving strategies are insufficient to ensure inactivation of *Clostridium thermohydrosulfuricum* spores to an acceptable level (Hyun *et al.*, 1983). They recommend a minimum of 30 minutes at 121°C for preparation of culture materials to grow thermophilic bacteria. It is conceivable that other unstudied extreme thermophiles might produce spores with a still greater thermal tolerance than those of *C. thermohydrosulfuricum.*

1.8 AIMS OF THIS STUDY

(i) To find and cultivate extremely thermophilic cellulolytic bacteria capable of growth at 75°C and beyond. If such bacteria existed, it seemed likely that their cellulases would be more thermostable than any previously reported. When we began this project there were no published reports of any cellulolytic bacteria with growth optima above 60-65°C and maxima above 70°C.

(ii) To isolate any such cellulolytic bacteria in pure culture.

(iii) To evaluate the cellulolytic activity of the bacteria by direct comparison with that of *Clostridium thermocellum*, the organism currently being evaluated by several groups involved in cellulose-to-ethanol research and development. It was recognized as the most thermophilic of the few bacterial producers of "complete" cellulase activity.

(iv) To fractionate the cellulase complex of one selected organism and to purify and characterise any component found to be active towards crystalline cellulase.

(v) To test for synergisms between the various components of the

cellulase complex of this organism in the hydrolysis of crystalline cellulose. It was hoped that this approach might yield valuable insight into the mechanism of cellulose hydrolysis. No such synergisms had been demonstrated for a bacterial system at that stage.

CHAPTER TWO

METHODS AND MATERIALS

2.1 CULTURE MEDIA

Cultures were grown in 30ml Universal or McCartney bottles in 10ml of medium for aerobes, while, for anaerobes, bottles were either filled completely with medium or else any head-space was filled with CO_2 or N_2 A modified version of the basal mineral salts medium of Zeikus *et al.*, (1979), supplemented with vitamins, trace elements, carbon sources and reductants as outlined below, was used for both aerobes and anaerobes.

Basal Medium

	(Quantities per litre)
NH_4Cl	0.9g
NaCl	0.9g
$MgCl_2.6H_2O$	0.2g
KH_2PO_4	0.75g
K_2HPO_4	1.5g
$FeSO_4.7H_2O$	0.06ml of fresh 5% solution
Vitamins Stock	5ml
Trace Elements Stock	9ml

Vitamins Stock

(Quantities per litre)

Biotin	2mg
Folic Acid	2mg
Pyridoxine HCl	10mg
Riboflavin	5mg
Thiamine-HCl	5mg
Nicotinic Acid	5mg
Pantothenic Acid	5mg

Trace Elements Stock

Nitrilotriacetic acid	12.8g
Neutralize to pH6.5 with KOH	
$FeCl_3.6H_2O$	0.2g
$MnCl_2.4H_2O$	0.1g
$CoCl_2.6H_2O$	0.17g
$CaCl_2.2H_2O$	0.1g
$ZnCl_2$	0.1g

Vitamins Stock (contin.)

Vitamin B-12 0.1mg
p-aminobenzoic acid 5mg

Trace Elements Stock (contin.)

$CuCl_2$ 0.02g
H_3BO_3 0.01g
$Na_2MoO_4.2H_2O$ 0.01g
NaCl 1.0g
Na_2SeO_3 0.02g
Finally adjust to pH6.75 with KOH.

Carbon sources, such as cellobiose, CMC, microcrystalline cellulose, MN300 amorphous cellulose or wood pulp were added to the medium at between 2 and $10g.l^{-1}$.

Yeast extract (0.6 - $3g.l^{-1}$), trypticase peptone (BBL) ($10g.l^{-1}$) and MOPS buffer ($10g.l^{-1}$) were sometimes included as supplements as described in the text.

The anaerobic culture medium additionally contained sodium thioglycolate ($1g.l^{-1}$) as reductant and methylene blue ($2mg.l^{-1}$). The reductant was changed in later experiments to cysteine-hydrochloride ($1g.l^{-1}$) and the redox indicator to resazurin ($1mg.l^{-1}$), since both were considered less likely to be toxic to the organisms. TP8.T6.3.3.1 was finally grown without any reductant, in which case the medium was not allowed to re-aerate after autoclaving, and the culture was not shaken until growth had become established and the resazurin had been decolourised.

The pH of all media was adjusted to 7.2 with NaOH prior to autoclaving.

All types of media were autoclaved for at least 30 minutes at 121°C. Twenty litre volumes were autoclaved for 2-3 hours. Anaerobic medium was autoclaved in full bottles, with their lids loosened slightly. The lids were tightened while the medium was still hot after autoclaving. This practice was sufficient to maintain the reduced state of the redox indicator, provided a reductant was present, and no subsequent addition of reductant prior to inoculation was necessary.

Aerobic medium (10ml) was autoclaved in tightly capped Universal bottles, leaving a 20ml gas head-space.

2.1.1 Preparation of 600 litres of sterile anaerobe medium

Some modifications to the above methods were necessary to allow preparation and sterilization of growth medium on a 600-litre scale.

A concentrate of all the inorganic salts of the basal medium, including 400g of Sigmacell 50, and another containing the trace elements were autoclaved separately before being added to the presterilized 600 litre fermentor. The 600 litres of mains supply tap-water and the potentially heat-labile components (the peptones, yeast extract and vitamins concentrate) were filter-sterilized before pumping into the fermentor.

The final levels of trypticase peptone, yeast extract and Sigmacell 50 were 0.3%, 0.08% and 0.07% respectively. No reductant was included.

The complete medium was brought to the boil for 30 minutes to reduce the dissolved oxygen content, while the head-space was flushed with filter-sterilized oxygen-free nitrogen. The medium was then cooled to 70°C and inoculated immediately.

2.1.2 Preparation of solid anaerobe medium

To produce solid anaerobe medium, agar (Oxoid No.1) was added to the basal anaerobe medium ($15g.l^{-1}$) and mixture was boiled, with constant stirring, until a clear solution resulted. If cellulose agar was required, 30ml Universal bottles were primed with a 3ml slurry of basal medium containing 5% MN-300 (amorphous cellulose) which had been blended in a Waring blender to remove lumps. The basal medium-agar solution was then added to fill the bottles, producing a final cellulose concentration of 0.5%. Yeast extract (at 0.3%) was included in the basal medium of all solid media.

To produce cellobiose agar, cellobiose ($2g.l^{-1}$) was dissolved in the molten basal medium-agar solution and 30ml Universals were then filled.

2.2 METHODS OF ISOLATING CELLULOLYTIC ANAEROBES

Universal bottles (30ml) containing ca.27ml of reduced, molten, basal medium-agar plus 0.5%(w/v) MN300 cellulose (see Section 2.1.2) were preheated to 80°C and thoroughly shaken to break up any lumps of cellulose before removal into an aquarium which was being flushed with CO_2 in the laminar flow cabinet. The CO_2 bath was shown to extinguish a flame lowered into it before the lids were removed from all the

Universals which were to form the dilution series. A 3ml inoculum was added by hand-held pipetter to the first bottle. This would normally fill the bottle completely, occupying the space due to losses incurred during autoclaving of a full bottle. The contents were rapidly mixed by pumping the pipetter several times while the tip was immersed and then a 3ml representative sample was transferred to the next Universal in the series. The procedure was repeated to produce a dilution series of 10 10-fold steps. Ideally a fresh sterile pipetter tip should have been used for each transfer to ensure that a truly representative sample was transferred, but to minimize risks of oxygenation and setting of the medium, a single tip was normally used so that the whole series was produced as rapidly as possible.

Once tightly capped, the Universals were given two quick inversions (to resuspend the MN300 cellulose without too much aeration of the medium by any gas bubbles caught under the lid) and placed in a cold water bath to set rapidly. The cellulose particles were thus trapped in suspension throughout the agar.

After incubation at 70°C for several days, the dilution series was examined for the growth of colonies and clearing of surrounding MN300 cellulose (Fig. 2.1).

A Universal from the dilution series which contained fewer than 10 colonies (typically about 2mm in diameter, with a cleared zone extending several mm beyond the colony borders) would be selected for colony removal. The lid was removed while the bottle was standing in a bath of CO_2 in the laminar flow cabinet, and the water film (usually about 0.5ml) was sucked off the agar surface with a sterile pasteur pipette. A sterile No.2 cork-borer was pushed down into the agar so as to enclose a particular colony, and the plug containing the colony was removed by sealing the end of the borer with a finger and pulling it upwards with a slight twist. A sterile glass rod was then used to push the agar plug back out of the borer to the point where the colony had just emerged. A 3-4mm section of the plug containing the colony was cut with a sterile scalpel and dropped into a Universal containing liquid cellobiose medium. The colony was then fragmented while submerged in the liquid medium by repeated stabbings of the agar disc with a sterile nichrome wire, and the Universal was then sealed. The whole of the above operation was performed under a CO_2 "blanket" in the bottom of an aquarium. Each colony dissected from the agar was placed in a separate Universal of liquid medium.

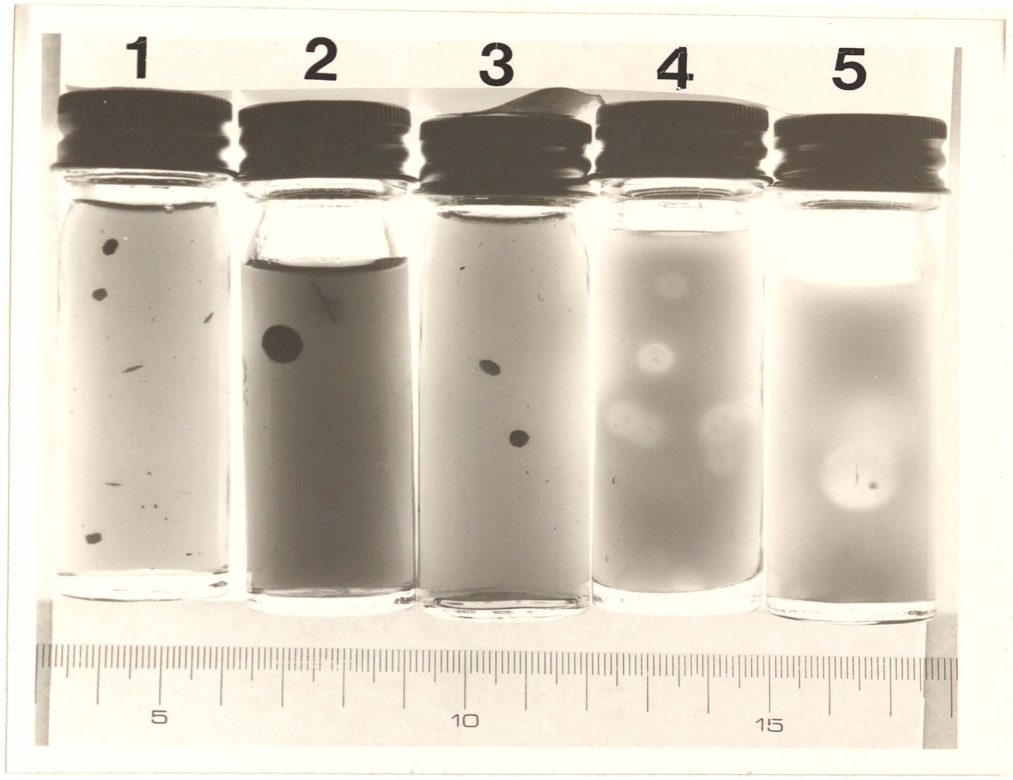


Figure 2.1 Colonies of cellulolytic, thermophilic anaerobes growing in solid agar.

Universal bottles 1-3 contained cellobiose agar.
Universal bottles 4-5 contained MN300 cellulose agar.
Cellulolytic activity produced clearing of the cellulose surrounding the colonies.
These cultures had been incubated at 75°C for three weeks and then stored at 4°C for several months before photographing.

At the first sign of turbidity in the liquid medium (normally after 24 hours of incubation at 75°C), a sample was taken to establish a second agar dilution series, this time in clear cellobiose agar, which made it possible to see any differences in colony morphology (Fig. 2.1). When more than one type of colony was noticed, representatives of each type were excised from an appropriate dilution and cultured in liquid medium containing crystalline cellulose. If CMCase activity could be detected in the culture supernatant after 3-4 days' growth, a further cellobiose agar dilution series would be established from this culture, and the process continued until colonies of a single morphology in cellobiose agar were achieved.

2.3 PRESERVATION OF ISOLATES

Cells were collected from actively growing cultures by mild centrifugation (ca. 3000*g*) and then were resuspended in 5ml of either:

(i)

Meso-inositol	5.0g	(Quantities per 100ml)
Beef-extract	10.0g	
Peptone	10.0g	The mixture was
NaCl	1.0g	sterilized by autoclaving.

or

(ii)

Bovine serum	50ml
Maltose (15% w/v)	50ml

Mix and filter-sterilize. Store frozen until required.

The cell suspension was then added to sterilized vials in quantities just sufficient to saturate a prepositioned strip of sterile blotting paper, which also served as the label. The vial was plugged with cotton wool and attached to a freeze-drier before being finally sealed while still under vacuum. To reconstitute the organism, the paper strip was removed from the vial and added to the normal growth medium, which was incubated at 75°C. First indication of growth was given by accumulating bubbles of gas within the layers of the paper. As growth of cellulolytic isolates progressed, the paper disintegrated (Fig. 2.2).

2.4 ASSESSMENT OF CELL GROWTH

Measurement of cell growth in the presence of fine insoluble cellulose crystals is difficult. Turbidimetric measurement or Coulter counting of cells in the unshaken culture medium is complicated by the possibility that cellulolytic action might create very fine cellulose particles which could move into permanent suspension, and by the binding of cells to cellulose.

Application of other common growth-estimation techniques also proved difficult. ATP estimation by bioluminescence was interferred with by some medium component, possibly the cellulose (95% loss of added ATP). A solution to this problem appears to have been found recently (Cochet *et al.*, 1984).

Methods based on estimation of protein or nucleic acid content were inappropriate since our growth medium generally contained yeast extract and trypticase peptone. In addition, solubilization of cellulose-bound protein would have been necessary in order to provide an accurate

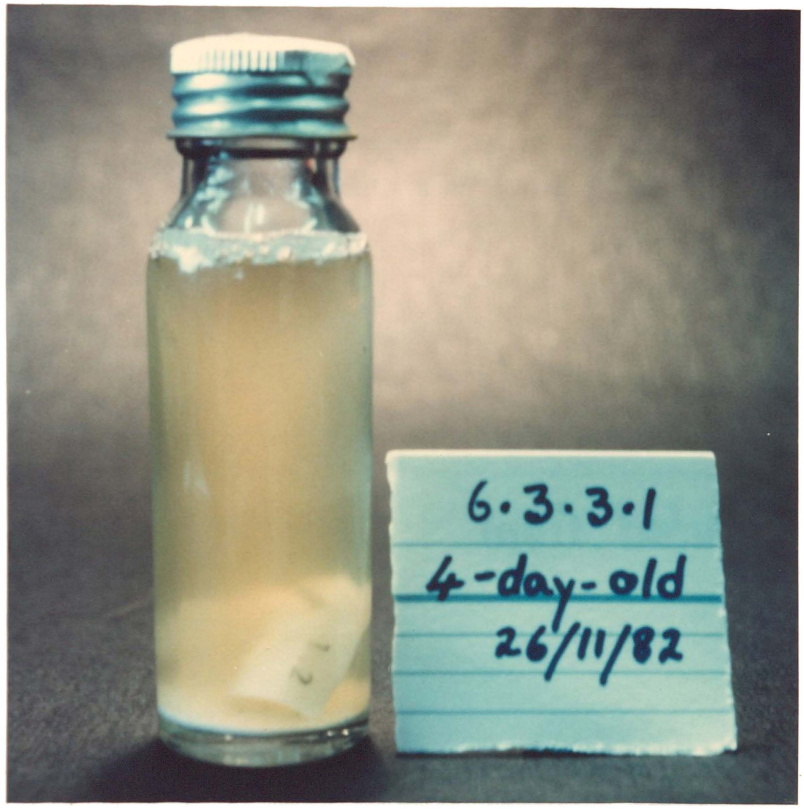


Figure 2.2 Reconstitution of freeze-dried bacteria

The paper strip bearing the freeze-dried cells was placed in a McCartney bottle containing the normal growth medium and incubated (without shaking) at 75°C. Turbidity, gas production and breakdown of the paper strip heralded the successful reconstitution of cellulolytic bacteria.

estimation of culture growth.

Since we required a simple, rapid and non-destructive means of assessing growth in our cellulose-containing cultures, we settled for a visual estimation of turbidity above the unshaken cellulose layer. The scoring system eventually evolved into a scale of 0 to 10, with an approximate doubling in turbidity between ranks. Step 5 was equivalent to 0.2 to 0.3 absorbance units at 600nm. The presence of a floc of cells would raise the growth score by 1 or 2 units.

2.5 CELLS AND SPORE STAINING

Gram staining was performed on heat-fixed cell smears using the method of Preston and Morrell (1962), modified by increasing the concentration of carbolfuchsin to 0.1% (w/v).

The spore-stain of Fleming (Gurr, 1957) was used in attempts to detect spore production.

2.6 GAS CHROMATOGRAPHY OF VOLATILE END-PRODUCTS

Volatile end-products were assayed by gas-liquid chromatography, using a Pye-Unicam GCD chromatograph equipped with a flame-ionization detector and connected to a 3390A Hewlett Packard integrator.

0.002ml samples of cell-free culture supernatants were injected into a glass column (1.8m x 4mm internal diameter) packed with Chromosorb 101, 100-120 mesh. The oven temperature was 200°C for rapid semiquantitative analysis and 160°C for slower but more precise quantitative analysis, in which propionic acid was used as an internal standard. The flow rate of the carrier gas (nitrogen) was 40ml/min. Acetate peak areas were multiplied by a factor of 1.7 to compensate for the lower sensitivity of the detector to acetate than to ethanol. Other volatile products with longer column retention times were not identified but in almost all cases they were minor products relative to ethanol and acetate.

2.7 PROTEIN ESTIMATION

A modification of the Lowry method (Peterson, 1977) was employed in most protein estimations, using bovine serum albumin (BSA) to produce a calibration curve. Where a degree of buffer interference was expected on the basis of the reviews of interfering substances (Peterson 1979, 1983), the buffer was included with the various BSA concentrations in preparing the calibration curve. A straight line relationship between the log A_{750nm} and the log [BSA] was still obtained.

Protein elution profiles in column chromatography were monitored by measuring absorbance at 280nm using an ISCO model UA-5 monitor.

2.8 MOLECULAR WEIGHT DETERMINATION

Molecular weights were determined on the basis of relative mobilities during gel filtration in Ultrogel AcA44 or after SDS-PAGE. The Ultrogel column and the two types of SDS-PAGE gel used had been previously calibrated using proteins of known molecular weights (Figs 2.3 - 2.5).

Figure 2.3 Elution volumes of molecular weight standard proteins from the Ultrogel AcA44 column.

Proteins applied were myoglobin (17,200), chymotrypsinogen (25,600), ovalbumin (45,000) and bovine serum albumin (66,000).

Column dimensions: 85cm x 2.6cm.

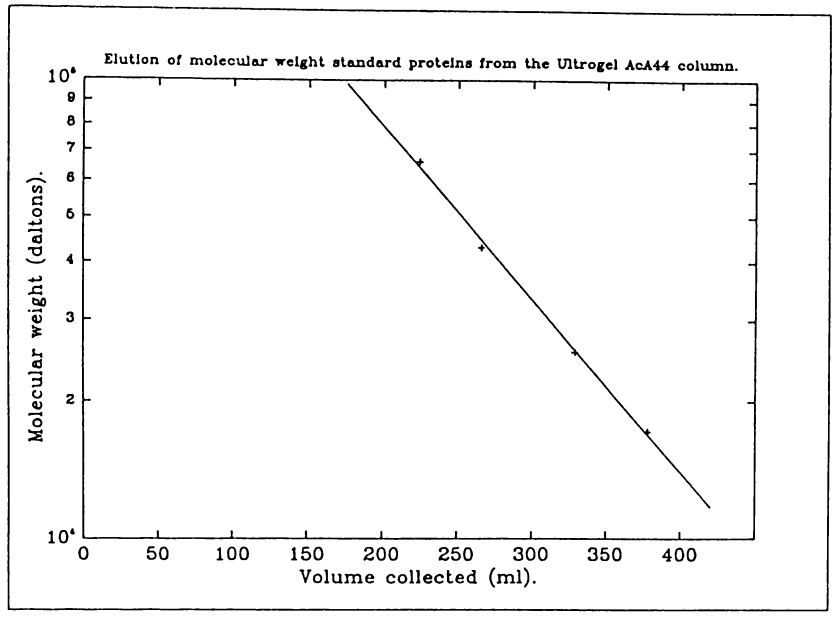
Flow rate: $33ml.h^{-1}$

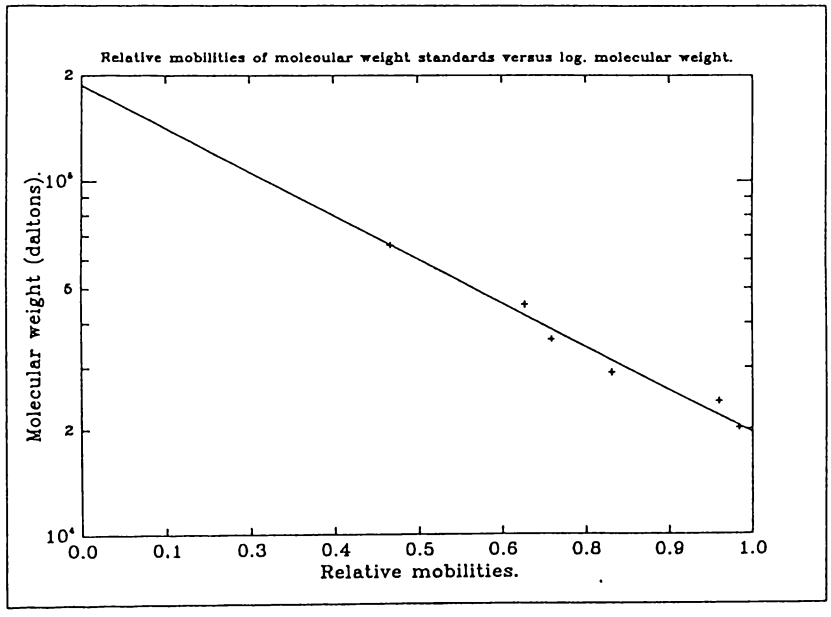
Figure 2.4 Relative mobilities versus log. molecular weight of standard marker proteins after SDS-PAGE using a 7.5%(w/v) acrylamide separating gel.

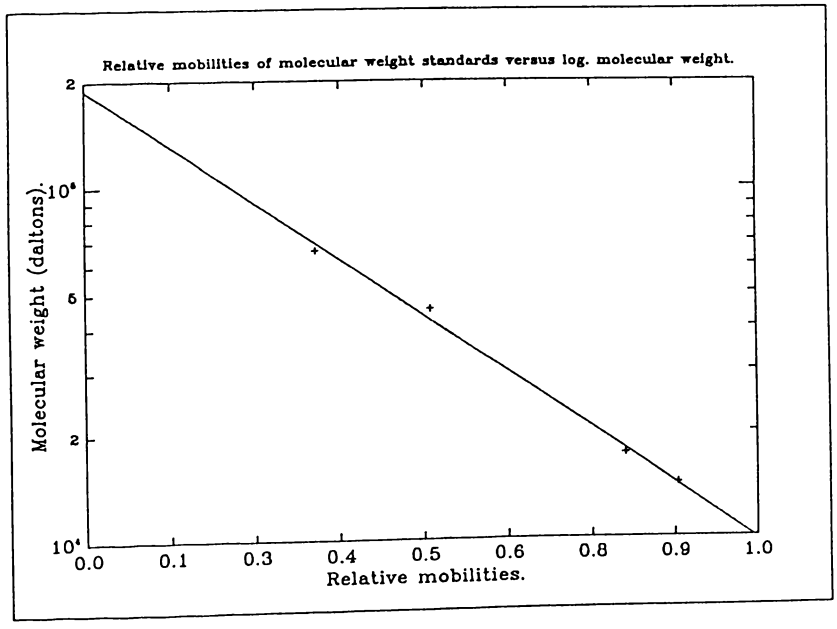
Proteins applied = trypsin inhibitor (20,100), trypsinogen (24,000), carbonic anhydrase (29,000), glyceraldehyde-3-phosphate (36,000), ovalbumin (45,000) and bovine serum albumin (66,000).

Figure 2.5 Relative mobilities versus log. molecular weight of standard marker proteins after SDS-PAGE using a 10%(w/v) acrylamide separating gel.

Proteins applied = lysozyme (14300), myoglobin (17200), ovalbumin (45,000) and bovine serum albumin (66000).


Elution of molecular weight standard proteins from the Ultrogel AcA44 column.
Molecular weight (daltons).
10⁶
9
8
7
6
5
4
3
2
10⁵
0
50
100
150
200
250
300
350
400
Volume collected (ml).


Relative mobilities of molecular weight standards versus log. molecular weight.
Molecular weight (daltons).
2
10⁶
5
2
10⁵
0.0
0.1
0.3
0.4
0.5
0.6
0.8
0.9
1.0
Relative mobilities.


Relative mobilities of molecular weight standards versus log. molecular weight.
Molecular weight (daltons).
2
10⁶
5
2
10⁵
0.0
0.1
0.3
0.4
0.5
0.6
0.8
0.9
1.0
Relative mobilities.

2.9 ELECTROPHORESIS

2.9.1 Equipment

In early work a home-made tube gel apparatus was used, capable of running 8 gels (80mm x 3mm diameter) simultaneously at up to 4mA/gel. The majority of the work however was done using an LKB 2001 Vertical Electrophoresis Unit, using slab gels 1.5mm or 0.8mm thick, cast between two glass plates measuring 16cm x 18cm.

2.9.2 Compositions of gels and buffers

Gel and buffer compositions were in accord with the published methods noted in the text, with the exception of the system used for non-denaturing electrophoresis of the ß-glucosidases, which employed an unpublished discontinuous system devised by a colleague, Mark Patchett. Details of this method are:

Top Electrode Buffer (Cathode)	33mM morpholinopropane-sulfonic acid 12.3mM histidine Adjust pH to 6.41 if necessary by further addition of one of the above buffers.
Bottom Electrode Buffer (Anode)	5mM histidine Adjust pH to 6.32 with HCl.
Stacking Gel Buffer Stock	140mM histidine pH to 5.5 with HCl
Resolving Gel Buffer Stock	140mM triethanolamine pH to 7.07 with HCl
Stacking Gel Acrylamide Stock	5% (w/v) acrylamide 1.26% (w/v) methylene-bis-acrylamide
Resolving Gel Acrylamide Stock	16% (w/v) acrylamide 0.32% (w/v) methylene-bis-acrylamide.

Resolving Gel Preparation

Mix:

7ml resolving gel buffer stock
7ml resolving gel acrylamide stock

Add:

10µl TEMED
0.5ml of fresh 1.3% (w/v) ammonium persulphate
10mg p-nitrophenol-ß-D-glucopyranoside
Setting was complete in 20 minutes if overlaid with water.

Stacking Gel Preparation

Mix:

1.5ml stacking gel buffer stock
2ml stacking gel acrylamide stock
Degas thoroughly

Add:

10µl TEMED
0.5ml saturated riboflavin freshly prepared in stacking gel buffer stock.
Allow to set in direct sunlight.

Sample Buffer

Mix: 1.2ml stacking gel buffer stock
0.8ml glycerol
80µl 0.1% (w/v) bromophenol blue

2.9.3 Sample preparation

For the above non-denaturing PAGE system of Patchett, 3 parts of sample were mixed with 1 part of sample buffer.

For the non-denaturing PAGE system identical to that of Laemmli (1970) but without SDS, 9 parts of sample were mixed with 1 part of saturated sucrose.

For the SDS-PAGE System of Laemmli (1970), samples were heated to 100°C for 10 minutes in the presence of mercaptoethanol (5% v/v), SDS (2% w/v) and bromophenol blue (0.002% w/v) in sealed

vials.

2.9.4 Running Conditions For the above system of Patchett; an initial 200V was maintained for the first 15 minutes, and then increased to 270V, for the rest of the run. This produced a current of 40mA/gel, falling to 25mA/gel by the end of the run.

All other types of gel were run at a constant current of 40mA per gel, with cooling by way of a flow of tap water through the central cooling tubes of the apparatus, assisted by constant magnetic stirring of the lower electrode buffer.

2.10 ISOELECTRIC FOCUSSING (IEF)

2.10.1 Equipment

A Pharmacia Flat Bed apparatus FBE-3000 was used, with the cooling plate raised by 2mm with glass spacers to allow adequate electrode pressure to be exerted on ultrathin gels. The cooling plate was chilled in our initial work by circulating iced water, and in later work a controlled temperature (10° or 15°C) was maintained by a Grant FH15 temperature controller.

An ISCO Model 494 power supply was used. Running conditions and times were based around manufacturers' recommendations supplied with the pre-made gels used.

2.10.2 Gel Compositions

Broad-range I.E.F. was performed initially on 1mm-thick LKB PAG premade plates, with a total acrylamide content of 5%, (3% of which was methylene-bis-acrylamide) and containing 2.4% (w/v) Ampholine, pH range 3.5-9.5. For later I.E.F. we used ultra-thin Servalyte Precoats No. 42965, pH3-10, from Serva. These gels were easy to handle and rapid to stain due to their permanently bonded plastic backing and ultra-thin gel layer.

I.E.F. over narrow pH ranges was performed on polyacrylamide gels which we made ourselves. Normally 6 ml of gel mixture was sufficient for one ultrathin gel (0.03 x 10 x 18cm), and was produced as follows:

Mix: 1ml stock acrylamide (30% (w/v) acrylamide, 0.08% (w/v) methylene-bis-acrylamide) (Both BDH electrophoresis grade).
0.3ml ampholine mixture (40% w/v)
0.6ml glycerol
3ml water (or saturated urea solution)
Degas thoroughly

Add: 1.1ml saturated riboflavin solution (degassed separately) and

0.01ml TEMED

Ampholines used were LKB pH4-6, LKB pH5-7, LKB pH3-5, Servalyte analytical grade 4.5-5.0. The LKB Ampholines were on one occasion mixed to produce a range covering pH3-7 with the 4-6 range expanded by including 0.1ml pH3-5, 0.2ml pH4-6 and 0.1ml pH5-7 in the above mixture. This produced a gel containing 2.6% (w/v) ampholine, whereas in all other cases a final ampholine concentration of 2% (w/v) was obtained. In cases in which the required pH range (i.e. 4.5-5) did not extend to pH7, ampholines of the pH range 4-9 were included so as to constitute 20% of the total ampholine content, as recommended by Serva.

2.10.3 Moulding, pouring and setting of ultrathin polyacrylamide gels

A gel mould was produced by sticking two 1cm wide strips of Leucoplast to a glass plate so that they lay parallel with the longest edges and were separated by 9.5cm. A second glass plate was covered with Gel-Fix plastic (Serva), using a water film to provide adherence, with at least 1cm of the Gel-Fix left protruding beyond one end of the glass. The first plate was placed on top of the Gel-Fix, with the Leucoplast strips acting as spacers between the top glass and the Gel-Fix. The mould was then fastened with bulldog clips along its sides, set horizontally with the Gel-Fix side downwards,and the degassed gel mixture was applied gradually from a pipette onto the protruding Gel-Fix at the point where it entered the glass plate sandwich. If applied centrally and not too rapidly, the gel would flow by capillary action to completely fill the mould, without any need to tilt the mould. Air bubbles, which on occasions became trapped by the advancing gel, were generally attributable to poorly cleaned plates. If any air bubbles were trapped within 5cm or so of the ends, they could be pulled out with a hook shape cut from a celluloid sheet.

The gel was then placed in direct sunlight for several hours to set, and then the whole mould was transferred to 4°C for several hours at least. This cooling appeared to facilitate subsequent clean separation of the gel from the top glass.

Removal of the glass plates was best done immediately after removal from the cold room, with the bottom plate being simply wedged off to break the water film adhering it to the underside of the Gel-Fix. Separation of the top glass plate from the gel

surface was then initiated by inserting a scalpel blade at one end and the glass was lifted off the gel whilst the plastic backing was held taut with the other hand.

The gel surface was covered with a non-stick plastic sheet (Gel-Cover, FMC), and the resulting plastic sandwich was stored in a humidified box at 4°C until required.

2.10.4 Preparative IEF The same ultra-thin polyacrylamide IEF gels as used for analytical IEF were used in a preparative role. A volume 0.4-0.6 ml of a mixture of concentrated sample and ampholines (final concentration 2% w/v and covering the pH range used in the gel) was applied to a glass fibre wick (8.5cm x 0.5cm x 0.2cm) positioned across the gel at about 2cm from the cathode. Running conditions and electrode buffers used were varied and are thus described for the individual runs in the captions which accompany the activity zymograms produced from each gel. The degree of focussing which had been achieved was conveniently monitored by making an activity zymogram (see Section 3.5.4). If this revealed incomplete focussing, the run was continued until sharp zymogram bands were achieved. This replica gel was then used as a template to allow separate excision of the cellulolytic bands from the IEF gel (see Section 3.5.4.4).

2.11 STAINING OF PROTEINS ON POLYACRYLAMIDE GELS

2.11.1 Coomassie Blue staining

Fixing and staining were performed simultaneously in a solution containing 50% (v/v) methanol, 10% (v/v) acetic acid and 0.27% (w/v) Coomassie Brilliant Blue R-250 (Sigma). Gels were soaked in this solution overnight before destaining in several changes of a reagent containing 5% (v/v) methanol and 7.5% (v/v) acetic acid.

Gels were photographed using transmitted light from a light-box.

2.11.2 Silver Staining

Slab gels were silver stained using the photochemical method of Merril *et al.* (1981 and 1982). The volumes of each reagent required and exposure times most appropriate for the various gel thicknesses were taken from Bio-Rad Bulletin 1089 (1982). During each step, the gel and bathing solution were shaken gently on the trolley of an empty shaking water-bath.

Several different types of plastic container were tried, but

none gave results which were as satisfactory as those obtained using a glass container. The silver tended to adhere to the plastic, resulting in lower sensitivity of staining and surface smudging on the gel.

The importance of exposure to bright light during the silver nitrate step (Merril *et al.*, 1981) or during the subsequent development (Merril *et al.*, 1982) has been disputed by Bio-Rad researchers who have studied and adapted the technique (personal communication). In any case, we continued to illuminate the gels with a fluorescent lamp positioned about 10cm above them, but performed no experiments to test whether or not this resulted in improved sensitivity.

We chose the above method from the numerous silver stain techniques published over the last five years because it is the most rapid, uses minimal amounts of silver nitrate and can readily be completely destained and restained, normally with improved sensitivity resulting, if the background is allowed to become too dark. In addition, this particular method has been well studied by Bio-Rad, who have commercialised it, and their Bulletin 1089 provided more practical information and trouble shooting hints than were available for any other silver staining method. Further, at no stage are the reagents potentially explosive.

The destaining technique of Switzer *et al.* (1979) was employed with success in completely destaining overstained gels, but was found to be very difficult to control if mere destaining of the background was required. Modifications made to this destaining procedure (Marshall, 1984) are reported to eliminate this problem.

Photography of gels with the darker backgrounds was most successful using transmitted light from a light-box, whereas the lighter stained gels photographed best using reflected light from a white background.

Densitometer traces were produced using a Helena Quick-Scan 2020 Densitometer.

The silver-stained gels were perfectly stable for at least 18 months if kept moist in sealed plastic bags in the dark.

2.12 SUNDRY EQUIPMENT

Centrifugation on a small scale employed the Runne Model RS85-1 microfuge with a fixed horizontal rotor accommodating 1.5ml Eppendorf tubes at a maximum of 7,000*g*. For larger volumes an MSE bench centrifuge with a swing-out rotor or Sorval models SS3, RC2-Band RC-5B were used. To separate cells or precipitated proteins from volumes of 20-600 litres, continuous flow industrial centrifuges (Sharples Types A-D1707C 1.0 and MV-12-16Y-1JY) were used.

Absorbance measurements were performed using a Beckman Model 24 double beam spectrophotometer, apart from U.V. monitoring of column effluents with an ISCO Type 6 optical unit.

Ultrafiltration employed a variety of stirred cells (Amicon and Millipore) using Millipore PT Series polysulfone membranes with a molecular weight cut-off of 10,000. For volumes of 20 litres or more we used a Millipore Pellicon High Volume recirculating cell system incorporating Pellicon ultrafilter cassettes which produced a tangential flow across a polysulfone membrane with a 10,000 MW. cut-off.

CHAPTER THREE

CELLULASE ASSAY METHODS

All cellulolytic enzymes attack ß-1,4-glucosidic linkages, and differ only in specificity regarding the structures surrounding the linkage. These differences in specificity are not absolute but are merely relative differences in effectiveness in hydrolysing these bonds in the various "niches" of the cellulose structure.

Different initial hydrolysis products also distinguish some of the types of cellulolytic enzymes and may be used as identifying features provided these often transient intermediates are ever present in detectable amounts.

There are two basic categories of cellulase assay, reflecting the uses to which they are put. Firstly, there is measurement of the activities of individual component enzymes of the complex. This is normally required for biochemical studies and is accomplished by one or more of the following techniques:

(i) Use of a specific substrate, if one exists.

(ii) Measurement of the formation of a hydrolysis product peculiar to a particular component.

(iii) Selective inhibition of other components in the complex.

(iv) Addition of several measured amounts of an interfering type of activity (in purified form) so as to allow calculation of the original level of this type of activity by backward extrapolation.

(v) Immunological methods in which specific antisera, prepared using previously purified cellulase components as antigens, are used for subsequent specific detection of these components in enzyme mixtures. For examples of the application of specific immunological detection methods see Fagerstam and Pettersson, (1979); Nummi *et al.*, (1980), Bayer *et al.*, (1983).

(vi) Purification of the component under study, thus allowing the use of non-specific substrates for quantitative assays.

The second basic category of cellulase assays entails measurement of the cellulose solubilizing capacity of the complex as a whole and is used mostly in microbiological studies aimed at producing fermentable sugars from cellulose. The substrates commonly chosen, (e.g. filter paper, powdered crystalline cellulose) are in fact only hydrolysed to a significant extent when a "complete" cellulase complex is used. Synergisms between component enzymes of the complex can result in the "solubilizing activity" measured being greatly influenced by the proportions in which the various enzymes are present.

These two categories of assays will now be discussed in greater depth.

3.1 ASSAY OF INDIVIDUAL ENZYMES OF THE CELLULASE COMPLEX

3.1.1 Endoglucanase assays

Five basic types of endoglucanase assay method are available. These are:

(i) Measurement of changes in viscosity of soluble substituted celluloses, e.g. carboxymethylcellulose (CMC).

(ii) Detection of chain cleavage in agar/CMC plates.

(iii) Reducing sugar measurement after incubation with CMC.

(iv) Measurement of chromophores or fluorescent groups released from modified CMC.

(v) Quantitative measurement of changes in the concentration of soluble substrates and hydrolysis products by HPLC.

3.1.1.1 The viscometric method. Methods for the viscometric determination of endoglucanase activity in absolute units (recommended by the International Union of Biochemistry (1973) as being the activity which leads to the conversion of 1µmole of substrate in one minute under given conditions) have been developed by Almin and Eriksson(1967, 1968), Almin *et al.* (1967, 1975) and Manning (1981). These methods require fairly complex mathematical treatment of extended sections of the curve of the change in viscosity with time.

A simplification based on determination of the initial rate of enzymatic hydrolysis of CMC appears to offer some advantages apart from relative mathematical simplicity (Klesov *et al.*, 1981a). They claim that assumptions generally introduced in converting viscometric changes in solutions to µmoles of glucosidic bonds cleaved are most exactly satisfied during the initial period of the reaction. In addition, the

influence of any exocellobiohydrolases present will be least significant during the initial period of decrease in substrate viscosity.

Comparisons have been made between the viscometric method and measurement of reducing sugars hydrolysed from CMC, since both methods are sometimes regarded as endoglucanase assays. Klesov *et al.* (1981a) performed this comparison on fourteen different cellulase preparations and concluded that the methods were in reasonable agreement when both were expressed in terms of μmoles glycosidic bonds cleaved per minute per gram of enzyme. In contrast, Canevascini and Gattlen, (1981) found that viscometric measurements indicated fewer glucosidic bonds broken per unit time than did the measurement of increased reducing power. They attribute this discrepancy to the predominant influence of exoglucanase components in the latter method.

Ionic-substituted celluloses, such as the commonly used CMC, are less than ideal substrates when used in viscometric assays, since their viscosity is dependent upon pH, ionic strength and polyvalent cations. Non-ionic substituted celluloses such as hydroxyethylcellulose (HEC) don't exhibit such limiting features. Child *et al.* (1972) have published a viscometric method for endoglucanase determination using hydroxyethylcellulose as the substrate, but the method has not been generally taken up, probably because the substrate is not widely produced commercially.

Measurement of endoglucanase in large numbers of samples in quick succession is impractical by viscometry unless numerous cheap and quick-to-operate viscometers are available. The effects of temperature on CMC viscosity are very significant. Heat-induced viscosity reduction is not completely reversed by a return to room temperature, so obviously considerable care must be exercised in preparation of the substrate to obtain a reproducible product, especially if heating is used to speed dissolving.

3.1.1.2 The CMC-plate-clearing assay Solid media containing soluble sodium salts of CMC were developed by Hankin and Anagnostakis (1977) as a means of detecting endoglucanase activity around colonies of micro-organisms. Undegraded CMC is visualised by precipitation with aqueous hexadecyltrimethyl ammonium bromide (Hankin *et al.*, 1971), so degraded zones, where chain lengths are significantly shortened, appear almost immediately as circular clearances surrounded by an opaque white precipitate.

We modified this method by excluding nutrients, incorporating a buffer and increasing the agar content to 1.5% (thereby improving water-holding capacity at 75°C). Further details are given in Section 3.5.1.1. Enzyme concentration was found to relate by a logarithmic relationship to cleared diameter. The outline of cleared zones would become diffuse within ten minutes of removing the precipitating reagent, so the plates could not be kept as a permanent record.

An improved method of detecting CMC hydrolysis on CMC-agar plates (Wood, 1981, Teather and Wood, 1982) utilizes an observation by Wood (1980) that Congo Red will complex with polysaccharides containing 5 or more contiguous (1-4)-ß-linked D-glucopyranosyl units. Complex formation with Congo Red was also shown with (1-3),(1-4)-ß-D-glucan and hemicellulosic galactoglucomannans, but most polysaccharides showed little or no interaction (Wood, 1980a,b, Wood and Fulcher, 1978, 1983, Wood *et al.*, 1983).

Decrease in viscosity was clearly related to loss of interaction with Congo Red during hydrolysis of oat ß-D-glucan by ß-D-glucan endohydrolase, whereas the level of reducing sugars released was not as clearly related to the viscosity decrease (Wood, 1981). Therefore the Congo Red plate assay method should be a more reliable measure of endoglucanase activity than is measurement of reducing sugars released from CMC. However, it is possible that exo-acting enzymes might eventually produce chain lengths of less than 5 units and be mistaken for endoglucanases by this assay.

The mechanism of complex formation between Congo Red (an anionic dye) and the above categories of neutral or anionic polysaccharides is not yet certain. Clearly the driving force

is not electrostatic. Wood (1982) suggested that binding results from partition of dye into regions of decreased polarity (such as the hydrophobic interior of the polysaccharide helix). He found, in support of this theory, that the presence of salt favoured complex formation (as evidenced by a shift in the Congo Red absorbance maximum) whereas 20% (w/v) sucrose and 7M urea inhibited binding. With polyanions, such as CMC, the presence of salt was vital for the wavelength shift in Congo Red. Presumably the salt screens the electrostatic repulsions between the anionic dye and the negative sites on the polysaccharide. A secondary precipitation reaction may occur, depending on the concentrations of dye, polysaccharide and salt (Wood, 1982).

The dye-binding technique is an extremely sensitive means of detecting certain polysaccharides. Complex formation by Congo Red with as little as 0.5μg.ml^{-1} oat ß-D-glucan produced a detectable bathochromic (red) shift in the Congo Red absorption spectrum, and this shift reached a maximum of 30-35nm between 6 and 10μg.ml^{-1}. Higher glucan concentrations produced an approximately constant shift of 25nm (Wood *et al.*, 1983). An increase (12-13 fold) in fluorescence intensity of Congo Red was also noted.

The plate diffusion assay utilising Congo Red to visualise areas of enzymatic degradation of various polysaccharides was proposed by Wood (1981) as being preferable to any attempt to relate wavelength shift or fluorescence intensity to loss of substrate. The latter techniques, although extremely sensitive, presented the problems of narrow linear ranges of interaction with polysaccharides, a dependence on salt concentration and pH, and a tendency towards precipitation. The plate assay method however has been found to be well suited to monitoring large numbers of samples, and, because of the logarithmic relationship between enzyme concentration and clearing diameter, virtually any enzyme concentration will give a measurable result. However, relatively small enzyme concentration differences (e.g. less than 2-fold) may not be apparent.

Contrast of clearance zones against the background is

much better by the use of Congo Red than by precipitation with hexadecyltrimethylammonium bromide, and can be improved still further by acidification, which turns the background blue. The low pH is sufficient also to prevent any further enzyme action, and plates can be kept for several weeks before photographing. The intense colour of the Congo Red - polysaccharide complex confers further advantages by allowing the use of very low substrate concentrations and a corresponding decrease in the time required to detect lower levels of activity. Refer to Section 3.5.1.1 for specific details of the CMC-plate-clearing assay which we used.

3.1.1.3 Measurement of reducing sugars from hydrolysis of CMC

This is the endoglucanase assay from which activity is most easily expressed in terms of µmoles of glucosidic bonds cleaved. However the method is fraught with a number of pitfalls for the unwary, which are well described by Lindner *et al.*, (1983). In essence, the basic problems are:

(a) The assay may not be specific for endoglucanases, since exo-ß-(1,4)-glucanases and exo-ß-(1,4)-glucosidases have been shown to hydrolyse CMC to some extent (Klesov *et al.*, 1981a). Therefore the frequently used term "CMCase", i.e. the ability to produce reducing sugars from CMC, should not be considered synonymous with "endoglucanase".

(b) Product estimation by most colorimetric reducing sugar assays is non-stoichiometric, since the aldehyde group reacts differently depending on the residue to which it is attached (Aminoff *et al.*, 1970). Therefore, a strictly proportionate increase in product will only be measured where the composition of the product remains invariant. The array of products will almost certainly undergo changes during the reactions of endoglucanases on CMC, and a non-stoichiometric assay system is therefore unreliable.

(c) The "CMCase" progress curve rapidly becomes non-linear, due largely to substituents making some glucosidic bonds less susceptible to hydrolysis than others.

(d) Measurement of endoglucanase activity in a crude cellulase complex presents special additional problems. Most cellulolytic organisms produce a "family" of CMCases, physiochemically different enzymes which may differ in many

ways, including their modes of attacking CMC, their stabilities under various incubation conditions and in their susceptibilities to end-product inhibition. The substrate CMC is also heterogenous with respect to the accessibility of its glucosidic linkages. It is not surprising therefore that enzyme dilution curves and time course curves employing crude CMCase preparations are frequently non-linear (Miller *et al.*, 1960a, Galas *et al.* 1981, Lindner *et al.*, 1983).

Comparisons of CMCase activities from different sources are normally based on the unsound assumption that respective enzyme dilution curves are linear. Similarly, calculation of recovery, specific activity and purification data during the isolation of a single CMCase generally involves an assumption that measured activity is due to a single enzyme species, and that, consequently, the reaction velocity is linearly related to enzyme concentration. This assumption is clearly invalid for systems containing multiple CMCases. The manifestation of synergistic effects amongst CMCases (Kanda, 1976b, 1979) further complicates the problem.

The validity of much of the endoglucanase purification data published to date must be questioned, since in most cases no explicit evidence has been offered to demonstrate that any of the above complicating factors have been dealt with. Similar problems apply to exoglucanase studies, but to an even greater degree, and hence they have been more generally recognised.

Linear CMCase dilution curves can be obtained, according to Lindner *et al.*, (1983), provided they are constructed using true initial velocities, measurement of which generally requires (i) dilute enzyme preparations, (ii) a substrate of low D.S. (e.g. 0.4) to minimise cryptic substrate inhibition, (iii) short incubation periods (e.g. 2-4 minutes), (iv) a sensitive reducing sugar detecting reagent (e.g. PABAH or Nelson-Somogyi - see Table 3.1)

The dinitrosalicylic acid-based method (Miller, 1960) is a widely used reducing sugar assay with the merits of simplicity (a single reagent addition) and stability of reagent and products. It doesn't however exhibit a definite stoichiometry of reaction with various reducing sugars

Table 3.1 Sugar Assay Systems used to Determine Solubilisation of CMC and Cellulose

Assay System	Relative Sensitivity+	Reactive towards:	Further Details
Dinitrosalicylic Acid (DNSA)	1	Reducing Sugars	Miller (1959, 1960) and Section
Para-hydroxybenzoic Acid hydrazide (PABAH)	12	Reducing Sugars	Lever, (1972, 1973) and Section
PABAH- (Fluorometric)	120	Reducing Sugars	Lever (1973)
Copper - arsenomolybdate	2	Reducing Sugars	Nelson (1944) Somogyi (1952) Klesov et al. (1981a)
Ferricyanide	54	Reducing Sugars	Halliwell (1974)
Ferricyanide Simplification	12	Reducing Sugars	Halliwell and Riaz (1970)
Phenol/H_2SO_4 *	6	Total carbohydrate	Dubois et al., (1956) and Section

+ Calculated on the basis of µg glucose required to produce an absorbanceof 1.0 in 1.5ml final volume (115µg for DNSA)

* Not appropriate for use when soluble CMC is present. It is, however, useful for measuring total solubilized carbohydrate if insoluble residual cellulose is first separated out (Section 3.2.2).

produced in CMC hydrolysis. Cellobiose is partially hydrolysed (see Section 3.5.1.2), to a degree presumably dependent upon the length of exposure to 100°C. Also, the method is relatively insensitive (Table 3.1).

The Nelson-Somogyi method (Nelson, 1944 and Somogyi, 1952) doesn't produce this partial hydrolysis of cello-oligosaccharides but involves two reagent additions and a longer boiling time.

Para-hydroxybenzoic acid hydrazide (PABAH) (Lever, 1972), is a very sensitive reducing sugar assay (see Table 3.1) and sensitivity can be increased by a further factor of 10 by a fluorometric adaptation (Lever, 1973). The PABAH reagent is unstable in air and must be stored under N_2 or paraffin, or made fresh daily.

The alkaline ferricyanide method (Halliwell, 1974) is extremely sensitive, but requires addition of two reagents (one of which is highly toxic). A less sensitive simplification has been published by Halliwell and Riaz (1970) in which absorbance due to residual ferricyanide is inversely proportional to reducing sugar levels.

3.1.1.4 The measurement of chromophore and fluorescent group release from modified CMC A wide range of chemically modified forms of cellulose have been prepared which release soluble coloured or fluorescent groups when their glucosidic bonds are hydrolysed. Since substantial hydrolysis of most of these substrates requires the combined action of several or all of the components of a complete cellulase complex, use of such substrates is discussed in Section 3.2.3. However, trinitrophenyl-CMC has been used in the study of a purified endoglucanase which exhibited very little activity towards crystalline cellulose (Petre *et al.*, 1981). The addition of further substituents to CMC might be expected to further impair hydrolysis by exo-acting enzymes, and thus make TNP-CMC a selective substrate for randomly-acting endoglucanases. However, an endoglucanase has been purified which exhibits no activity towards TNP-CMC (Creuzet and Frixon, 1983), and Beguin *et al.*, (1983) purified another which had only slight TNP-CMCase activity. The latter enzyme is produced by the same organism (*Clostridium thermocellum*) as produces the very

active TNP-CMCase purified by Petre *et al.* (1981). Obviously a lack of TNP-CMCase activity is insufficient grounds on which to rule out the presence of an endoglucanase in a system.

The synthesis and use of TNP-CMC and another CMC-derivative, Fluram-cellulose, is described by Huang and Tang (1976). They obtained linear relationships between amounts of soluble TNP- or Fluram-oligosaccharide released and the amounts of cellulase present in the reaction mixture. The TNP derivative, when used at a 1% level, is claimed to allow a 25-fold improvement in sensitivity towards endoglucanase relative to measurement of reducing sugars released from normal CMC by the DNSA method, and the fluorescent product from Fluram-oligosaccharide gives a further 10-fold increase in sensitivity (Huang and Tang, 1976).

A further advantage conferred by these methods is that they allow measurement of endoglucanase activity in the presence of sugars and various other substances which would interfere with reducing sugar and viscometric assays.

3.1.1.5. The use of high performance liquid chromatography to measure substrates and products Several groups have shown that HPLC is a valuable method in the study of the activities of prepurified components of cellulase complexes (Gum and Brown, 1977a, 1977b, Shoemaker and Brown, 1978, Bissett, 1979). Transient products such as cellooligosaccharides can be detected, provided a means of stopping the hydrolysis at various stages is available, and by this method the presence of an endoglucanase in the midst of a cellulase complex may be demonstrated.

Unsubstituted cellooligosaccharides in HPLC effluents are usually measured by refractive index. Preparation of a chromophoric homologous series of 4-methylumbelliferyl glycosides of cellooligosaccharides (MeUmb ß-(Glc)n; n=2-6) has made possible a simple and sensitive photometric determination at 313nm to quantify HPLC effluent peaks (van Tilbeurgh *et al.*, 1982).

Gas chromatography and thin layer and paper chromatography have also been used in identification of cellulase end products, but have been out-dated by the advent of extremely sensitive refractive index sugar detectors for

HPLC systems.

3.1.2 Exo-ß-D-glucan cellobiohydrolase assay methods

There is no generally accepted method for measuring the activities of 1,4-ß-cellobiohydrolases, also known as exocellobiohydrolases, in cellulase complexes. By definition, an exocellobiohydrolase cleaves cellobiose from the non-reducing end of a cellulose or cellooligosaccharide molecule. In the presence of ß-glucosidases, cellobiose is a transitory product. Also, endoglucanase action has been shown to produce some cellobiose (Petre *et al.*, 1981), so measurement of cellobiose levels present during cellulose decomposition by a cellulase complex is of dubious value in assessing exocellobiohydrolase activity.

Klesov *et al.* (1981a) claim to have developed a graphical method of determining exocellobiohydrolase activity in admixture with other components of the complex. Their method, based on the addition of various known amounts of a purified ß-glucosidase and backward extrapolation to zero ß-glucosidase appears to enable allowance to be made for ß-glucosidase action in the original complex. However the contribution of endoglucanases to the cellobiose pool is ignored, a factor likely to be made especially significant by their use of CMC as the substrate.

A method for selective determination of exocellobiohydrolase activity in a mixture of cellulolytic enzymes has recently been published (Deshpande *et al.*, 1984) using the synthetic substrate p-nitrophenyl-ß-D-cellobioside. Exoglucanases, especially exocellobiohydrolases, specifically act on the agluconic bond (between p-nitrophenyl and the disaccharide moiety) and not on the holosidic bond (between the two glucose units of cellobiose). The interfering effect of ß-glucosidase, which acts on both agluconic and holosidic bonds, is overcome by the addition of D-glucono-1,5-δ-lactone, a specific inhibitor of ß-glucosidases. Interference by endoglucanases, which also act on both agluconic and holosidic bonds, can be compensated for by prior standardization of the assay procedure with purified endoglucanases from the cellulase complex under scrutiny. Having predetermined the ratio of agluconic:holosidic bond cleavage for the purified endoglucanase activity, the change in the ratio in favour of the hydrolysis of the agluconic bond when exoglucanases are also present can be used to calculate the activity of the exoglucanase

alone. This assumes that the action of the exoglucanase does not influence the ratio of products of the endoglucanase action. Further, the problem of assessing the effects of exoglucosidases is not dealt with, since they are not inhibited by glucono-lactone (Reese *et al.*, 1968).

Another major drawback with the method of Deshpande *et al.* (1984) is that purification of the endoglucanases is necessary for the standardization, so it would not be appropriate for screening of cellulolytic organisms.

Unequivocal assessment of exocellobiohydrolases has so far only been possible after their isolation and purification to homogeneity (Halliwell and Griffin, 1973; Emert *et al.*, 1974, Berghem *et al.*, 1975, Gum and Brown, 1976, Wood and McCrae, 1977, Nummi *et al.*, 1983). Once purified, exocellobiohydrolases that are confirmed as such by qualitative product analysis can be assayed quantitatively by measurement of reducing sugar production from cellulose, or by the release of p-nitrophenol and cellobiose from p-nitrophenyl-ß-D-cellobioside.

3.1.3. Exo-ß-1,4-glucosidase assay methods

The possible existence of components in cellulase complexes which cleave glucose from the non-reducing ends of ß-1,4-linked glucose polymers or oligomers has been largely ignored in most studies. This has doubtless been due to the lack of a completely unequivocal means of detecting such activity in the presence of endoglucanases and ß-glucosidase, which may also produce glucose.

Reese (1969) published a method based on the differing susceptibilities of cellotetraose and cellobiose to hydrolysis. High activity towards cellotetraose relative to cellobiose was taken as indicative of a predominance of exoglucosidase activity, since purified ß-glucosidases have been shown to exhibit higher specific activities towards cellobiose than cellotetraose (Sternberg *et al.*, 1977, Ait *et al.*, 1982, Schliemann, 1983). The endoglucanase contribution to cellotetraose hydrolysis appeared insignificant for a variety of different fungal systems tested by Reese (1969) since he found that variation of the endoglucanase levels didn't affect the rate of hydrolysis of cellotetraose. Sinitsyn and Klesov, (1981) also reached the same conclusion. However, in his 1977 review, Reese subsequently conceded that although some endoglucanases have no activity on substrates smaller

than 5 units, others can act on tetramers and trimers. The presence of such endoglucanases would invalidate his earlier described method of determining exoglucosidase activity in a complex.

An alternative method utilizes glucono-lactone, which is a potent inhibitor of ß-glucosidase but which is without effect on exo-ß-1,4 glucosidase (Reese *et al.*, 1968). Glucose liberated from cellotriose or cellotetraose is attributed then to the action of exo-ß-1,4 glucosidase. Using this method, Sinitsyn and Klesov (1981) concluded that exoglucosidases in fact play the major role in the formation of glucose during the initial stages of hydrolysis of cellulose by cellulase complexes of many different origins. Note however that the assumption of endoglucanase inactivity towards cellotriose and cellotetraose is basic to this method also, and may on occasions be invalid (Reese, 1977).

A third approach is based on study of the rate of formation of D-glucose in the presence of various concentrations of added ß-glucosidase and extrapolation of the results obtained back to a theoretical complex containing no ß-glucosidase, in which the rate of glucose formation would be thus attributable entirely to exoglucosidase action (Klesov *et al.*, 1981a). The assumption is made that endoglucanases do not produce significant levels of glucose from the chosen substrate (CMC). However, a purified endoglucanase has been shown to produce glucose from CMC (Petre *et al.*, 1981). Cellotetraose would have been a better choice of substrate for this method.

3.1.4 ß-glucosidase assay methods

It is frequently but incorrectly assumed that hydrolysis of an aryl-ß-glucoside (e.g. p-nitrophenol-ß-1,4 glucopyranoside or pNPG) is a reliable indication of a system's ability to hydrolyse cellobiose to glucose. In recent years, 3 different types of ß-glucosidases have been identified (Bucht and Ericksson, 1969, Rodionova *et al.*, 1977, Klesov *et al.*, 1981b, Enari, 1983).

(i) cellobiases cleaving cellobiose exclusively and having no action on aryl-ß-glucosides.

(ii) ß-glucoside hydrolases possessing broad specificity in relation to the aglycone

(iii) aryl-ß-glucosidases which cleave only ß-glucosides in which the aglycone is an aryl alcohol, e.g. phenol, o-

or p-nitrophenol, saligemin etc.

Therefore any study of ß-glucosidase activity by the most simple and commonly used method utilizing an aryl-ß-glucoside should be complemented at some stage with measurement of glucose production from cellobiose.

3.2. MEASUREMENT OF THE ACTIVITY OF THE TOTAL CELLULASE COMPLEX

Some of the methods described in the preceding section as being of use in the study of individual component enzymes are also appropriate to measurement of the overall activity of a cellulase complex, e.g. the use of chemical or HPLC end-product analysis. Some of the substrates, such as CMC and TNP-CMC, may be attacked by exo-acting cellulases as well as by endocellulases, and therefore provide some measure of combined cellulase activities. Hydrolysis of native crystalline cellulose is however a much more stringent measure of the activity of a "complete" cellulase complex. The following is a list and evaluation of various types of assay which can be used to measure "total" or "complete" cellulase activity.

3.2.1 Measurement of residual cellulose

Residual insoluble cellulose can be measured by weight or by chemical means (Halliwell, 1958). This can be a reliable method but is insensitive and tedious. Care must be taken to firstly remove adsorbed proteins and bacteria from the cellulose.

3.2.2 Determination of sugars produced from insoluble cellulose

This has been the most commonly used method (Mandels and Weber, 1969, Halliwell and Riaz, 1970, Berghem and Pettersson, 1973, Mandels *et al.*, 1976). Such determinations normally employ one of the systems of Table 3.1 which detect total reducing sugars.

Unfortunately, the formation of reducing sugars or glucose is not necessarily proportional to the solubilizing effect. The final ratios of the various soluble products, normally cellobiose, glucose and lesser amounts of cellooligosaccharides, will affect the result to varying extents depending upon which particular reducing sugar assay method is used. Therefore the absence of ß-glucosidase could give the impression that a particular system had only half the cellulose-solubilizing capacity of a second system identical in all activities other than ß-glucosidase.

The addition of an excess of ß-glucosidase may quash this objection, provided such an enzyme is available which is free of contaminating cellulases and which can operate effectively under

the desired reaction conditions. Alternatively the levels of glucose and total reducing sugars may be separately measured but enzymatic assays specific for glucose may be inhibited by conditions prevailing in the cellulose hydrolysis mixture.

Total soluble carbohydrate can be determined by methods such as the phenol-sulphuric acid assay of Dubois *et al.*, (1956). Use of this method in a "total" cellulase assay is fully described in Section 3.5.3.1. Since total soluble carbohydrate is measured, the results are less dependent upon the form of the soluble sugars than are any of the other sugar assays listed on Table 3.1.

HPLC analysis, which can provide complete qualitative and quantitative analysis of end-products of crystalline cellulose hydrolysis, is becoming an attractive alternative to test-tube assays as sugar detector sensitivities are improved.

3.2.3 Measurement of dye release from insoluble dyed forms of cellulose

This alternative assay method, strongly advocated by Leisola and Linko, (1976), is extremely simple and relatively unambiguous, but requires calibration by some form of sugar assay in order that results may be expressed in terms of International Units (i.e. μmoles of substrate ß-1,4 glucosidic bonds cleaved per minute). Details are available for preparation of dyed forms of cellulose ranging from crystalline (Leisola *et al.*, 1975) to amorphous acid-swollen (Fernley, 1963). The crystalline dyed celluloses offer the most rigorous test for a cellulase complex, but dye release is more rapid from the more amorphous forms such as the commercially available "cellulose azure", and thus the sensitivity of the assay is improved. Sections 3.5.3.2 and 3.5.3.3 detail their use.

Non-linearity of dye release against time of incubation is likely to become apparent if a substantial proportion of the substrate is degraded, since dye will not be uniformly distributed throughout the cellulose structure, and also because the rate of cleavage of glucosidic bonds can be expected to decrease once the most reactive bonds have been hydrolysed and the most crystalline portions of the substrate are encountered.

3.2.4 Measurement of isotope release from ^{14}C-labelled cellulose

Monitoring the appearance of soluble ^{14}C-labelled sugars (Weimer amd Zeikus, 1977) provides completely unequivocal evidence of cellulose solubilization. Drawbacks are the poor range of ^{14}C-labelled celluloses available and their expense.

3.2.5 Turbidometric methods

Decreasing optical density of an aqueous suspension of amorphous cellulose has been related to cellulase activity (Nummi *et al.*, 1981a; Johnson *et al.*, 1982). Not all cellulase complexes can be studied by this technique. We found that our cellulase complex produced an initial increase rather than a decrease in optical density, presumably as a result of cleavage of crystals by predominantly endo-acting enzymes producing more numerous smaller particles (Section 3.5.3.4).

3.2.6 Clearance of cellulose-agar

Clearance of cellulose suspensions in solid agar has been used in plate assays (McBeth, 1916, Toyama, 1963, Montenecourt and Eveleigh, 1977, Creuzet *et al.*, 1983) and as an indication of cellulolytic activity of colonies in culture tubes (Rautela and Cowling, 1966; Tansey, 1971). As a compromise, non-crystalline cellulose, (e.g. MN300, or phosphoric acid-swollen cellulose) is normally used since clearance zones develop more rapidly than in crystalline cellulose (Montenecourt and Eveleigh, 1977). Even after this compromise of substrate and thus of reliability as an assay of "complete" cellulase activity, incubation periods of 24 hours or longer are normally required for clearing to become visible by published methods. Toyama (1963) improved the visibility of the zone of cellulose degradation by a post-incubation treatment with $ZnCl_2$, I_2 solution and $NaNO_2$-Na_2HPO_4. However, activities measured by Toyama using this method did not correlate well with activities obtained on the basis of the filter paper decomposition.

We have found that more rapid development of clearance can be achieved by substantially lowering the substrate level, even to the point where turbidity due to suspended cellulose is not visible (viz. 0.002% w/v). Post-incubation staining of undegraded cellulose with Congo Red can be used to visualize the zones of clearance. By lowering the cellulose concentration in the agar, and thus shortening the assay time required, one can avoid the need

to compromise the stringency of the assay by using a more rapidly hydrolysed substrate. Section 3.5.3.5 details our Congo Red stained avicelase plate assay.

3.2.7 Decrease in tensile strength

Measurement of loss of tensile strength of strips of cotton (Reese and Levinson, 1952, Rautela and Cowling, 1966) requires a substrate of uniform tensile strength and reasonably complex apparatus, and has not been used widely.

3.2.8 The filter paper assay: a proposed standard assay

The large number of different methods which have been employed in studying the total cellulase activity of a complex has hindered communication between the various groups, and there has been no lasting agreement on which particular assay should be accepted as the international standard. This is partly due to different substrate "preferences" of the cellulase complexes from widely differing sources, but methods finally selected also reflect the researcher's personality and the scale of the work.

A technique which measures the reducing sugars released from filter paper, proposed by Mandels and Weber (1969) and modified by Mandels, Andreotti and Roche (1976), has come closest to gaining general acceptance as a standard measure of the activity of a cellulase complex.

The method's good features are:

(a) The substrate is widely available and uniform.

(b) It can be easily measured out on an area basis. Paper antibiotic discs, small enough to drop into a test-tube, have been advocated as a substitute for the filter paper by Montenecourt and Eveleigh (1978).

(c) It is moderately susceptible to most "complete" cellulase complexes.

(d) Hydrolysis can be followed visually once free-fibre formation begins and the structure collapses.

(e) Removal of the paper prior to addition of the DNSA reagent is unnecessary.

In order to circumvent the interpretation problems which are inherent in the use of a reducing sugar detection system, (see Section 3.2.2) dyed filter paper (Poincelot and Day, 1972) may prove helpful. Difficulties in attaining uniform dying of filter paper sheets would be surmountable if commercial production was

undertaken.

Powdered crystalline cellulose was preferred by us as a substrate rather than filter paper, for the following reasons:

(a) Powdered cellulose could be more cleanly separated by centrifugation from the supernatant since it formed a less bulky, tighter pellet than did free fibres of filter paper.

(b) It could be rapidly dispensed volumetrically as a slurry.

(c) Crystalline cellulose is generally considered to be a more recalcitrant substrate and thus a more stringent test of "total" cellulase activity than is filter paper.

We used a fairly coarse grade of microcrystalline cellulose (Sigmacell 50), which is very similar to Avicel, as substrate in our assays of total cellulolytic activity which were based on measurement of total soluble carbohydrate release. Such activity against Sigmacell 50 was termed "Avicelase" (see Section 3.5.3.1 for details).

It is highly unlikely that any substantial solubilization of crystalline or semi-crystalline cellulose will proceed linearly with time due to the varying accessibilities of glucosidic bonds in different regions of the fibre. A decrease in hydrolysis rate or, in the case of some cellulose complexes, a complete cessation of hydrolysis, can be expected as the most reactive bonds are hydrolysed. Therefore if a substrate such as filter paper is to be used as an assay for comparing various cellulase complexes, the extent to which hydrolysis is allowed to proceed before terminating the assay must be standardized. Mandels *et al.* (1976) suggested that activity should be calculated from the enzyme dilution necessary to produce 2mg of glucose in one hour, using 0.5ml of enzyme and 50mg of filter paper.

3.3. DETECTION OF CELLULOLYTIC ACTIVITY ON POLYACRYLAMIDE GELS

It is surprising that amongst the numerous groups involved in cellulase research, only very few have employed techniques for detecting cellulolytic activity on polyacrylamide gels. "Blind" parallel sectioning has been used successfully in obtaining purified cellulases from electrophoresis polyacrylamide disc gels (Umezurke, 1979; Tong *et al.* 1980) but the method is laborious and less likely to result in clean band separation when applied to slab gels. Some method for visualizing focussed cellulase bands becomes essential for preparative isoelectric focussing, particularly on polyacrylamide, since the bands

produced are not normally perfectly straight.

Eriksson and Pettersson (1973) developed a technique in which flat-bed IEF gels were sprayed upon completion of the run with a viscous CMC/buffer solution. The reducing sugar products of the CMC hydrolysis were then blotted onto a sheet of filter paper and detected by spraying the paper with p-anisidine-hydrochloride in ethanol. We didn't try this technique since the sharpness of the zymogram bands is largely dependent on the high viscosity of the CMC coating, which would be greatly reduced by incubation at the high temperatures required for the activity of our cellulases. In addition, the p-anisidine spray is toxic. The method is readily adapted to the detection of a wide range of carbohydrases, provided the substrates are viscous or else can be readily combined with an inert viscous matrix.

Nummi *et al.* (1980) utilized agarose overlay gels containing amorphous cellulose (0.5% w/v). Cellulase activity was detected by the development of clear zones in the turbid gel. The major drawback with this technique is the long incubation (24h) required before clear zones appeared.

Lamed *et al.* (1983) overlaid their electrophoresis gels with agarose containing 1.4% CMC in buffer, incubated the two gels together for 3 to 20 hours and then detected CMC degradation by immersing the overlay in 2-propanol. Zones of clearance stabilized within 2 to 3 hours. Overall the technique took between 5 and 23 hours, depending on the levels of activity. Band definition on the resulting zymogram was much poorer than that which was demonstrated by Eriksson and Pettersson (1973) and by Beguin (1983) (described later).

Sprey and Lambert (1983) utilised a 0.5-1.0mm thick agarose gel containing 1% (w/v) CMC, backed by a Gel-Bond plastic sheet. This agarose was then hot-air dried before being sandwiched against the IEF gel for 15 minutes at 40°C. Clearance zones were visualized by treating the dried foils as photographic negatives. Band definition was not as good as that obtained by protein staining, but the technique was relatively rapid.

McHale and Coughlan (1981c) surrounded their disc gels after electrophoresis with agar containing 5% (w/v) phosphoric acid-swollen cellulose azure. During a 1 hour incubation, the dye released from the cellulose azure diffused into the disc gel at locations corresponding to the enzymes that effected its release. Thus the original gel became marked directly and the active bands could be sectioned from it without

risk of mislocating them due to incorrect positioning of the zymogram template required by other methods. McHale and Coughlan also suggest that cellotetraose could be substituted for cellulose azure, since cellotetraose is claimed to be a substrate for both endo- and exoglucanases (Mandels *et al.* 1976). The activity could be located with a solution containing ß-glucosidase, glucose oxidase, peroxidase and a suitable dye. Nobody has yet published work in which this suggestion has been tested, possibly largely because cellotetraose is not commercially available. Other possible substitutes for the cellulose azure in the McHale and Coughlan method are trinitrophenyl cellulose (Huang and Tang, 1976) and dyed crystalline cellulose (Leisola and Linka, 1976).

The application of the Congo Red staining technique as a ß-D-glucanase assay by Wood (1981) and demonstration of its usefulness and sensitivity in a plate overlay cellulase detection system (Teather and Wood, 1982) led to Beguin (1983) publishing a system for the detection of cellulase activity in polyacrylamide gels using Congo Red-stained agar replicas. CMC, supplied at a comparatively low concentration (0.1% w/v) in the overlay gel, complexes with Congo Red, except of course in areas where the CMC has been degraded, which finally appear as colourless bands. After having tried the Beguin technique, we modified it by substituting polyacrylamide or agarose for the agar, by reducing the thickness of the overlay gel to 0.3mm and supporting it on a plastic backing sheet. The method which we finally used routinely is detailed in Section 3.5.4.3.

Our modifications conferred the following advantages over the original Beguin technique:

(i) Much shorter gel contact, incubation and staining times became possible due to the thinness of the overlay gel. A completed zymogram was produced within 20 minutes of ending an SDS electophoresis run c.f. 4-6 hours by the Beguin technique. Two hours were saved by eliminating the four washes designed to promote renaturation, since our cellulases retained sufficient activity in the presence of 0.1% SDS to be readily detected.

(ii) Better band definition was possible due to the substitution of polyacrylamide for agar as the gel matrix, since the reduced pore sizes restricted lateral diffusion. Shorter contact and incubation times necessary would also have been contributing factors.

(iii) Improved sensitivity resulted since less CMC degradation was required to produce a band of "clearance" right through a thinner gel. We obtained sensitivity and band definition equalling that achieved by silver staining the proteins in the original gel.

(iv) Wrapping the overlay gel with plastic film was unnecessary during the very short incubation required since desiccation was not a problem. This avoided the associated risk of smudging enzyme bands.

(v) Greater mechanical strength was conferred by the plastic backing, making handling and storage simple and preventing shrinkage during salt treatment. This retention of the original size was vital if the zymogram was to be used as a template for active band excision.

Congo Red forms complexes with several other polysaccharides besides those with contiguous (1,4)-ß-D-glucopyranosyl groups, such as (1,3)-ß-D-glucans, (1,3),(1,4)-ß-D-glucans and hemicellulosic galactoglucomannans (Wood, 1980a; Wood *et al.*, 1983). Thus a wide range of enzymes which exhibit activity towards one or more of these classes of polysaccharide can be detected by overlaying with gels containing the appropriate substrate and subsequently staining the residual substrate with Congo Red.

Congo Red will not form a complex with oligosaccharides of less than 5 contiguous (1-4)-ß-linked D-glucopyranosyl units (Wood, 1980a) so enzymes which produce the most rapid reduction in chain length (i.e. endoglucanases) will be the most sensitively detected by the Congo Red-stained zymogram technique.

No zymogram method has yet been published which allows specific detection of avicelase activity in polyacrylamide gels. However, three options appear feasible:

(i) Overlaying with a viscous slurry containing crystalline cellulose, producing a paper replica by blotting, and finally spraying the paper with a sensitive reducing sugar-detecting reagent (i.e. the method of Eriksson and Pettersson (1973) with a change of substrate).

(ii) Overlaying or preferably surrounding the gel with agar containing dyed crystalline cellulose. The dye released should penetrate the polyacrylamide in the area containing the enzyme effecting the hydrolysis (i.e. the method of McHale and Coughlan (1981c) with a change of substrate).

(iii) Overlaying with a gel containing an extremely dilute suspension of crystalline cellulose "fines", followed by incubation and staining with Congo Red. As little as 0.002% (w/v) cellulose will produce a distinct red background with Congo Red ($1mg.ml^{-1}$), despite being virtually undetectable as turbidity prior to staining. A plate assay which utilizes this very sensitive technique of avicelase detection is described in Section 3.5.3.5, but the applicability of this method to zymogram production has yet to be tested.

3.4. DETECTION OF ß-GLUCOSIDASE ACTIVITY IN POLYACRYLAMIDE GELS

Several very sensitive and rapid zymogram techniques are available for the detection of ß-glucosidase activities in polyacrylamide gels, utilizing the substrates 4-methylumbelliferyl-ß-D-glucopyranoside (Fahnrich and Irrgang, 1984), 6-bromo-2-napthyl-ß-D-glucoside (Rissler, 1983) and ortho- or para-nitrophenyl-ß-D-glucopyranoside (Verachtert *et al.*, 1978, Ait *et al.*, 1982). Whereas detection of cellulases in gels depends upon diffusion of some of the enzyme out of the gel in order to come into contact with an insoluble or very high molecular weight substrate surrounding it, the substrates for ß-glucosidase detection are water soluble and readily penetrate the polyacrylamide to contact the enzyme quickly.

However, the low molecular weight fluorescent or coloured products also diffuse rapidly away from their points of production. A solution to this problem may be provided in the method of Rissler, (1983), in which the bromonapthanol from 6-bromo-2-napthyl-ß-D-glucoside released is complexed by o-amino azotoluene diazonium salt (Fast Garnet) to form a water insoluble red precipitate, which has an affinity for protein, and thus would seem likely to adhere to the band of enzyme effecting its production. Rissler applied this technique to detection of ß-glucosidase-producing fungal colonies.

The technique of Verachtert *et al.* (1978) is somewhat novel in that the o-nitrophenyl-ß-D-glucopyranoside substrate, lacking any net charge, is incorporated into the gel at a low concentration before electrophoresis. The o-nitrophenol released as the enzyme migrates down the gel runs towards the anode, serving to mark the front. When the run is complete, a brief incubation at an elevated temperature produces yellow bands in the final positions of the enzymes. The system is particularly suited to thermophilic ß-glucosidase detection since very little substrate hydrolysis occurs until the final incubation when the

run is complete. We employed this technique successfully (see Section 2.9.2 for details).

3.5. DETAILS OF CELLULASE ASSAY METHODS USED IN THIS THESIS

3.5.1 Endoglucanase assays

3.5.1.1 The CMC plate-clearing assay 5g 7HF-CMC (Hercules) and 15g Oxoid No. 1 agar were added gradually, with stirring, to 1 litre of hot 0.1M pH6 sodium citrate buffer. The suspension was brought to the boil and simmered, while being stirred constantly, until a clear solution formed. After autoclaving, 20*ml* aliquots were dispensed into petri dishes. Such assay plates could be stored for several months in sealed bags at 4°C.

Immediately prior to use, 4mm-diameter wells were cut with a No.1 cork-borer connected to an aspirator. Enzyme samples (20µl) were added to the wells. Plates were then wrapped in plastic film (Glad-Wrap) or sealed with masking tape, inverted and incubated at 75°C for 16 to 18 hours.

Enzyme activity was made visible in our earlier work by precipitation of undegraded CMC with 1% (w/v) aqueous hexadecyltrimethylammonium bromide (Hankin and Anagnostakis, 1977). Zones which remained clear were indicative of endoglucanase action having caused considerable shortening of CMC chain lengths. Clear zones became visible, particularly when viewed against a grey background, within 5 minutes of adding the precipitating reagent. After 20 minutes the boundaries of the zones became progressively less distinct and the plates became of no value as a permanent record.

A superior method of visualizing endoglucanase action involved bathing the incubated plate with Congo Red ($1mg.ml^{-1}$) aqueous solution for 30 minutes, followed by 1M-NaCl which removed dye from areas where the CMC had been hydrolysed (Teather and Wood, 1982). If hydroxyethyl cellulose is used in the plates in place of CMC, the NaCl is not necessary (Wood, 1981).

A central purple zone would appear within the decolourised zone ca. 2 hours after adding the 1M NaCl (Fig. 3.1). This zone was evidently related to enzyme activity since its diameter bore a logarithmic relationship to enzyme concentration. The purple zone boundary was sharper

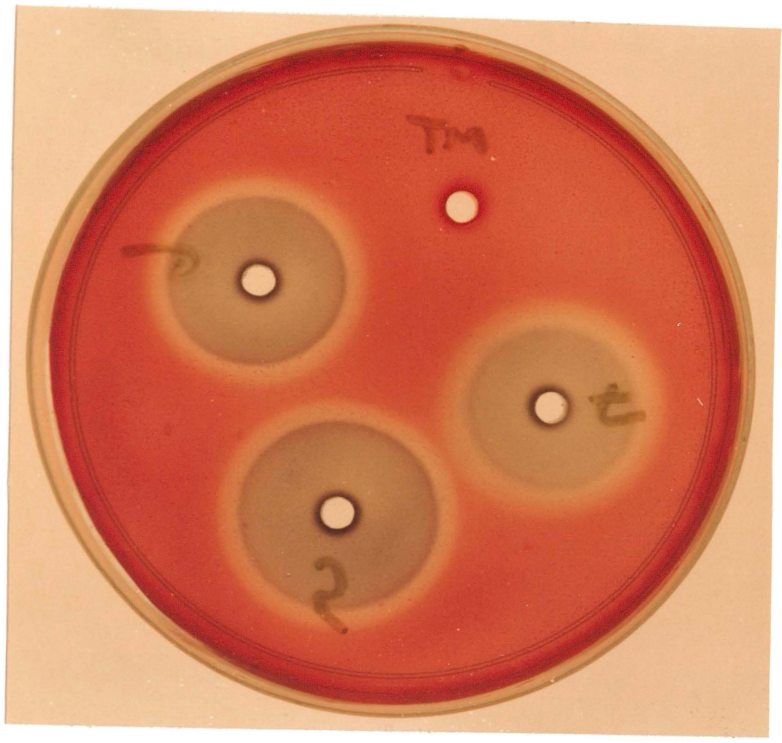


Figure 3.1 A CMC-plate-clearing assay using the Congo Red detection system.

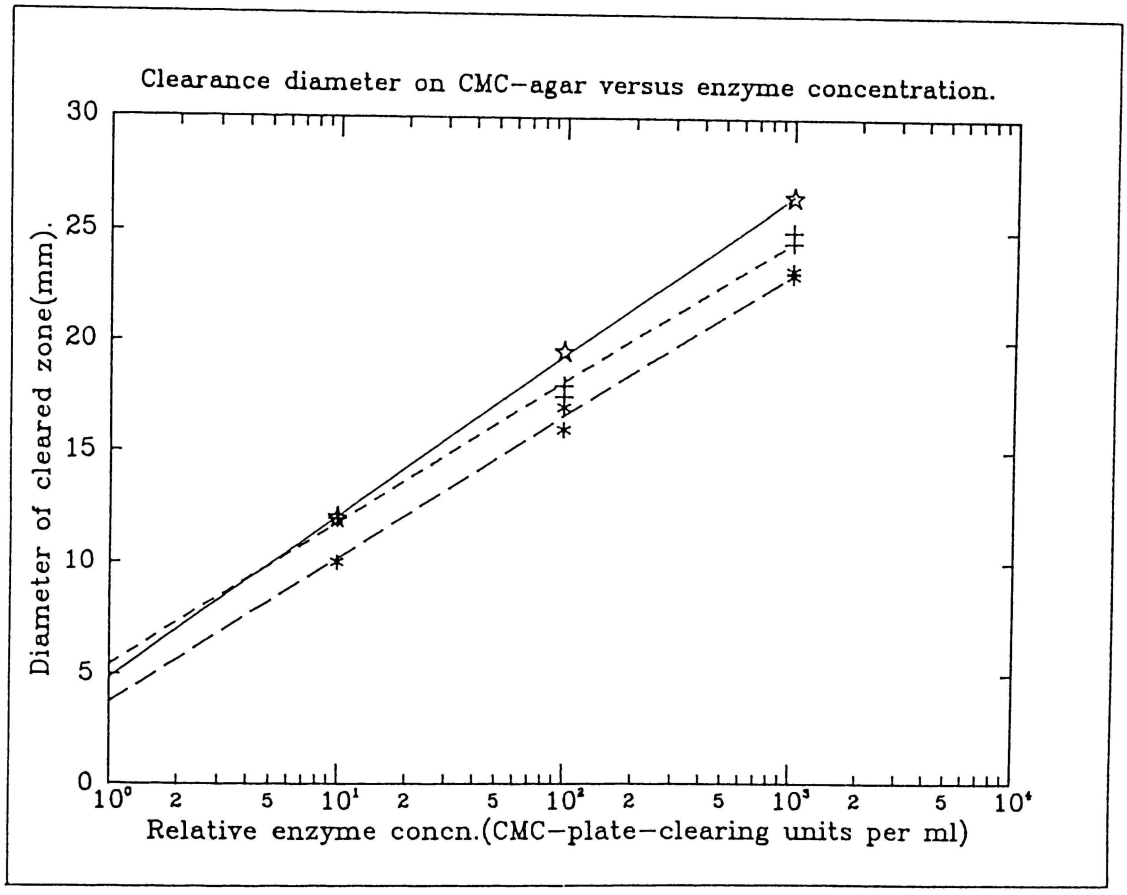


Figure 3.2 The effect of enzyme concentration and incubation time on the clearance diameter of CMC-agar plates.

* = 19h incubation at 75°C.

+ = 21h incubation at 75°C.

☆ = 24h incubation at 75°C.

and more distinct than the outer boundary of the decolourised zone. However, measurement of the larger diameter of the decolourised zone conferred greater sensitivity since certain low levels of enzyme would produce only a decolourised zone, with no central purple area.

No mention of any similar concentric zone has been made in the relevant literature. It didn't develop around our boiled enzyme or buffer blanks, nor in decolorized zones in our avicelase plate assay (Section 3.5.3.5) in which the same enzyme was employed. Neither did any purple zones appear within bands of clearance on our activity-detecting gel overlay, in which a different CMC and buffer were used and polyacrylamide replaced the agar (Section 3.5.4.1). A maximum red shift in the absorption spectrum of the Congo Red is induced by very low concentrations of ß-D-glucan (6-10 $\mu g.ml^{-1}$) and both higher and lower levels produce smaller shifts (Wood *et al.*, 1983). One might therefore speculate that the purple zones contained CMC at concentrations within the range that produced the maximum red shift (i.e. a shift to absorbance of longer wavelengths). This would suggest that hydrolysis of the CMC had been incomplete, perhaps being unable to continue beyond a certain minimum chain length or past a certain pattern of substitution. The colourless "halo", marking the extremities of detectable enzyme action, may have contained CMC in a concentration higher than that which produced the largest wavelength shift (i.e. in the inner purple zone) but which was too low to produce the substantial bindng (and perhaps precipitation) of the Congo Red as seen across the rest of the plate.

Acidification with 0.5M acetic acid changed the background to blue, providing better contrast with the clearance zones, and also served to inhibit any further cellulase activity. The inner purple zone still remained quite distinct.

Congo Red-stained plates would keep for weeks with only slight fading provided the lids were on them to limit desiccation.

A variation of the above procedure which eliminated the 30 minute wait during the Congo Red staining after incubation

was to stain the plates before adding the enzyme. Visualization of activity only required a brief wash with 1M-NaCl. However before relying on this alternative, a comparison of clearance diameters obtained by both pre- and post- incubation staining is advisable in case a particular enzyme is inhibited by Congo Red.

Enzyme concentration was related by a logarithmic relationship to clearance diameter. Calibration of plate clearing diameters in enzyme concentration terms was based on clearance diameters obtained with a dilution series produced from crude concentrate of TP8.T 6.3.3.1 cellulase. The undiluted concentrate was given an arbitrary value of 1000 plate clearing units.ml^{-1}. Diameters of clearance produced after incubations of 19, 21 and 24 hours at 75°C were calibrated on this basis (Fig. 3.2.).

In subsequent experiments plate clearance diameters were converted to plate clearing units.ml^{-1} by applying the equation of the line from the calibration experiment with the most similar incubation period.

3.5.1.2. Assay of carboxymethyl cellulose hydrolysis (CMCase) by reducing sugar measurement A modification of the method of Weimer and Zeikus, (1977) was used. 0.1ml of enzyme was mixed with 0.4ml of 2% (w/v) CMC (sodium salt, low viscosity, Sigma), in 0.1M sodium citrate buffer, pH6. The mixture was vortexed, incubated at 75°C for 15 to 30 minutes (depending on the activity) and stopped by addition of 1.5ml of DNSA reagent (Miller, 1960) or 1.0ml of the concentrated form of the PABAH reagent (Lever, 1973). After boiling in capped tubes for 15 minutes (DNSA) or 5 minutes (PABAH), the tube contents were cooled to room temperature in tap water, vortexed and their absorbances were read at 575nm (DNSA) or 420nm (PABAH). Absorbance readings could be made up to 24 hours after colour development provided glucose standards were treated similarly.

As standards, 0.1ml of a range of glucose concentrations (0-20mM for DNSA and 0-1mM for PABAH) were substituted for the enzyme sample in the above mixture.

Blanks, both incubated and unincubated, in which water or uninoculated growth medium was substituted for the enzyme, were included in each set of assays. This allowed

compensation for any apparent CMCase activity which could have been due to medium decomposition during the incubation at high temperature.

Allowance was made for background reducing sugar levels by adding the highly alkaline DNSA or PABAH to the enzyme before the CMC substrate, thereby preventing any enzymatic hydrolysis of the CMC. These tubes were not incubated.

A unit of "CMCase" activity was defined as that which released 1µmol glucose equivalents per minute under the above incubation conditions.

A "lag" in the absorbance versus glucose concentration curve produced with DNSA (Fig. 3.3), due to oxidation of a portion of the glucose, can be prevented by a variety of methods. These include (i) gassing tube contents with nitrogen prior to boiling (ii) addition of the Rochelle salts immediately after boiling but prior to rapid cooling (Miller, 1959), or (iii) "doping" the CMC stock with an appropriate level of glucose to compensate (Miller, 1960). The first two alternatives require increased manipulation and tube handling and are thus less suited to experiments involving large numbers of tubes than alternative (iii). The latter, however, presents the potential complication that glucose added might inhibit cellulase activities. Therefore, we chose to add glucose to the DNSA reagent (1ml of 0.01M glucose per 100ml of reagent). This doping had to be performed within a few hours prior to using the reagent, or else the lag was reinstated. The PABAH reagent didn't produce any lag, so was not doped with glucose.

The period of boiling required for maximum colour development with DNSA and glucose was investigated (Fig. 3.4). The addition of CMC and anaerobe medium, both separately and in combination, delayed the development of maximum colour during boiling. A 15 minute boiling time was decided upon and adhered to particularly strictly whenever anaerobe medium was included, since the latter caused a constant increase in absorbance with increased boiling time whereas a plateau was attained in its absence after 15 minutes boiling.

For the PABAH assay, boiling periods of 5 and 10 minutes were found to produce identical absorbances with glucose. CMC

Figure 3.3 The response curve of the DNSA reagent to reducing sugars.

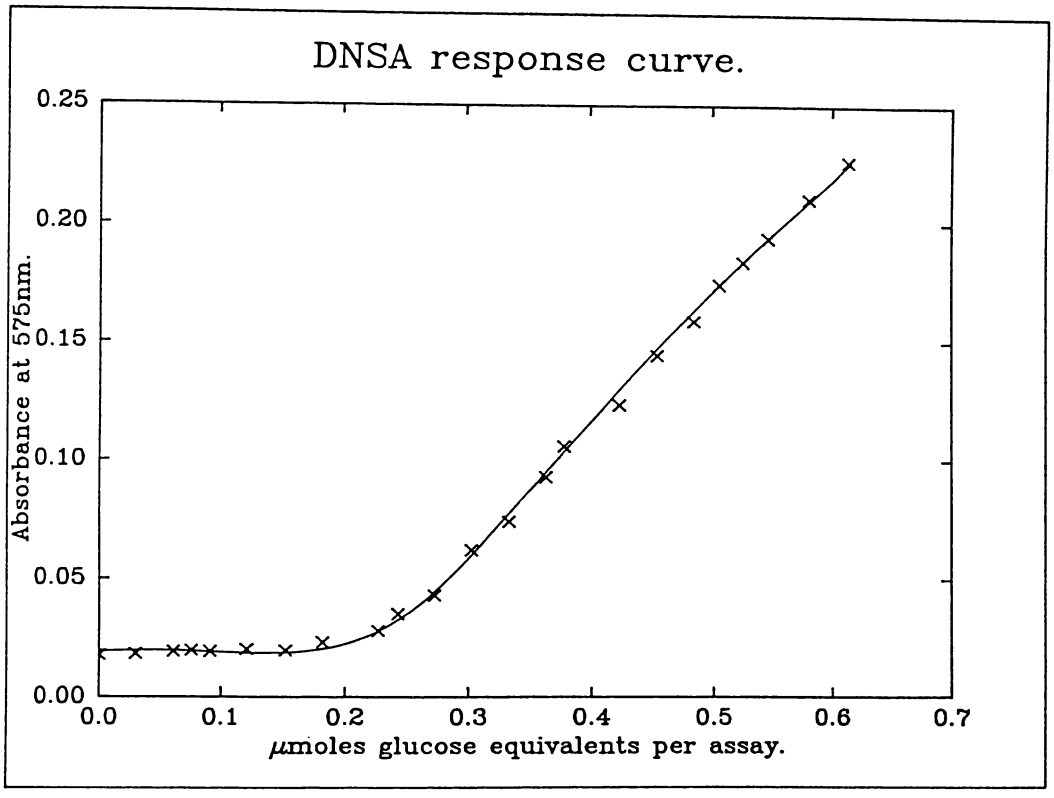
Data shown was obtained from the time zero unincubated controls of the enzyme dilution series employed in Fig. 3.5.

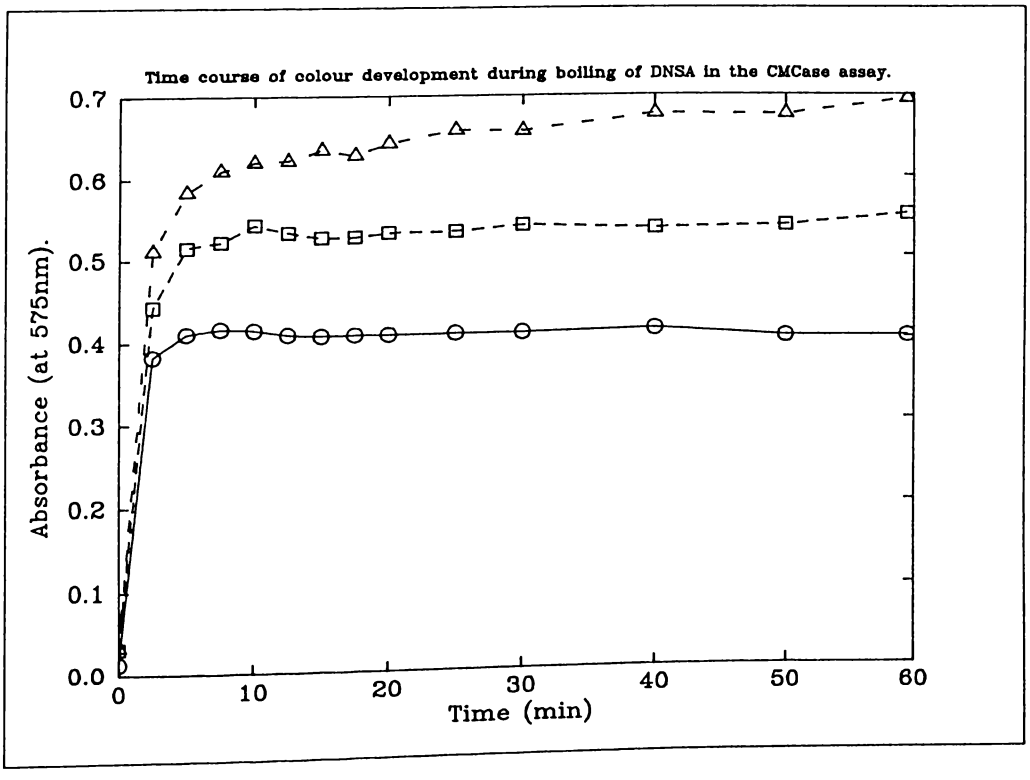
Figure 3.4 The effects of CMC, citrate buffer and growth medium on the time course of the DNSA colour development during heating in boiling water.

○ = 0.2ml 1.35mM glucose + 1ml water + 3ml DNSA.

□ = 0.2ml 1.35mM glucose + 0.8ml 2%(w/v) CMC in 0.1M sodium citrate buffer pH5 + 0.2ml water.

△ = 0.2ml 1.35mM glucose + 0.8ml 2%(w/v) CMC in 0.1M sodium citrate buffer pH5 + 0.2ml of basal growth medium supplemented with 0.2%(w/v) cellobiose.


DNSA response curve.
Absorbance at 575nm.
0.25
0.20
0.15
0.10
0.05
0.00
0.0
0.1
0.2
0.3
0.4
0.5
0.6
0.7
μmoles glucose equivalents per assay.


Time course of colour development during boiling of DNSA in the CMCase assay.
Absorbance (at 575nm).
0.7
0.6
0.5
0.4
0.3
0.2
0.1
0.0
0
10
20
30
40
50
60
Time (min)

at the level used made a substantial contribution to the absorbance of the blanks, but didn't affect the linearity of glucose calibration standards.

Equimolar concentrations of glucose and cellobiose produced different absorbances by the DNSA assay, with one molecule of cellobiose producing a response equivalent to that of 1.5 glucose molecules. Presumably this was due to hydrolysis occurring during the boiling, so the precise value can be expected to depend on the exact boiling time. The precise temperature of the boiling water bath can also have a large effect on the relative absorbances produced by glucose and disaccharides (Luchsinger and Cornesky, 1962). In contrast, the PABAH reagent produced equal (i.e stoichiometric) absorbances with equimolar concentrations of glucose and cellobiose.

An enzyme dilution curve for the reaction of crude TP8T supernatant concentrate with CMC under the conditions described above (30 min incubation) was linear up to the highest concentration of enzyme tested (Fig. 3.5). DNSA was used to measure reducing sugars released.

A time course or "progress" curve for the reaction of a 15-fold concentrate of TP8T supernatant with CMC in the conditions described above appears in Fig. 3.6. Linearity was maintained for 10 minutes, by which time an absorbance of 0.55, corresponding to a glucose concentration of 1mM, had been produced. Thereafter, the reaction rate decreased with increased time of incubation.

There are numerous difficulties to be expected in attempting to establish a standardized set of CMCase assay conditions which will permit linear enzyme dilution and progress curves (Section 3.1.1.3). Since we employed this assay mainly in the comparison of crude supernatants which were highly likely to each contain multiple CMCase components, it was unlikely that conditions would be found which might allow linear enzyme dilution and progress curves in all cases. We simply settled for incubating our CMCase assays for the shortest period which would produce a reliable absorbance change (of at least 0.2) when DNSA was used to measure reducing sugars produced. In retrospect, the PABAH reagent,

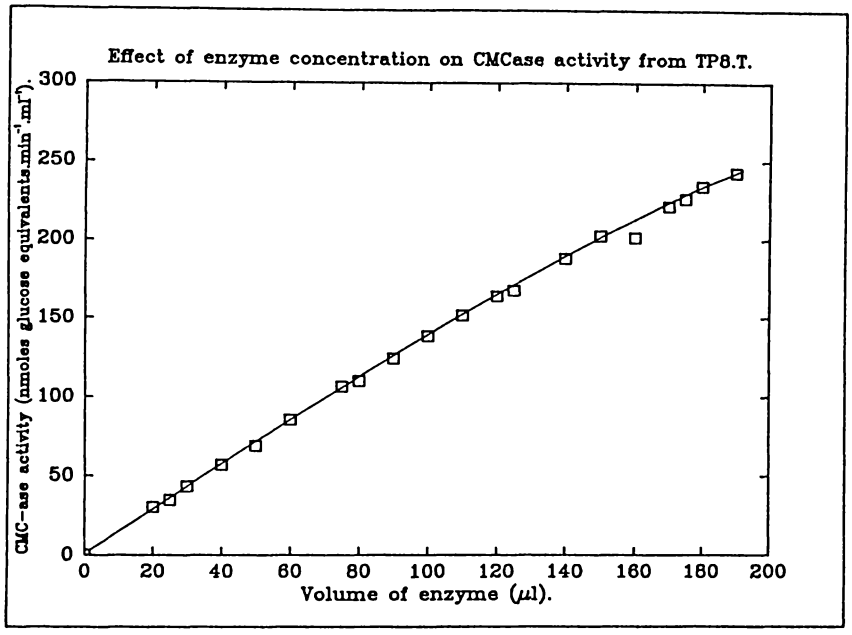
Figure 3.5 Effect of TP8.T enzyme concentration on CMCase activity.

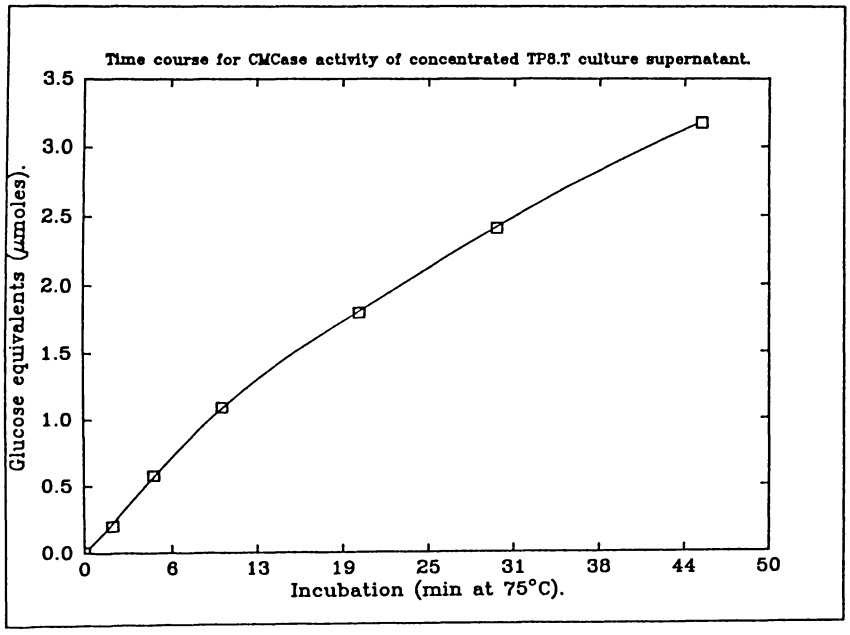
The volumes of crude concentrate of TP8.T culture supernatant shown were diluted with sufficient water to produce a sample volume of 0.2ml. Each dilution was then subjected to the CMCase assay procedure described in Section 3.5.1.2, but with all volumes doubled.

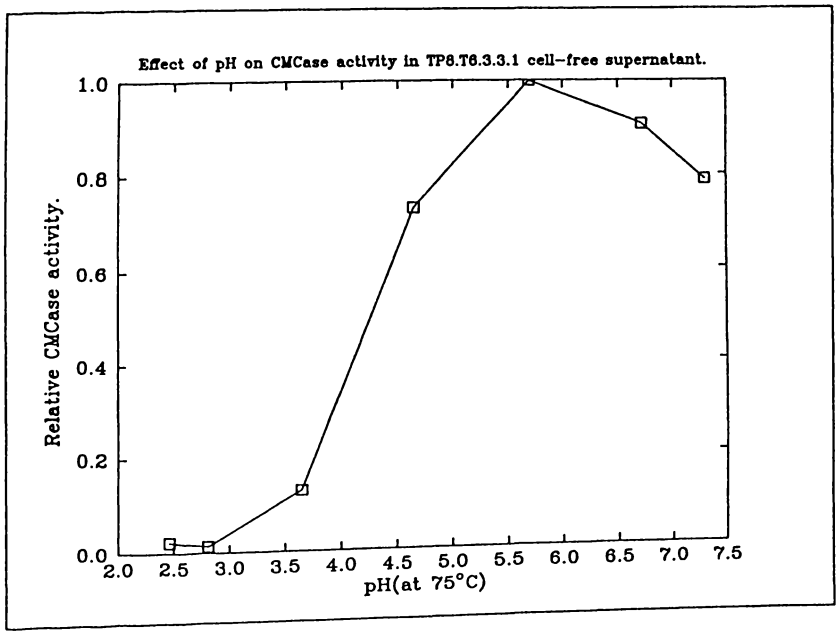
Figure 3.6 Progress curve for the reaction of a crude 15-fold concentrate of TP8T supernatant with CMC.
Reaction conditions were as described in Section 3.5.1.2 but with the incubation time varied as shown.

Figure 3.7 Effect of pH on CMCase activity in TP8.T6.3.3.1 cell-free supernatant.

The various pH conditions were all produced using McIlvaine's citric acid/Na_2HPO_4 buffers as described by Dawson et al. (1974). pH values plotted were measured at the incubation temperature (75°C).


Effect of enzyme concentration on CMCase activity from TP8.T.
CMC-ase activity (nmoles glucose equivalents.min^{-1}.ml^{-1}).
300
250
200
150
100
50
0
0 20 40 60 80 100 120 140 160 180 200
Volume of enzyme (µl).


Time course for CMCase activity of concentrated TP8.T culture supernatant.
Glucose equivalents (µmoles).
3.5
3.0
2.5
2.0
1.5
1.0
0.5
0.0
0 6 13 19 25 31 38 44 50
Incubation (min at 75°C).


Effect of pH on CMCase activity in TP8.T6.3.3.1 cell-free supernatant.
Relative CMCase activity.
1.0
0.8
0.6
0.4
0.2
0.0
2.0 2.5 3.0 3.5 4.0 4.5 5.0 5.5 6.0 6.5 7.0 7.5
pH(at 75°C)

being 12-times as sensitive as the DNSA reagent (Table 3.1) would have been a better choice. It would have permitted shorter incubations, thus allowing truer estimation of initial reaction velocities.

The pH curve for CMCase activity in crude concentrated supernatant (Fig. 3.7) showed pH variation to have only a slight effect over the range 5.7 to 6.7. A pH of 6 was selected at which to perform all CMCase assays.

3.5.1.3. Hydrolysis of trinitrophenol CMC Trinitrophenol-CMC (TNP-CMC) was prepared by the method of Huang and Tang (1976), excepting that Whatman CM11 was the starting material. The same batch of substrate was used throughout. The TNP-CMC was ground to pass through a 60 mesh to allow it to be dispensed as a slurry.

0.2ml volumes of 1.5% (w/v) TNP-CMC suspension in 0.25M sodium acetate buffer, pH 5.7 were added to 0.1ml enzyme samples. The mixtures were incubated at 75°C in tightly capped 1.5ml Eppendorf reaction tubes. When dye release was visible (in most cases after 5 to 18 hours incubation), 1.0ml water was added, the tube shaken to rehomogenize the contents and the residual TNP-CMC was then removed by centrifugation (7,000*g*. for 5 min). The absorbance of the supernatant at 349nm was measured using a "Sipa-cell" attachment and a flow-through micro-curvette.

Blanks, in which water or buffer was substituted for the enzyme, were incubated with each batch of samples. An increase in blank absorbance with time was noticed when incubating at 75°C, making the inclusion of blanks particularly important in experiments involving long (e.g. 24h) incubations.

A unit of TNP-CMCase activity was arbitrarily defined as activity causing an increase of 10^{-5} absorbance units per minute, with the absorbance being measured in a 1.3ml final assay volume and corrected for any absorbance which developed in the incubated blank.

3.5.2 ß-glucosidase assays

A modification of the method of Tong *et al.* (1980) was used. Enzyme solution (0.1ml) was incubated at 75°C with 0.4ml of 1mM p-nitrophenyl ß-1,4 glucopyranoside (Sigma) in 50mM acetate buffer pH 5.6. After 15 or 30 minutes, the reaction was stopped by addition of 1ml of 1M sodium carbonate and the absorbance at 400nm was measured. A millimolar extinction coefficient of 18.3 was used in calculating the amount of p-nitrophenol produced.

Yellowing of the medium frequently occurred during growth of our anaerobes, and thus it was necessary to include,as controls, unincubated mixtures in which the 1M sodium carbonate was added to the enzyme solution before the substrate.

Blanks (both incubated and unincubated) in which water was substituted for the enzyme were included in each batch of assays.

A unit of ß-glucosidase activity was defined as that which produced 1µmol p-nitrophenol.min^{-1} under the above conditions.

An enzyme dilution curve (Fig. 3.8) was linear up to the highest concentration of ß-glucosidase tested (23.5 x 10^{-3} units.ml^{-1}.).

A pH curve for ß-glucosidase activity from concentrated culture supernatant (Fig. 3.9) led to the choice of pH5.6 as the standard pH for future ß-glucosidase assays.

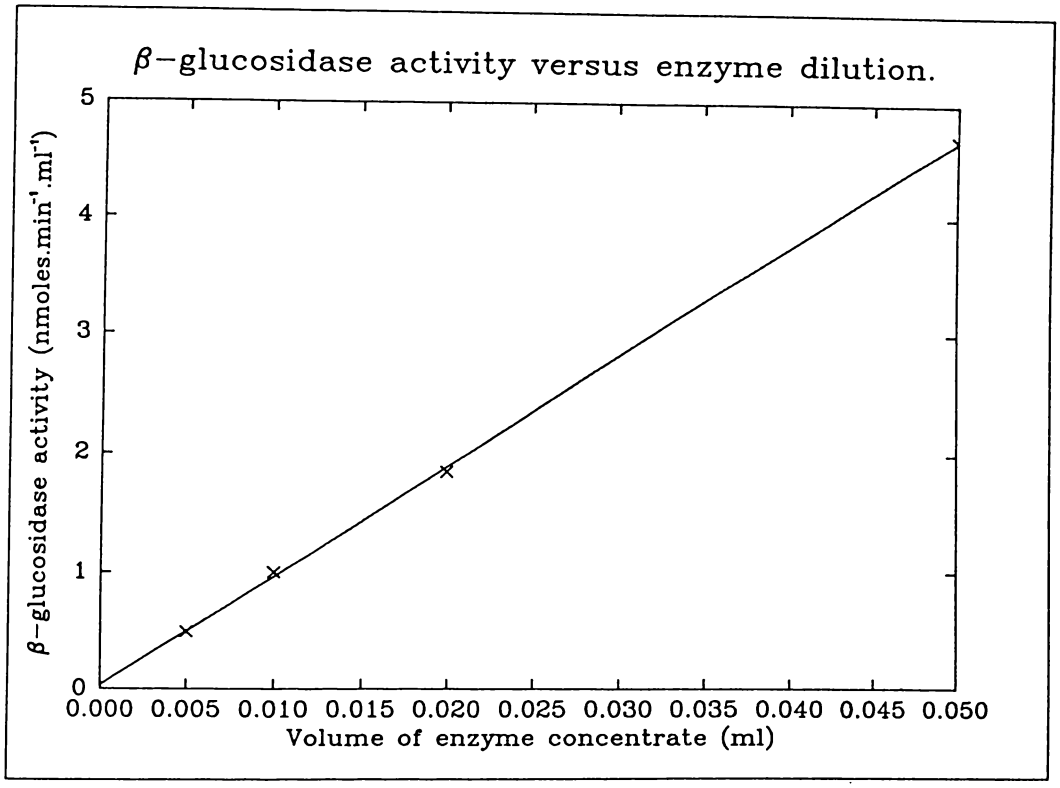
An alternative ß-glucosidase assay was also used in which cellobiose hydrolysis to glucose was measured by detecting glucose specifically with a glucose oxidase/peroxidase/chromatogen reagent (GOD-Perid test kit, Boehringer). 0.05ml samples of enzyme or buffer blank were added to 0.5ml cellobiose (10mM) in 0.25M sodium acetate buffer, pH 5.6 and incubated for 40 min at 75°C. After cooling the tubes to R.T., 100µl samples were removed from each into a second set of tubes, to which 1.5ml of the glucose oxidase/buffer/chromatogen was added. Following a further incubation for 50 min at 25°C, absorbances were read at 436nm. 100µl samples of glucose standards (0.02 - 0.3mM) were given exactly the same treatment as the 100µl samples taken from the cellobiose incubation.

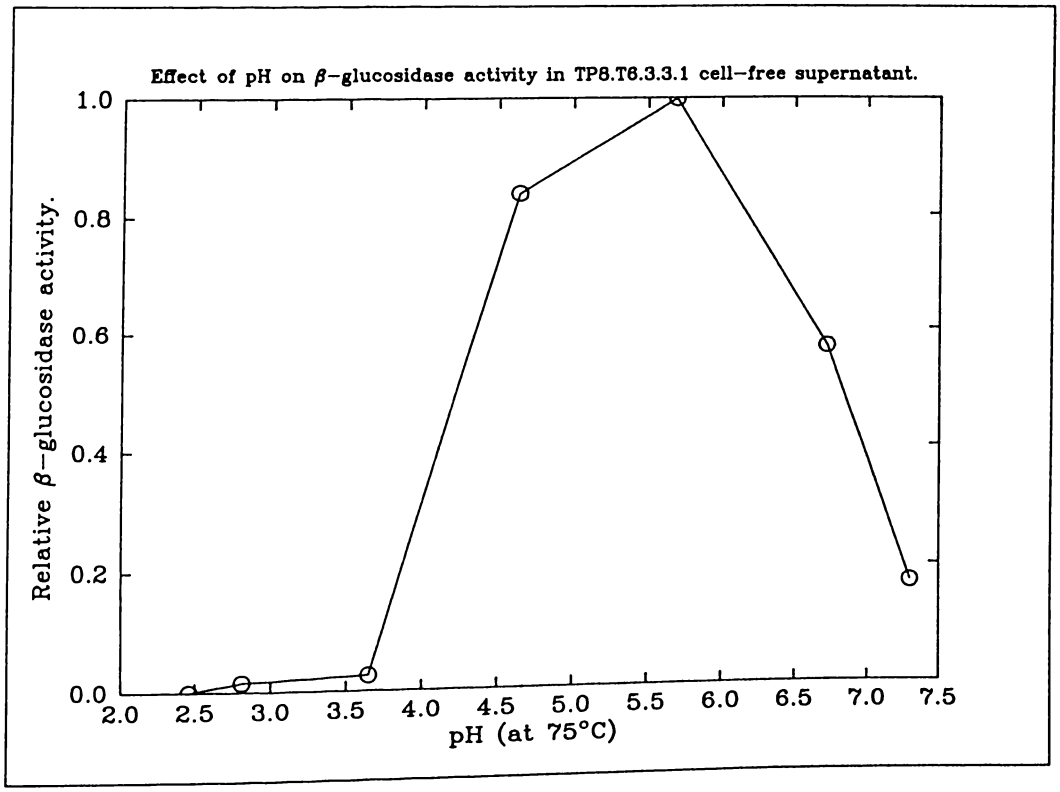
Figure 3.8 ß-glucosidase activity versus concentration of TP8.T6.3.3.1 culture supernatant.

The volumes of concentrated culture supernatant indicated, plus water to make a combined volume of 0.2ml, were incubated with 0.3ml of McIlvaine's citrate/phosphate buffer pH5.7) (Dawson et al, 1974) and 0.5ml 2mM p-NPG for 30 min at 75°C, and the absorbances at 400nm measured.

Figure 3.9 Effect of pH on ß-glucosidase activity in TP8.T6.3.3.1 cell-free supernatant.

The various pH conditions were all produced using McIlvaine's citric acid/Na_2HPO_4 buffers as described by Dawson et al. (1974). pH values plotted were measured at the incubation temperature (75°C).


β−glucosidase activity versus enzyme dilution.
β−glucosidase activity (nmoles.min⁻¹.ml⁻¹)
0
1
2
3
4
5
0.000 0.005 0.010 0.015 0.020 0.025 0.030 0.035 0.040 0.045 0.050
Volume of enzyme concentrate (ml)


Effect of pH on β−glucosidase activity in TP8.T6.3.3.1 cell−free supernatant.
Relative β−glucosidase activity.
0.0
0.2
0.4
0.6
0.8
1.0
2.0 2.5 3.0 3.5 4.0 4.5 5.0 5.5 6.0 6.5 7.0 7.5
pH (at 75°C)

3.5.3 Measurement of total cellulose-solubilizing activity

3.5.3.1 Micro-crystalline cellulose hydrolysis ("Avicelase" activity) 50µl of enzyme solution was mixed with 1.1ml of a 1.05% (w/v) slurry of Sigmacell 50 (average particle size 50µm) in 0.2M sodium acetate pH 5.6 buffer. The mixture was incubated in tightly capped Eppendorf reaction tubes for 4-15 hours, cooled, shaken to ensure homogeneous sugar distribution, and centrifuged at 7,000*g* for 5 minutes. Total soluble carbohydrate in the supernatant was then measured by a modification of Dubois *et al.*, (1956). Two 0.5ml aliquots of supernatant were transferred to two thick-walled pyrex tubes (1.2cm internal diameter), containing 0.5ml of 7% phenol (w/v) in water. After vortexing, 2.5ml of concentrated H_2SO_4 was added directly onto the surface of the liquid, in a reproducible manner, from a glass syringe. The heat generated by the forced mixing of acid and water drove the hydrolysis, and thus it was important to maintain uniformity of mixing and rate of cooling. After standing for 10 minutes in close-fitting holes in a wooden block, the tubes were vortexed and cooled to room temperature in a water bath.

The background level of soluble carbohydrates was assessed by mixing 22µl of each enzyme solution with 478µl of 0.2M sodium acetate buffer pH 5.6 directly in the acid hydrolysis tube. This 0.5ml mixture contained enzyme and buffer in the same concentrations as in the incubation mixture, but they were not incubated with cellulose. Instead, the mixture was assayed directly by the above phenol/H_2SO_4 method.

Blanks (both incubated and unincubated), in which water replaced the enzyme solution, were included with each batch of assays.

Standards in which 0.5ml of 0 - 500 µM glucose was subjected directly to the phenol/H_2SO_4 assay, produced a linear standard curve, and were also included in each batch of assays.

Absorbance was measured at 490nm, using a Sipa-cell attachment and a flow-through curvette.

A unit of "avicelase" activity was defined as the release of 1µmol glucose equivalents per minute under the above

conditions. Increased sensitivity could be achieved by incubating 1ml of the enzyme solution mixed with 0.1ml of 11% Sigmacell 50 in concentrated buffer (e.g. 0.5M sodium acetate, pH 5.6). In addition, the sensitivity of the assay of the products could be almost doubled by mixing 0.9ml of the supernatant with 0.1ml of 35% (w/v) phenol solution before adding the 2.5ml of acid as normal.

A much simpler, safer and more sensitive alternative assay of the products of cellulose hydrolysis involved the measurement of the increase in reducing sugars using the PABAH reagent (Lever, 1973). 0.5ml of the supernatant was added to 1ml of the PABAH reagent (the concentrated form of Lever (1973)) and the mixture was boiled for 5 minutes. After cooling to room temperature under running water, absorbance was measured at 420nm.

Freshly made PABAH reagent offers twice the sensitivity to glucose of the phenol/H_2SO_4 method. However, unlike the phenol/H_2SO_4 reagent, which measures all forms of soluble carbohydrate in the supernatant, the PABAH reagent only reacts with exposed reducing aldehyde groups. Consequently use of the PABAH reagent in the "avicelase" assay may give anomalously high values for preparations rich in ß-glucosidase since glucose gives twice the absorbance of an equal mass of cellobiose.

The pH curve for avicelase activity in crude culture supernatant concentrate (Fig. 3.10) showed a broad optimum around 4.6-5.7, but increasing the pH to 7.3 only reduced the activity by 25%. A pH of 5.6 was selected for use in future avicelase assays.

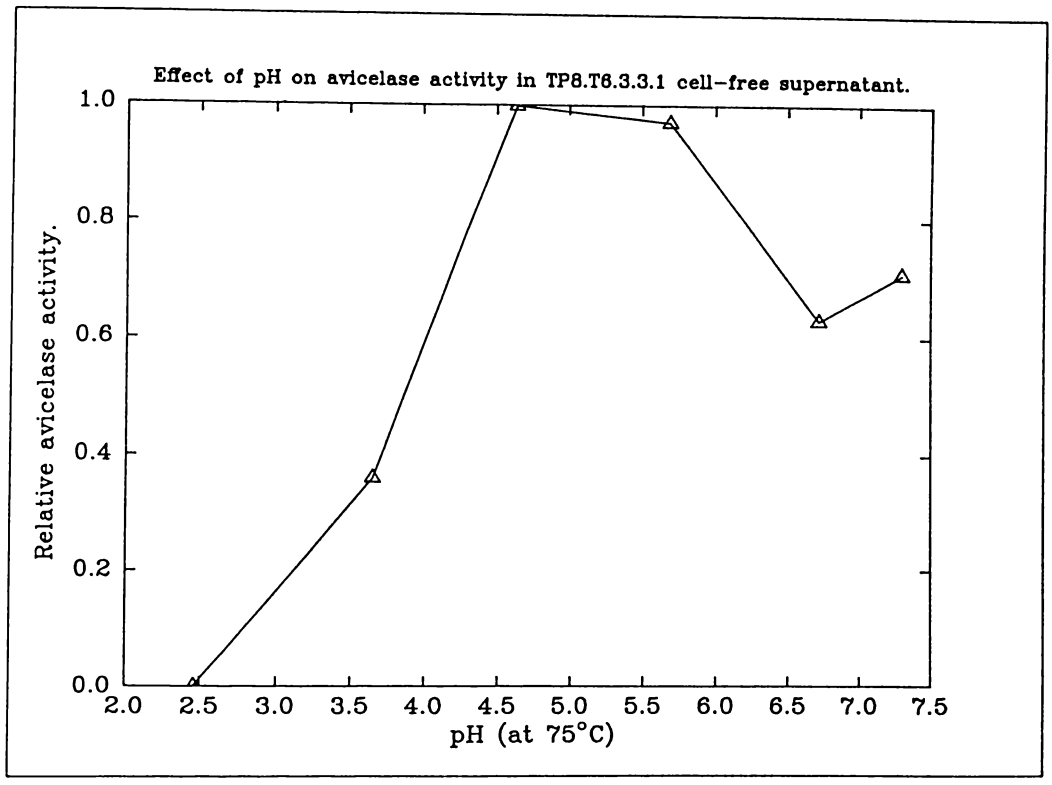
A time-course or "progress curve" for avicelase activity of a 15-fold concentrate of crude TP8T supernatant is shown in Fig. 3.11. Linearity was maintained for the first hour only, and thereafter the rate of hydrolysis began to decline. Both the time course and the enzyme dilution curves for avicelase reactions can be expected to deviate from linearity for a whole host of reasons, so no one set of standard conditions will be likely to produce this linearity for all the cellulase complexes studied. It was concluded that avicelase incubations should be kept as short as possible, so as to

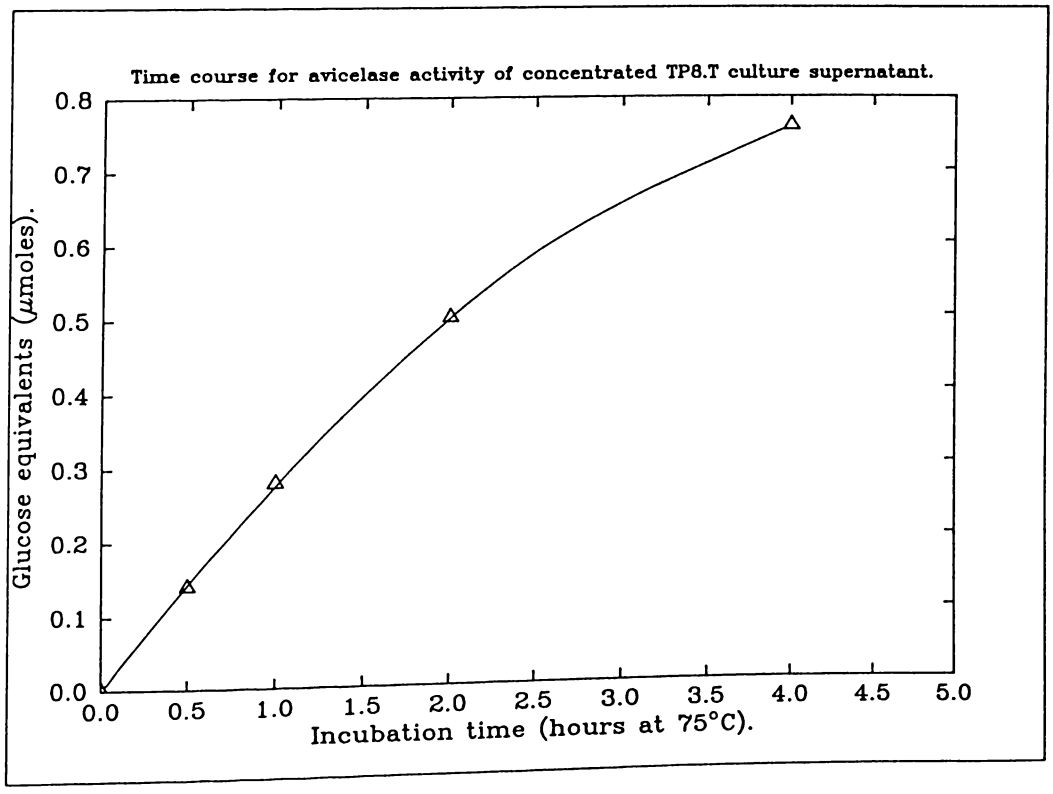
Figure 3.10 Effect of pH on avicelase activity in TP8.T6.3.3.1 cell-free supernatant.

The various pH conditions were all produced using McIlvaine's citric acid/Na_2HPO_4 buffers as described by Dawson *et al.* (1974). pH values plotted were measured at the incubation temperature (75°C).

Figure 3.11 Time course for avicelase activity of a crude 15-fold concentrate of TP8T supernatant.

The assay procedure of Section 3.5.3.1 was used, excepting that the incubation time was varied as shown and DNSA was used to measure the reducing sugars produced.


Effect of pH on avicelase activity in TP8.T6.3.3.1 cell-free supernatant.
Relative avicelase activity.
1.0
0.8
0.6
0.4
0.2
0.0
2.0 2.5 3.0 3.5 4.0 4.5 5.0 5.5 6.0 6.5 7.0 7.5
pH (at 75°C)


Time course for avicelase activity of concentrated TP8.T culture supernatant.
Glucose equivalents (μmoles).
0.8
0.7
0.6
0.5
0.4
0.3
0.2
0.1
0.0
0.0 0.5 1.0 1.5 2.0 2.5 3.0 3.5 4.0 4.5 5.0
Incubation time (hours at 75°C).

lessen the risk of their running into the non-linear section of the time-course.

3.5.3.2 Hydrolysis of dyed microcrystalline cellulose

Sigmacell 50 microcrystalline cellulase was dyed with Remazol Brilliant Blue R Reactive Blue 19 (Sigma) by the method of Leisola *et al.* (1975), as modified to a smaller scale by Ng and Zeikus (1980).

Enzyme solution (0.2ml) was mixed with 0.1ml of a 5% slurry of the dyed cellulose in 50mM pH 5.6 acetate buffer. The mixture was incubated at 75°C in a capped Eppendorf tube until the liquid phase was visibly blue. 1 ml of water was added and then the tube contents were mixed to distribute the dye evenly, followed by 5 minutes of centrifugation at 7,000*g* and measurement of the absorbance of the supernatant at 595nm, using a Sipa-cell attachment and a flow-through micro-curvette to avoid disturbing the pellet of dyed cellulose.

As blanks, water or buffer were incubated in place of the enzyme solution.

Highly active "avicelase" preparations have been shown to produce linear dye solubilization with time (Leisola and Linko, 1976), but such linearity should not be assumed to apply to all cellulase preparations, since the dye will be attached to a variety of sites of varying accessibilities on the cellulose molecule.

An enzyme dilution curve, using the partially purified avicelase preparation of the TP8.T6.3.3.1 cellulase complex, was produced for the reaction with Remazol Brilliant Blue R dyed cellulose (Fig. 3.12). At greater dilutions the enzyme exhibited increased specific activity, as indicated by the increased gradient in Fig. 3.12. The shapes and linearity of these curves will no doubt be very dependent on the particular cellulase complex under study, on the ratio of enzyme to substrate, and on the extent to which hydrolysis of the substrate is allowed to proceed.

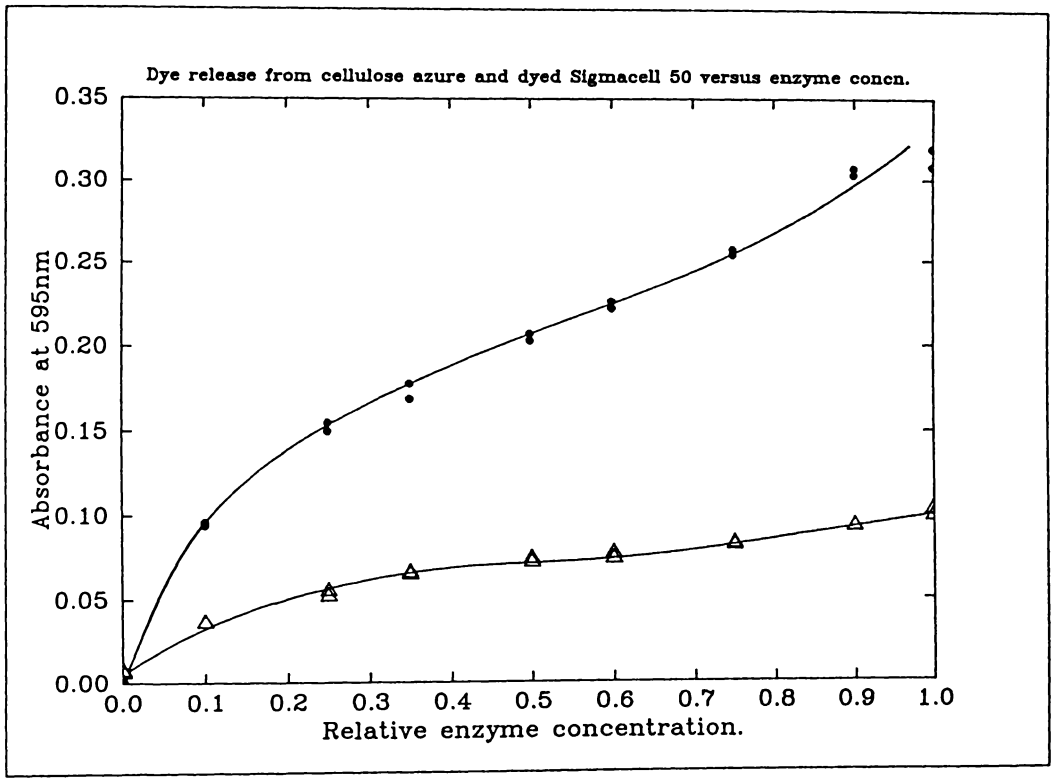
Comparison of avicelase data obtained by the above dyed cellulose method with values obtained for the same enzymes samples using the phenol/H_2SO_4 method revealed a strong correlation (r=0.99) across a 5-fold range in avicelase activities.

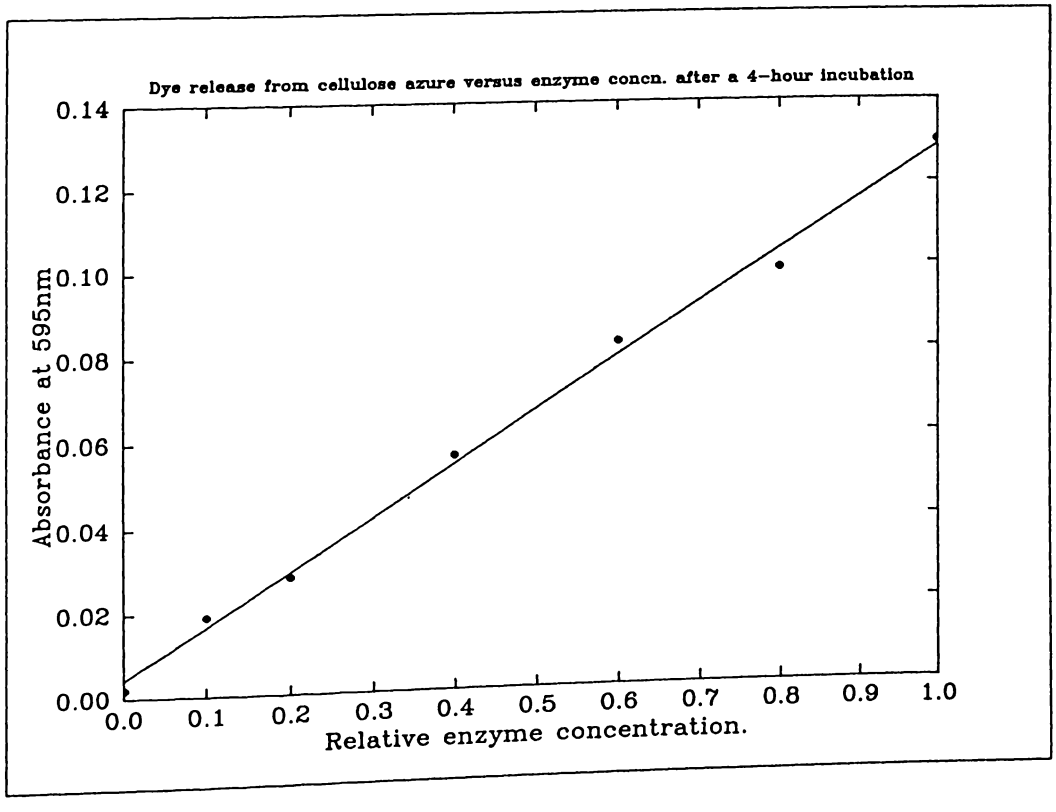
Figure 3.12 Enzyme concentration versus dye release from Remazol Brilliant Blue R-dyed Sigmacell 50 and cellulose azure.

50µl of each of a range of dilutions prepared from a crude concentrate of TP8.T6.3.3.1 culture supernatant was mixed with 50µl of 200mM sodium acetate buffer containing either Remazol Brilliant Blue-dyed Sigmacell 50 at 10%(w/v) (△) or cellulose azure at 5%(w /v) (●). Following incubation at 75°C for 15h., each mixture was diluted with 1.3ml water, centrifuged at 7000*g* for 5min, and the absorbance of the supernatant at 595nm was measured.
Each assay was performed in duplicate.

Figure 3.13 Enzyme concentration versus dye release from cellulose azure after a short (4h) incubation.

100µl of each of a range of dilutions prepared from a partially purified cellulolytic component of TP8.T6.3.3.1 culture supernatant was mixed with 100µl of 200mM sodium acetate buffer containing a suspension of 5%(w/v) cellulose azure. Following incubation at 75°C for 4h, 1.3ml of water was added and the mixture was centrifuged at 7000*g* for 5 min. The absorbance of the supernatant was then measured at 595nm.


Dye release from cellulose azure and dyed Sigmacell 50 versus enzyme concn.
Absorbance at 595nm
0.35
0.30
0.25
0.20
0.15
0.10
0.05
0.00
0.0 0.1 0.2 0.3 0.4 0.5 0.6 0.7 0.8 0.9 1.0
Relative enzyme concentration.


Dye release from cellulose azure versus enzyme concn. after a 4-hour incubation
Absorbance at 595nm
0.14
0.12
0.10
0.08
0.06
0.04
0.02
0.00
0.0 0.1 0.2 0.3 0.4 0.5 0.6 0.7 0.8 0.9 1.0
Relative enzyme concentration.

The unit adopted for the dye release avicelase assay was arbitrarily defined as the activity sufficient to cause an increase of 10^{-5} absorbance units per minute, utilizing the above incubation conditions and with the absorbance being measured at 595nm in a 1.3ml final assay volume.

A variety of other dyes can be used to dye crystalline cellulose (Leisola and Linko, 1976). Remazol Brilliant Blue R did not appear appropriate for demonstrating avicelase activity in growing liquid cultures of our anaerobes, since any released dye was decolourised, with only a faint yellow tinge resulting.

3.5.3.3. Hydrolysis of commercial cellulose azure preparations Commercially available "cellulose azure" substrates are various forms of acid-swollen cellulose, dyed with Remazol Brilliant Blue R or a related dye. Being more amorphous, they are more readily hydrolysed than crystalline cellulose, so do not always give a reliable indication of a system's capacity to hydrolyse crystalline cellulose.

Type I Cellulose Azure from Sigma is fluffy and had to be ground to pass through a 60 mesh before it could be pipetted as a slurry. It was then utilized in an identical manner to dyed Sigmacell 50 (see Section 3.5.3.2).

An enzyme dilution curve for the dye release from Type I cellulose azure was found to be linear after a 4-hour incubation (Fig. 3.13) but another dilution series incubated for 14.5 hours (Fig. 3.12) was markedly non-linear. This difference may be due to the more complete hydrolysis of the substrate which occurred in the latter case, and possibly to differences between the two enzyme preparations also.

Dye release from cellulose azure was several times more rapid than from dyed Sigmacell 50 subjected to the same range of cellulose concentrations (Fig. 3.12).

A unit of "cellulose azurase" activity was arbitrarily defined as that which produced an increase of 10^{-5} absorbance units per minute, utilizing the above incubation conditions and with the absorbance being measured at 595nm in a final assay volume of 1.3ml.

Type II Cellulose Azure from Sigma is prepared by a method of acid hydrolysis and reprecipitation based on the

method of Fernley, (1963), and consequently contains considerable quantities of dyed cellodextrins which are soluble in water. These were removed by exhaustive water washing on a Buchner funnel and the substrate could then be used in the same manner as was dyed Sigmacell 50. Alternatively, if the soluble, dyed cellodextrins had been included in the assay mixture, those cellodextrins remaining that contained more than 20-30 anhydroglucose residues could have been precipitated out by stopping the reaction with 2.4M K_2HPO_4 adjusted to pH 9.0 with HCl, followed by centrifugation (Fernley, 1963).

Type II Cellulose Azure should probably be grouped with TNP-CMC as a substrate for endoglucanase assay since Fernley (1963) found evidence that most dye solubilization resulted from random cleavage of cellodextrin chains.

3.5.3.4. Turbidometric methods Comparisons were made between avicelase data obtained from measurement of changes in turbidity due to suspended cellulose and from the measurement of avicelase activity by the phenol/H_2SO_4 assay of total soluble carbohydrates. Enzyme samples with differing avicelase activities were taken for the comparison from 6 supernatants of TP8.T6.3.3.1 cultures. 0.2ml of each enzyme sample and a buffer blank were incubated for 15 hours at 75°C in 1% (w/v) Sigmacell 50 (crystalline cellulose, average particle size 50μm) in citrate/phosphate buffer pH6 (McIlvaine, 1921). The total incubated volume was 1.2ml. After centrifugation, 0.6ml of supernatant was withdrawn for the assay of total solubilized carbohydrate, and 4.2ml of water was then added to the remainder in each tube. This resulted in a 0.21% (w/v) cellulose suspension in the blank. The capped tubes were then shaken vigorously to homogenize the cellulose distribution, immediately inserted into a Spectronic 20 spectrophotomer (Bausch and Lomb) and their absorbances at 660nm were read 5 seconds after shaking was stopped. The A_{660} of each tube was remeasured exactly 7 minutes after the first reading, without any intervening agitation. The tubes were then returned to the 75°C. The two A_{660} values were remeasured by the same method at 6 further intervals over the following 10 days, and finally after 36 days.

A_{660} measured immediately after shaking following a 15 hour incubation correlated well with avicelase activity as measured by total carbohydrate release (r=0.99)(Fig. 3.14a). Note that avicelase activity initially resulted in increased rather than decreased cellulose turbidities when shaken. However, with longer incubations, the correlation coefficient decreased substantially (r=0.76 at 39h), since by that stage turbidities in the tubes containing the more active cellulases had passed through their maxima and were beginning to decrease. Turbidities of some tubes had fallen below that of the blank after 144 hours at 75°C, while others containing less avicelase were still increasing. Therefore, to obtain a good correlation between turbidity and avicelase activity it is necessary to measure all turbidities before the turbidity maximum is reached.

Cellulose particle sizes in the incubations were compared on the basis of the rates at which they settled after shaking. The A_{660} measured exactly 7 minutes after shaking was expressed as a percentage of the A_{660} measured immediately after shaking. There was a strong correlation (r=0.99) between these percentages and the avicelase activities after a 15 hour incubation. However, once again this correlation deteriorated with continued incubation as there appeared to be a common limit to the reduction in particle size attainable by the various concentrations of enzymes. Despite this, an increase in cellulose settling time remained a reliable indicator of cellulase activity throughout the incubation period. A rack of tubes could be quickly scanned for avicelase activity by inverting the tubes simultaneously to resuspend the cellulose and then visually comparing the settling rates.

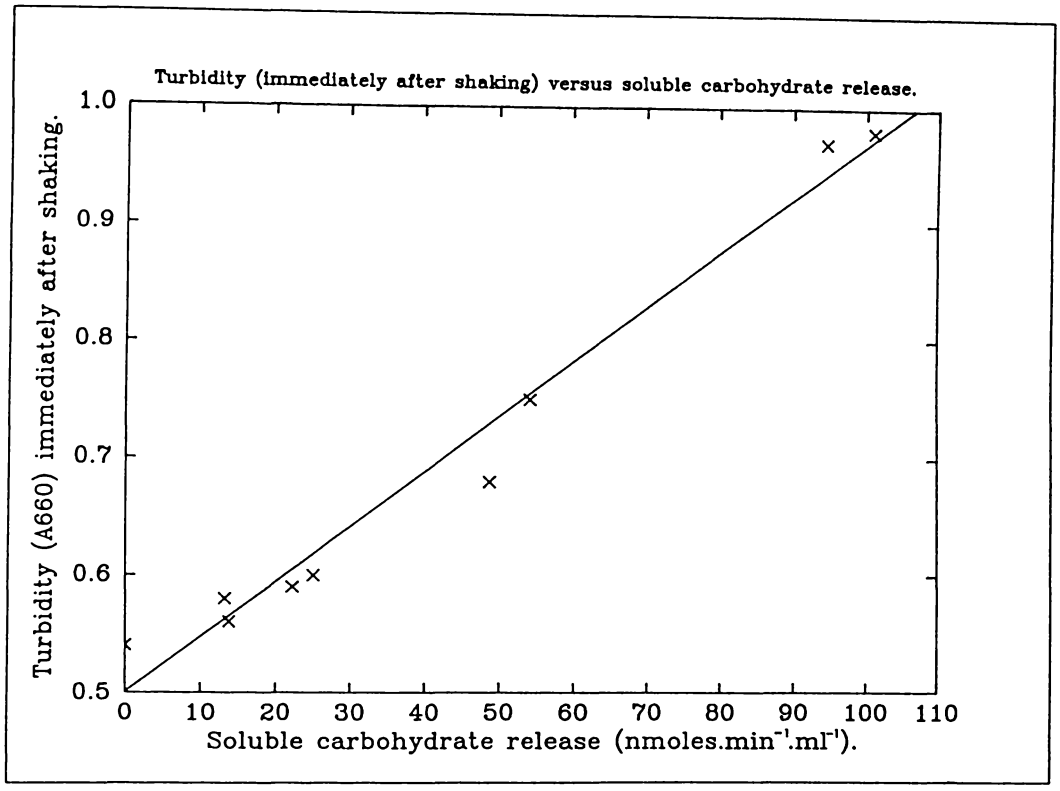
Published turbidimetric cellulase assay methods (Nummi *et al.*, 1981a, Johnson *et al*, 1982) make no mention of any turbidity increases, but rather relate the rate of decrease in cellulose turbidity to cellulase action. This difference can probably be attributed to their use of more finely powdered cellulose. However, the initial increase in turbidity which we observed with the TP8.T6.3.3.1 cellulase might have been a characteristic which was peculiar to this particular complex.

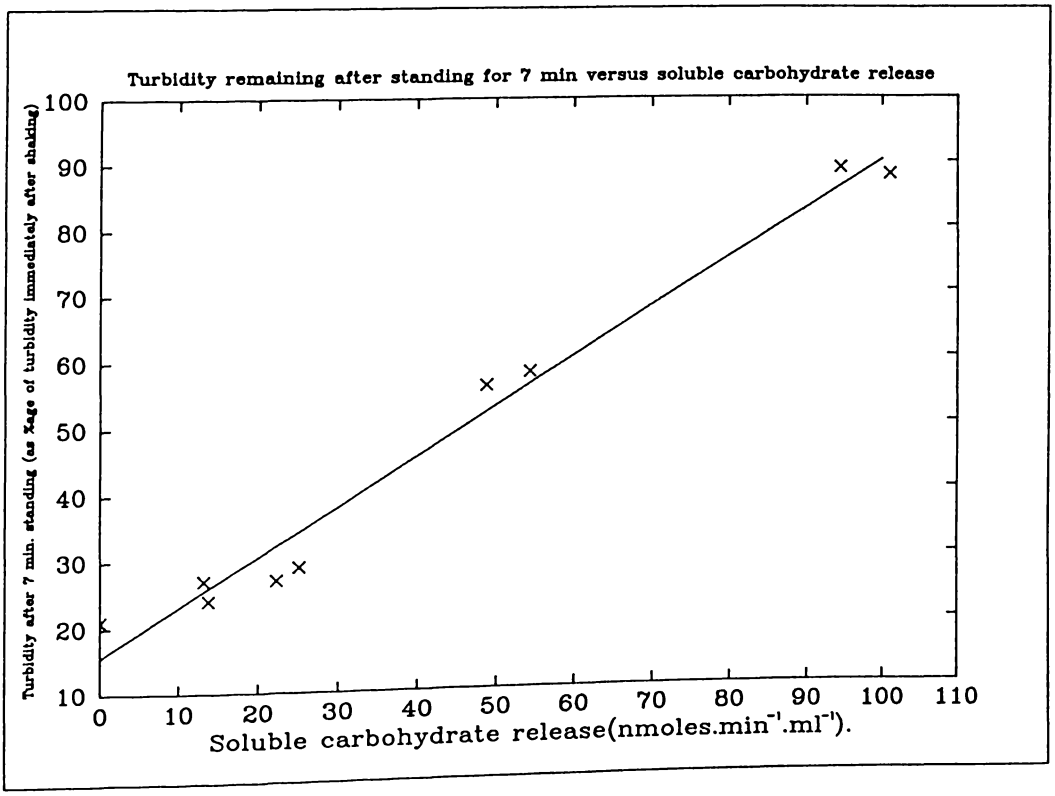
Figure 3.14 Turbidometric versus phenol/sulphuric estimation of avicelase activity in culture supernatants.

Figure 3.14a Turbidity measured immediately after shaking versus avicelase activity assessed from soluble carbohydrate release using the phenol/H_2SO_4 method.

Figure 3.14b Turbidity measured after shaking and allowing to settle for 7 min (as a percentage of the turbidity measured immediately after shaking) versus avicelase activity assessed from soluble carbohydrate release using the phenol/H_2SO_4 method.

All measurements were performed on the same set of tubes after incubation of various culture supernatants for 15h at 75°C in 0.1M citric acid/phosphate pH5.7 buffer (Dawson et al, 1974) containing 0.2%(w/v) Sigmacell 50.


Turbidity (immediately after shaking) versus soluble carbohydrate release.
Turbidity (A660) immediately after shaking.
1.0
0.9
0.8
0.7
0.6
0.5
0 10 20 30 40 50 60 70 80 90 100 110
Soluble carbohydrate release (nmoles.min-1.ml-1).


Turbidity remaining after standing for 7 min versus soluble carbohydrate release
Turbidity after 7 min. standing (as %age of turbidity immediately after shaking)
100
90
80
70
60
50
40
30
20
10
0 10 20 30 40 50 60 70 80 90 100 110
Soluble carbohydrate release(nmoles.min-1.ml-1).

3.5.3.5. The avicelase plate assay 0.1g Sigmacell 20 microcrystalline cellulose (average particle size 20μm) was ground in 5ml water in a preweighed Uniform teflon/glass tissue homogenizer for several minutes. The resulting suspension was left to stand for two hours to allow the larger particles to settle out, and the stable suspension of "fines" was drawn off by pipette for incorporation into the gel. Subsequent dry-weighing of the particles which had settled out revealed that only 20mg of cellulose was present in the suspension of fines, which was added slowly, with stirring, to one litre of a boiling agar solution, buffered at pH 6 (2.415g citric acid, 26.03g trisodium citrate, 15g Oxoid No. 1 agar per litre). After autoclaving and cooling to 60°C, 20ml aliquots were dispensed into petri dishes in the laminar flow hood. The plates were then stored in sealed plastic bags at 4°C until required.

Enzyme samples (20μl) were placed in 4mm diameter wells cut in the agar with a No.1 cork borer connected to an aspirator. The plates were sealed with masking tape and incubated inverted for 18 hours at 75°C. They were then flooded with Congo Red ($1mg.ml^{-1}$) for 60 minutes followed by a 1M NaCl wash for 15 minutes, then a further Congo Red wash for 60 minutes and finally a second 1M NaCl wash for at least 60 minutes. The zones appeared as light pink or yellowish areas as the background changed from orange/red to mauve during the final NaCl wash. Unlike the CMC plate assay (Section 3.5.1.1), acidification to produce a dark purple background was a hindrance to obtaining maximum contrast.

The need for the above sequence of Congo Red and NaCl washes is difficult to understand. If the procedure was simplified by omitting either (i) the initial Congo Red wash, or (ii) the initial NaCl wash, the areas of cellulolytic activity either contrasted poorly against the background (simplification (i)) or were rendered completely invisible (simplification (ii)).

We concluded that the zones of "clearance" which developed in this assay were genuine evidence of some sort of enzyme activity on the basis of the following observations:

(i) Boiling the cellulase (10min at 100°C) produced a

dramatic reduction in zone diameter.

(ii) Buffer or water blanks alone produced no such zones.

(iii) The diameter of "clearance" related directly to the log. of the enzyme concentration.

We have not yet performed comparisons between our "avicelase plate assay" and the generally accepted test-tube avicelase assays. A disturbing feature is that the plate "clearance" is not a complete absence of Congo Red staining, such as occurs in the CMC plate clearing assay, but rather is an area of less intense staining, and of a different hue. It is possible that some enzyme-induced changes to the crystalline cellulose, which may not constitute complete degradation, are nonetheless sufficient to lessen its reaction with Congo Red or to alter the wavelength shift produced when the dye binds with the cellulose. Microscopic examination of the zones of "clearance" should reveal any persistence of cellulose crystals.

It is regrettable that the above method was devised so late in the course of these studies, since it would have been very useful in our initial screening for "avicelase" producing bacteria, and also as a simple means of looking for synergism amongst a large number of different combinations of the various components of the cellulase complex.

We compared the sensitivity of this technique, which used agar containing a mere 0.002%(w/v) crystalline cellulose, with that employed by Creuzet *et al.* (1983). The latter utilized visibly turbid plates containing finely dispersed MN300 amorphous cellulose at the relatively high concentration of 0.1% (w/v). The same enzyme sample which by the latter method produced a clearance of 6mm diameter (4mm occupied by the central well), after an 84 hour incubation at 75°C, gave a "clearance" of 16mm after only an 18 hour incubation at 75°C using our plate assay. In addition to being more sensitive, our assay employs crystalline cellulose as the substrate, which is preferable to the use of MN300 in assessing avicelase activity.

3.5.4 Detection, excision and elution of cellulases in polyacrylamide gels.

The zymogram gel-overlay method of Beguin (1983), which employs the same principle of cellulase detection as does the CMC-plate-clearing assay (Section 3.5.1.1) was modified to improve the sensitivity and to shorten the times necessary for enzyme transfer and incubation. These improvements were achieved through the use of ultra-thin overlay gels cast on plastic backing sheets. Polyacrylamide or agarose were substituted for the agar used by Beguin (1983). Polyacrylamide was found to be the best matrix since it permitted less band-spreading than did agarose (which had larger pore sizes) and was less prone than agarose to separate from the backing plastic during agitation in staining and destaining. Also, agarose gels became permanently marked (visible only when subsequently stained) by localised heating from bars of the oven rack, whereas polyacrylamide did not. Agarose gels tended to change colour in patches during storage, whereas polyacrylamide gels retained an even coloration indefinitely.

3.5.4.1 Polyacrylamide overlay composition To make a gel of dimensions 18cm x 9.5cm x 0.3mm:

1ml acrylamide solution (30% (w/v) acrylamide, 0.8% (w/v) methylene-bis-acrylamide).

4ml 0.15% CMC (medium viscosity sodium salt, Sigma) in 0.05M disodium hydrogen phosphate, 0.0125M citric acid, pH6.3.

The above were mixed, degassed and added to 1ml of saturated riboflavin solution which had been degassed separately. Finally 5µl TEMED was added and mixed in well by swirling the mixture. Methods of gel moulding, pouring and setting were as for the preparation of IEF gels (Section 2.10.3).

3.5.4.2. Agarose overlay gel composition To make a gel of dimensions 18cm x 9.5cm x 0.3mm:

0.115g agarose (Sigma Type 1)

2.5ml water

4.5ml of the same CMC/buffer solution as used in the polyacrylamide overlay (Section 3.5.4.1).

The mould was prepared as for a polyacrylamide IEF gel (Section 2.10.3) except that Gel-Bond agarose-coated plastic

sheeting (Pharmacia) replaced the Gel-Fix, which would not adhere to agarose. The mould was preheated to 75°C.

The gel mixture was heated at 100°C, with constant agitation, until all the agarose had dissolved and was poured while still very hot into the preheated mould, using a pipette that had been preheated to 100°C. The mould was supported at a 45°C angle during pouring and was then placed horizontally while the gel set.

The adherence of the agarose gel to the backing sheet became more dependable if the whole sandwich was cooled, once the gel had set, to 4°C overnight. Separation from the glass plates was performed by the method described in Section 2.10.3 for polyacrylamide gels.

Both agarose and polyacrylamide overlay gels could be stored for at least a week by covering them with a non-stick plastic sheet (Gel-Cover, Pharmacia) and placing them in a humidified box at 4°C. The inclusion of azide in the gel mixtures should allow storage for much longer periods if required.

3.5.4.3 Replication of the polyacrylamide gel and activity staining The same technique was applied to the detection of cellulases on both ultra-thin isoelectric focussing gels and 0.75mm or 1.5mm thick electrophoresis gels. None of the washing to renature or wiping of the original gels, as advocated by Beguin (1983), proved necessary even after SDS electrophoresis. Condensation which collected on the surface of IEF gels rapidly evaporated when they were removed from the cooling plate. This avoided the risk of smudging enzyme bands by any wiping procedure.

Locating spots were marked around the boundaries of the Gel-Fix bearing the IEF gel or on the glass plate backing the electrophoresis gel. The overlay gel was then placed, with a rolling motion so as not to trap air bubbles, onto the surface of the "original" gel, while being held firmly at the edge where contact between the gels was first made. The locating spots were then traced through onto the back of the plastic backing of the overlay and the positions of the gel interface and the marker dye front could also be marked.

Having remained in contact with the enzyme-containing gel

for 10-20 seconds, the overlay was then peeled back in the exact reverse of the application procedure, and was incubated for 3 minutes at 75°C, gel side up. It was not necessary to wrap the gel in plastic film during this short incubation, thus avoiding the risk of smudging enzyme on the gel surface. Longer contact and incubation times generally produced poorer band definition, but were appropriate if well separated bands of relatively low activity were expected.

The overlay was then bathed in Congo Red solution ($1mg.ml^{-1}$) for 10 minutes, with agitation to ensure that all parts of the gel received equal exposure. Next followed a rinse in 1M-NaCl for as long as was required to remove the dye from the bands where the CMC had been hydrolysed. Finally, a 5% (v/v) acetic acid wash served both to improve the contrast of the bands, by turning the undegraded background dark blue, and also to prevent continued enzyme action.

The completed zymogram was available within 20 minutes of making contact between the overlay and the original gel.

The sensitivity and band sharpness of the zymogram method of cellulase detection was equal to that which we were able to achieve using the protein silver stain technique of Merril *et al.*, (1981) (Fig. 6.30 cf. Fig. 6.29). Sensitivity should probably be further improved by lowering the CMC content of the overlay.

3.5.4.4 Use of the zymogram as a template in excision of cellulase bands from polyacrylamide gels The zymogram was stuck upside down under the original gel, with a glass sheet separating them, so that the locating spots were superimposed. The glass sheet was supported at its edges, over a white background, and slivers of polyacrylamide were cut using a fine-tipped scalpel from the original gel at positions directly above the bands of clearance on the zymogram. Incisions were made right through the plastic backing sheet of an IEF gel. Although this made slicing more difficult, particularly when curved bands had to be followed, subsequent handling of the gel slivers was greatly facilitated by the plastic, which was cut so as to extend beyond the edge of the gel for handling with tweezers.

In order to check for possible shrinkage of

electrophoresis gels once the top glass had been removed, the positions of the corners of each gel were marked on the underside of the glass beneath it. No shrinkage was ever detected during the 1 to 2 hours taken to develop the zymogram template and then to carefully excise the active bands.

Diffusion of cellulases in polyacrylamide appeared to be so slow as to be no cause for concern over the time-span involved in the band detection and excision process. An SDS electrophoresis gel was loaded with the same set of samples duplicated on opposite halves of the gel. After running, one half was protein-stained immediately while the other half was subjected to the lengthy (3 hours total) washing and incubation to produce a zymogram by the procedure of Beguin (1983). The latter half-gel was then also stained for protein and produced bands which were virtually identical in sharpness with those of the other half which had been fixed and protein-stained immediately. Clearly there was no need to rush the excision of the gel slices, and due attention could be paid to such details as ensuring to wash the scalpel blade and tweezers between slices. A clear plastic ruler, supported so as not to actually contact the gel surface, was helpful in producing straight and parallel slices. Two further rulers were positioned along each side of gel so the position of each slice removed could be noted relative to some reference point (e.g. the top of the separating gel). In most cases 2-3mm wide strips were cut, since this width was normally necessary to encompass a band of clearance on the zymogram.

Special attention was paid to firstly removing cleanly the gel strips which corresponded to bands of clearance on the zymogram. After firstly excising the bands revealed by the zymogram, the entire remainder of the gel was subsequently sliced and all the slice washings were assayed by a variety of cellulase assay techniques. This was done as a precaution in case cellulases were to be found which had little or no activity towards CMC and which therefore would not be expected to produce bands on the zymogram.

Zymograms could be stored for months if sealed in a plastic bag with a few drops of water. The acetic acid was sufficient to prevent growth of microorganisms. Photography

was most successful if reflected light from a white background rather than transmitted light was used.

3.5.4.5 Elution of enzymes from polyacrylamide gel strips

Enzymes were eluted from the gel strips by immersing them for several hours in 5ml of 0.25M sodium acetate buffer, pH5.7 at 4°C. Typical recoveries of activities of 50% or more showed that little would be gained by fragmenting the gel or by attempting electrophoretic elution.

3.6 H.P.L.C. ANALYSIS OF END PRODUCTS OF CELLULOSE HYDROLYSIS

A Waters Sugar-PAK1 ion exchange column, coupled to a Waters high pressure pump system and a Waters refractometer, were kindly made available for our use at the Forest Research Institute, Rotorua, but unfortunately very near the end of this study.

The column was operated at 92°C and was eluted with deionized water at 0.6ml.min^{-1} at pressures between 14150 and 14900 KPa. The sample volume injected was 20µl.

The refractometer response was calibrated with glucose and cellobiose standards over the range 1.7µg - 18µg. The slower eluting standard (glucose) had emerged completely within 10 minutes of injecting it. However our samples contained an additional component(s) which eluted as a broad peak between 18 and 30 minutes after injection (probably protein). This didn't interfere with the sugar peaks provided at least 40 minutes was allowed between sample injections. No attempt had been made to deproteinate the samples, which may account for this large and unexpected slow moving peak.

CHAPTER FOUR

DISCOVERY, ISOLATION AND PARTIAL CHARACTERIZATION OF EXTREMELY THERMOPHILIC CELLULOLYTIC BACTERIA

4.1 INTRODUCTION

The advent of genetic engineering techniques allowing gene amplification (and hence increased enzyme yield) has allowed the primary aim of bacterial selection programmes to move from the pursuit of improved enzyme yields to instead an emphasis on enzyme properties, since the latter are much more difficult to improve. We selected our cellulolytic strains primarily on the basis of the thermal stabilities of their cellulases. Emphasis was also placed on the production of a "complete" cellulase complex, capable of completely hydrolysing crystalline cellulose. Specific activity, another important enzyme attribute, proved difficult to assess reliably in the growth medium used in the screening programme.

The ability to produce high cellulase levels, though crucial to efficient fermentation systems, was a secondary criterion in the selection programme, since improved yields should be attainable by genetic manipulation. Reliable, rapid growth on the particular media which we tried, plus the capacity to survive repeated subculturing were, however, characteristics of the bacteria which inevitably became involved in our final selection of TP8.T6.3.3.1 as the strain upon which to perform further biochemical studies for this thesis.

4.2 SOURCES OF THE BACTERIA

At the outset of the project we had no indication of the frequency of occurrence (or even proof of the existence) of extremely thermophilic cellulolytic bacteria, so a wide range of types of natural thermal pools were sampled. Initially we sampled 47 sites, ranging in temperature from 48°C - 100°C. These were in the Taupo-Rotorua area, at 4 locations separated by 30-60km i.e. Tikitere, Waimangu, Taupo and Tokaanu.

Samples of pool water were transported back to the lab. in near-full, tightly-capped bottles. Bottom sediments were included where

possible, and in some cases this material contained pieces of decomposing plant material. One sample (from a Taupo site named TP8) consisted of the rotting end of a *Pinus radiata* plank found in a creek fed by some very hot springs upstream. This was brought back to the lab. wrapped in plastic to prevent it drying out, and was to yield the organism TP8.T6.3.3.1 which we eventually selected for biochemical investigation of its cellulase complex.

Mineral analyses were performed on the pool water samples (Table 4.1). There was no obvious connection between finding extremely thermophilic cellulolytic bacteria and mineral composition, ionic strength or temperature of the sites. None of the pools of pH lower than 5.6 yielded cellulolytic bacteria, but this may have been because our enrichment medium was buffered at pH7. Tikitere was noteworthy in that from the 18 locations sampled, ranging in pH from 1.9-6.5, no cellulolytic bacteria were obtained. Nine of these sites were at pH6 or higher. There was, however, much less vegetation around these pools than at other sites.

The main feature common to all the pools which yielded cellulolytic bacteria (Table 4.1) was a high content of organic matter, at least in the samples taken. Some pools contained algal mats (which have a growth maximum of 65°C) that should act as a continuous source of cellulosic materials. The sites sampled were in many cases subject to wide fluctuations in temperature, a feature which would favour the survival of bacteria capable of growth over a wide temperature range.

Some sites were included despite their relatively low water temperatures since they contained cellulosic material which was exposed to flow from a source at a much higher temperature. The range of clearly different extremely thermophilic cellulolytic bacteria obtained from the TP8 wood sample (which was at only 48°C) emphasizes the value of such "in-pool" enrichment.

We attempted an artificial "in-pool" enrichment in which pieces of cotton cloth (a lab. coat) were placed in a bottle left open, submerged and facing upstream towards the source of pool RT8, which is the rather variable outflow from a heat exchanger of a geothermally heated building.

After 3 months, the lab. coat had become covered with grey slime and was visibly breaking down. The full bottle was incubated at 75°C within 3 hours of its removal from the site. The bottle broke upon being incubated at 75°C, probably due to there being insufficient head

Table 4.1: Characteristics of pools which yielded cellulolytic bacteria

Pool	Temp. °C	pH at 25°C	Conductivity µS	Organic Matter	Cl^- mM	SO_4^{2-} mM	PO_4^{3-} µm	HCO_3^- mM	NO_3^- µm	SiO_3^{2-} mM	Na^+ mM	K^+ mM	Ca^{2+} mM	Mg^{2+} mM	NH_4^+ mM
TP10	72	6.8	570	++	1.16	0.07	0.68	0.61	4.64	0.09	1.04	0.07	0.05	0.01	1.12
TP8*	48														
TOK3	76	6.9	8782	+											
TOK4	89	5.6	7050	+++	54.0	1.3	2.6	0.25	11.6	1.6	60.5	7.3	1.2	0.04	0.86
TOK6	97	7.0	4004	+++	34.3	0.4	3.0	0.87	13.6	1.5	32.8	1.2	1.0	0.05	0.70
TOK8	75-80	5.6	8480	+	72.0	0.8	4.0	0.51	86.3	1.7	78.4	3.3	0.9	0.01	0.16
WAI21	82	6.3	5020	+	10.8	2.1	0.4	0.13	3.4	1.5	13.7	1.0	0.5	0.19	0.25
WAI24	70	7.7	5730	+	21.1	1.9	2.9	0.004	10.2	1.9	21.2	1.9	0.9	0.18	0.06
WAI25	67	6.8	3950	+	13.9	1.3	3.3	0.002	18.2	1.9	16.2	0.8	0.8	0.30	0.001
RT8	50-75	8.9	Variable	+++	13.8	0.7	1.9	8.7	-	73.3	25.7	1.0	0.04	0.003	0.01

* TP8 was a wood sample found 0.5 km downstream from site TP10.

space to allow for thermal expansion, and the residual cloth and sludge has almost dried completely before it was discovered and transferred to another bottle containing the normal enrichment medium. Since the fragmented state of the cloth clearly attested to the presence of cellulolytic organisms, a cellulose-agar dilution series was established from the freshly inoculated enrichment medium.

Cultures of extremely thermophilic anaerobes which were suspected of being cellulolytic were supplied to us by other workers in our group for inclusion in the cellulase screening programme. These originated from Mt. Erebus crater, in Antarctica, (E2), from an earlier sampling of Waimangu pools (W1, W2, W4, W5) and from a nearby duck-pond (3WUB).

4.3 ENRICHMENT OF HOT-POOL BACTERIA AND ASSESSMENT OF CELLULOLYTIC ACTIVITIES

Enrichment media for both anaerobes and aerobes was essentially that of Zeikus *et al.* (1979), and consisted of basal medium (Section 2.1) including 1% trypticase peptone, 0.3% yeast extract and either glucose, CMC (insoluble form) or Sigmacell 50 crystalline cellulose (all at 1%). No reductant was included in the aerobic enrichment media.

Pool samples were left sealed for 20 hours at room temperature in near-full bottles. 30ml Universal bottles containing 25ml or 10ml of the enrichment media were inoculated with 5ml of pool water (with any sediments resuspended) so as to produce completely full anaerobic culture bottles and half-full aerobic bottles. The decaying wood from site TP8 was homogenised in the pool water in which it was found and the resulting slurry used as an inoculum. All were incubated at 75°C.

Anaerobic Sigmacell-50 medium was also inoculated with 1ml samples of cultures of E2, W1, W2, W4, W5 and 3WUB (origins listed in Section 4.2). These were cultured at 65°C.

After 6 and 10 days incubation, the enrichment cultures were assessed for cell growth on the basis of visual ranking of the turbidities of unshaken cultures (Section 2). After 10 days, of the 47 anaerobic enrichments of pool water samples on each carbon source, 31, 32 and 29 of the glucose, CMC and crystalline cellulose-containing bottles had produced growth. Aerobic growth occurred in 28 of the 47 glucose-containing enrichments, but only 5 of each of the CMC and cellulose-containing aerobic sets showed growth.

Enrichment cultures which had shown growth after 14 days in the crystalline cellulose medium were subcultured into more of the same medium. After 6, 14 and 21 days, the cultures of this second enrichment

series were assessed for growth, and CMCase activity and reducing sugar accumulation were measured in the cell-free supernatants. This data for the nine cultures which had definite CMCase activity is displayed in Table 4.2.

Definite growth occurred in many of the cultures without CMCase activity. This was presumably through utilization of the 1% trypticase peptone and 0.3% yeast extract as carbon sources.

Accumulation of reducing sugars in the medium was only detected in three of the cultures (WAI21, TP8 and TP10), which also possessed the highest CMCase activities at days 14 and 21. Such sugar accumulation is a clear indication of the capacity to hydrolyse crystalline cellulose. The non-occurrence of sugar accumulation may, however, simply reflect efficient consumption of cellulose hydrolysis products by the cellulolytic organism, or possibly by other organisms in a mixed culture. Cellulose consumption was visible only in Wai21, TP8 and TP10.

Only one of the aerobic enrichment cultures containing crystalline cellulose (TOK6) showed CMCase activity when assayed after 22 days' growth. Unfortunately the cellulolytic organism could not be subcultured from this culture, nor from the glucose or CMC-containing enrichments.

Amongst the anaerobes, only those second enrichments which had produced CMCase activity were subcultured and incubated at 70°C, 75°C and 80°C on crystalline cellulose medium. Very similar levels of growth and CMCase production resulted at 70°C and 75°C, but no definite indication of growth was obtained at 80°C.

Culture 3WUB, originating from bottom sediments of a duck-pond, was the only one of the cultures supplied to us which was clearly cellulolytic when grown in our enrichment medium. Hence only 3WUB was included in the isolation programme. However the cellulolytic component was not extremely thermophilic, growing well at 65°C but very slowly at 70°C.

4.4 ISOLATION OF CELLULOLYTIC BACTERIA AND ASSESSMENT OF THEIR CELLULASES

4.4.1 Isolation of bacteria from single colonies

All but one of the cellulolytic enrichment cultures were used to inoculate cellulose-agar dilution series, as described in Section 2.2. TOK3 had failed to subculture and could not be retrieved. All the dilution series established from other cultures produced visible colonies within 8 to 14 days at 70°C, but only

Table 4.2: First analyses of cellulolytic activity obtained from cultures in the initial screening programme

Source	WAI21	WAI24	WAI25	TOK3	TOK4	TOK6 #	TOK8	TP8	TP10
Initial analysis at day 6									
(i) CMCase* (Incubated 60°C, 30 min)	104	31	65	39	58	ND	41	89	15
Analysis at day 21									
(i) CMCase* (Incubated 60°C, 30 min)	218	36	115	64	111	ND	60	151	71
CMCase* (Incubated 75°C, 30 min)	273	36	125	77	134	62	69	222	152
(ii) Accumulated reducing sugar (mM)	5.7	0	0	0	0	5.4	0	28.3	4.8
-as % (w/w) of initial cellulose	9.5	0	0	0	0	9.0	0	42.2	8.0
(iii) Visible cellulose loss	+	-	-	-	-	-	-	++	+
Maximum growth**	6	2	6	1	2	4	6	8	4

* Units = $nmol.min^{-1}.ml^{-1}$ of culture supernatant
** For scale see Section 2.4

TOK6 was the sole aerobic culture showing cellulolytic activity

TP10 yielded colonies which cleared surrounding MN300 cellulose.

Colonies were cut individually from the agar as described in Section 2.2, and used to inoculate liquid anaerobe basal medium supplemented with 1% crystalline cellulose, 1% trypticase peptone and 0.3% yeast extract.

4.4.2 CMCase and ß-glucosidase production by the isolates

After 20 days' growth on the above medium, the isolates were assayed for CMCase and ß-glucosidase activities. Table 4.3 lists the isolates which produced the most CMCase, and includes growth and levels of ß-glucosidase and reducing sugars in the growth media.

The isolates showed considerable variation in their ß-glucosidase: CMCase ratios, and also in the extent to which the ß-glucosidase was cell-associated. The WAI21 isolates, which possessed the most total ß-glucosidase activity, were found to have 97-98% of this activity cell-bound. This figure will be dependent on the stage of growth since eventual cell lysis will presumably liberate previously cell-bound enzymes. All of the cultures were studied after 3 weeks of growth at 75°C, but the extent of cell lysis was probably not uniform. Nonetheless, total ß-glucosidase levels were clearly lowest in the TOK4 isolates, and their non-accumulation of reducing sugars when grown on cellulose singled them out as being unlikely to possess a complete cellulase complex.

TP10 isolates were clearly in a class above the other isolates with regards to reducing sugar accumulation and CMCase levels (Table 4.3).

Each of the 4 or 5 colonies cut from TOK4, TOK8, WAI21 and TP10 agars gave rise to cultures with CMCase activity. In complete contrast, none of the 4 colonies cut from agars of WAI25 or TP8 enrichments produced CMCase in liquid medium, although growth clearly occurred. The cellulolytic organism(s) of WAI25 were not pursued any further, but since enrichment cultures of TP8 had shown the most substantial visible degradation of cellulose and the greatest reducing sugar accumulation (Table 4.2), a further two dilution series were set up in agar medium containing 1% cellulose (MN300) with and without 0.2% cellobiose. One very large colony (TP8T) was found to be clearing surrounding cellulose and was readily isolated and subcultured successfully to produce cellulolytic cultures. None of the other 8 colonies, which were

Table 4.3: CMCase and β-glucosidase activities in the main bacterial strains originally isolated

Isolate	Free reducing sugar (mM)	CMCase	β-glucosidase	β-glucosidase / CMCase	% Soluble β-glucosidase	Maximum growth
TP10.A.3	17.1	169	82	0.49	59	7
TP10.B.1	14.3	235	68	0.29	31	6
TP10.B.2	14.6	277	66	0.24	36	7
TOK8.1	1.8	76	22	0.29	15	4
TOK8.3	1.7	55	47	0.81	11	5
TOK4.3	0	77	16	0.21	43	3
TOK4.4	0	48	7	0.14	0	3
WAI21.3	2.0	66	103	1.57	3	5
WAI21.3	1.9	63	86	1.32	2	5

All these enzyme assays were at week 3 of growth, and incubated at 75°C for 30 minutes. Total β-glucosidase was assayed using the complete culture whereas soluble β-glucosidase and CMCase were assayed using cell-free culture supernatants. Units are $nmol.min^{-1}.ml^{-1}$.

selected on the basis of slight clearance of surrounding cellulose, produced cellulolytic liquid cultures. Details of the continued TP8 isolation programme are given in Section 4.4.8.

The lab. coat "in-pool" enrichment from pool RT8 (Section 4.2) was used to prepare an agar dilution series. Seven of the 12 colonies cut from this series gave rise to CMCase producers (Table 4.4). Four of them (LC7, 8, 9 and 10) were found to accumulate reducing sugars, indicating avicelase activity, and had CMCase activities on a par with TP8T. LC8 had converted over 60% of the 1% (w/v) cellulose supplied to reducing sugars within 50 days. Unfortunately the most cellulolytic LC isolates failed to subculture, and only LC4 and LC12 remained viable.

3WUB, the duck pond culture, was treated similarly but incubations were at 65°C. A variety of colony morphologies resulted, but none produced clearing of the cellulose nor gave rise to a cellulolytic liquid culture. However high levels of CMCase continued to be produced in subcultures of the parent mixed culture (grown at 65°C).

4.4.3 The effects of various modified media on growth and accumulation of reducing sugars and CMCase by TP10 isolates

In the early stages of our screening and comparison programme it was important to maintain high selective pressures in favour of cellulolytic organisms, particularly since most of the so-called isolates had at that stage only been through a single isolation step and were most probably not pure cultures. We were concerned that yeast extract and trypticase peptone (added to enrichment media to promote rapid growth of the very few cells from the pool water inocula, as advocated by Zeikus *et al.*, 1979) could have been acting as significant alternative sources of carbon to the cellulose or cellobiose, so an experiment was performed, using the TP10 isolates, to determine the importance of these additives in their cellulase production and growth (Table 4.5).

Removal of the 1% trypticase peptone (TP) resulted in lower CMCase and reducing sugar (RS) accumulation, poorer growth and a longer lag phase (medium 1 cf. medium 2).

Removal of both the 0.3% yeast extract (YE) and the 1% TP further slowed growth (medium 2 cf. medium 3, and medium 4 cf. medium 5), but there was no general effect on the CMCase levels. Reducing sugar accumulation as a result of cellulose

Table 4.4: CMCase and reducing sugar levels in the LC-series isolated from pool RT8 in-pool enrichment

Isolate No.	CMCase (nmol. $min^{-1}.ml^{-1}$)	Reducing sugar (mM glucose equivalents)
1	5	0.0
2	0	0.0
3	0	0.0
4	33	2.8
6	9	0.0
7	44	22
8	109	34
9	68	37
10	87	25
11	22	-*
12	19	-*

* LC-11 and LC-12 were inoculated into 0.2% cellobiose-containing cellulose medium and accumulated no reducing sugars.
All assays were performed at day 24 of growth. LC-5 failed to grow.

Table 4.5: The effects of various additions to the basal medium on growth and CMCase and reducing sugar production by TP10 isolates

Medium No.		1	2	3	4	5	6
Trypticase Peptone	1%	+					
Yeast Extract	0.3%	+	+		+		
Sigmacell 50	1%	+	+	+	+	+	
Cellobiose	0.2%				+	+	+
TP10.B.1	Growth	4.5	2.5	1.5	3.0	2.0	4.0
	CMCase	179	109	113	79	84	90
	ΔRS(mM)	+15	+12	+5	0	-2	-5
TP10.B.2	Growth	4.5	2.5	1.5	3.0	3.0	4.0
	CMCase	139	149	92	80	58	75
	ΔRS(mM)	+17	+9	+4	-2	-3	-5
TP10.A.3	Growth	7.0	1.5	3.5	4.0	1.5	6.0
	CMCase	147	77	77	80	60	106
	ΔRS(mM)	+15	+13	+6	-3	+1	-4

All media were inoculated with a 1% inoculum. Growth was monitored by a visual assessment of unshaken turbidity and the extent of flocculation (Section 2.3). Values shown were obtained after 30 days' growth. Units of CMCase are $nmol.min^{-1}.ml^{-1}$. ΔRS(mM) is the millimolar change in reducing sugars (glucose equivalents) in the growth medium compared with uninoculated blanks.

hydrolysis was clearly reduced still further by removal of the YE (medium 2 cf. medium 3), but nonetheless the fact that this accumulation was not prevented entirely in medium 3 clearly indicates that neither TP nor YE is indispensable for production of a "complete" cellulase complex.

Rapid and substantial growth and moderate levels of CMCase production occurred in medium 6, in which cellobiose was the only carbon source.

Cellobiose didn't prevent the production of a "complete" cellulase complex, as evidenced by the reduced consumption of cellobiose in media 4 and 5 (containing both cellulose and cellobiose) cf. medium 6 (in which growth was on cellobiose only). An increase in reducing sugar levels above those of the medium blank occurred in TP10A3 in medium 5, showing that release of sugars from cellulose more than equalled consumption of cellobiose supplied in the medium.

Substitution of cellobiose for yeast extract (medium 5 cf. medium 2) shortened the lag phase substantially (early growth data not shown) but caused some reduction in the CMCase levels finally achieved. This reduction in CMCase appeared to be due to the addition of cellobiose since CMCase levels did not return to the levels of medium 2 when yeast extract was added. This was in keeping with the findings of Section 4.4.6.

A general conclusion from the above experiment was that TP and YE improved growth and cellulase production by the TP10 isolates, and that growth on cellulose in the complete absence of these supplements was so slow as to be impractical for our purposes.

Rapid, reliable subculturing was vital for the maintenance of our cultures at that stage, since we were without a freeze-drier and no reliable alternative means of preserving our organisms had been found. Cellobiose was shown to promote, in particular, the rapid growth of organisms which did not possess "complete" cellulase complexes (Sections 4.4.5 and 4.4.6). Therefore we chose to continue our routine maintenance subculturing on cellulose (medium 2 of Table 4.5) in order to retain as many of the "complete" cellulase producers as possible. We retained the yeast extract (0.3%) but dispensed with the trypticase peptone as a compromise between the rapid, rather unselective growth conditions of medium 1 and the highly selective but slow growth environment of

medium 3.

Clostridium thermocellum is generally grown on media containing much higher levels of Mg^{2+} and Ca^{2+}, e.g. the GS-2 medium of Johnson *et al.*, (1981). We tried growing our best cellulolytic isolates and *C. thermocellum* on the GS-2 medium, modified so that yeast extract and cellobiose were lowered, at 0.3% and 0.2% respectively. *C. thermocellum* grew extremely well (at 60°C) but most of our strains grew poorly if at all, and sheet-like crystals tended to precipitate. TP8.T, TP10B.2 and TP10A.3 grew moderately, but none grew as well as in medium 2.

Wide variations in medium composition are reputed to have little effect on mesophilic anaerobic cellulolytic bacteria (Hungate, 1950). It seems from our results that the mineral composition of the medium may have a more pronounced effect on extreme thermophiles, in spite of the wide variation in pool composition (Table 4.1).

The only literature reference to growth media of extremely thermophilic cellulolytic anaerobes (Ljungdahl *et al.*, 1981) states that sulphate ions (0.1M) promote growth. We have not yet tested this on our isolates.

pH decrease is suspected of being a growth-limiting factor in the case of some of our isolates (see Table 4.6). Delaying or preventing the pH decrease by increased buffering (e.g. 1% MOPS (Johnson *et al.*, 1981)) or the addition of solid $CaCO_3$ (Guiliano *et al.*, 1983) has been shown to prolong culture life and improve cellulase levels of *Clostridium thermocellum*.

4.4.4 Accumulation of CMCase and reducing sugars during culture growth

The most promising isolates, plus the TP8 and 3WUB mixed cultures, were compared with *Clostridium thermocellum* with regards to rates of growth and accumulation of CMCase and reducing sugars (Fig. 4.1).

CMCase activity generally appeared in the medium during mid-exponential and stationary phases, and in most cultures reached a maximum by day 14. *C. thermocellum* produced CMCase activities which were 3-5 times higher than those of the other cultures, despite the use of a 75°C incubation for 30 minutes as part of the assay procedure, which would have favoured our more stable enzymes.

Substantial loss of CMCase activity occurred after day 14 in

Table 4.6: Avicelase activities and pH's of the culture supernatants after 9-days' growth of a selection of the most promising isolates and mixed cultures

	Clostridium thermocellum	3WU (mixed)	LC4 (isolate)	TOK8.1 (isolate)	WAI21.3 (isolate)	TP10.B.1 (isolate)	TP8T (isolate)	TP8 A (mixed)	TP8 B (mixed)	TP8 C (mixed)
Avicelase*	2.52	0	0	0	0	0.47	0.36	0.47	0.32	0.61
pH	5.8	6.7	6.7	6.7	6.7	5.4	5.9	5.2	5.5	5.2

See caption to Fig. 4.1 for growth conditions

* Units of avicelase = $nmols.min^{-1}.ml^{-1}$

Figure 4.1: Accumulation of CMCase and reducing sugars during growth of various isolates and mixed cultures.

6ml inocula were added to 300ml bottles filled with basal medium supplemented with 0.3%(w/v) yeast extract and 1%(w/v) crystalline cellulose and reduced with 0.05% sodium thioglycolate (pH 6.8 after autoclaving).

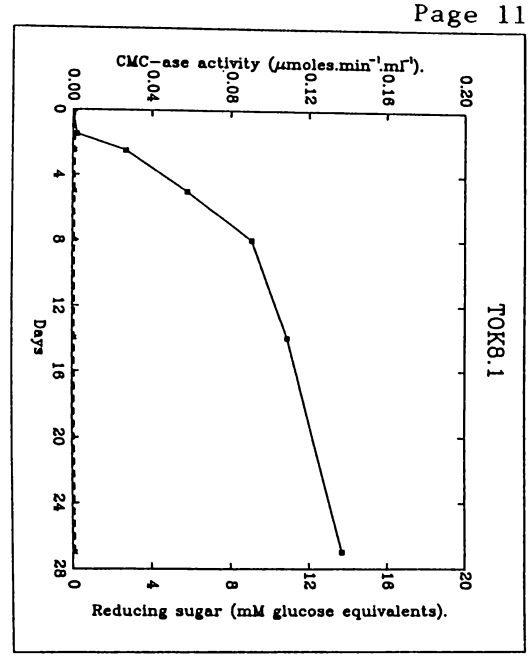
C. thermocellum was incubated at 60°C, 3WU at 70°C and all others at 75°C.

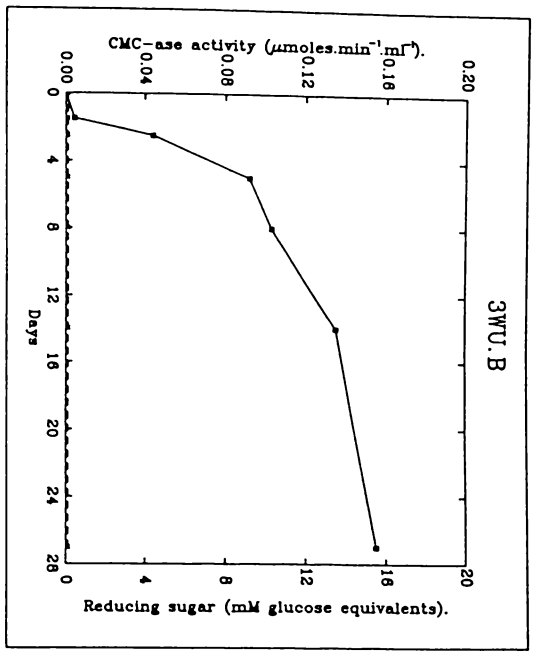
Representative 5ml portions were removed at the times shown, centrifuged at 27000*g* for 10 minutes, and reducing sugar levels and CMCase activities at 75°C (30 min incubation) were measured in the supernatants by the DNSA method. Note the use of different scales in the case of *C. thermocellum*.

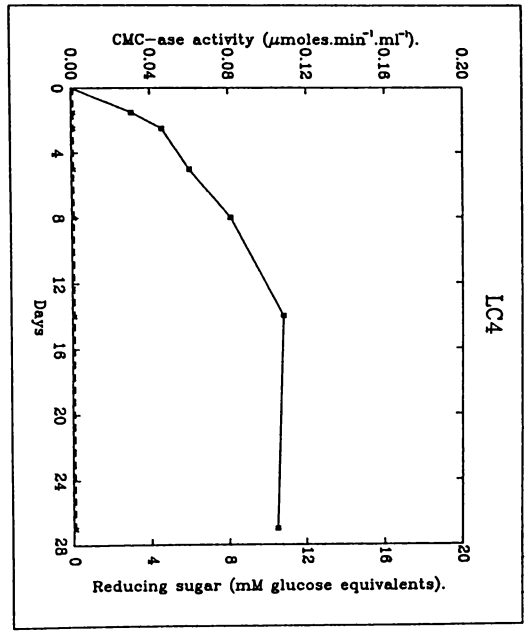
Avicelase activity and pH were measured in the supernatants at day 9 only (Table 4.6).

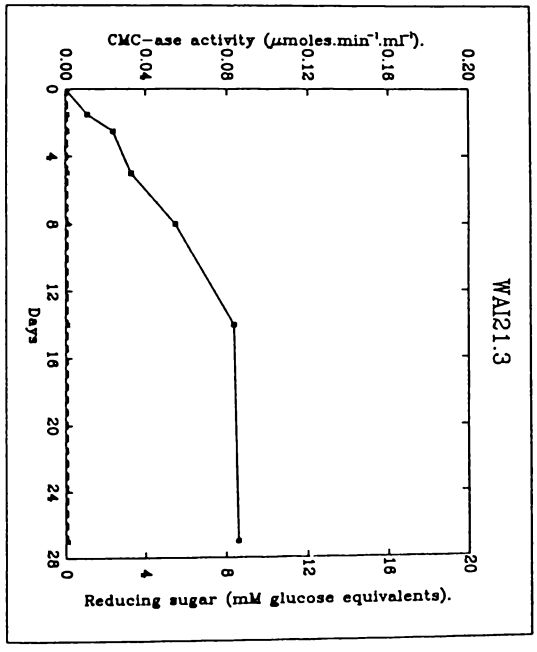
□ = CMCase activity

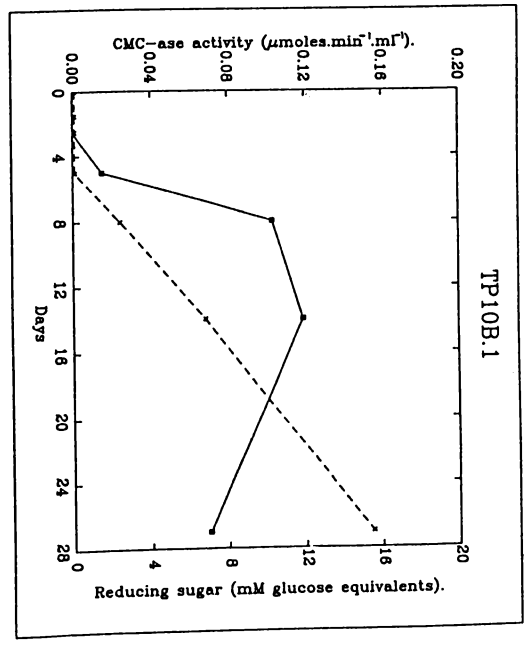
× = reducing sugars

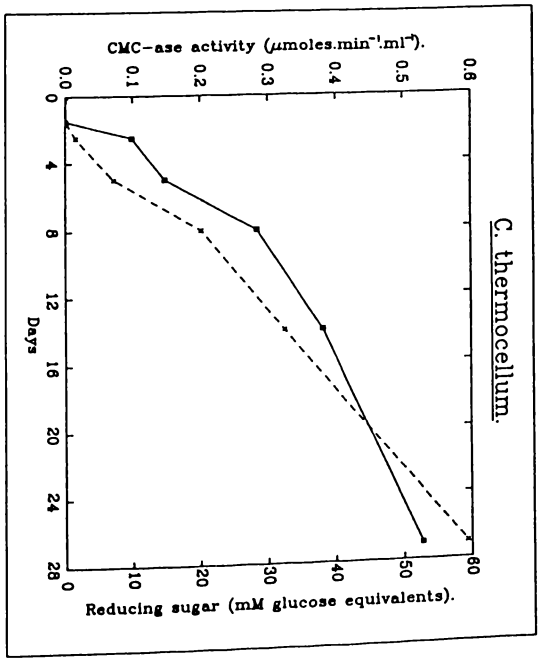

CMC-ase activity (μmoles.min⁻¹.ml⁻¹).
0.00 0.04 0.08 0.12 0.16 0.20
Days
0 4 8 12 16 20 24 28
Reducing sugar (mM glucose equivalents).
0 4 8 12 16 20
TOK8.1

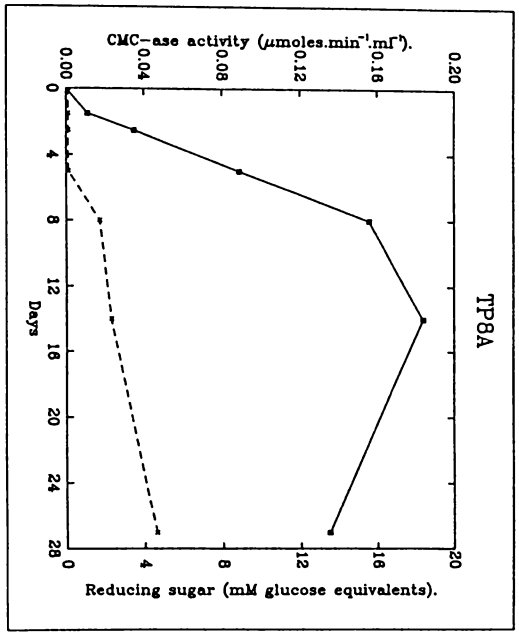

CMC-ase activity (μmoles.min⁻¹.ml⁻¹).
0.00 0.04 0.08 0.12 0.16 0.20
Days
0 4 8 12 16 20 24 28
Reducing sugar (mM glucose equivalents).
0 4 8 12 16 20
3WU.B

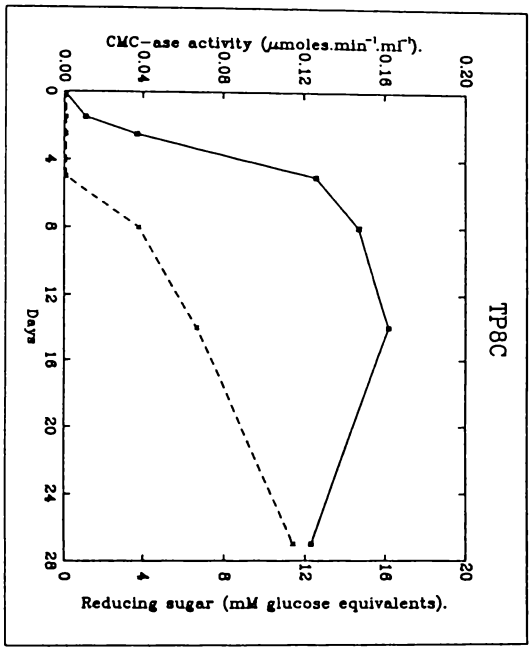

CMC-ase activity (μmoles.min⁻¹.ml⁻¹).
0.00 0.04 0.08 0.12 0.16 0.20
Days
0 4 8 12 16 20 24 28
Reducing sugar (mM glucose equivalents).
0 4 8 12 16 20
LC4

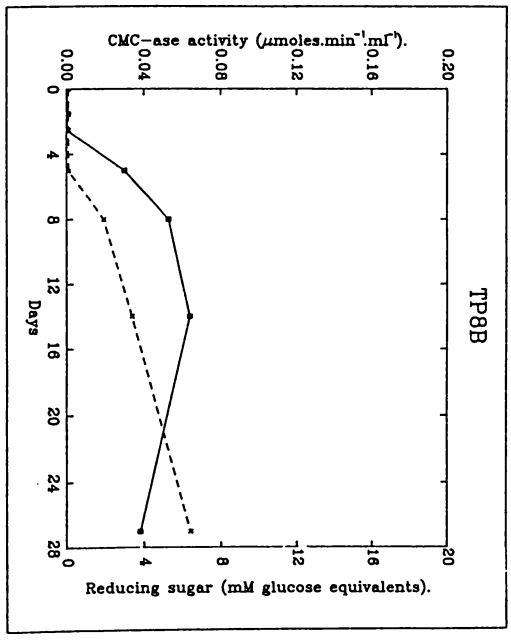

CMC-ase activity (μmoles.min⁻¹.ml⁻¹).
0.00 0.04 0.08 0.12 0.16 0.20
Days
0 4 8 12 16 20 24 28
Reducing sugar (mM glucose equivalents).
0 4 8 12 16 20
WAI21.3

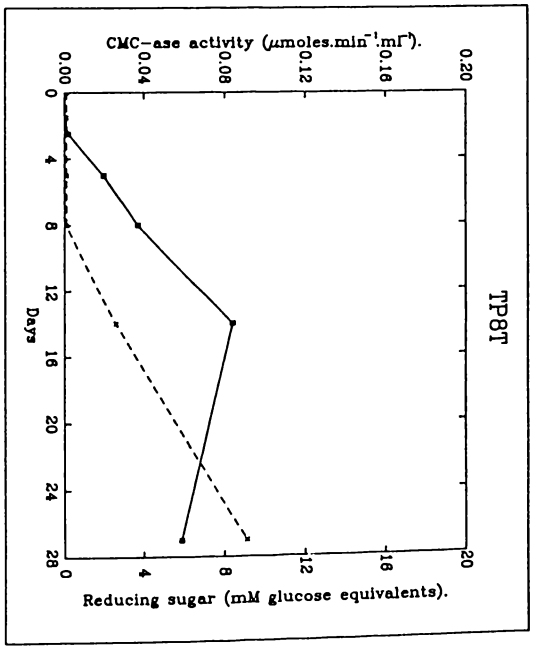

CMC-ase activity (μmoles.min⁻¹.ml⁻¹).
0.00 0.04 0.08 0.12 0.16 0.20
Days
0 4 8 12 16 20 24 28
Reducing sugar (mM glucose equivalents).
0 4 8 12 16 20
TP10B.1


CMC-ase activity (μmoles.min⁻¹.ml⁻¹).
0.0 0.1 0.2 0.3 0.4 0.5 0.6
Days
0 4 8 12 16 20 24 28
Reducing sugar (mM glucose equivalents).
0 10 20 30 40 50 60
C. thermocellum.


CMC-ase activity (μmoles.min⁻¹.ml⁻¹).
0.00 0.04 0.08 0.12 0.16 0.20
0 4 8 12 16 20 24 28
Days
0 4 8 12 16 20
Reducing sugar (mM glucose equivalents).
TP8A


CMC-ase activity (μmoles.min⁻¹.ml⁻¹).
0.00 0.04 0.08 0.12 0.16 0.20
0 4 8 12 16 20 24 28
Days
0 4 8 12 16 20
Reducing sugar (mM glucose equivalents).
TP8C


CMC-ase activity (μmoles.min⁻¹.ml⁻¹).
0.00 0.04 0.08 0.12 0.16 0.20
0 4 8 12 16 20 24 28
Days
0 4 8 12 16 20
Reducing sugar (mM glucose equivalents).
TP8B


CMC-ase activity (μmoles.min⁻¹.ml⁻¹).
0.00 0.04 0.08 0.12 0.16 0.20
0 4 8 12 16 20 24 28
Days
0 4 8 12 16 20
Reducing sugar (mM glucose equivalents).
TP8T

the four TP8 cultures and TP10B1. No proteolytic activity was found by the casein-agar plate assay (Cowan and Daniel, 1982b) in the 2-day-old culture supernatants, so the above decreases in CMCase levels can probably be attributed to enzyme instability at 75°C. The CMCases of TOK8.1 and WAI21.3, which showed no decline by day 27, despite declining cell numbers measured on a turbidity basis, were thus likely to be the most stable. This was later confirmed (Section 4.4.7). Levels of CMCase activity continued to increase in both 3WUB and *C. thermocellum* up to day 27, but this can't be taken as indicative of exceptional CMCase stabilities since they were subject to lower growth temperatures (70°C and 60°C respectively).

Avicelase activity in the 9-day-old *C. thermocellum* culture was 4-fold higher than that of the best of our cultures (Table 4.6) despite having been considerably disadvantaged by the assay conditions (14 hour incubation at 75°C). Of the strains included in this experiment, only those derived from TP8 and TP10.B1 possessed avicelase activity.

The pH of the culture medium after 9 days' growth (Table 4.5) also divided the cultures into two groups. One group, comprising the four which didn't produce avicelase, had maintained a medium pH that was very near that of uninoculated medium after autoclaving and an identical incubation (i.e. pH 6.8). The other group, which all possessed "complete" cellulase complexes, caused the medium pH to fall below six.

Reducing sugar accumulation began earliest in *C. thermocellum*, despite it having the longest lag phase and reached a final level almost four-times greater than that of TP10.B1, our best accumulator of reducing sugars (Fig. 4.1). Cultures which produced no accumulation of reducing sugars were also devoid of avicelase activity at day 9 (i.e. WAI21.3, TOK8.1, 3WUB and LC4).

Cell growth was very rapid and substantial in 3WUB and LC4, but both exhibited an early decline in turbidity. Similar early declines occurred in TOK8.1 and WAI21.3, which were also the slowest growing cultures. Growth of these short-lived cultures may have been supported largely by the 0.3% yeast extract. In contrast, turbidities of the other cultures (i.e. those which produced avicelase) continued to increase for a longer period and finally exceeded those of the faster-growing cultures.

C. thermocellum was the exception, reaching exceptionally high turbidity at day 5 and then the turbidity fell, possibly due to cell lysis or to settling of the cells making them difficult to distinguish from cellulose.

The yellow pigment characterising *C. thermocellum* growth on cellulose (Ljungdahl *et al.*, 1983) was peculiar to *C. thermocellum* and no obvious coloured equivalent was produced by any of the other cultures.

Cellulose degradation was virtually complete in the *C. thermocellum* culture after 27 days, whereas TP10B1 appeared to have consumed only ca. 30%, and the other cultures had made even smaller inroads into their cellulose layers.

The three TP8 mixed cultures were included in this comparison since their differing culture conditions appeared to have produced populations of diverging characteristics (Fig. 4.5 and Section 4.4.8.3). TP8B mixed culture produced very similar patterns of CMCase and reducing sugar release during growth to those of the TP8T isolate. TP8A and TP8C mixed cultures both produced higher levels of CMCase and avicelase activity, and lower medium pH's than the other TP8-derived cultures. Since it seemed likely that a better cellulolytic organism(s) than TP8T remained in the TP8 mixed cultures, efforts were continued to isolate it (them) (Figure 4.5 and Section 4.4.8.6).

4.4.5 Volatile end-products.

Gas chromatography was performed on cell-free supernatants of 2-week-old cultures of most of the cellulolytic isolates, plus a few mixed cultures. Provided the influences of the medium and the phase of growth were recognized, the ratios of the various volatile end-products showed considerable potential as a means of categorizing the cultures. On this basis they fell into 3 broad categories, each with sub-categories:

(i) those which produced approximately equal amounts of ethanol and acetate. These were:
   (a) all the TP8-derived cultures, including all isolates of Fig. 4.5.
   (b) all the TP10-derived isolates
   (c) LC9, LC11, LC12.

(ii) those which produced predominantly acetate. These were:
   (a) WAI21.3, TOK8.3, TOK4.4

(b) LC4, LC6, LC8, LC10.

(iii) those which produced several times more ethanol than acetate. These were:

(a) 3WUB mixed culture

(b) *C. thermocellum*

The basis for the divisions into subcategories will now be discussed. Types (ia) and (ib) were distinguished by the effects of cellobiose on the ethanol to acetate ratio. If added to a cellulose-containing medium, 0.2% (w/v) cellobiose lowered the ethanol: acetate ratio for type (ia) cultures whereas it raised the ethanol: acetate ratio for type (ib). All the TP8-derived cellulolytic isolates were type (ia),the only difference noticed amongst them being a markedly higher ethanol:acetate ratio in each of the TP8A.10.1, A.10.2 and A.10.3 isolates than in any of the others. This difference was not apparent at 9 days, but appeared after 15 days growth.

Type (ic) isolates produced substantial unidentified peaks with longer retention times than that of acetate whereas (ia) and (ib) did not.

Type (iia) organisms produced acetate as their major volatile product, provided cellobiose was supplied (0.2% w/v). When grown on basal medium and yeast extract plus cellulose, growth was slight and no significant volatile end-products accumulated.

Type (iib) organisms differed from type (iia) in two respects. Firstly they produced more acetate when grown on cellulose in the absence of cellobiose than when the latter was included. Secondly, they produced several significant peaks with retention times longer than that of acetate. In the LC6 culture, one of these unidentified peaks was the major end-product.

Type(iiia), i.e. 3WUB mixed culture, was similar to type (iia) in that it produced only traces of ethanol and acetate when grown on cellulose in the absence of cellobiose. In contrast, when 0.2% cellobiose was present, ethanol was the major product, and after 2 weeks' growth would accumulate to 10mM, which exceeded the highest concentration which we obtained with *C. thermocellum* grown on the same medium. The ethanol:acetate ratio was slightly higher than that of *C. thermocellum* growing on cellobiose.

Type (iiib), i.e. *C. thermocellum*, produced high ethanol:acetate ratios both with and without added cellobiose,

although a higher ethanol:acetate ratio resulted when cellobiose was supplied. It also differed from type (iiia) in accumulating more volatile products from cellulose in the absence of cellobiose than when it was added.

A high ratio of ethanol to acetate is generally considered desirable, although not vital, if a specialist fermenting organism is co-cultured with the cellulolytic organism. Unfortunately the only culture in the screening programme to compare favourably with *C. thermocellum* in this respect was 3WUB, which was a mixed culture from which the cellulolytic component was never isolated. Other significant demerits, such as a 70°C growth maximum, poor thermal stability of the CMCase activity and a lack of avicelase activity disuaded us from persisting in trying to obtain cellulolytic single colony isolates from it. The fermenting capacity and CMCase activity could well have proven to be attributes of different organisms in any case.

4.4.6 The effects of cellobiose on CMCase production

All of the isolates grew rapidly on media containing 0.2% cellobiose as a substitute for cellulose, but in general the addition of 1% crystalline cellulose prolonged culture life.

The effects of added cellobiose (0.2%) on CMCase production in medium containing 1% cellulose are shown in Table 4.7. For all our strains except TOK8.1, LC4 and 3WUB, the presence of 0.2% cellobiose reduced CMCase levels by around 50%. A more marked CMCase reduction (by about 90%) was seen in 3WUB, which also showed the greatest reduction in the level of reducing sugars and the highest ethanol production in the cellobiose-containing medium (data not shown). This clearly indicated good growth of 3WUB on the cellobiose-supplemented medium.

Growth was improved in all cases by the addition of cellobiose, so reduction of CMCase levels probably indicates repression of CMCase synthesis by cellobiose.

Exceptions were strains TOK8.1 and LC4, which had their CMCase levels boosted by the presence of 0.2% cellobiose. This may be a useful feature in applications in which cellobiose accumulation is expected, and also demonstrates TOK8.1 and TOK8.3 to be different strains. Levels of CMCase production by WAI21.3 and *C. thermocellum* were virtually unaffected by the 0.2% cellobiose.

It is quite possible that cellobiose initially present was

Table 4.7: The effect of a cellobiose supplement on CMCase and reducing sugar production by bacteria growing on crystalline cellose

Strain	CMCase (nmol.min^{-1}.ml^{-1})			Change in reducing sugar concentration relative to medium blanks (mM)	
	cellulose -only medium	cellulose + 0.2% cellobiose medium	+ cellobiase -cellobiose	cellulose -only medium	cellulose + 0.2% cellobiose medium
TOK4.4	126	64	0.51	0	-4.3
TOK8.1	123	164	1.33	0	-4.5
TOK8.3	92	52	0.57	0	-3.1
WAI21.3	50	47	0.94	0	-2.4
LC.4	70	134	1.91	0	-4.6
TP10.B.1	80	43	0.54	+ 8.7	-3.0
TP10.B.2	85	49	0.58	+ 4.8	-2.3
TP10.A.3	67	27	0.40	+ 6.7	-3.5
TP8.T	64	37	0.58	+10.8	-2.1
TP8 mixed culture A	135	53	0.39	+ 0.1	-7.5
TP8 mixed culture B	64	41	0.64	+ 5.4	-2.4
TP8 mixed culture C	104	72	0.69	+ 2.2	-5.3
3WU mixed culture	179	22	0.12	0	-6.9
C. thermocellum	314	296	0.94	+46.3	+19.9

The bacteria were grown for 14 days on basal medium containing 1% crystalline cellulose, 0.3% yeast extract, supplemented with 0.2% cellobiose where shown.

rapidly hydrolysed to glucose by ß-glucosidase and that the resulting glucose levels were responsible for the effects on CMCase production during the ensuing two weeks of growth. The effects of glucose, sucrose and cellobiose on CMCase and avicelase production during growth of TP8.T6.3.3.1 are dealt with in Section 5.2.

4.4.7 Thermal stabilities of CMCases

Stabilities of the CMCases in supernatants from the first set of cellulolytic isolates, the TP8A and TP8C and 3WUB mixed cultures are shown in Fig. 4.2. TOK8.1 and WAI21.3 were exceptionally stable, with half-lives at 85°C which were both too long to be accurately predicted by extrapolation. They appeared to be substantially in excess of 10 hours, which was the 85°C half-life of TOK8.3 CMCase, the next most stable.

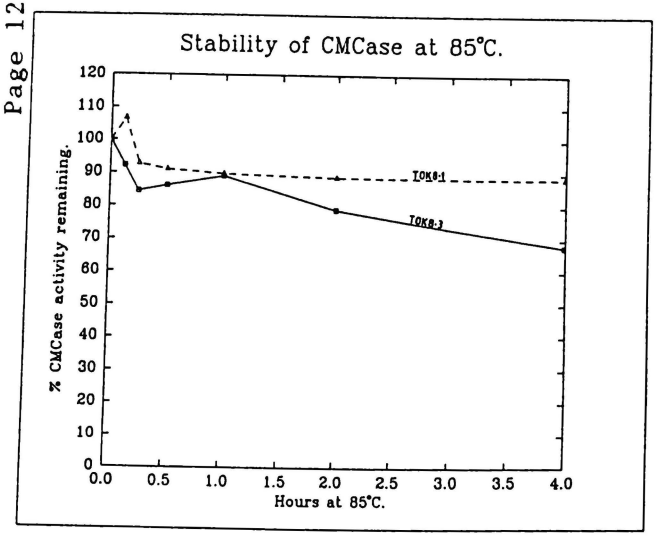
Thermal stability of CMCase activity served to distinguish between isolates WAI21.1 and WAI21.3, between TOK8.1 and TOK8.3 and between TOK4.3 and TOK4.4. The TP10 isolates all had relatively poor CMCase thermal stabilities, and since a high background sugar level reduced the assay accuracy, the differences between them (Fig. 4.2) are probably not significant. 3WUB, the duckpond enrichment, produced CMCase with similar thermal stability to the TP10 CMCases. The differing stabilities of CMCases from the TP8A and TP8C mixed cultures suggested a difference in their cellulolytic populations. This is further discussed in Section 4.4.8.3 with reference to their different culture conditions.

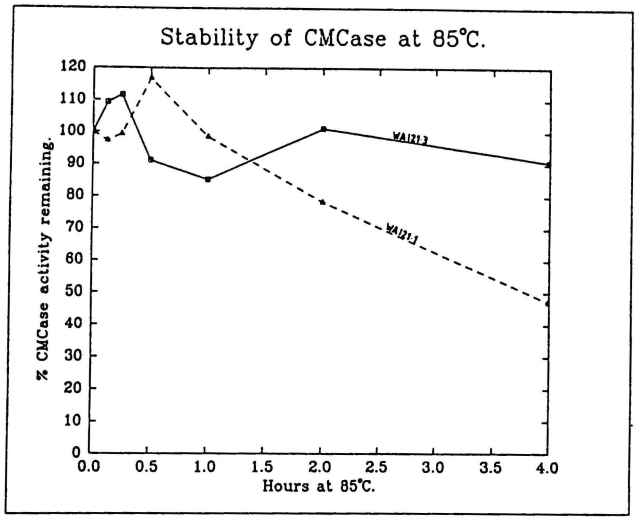
Thermal activation after short periods of incubation at 85°C appeared to occur in most cases. Alternatively there was a rapid, limited inactivation followed by a relatively constant rate of activity loss. Similar behaviour patterns have been published in thermal stability studies of *Thermoactinomyces* cellulases (Hagerdal *et al.*, 1979b) and of other thermophilic enzymes (Hickey and Daniel, 1979). Possible explanations include the creation of more active pre-denaturation conformational states (Galas *et al.*, 1981), the release or binding of modifying factors, the presence of multiple enzymes with CMCase activities and proteolytic action. We examined our cellulolytic strains for proteases by the casein plate assay method (Cowan and Daniel, 1982b) and only TOK4.4 showed proteolytic activity.

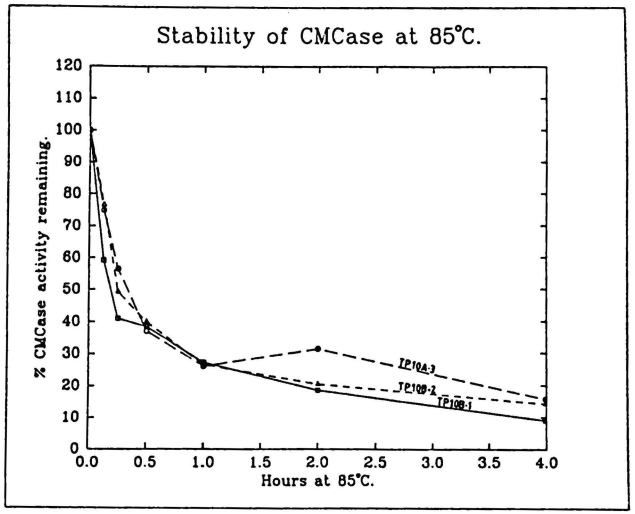
pH values of the various culture supernatants of Fig. 4.2 were not controlled during the preincubation at 85°C. Since this may

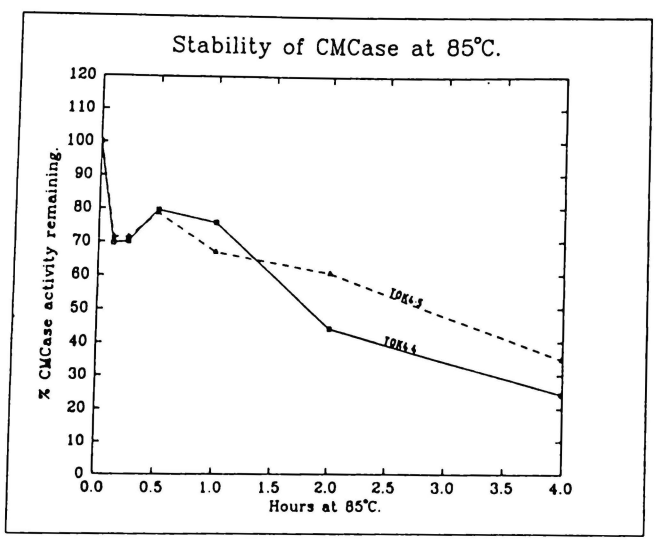
Figure 4.2: Endocellulase stability at 85°C of various isolates and mixed cultures of interest.

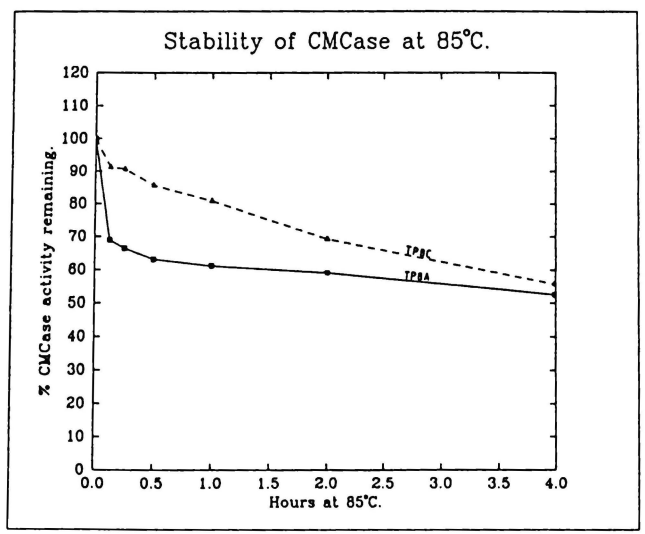
Unbuffered culture supernatants after three weeks growth were incubated at 85°C for the times shown. 0.2ml portions were then removed and CMCase activity measured over a 30 min incubation at 75°C.

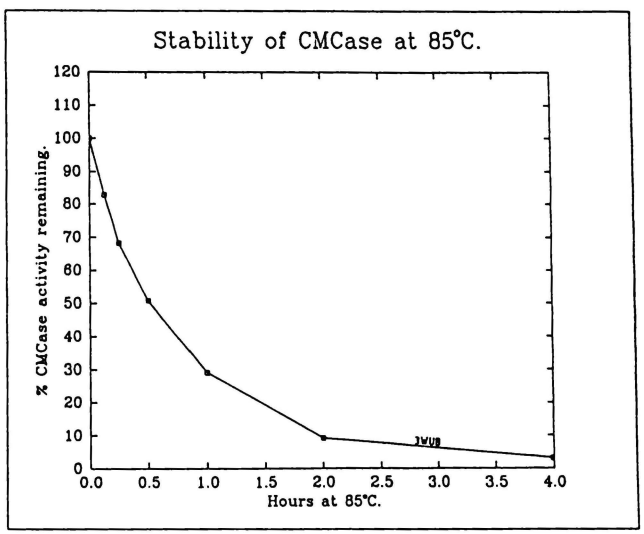

Stability of CMCase at 85°C.
% CMCase activity remaining.
Hours at 85°C.
TOK8-1
TOK8-3


Stability of CMCase at 85°C.
% CMCase activity remaining.
Hours at 85°C.
WA121-3
WA121-1


Stability of CMCase at 85°C.
% CMCase activity remaining.
Hours at 85°C.
TP10A-3
TP10B-2
TP10B-1


Stability of CMCase at 85°C.
% CMCase activity remaining.
Hours at 85°C.
TOK4-5
TOK4-4


Stability of CMCase at 85°C.
% CMCase activity remaining.
Hours at 85°C.
TP8C
TP8A


Stability of CMCase at 85°C.
% CMCase activity remaining.
Hours at 85°C.
TWUB

have had some influence on relative CMCase stabilities, a second series of experiments was performed involving preincubation at 85°C in 0.05M citrate-phosphate buffer (Dawson *et al.*, 1974) at pHs 5 and 7 (Fig. 4.3). *Clostridium thermocellum* was included in this experiment.

Compared with *C. thermocellum*, our organisms possessed extremely stable CMCases. The half-life of the *C. thermocellum* CMCase activity at 85°C was about 5 minutes at both pH5 and pH7.

In the majority of cases (excluding *C. thermocellum*, LC8 and TP8T) there was a definite difference in CMCase stability in the two buffers. This could have been solely a pH effect, or may have included some direct effects of the buffer components (Na^+, citrate and phosphate) or an effect of ionic strength.

Three different pH5 buffers, which were all at 50mM final concentration but which contained various buffering anions, were compared in their effects on the stability of LC12 CMCase (Fig. 4.4.). There were clear differences in their stabilizing effects, with citrate alone conferring the least stability whereas a citrate-phosphate combination resulted in the greatest stabilization.

It was apparent from the above that the effects of pH, ionic strength and buffer composition would have to be thoroughly investigated first before strictly comparative thermal stability data for these CMCases from different organisms could be obtained. However, amongst the isolates which were included in the above cursory comparison, TP10B1 produced CMCases which were clearly the least stable in the normally employed buffer and ionic strength conditions. They were also distinctive in being substantially more stable at pH7 than at pH5.

Considerably more stable CMCases were produced by the other two isolates in the above comparison which were known to produce "complete" cellulase complexes (TP8T and LC8). Unfortunately LC8 was lost in subculturing, as was the next best group of LC-derived organisms (LC7, 9 and 10). The TP10 isolates met the same fate several sub-culturings later, just prior to the commissioning of the freeze-drier.

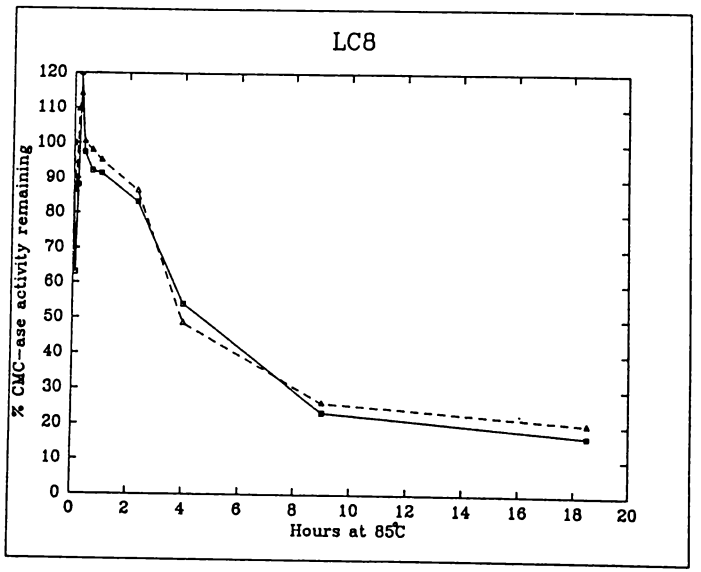
The continuation of the isolation programme which produced a range of different cellulolytic isolates from TP8 is described in Section 4.4.8.

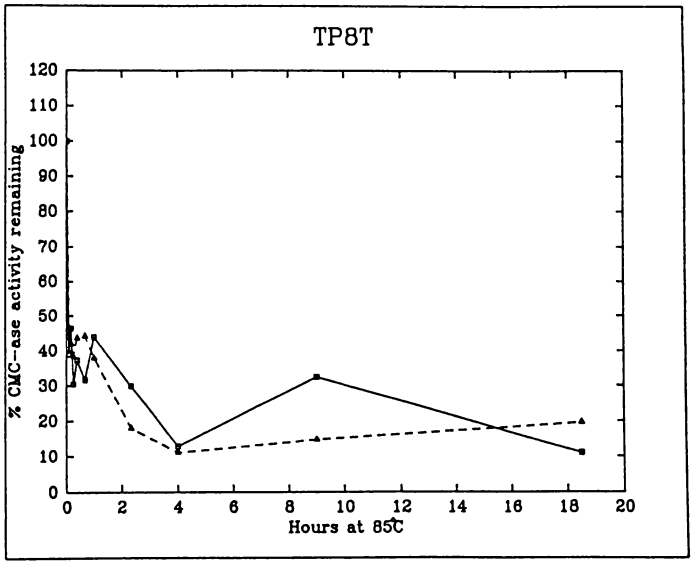
Figure 4.3: CMCase stability, at pH5 and pH7, of a range of our isolates and *C. thermocellum*.

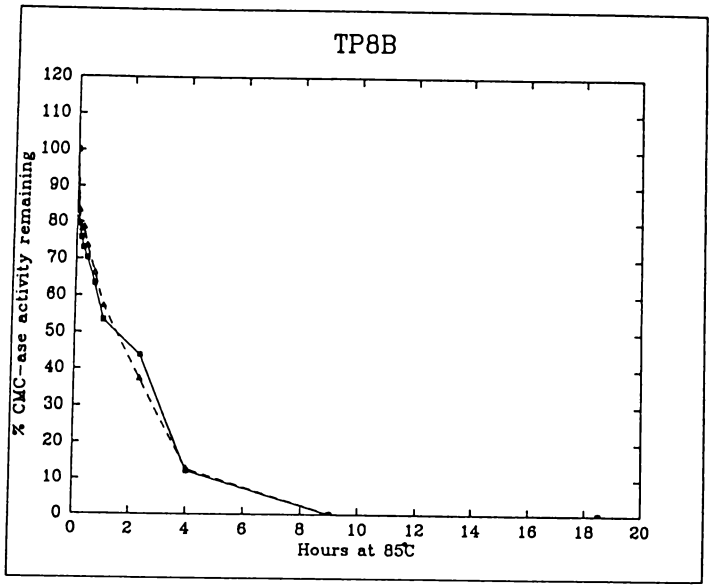
Culture supernatants (4 weeks growth) buffered to either pH5 or pH7 with 0.05M citrate-phosphate buffer (Dawson et al., 1974) were incubated at 85°C for the times shown and CMCase activity remaining was measured at 75°C.

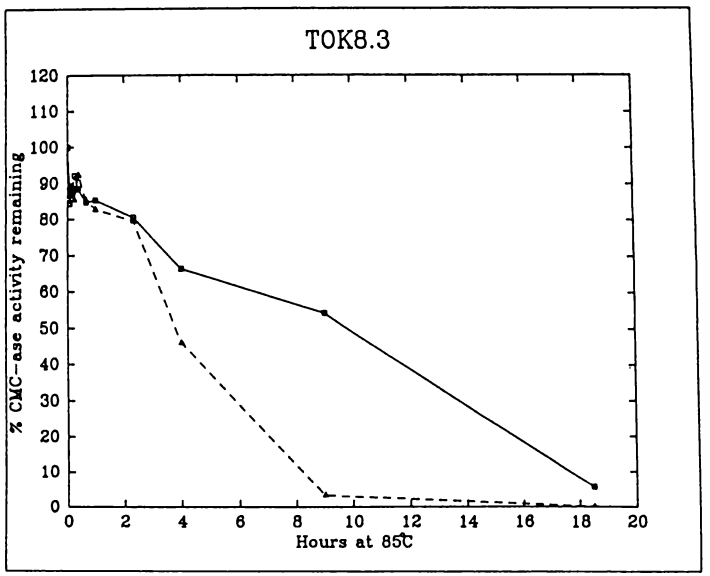
□ = pH5

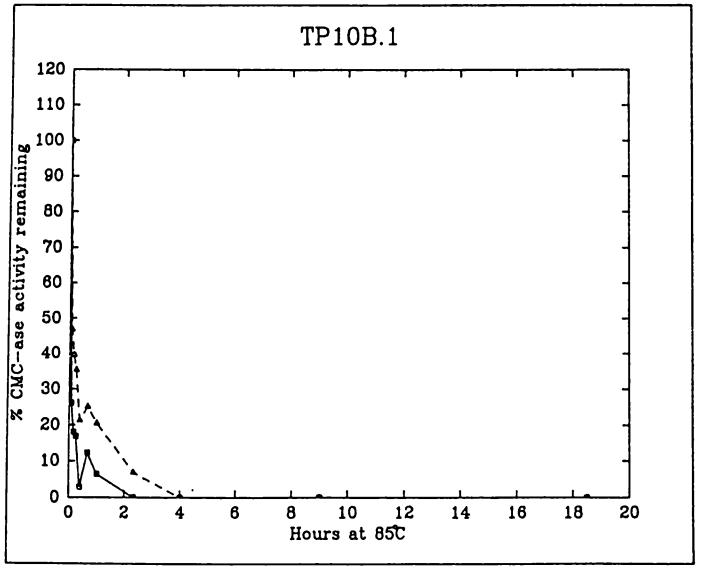
△ = pH7

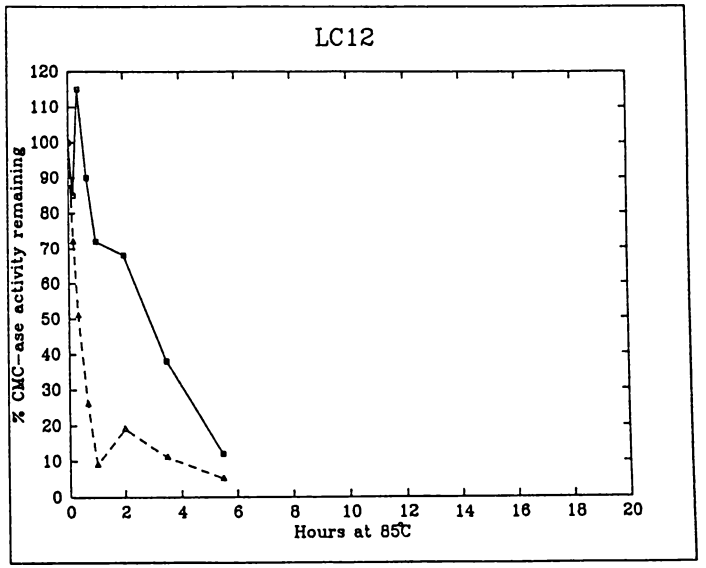

LC8
% CMC-ase activity remaining
Hours at 85C
0
10
20
30
40
50
60
70
80
90
100
110
120
0
2
4
6
8
10
12
14
16
18
20

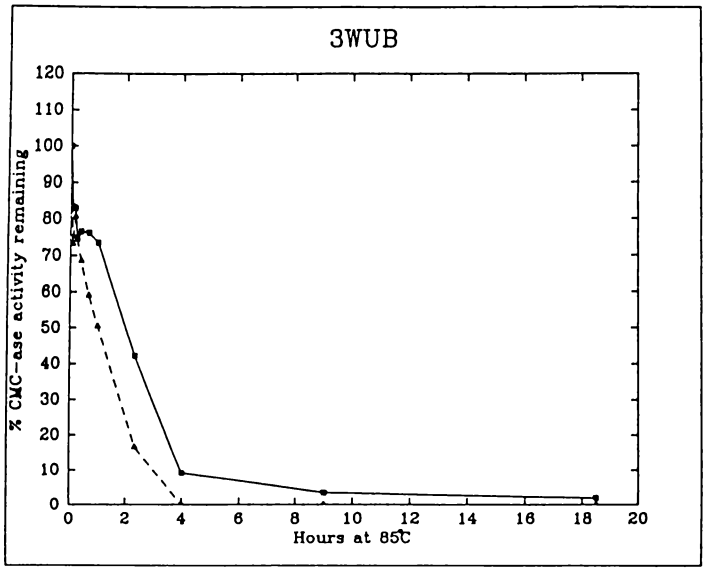

TP8T
% CMC-ase activity remaining
Hours at 85C
0
10
20
30
40
50
60
70
80
90
100
110
120
0
2
4
6
8
10
12
14
16
18
20

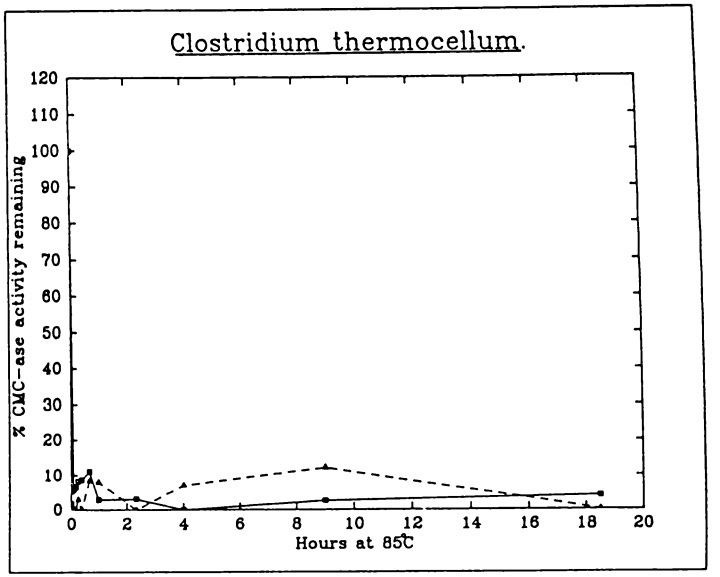

TP8B
% CMC-ase activity remaining
Hours at 85C
0
10
20
30
40
50
60
70
80
90
100
110
120
0
2
4
6
8
10
12
14
16
18
20


TOK8.3
% CMC-ase activity remaining
Hours at 85C
0
10
20
30
40
50
60
70
80
90
100
110
120
0
2
4
6
8
10
12
14
16
18
20


TP10B.1
% CMC-ase activity remaining
Hours at 85C


LC12
% CMC-ase activity remaining
Hours at 85C


3WUB
% CMC-ase activity remaining
Hours at 85C


Clostridium thermocellum.
% CMC-ase activity remaining
Hours at 85C

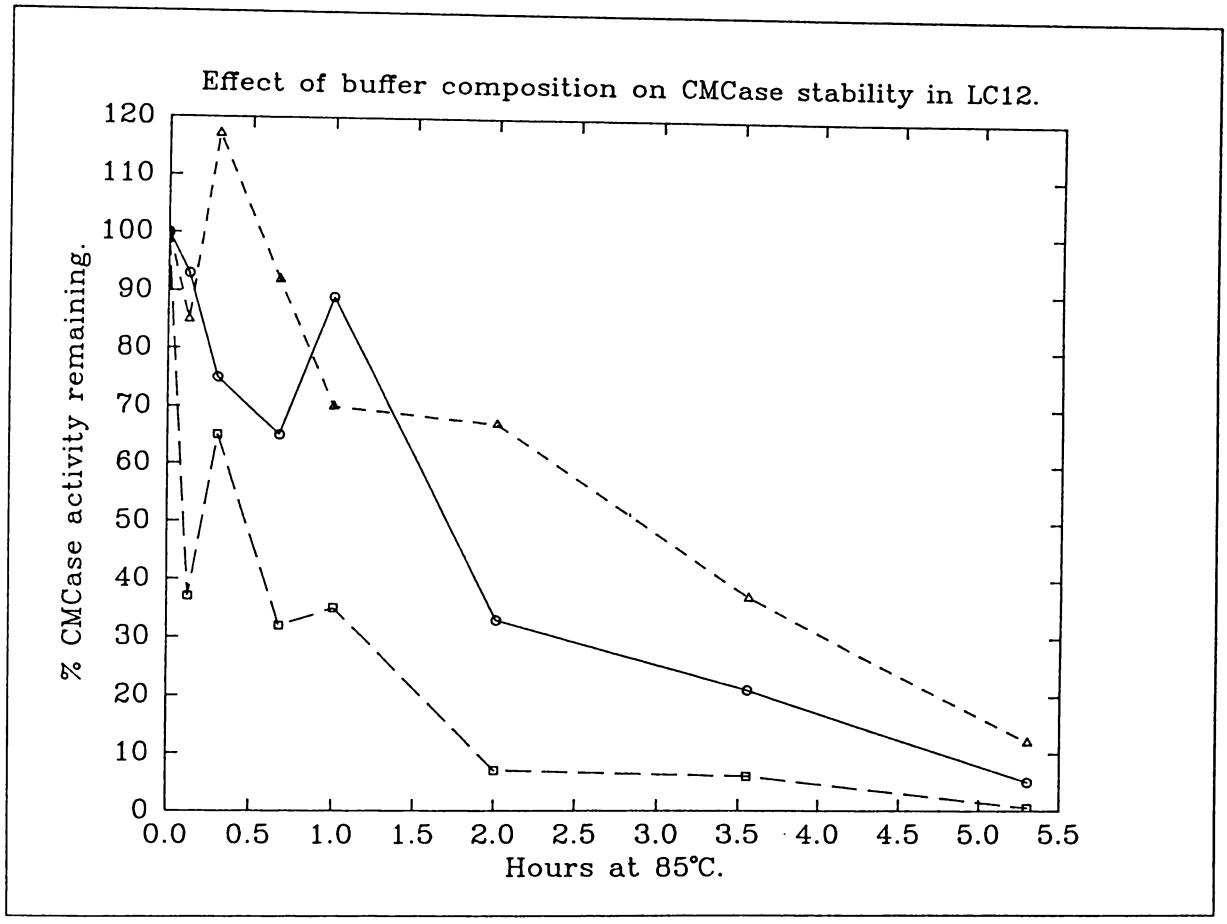


Figure 4.4: Effect of buffer composition on endocellulase stability in LC-12.

The culture supernatant was buffered at pH5 with the buffers shown at 0.05M final concentration, incubated at 85°C for the times shown and CMCase activity remaining was measured at 75°C.

△ = citrate-phosphate (Dawson et al.,1974)

○ = acetate-Na^+ (Dawson et al.,1974)

□ = citrate-Na^+ (Dawson et al.,1974)

TOK8.1, TOK8.3 and WAI21.3 were clearly worth keeping since, despite the absence of avicelase activity, their CMCases were certainly amongst the most stable ever reported. They were eventually freeze-dried without any further isolation steps. Attempted preservation in 50% glycerol at -20°C was unsuccessful.

4.4.8 Isolation and partial characterisation of cellulolytic bacteria from TP8 mixed cultures

Special emphasis was placed on isolation of the cellulolytic organisms of TP8 origin for the following reasons.

(i) They produced a "complete" cellulase complex (i.e. they had avicelase activity) (Section 4.4.4)

(ii) They had relatively stable CMCase activities (Section 4.4.7)

(iii) They survived the rigours of maintenance by subculturing.

4.4.8.1 Origins of TP8 The original TP8 enrichment culture inoculum was the homogenised decomposing end of a *Pinus radiata* plank, found submerged in a stream which was, at that point, only 48°C but which was fed by a much hotter source 500m upstream (site TP10). Since the plank had been comprising a splendid "in pool" enrichment substrate it was no surprise that a variety of different bacteria grew in the lab. enrichment culture.

4.4.8.2 Evidence of population heterogeneity. The first clear evidence of the heterogenous nature of the population in the TP8 enrichments was the failure of our first two attempts to culture cellulolytic organisms from individual colonies produced in cellulose and cellobiose agar dilution series inoculated with TP8 enrichment culture. In all cases growth which resulted from the transferred colonies was not cellulolytic, and presumably survived on the yeast extract (0.3%) or cellobiose (0.2%) present. In contrast, all isolated colonies cut from agar dilution series inoculated from WAI21, TOK4, TOK8 and TP10 proved to be cellulolytic to some extent.

4.4.8.3 Initial evidence for the existence of more than one cellulolytic strain in TP8 cultures. It was also clear that the original TP8 enrichment contained at least 2 different cellulolytic bacteria. This was first indicated by the different stabilities at 85°C of CMCases from TP8 mixed cultures which had different growth histories (Fig. 4.2).

Avicelase, Filter Paper and CMCase activities and particularly the ratios of these activities were also different for the two cultures (Table 4.8).

Table 4.8: Cellulase assays on the starting inoculum and on a sample of the fermentor culture resulting

Enzyme source	CMCase*	Avicelase*		Filter-Paper Activity*	
(Assay incubation time)	30 min	2h	22h	2h	22h
TP8 culture (see Fig. 4.5)					
TP8A (Starting inoculum)	148	2.9	0.52	4.13	0.52
TP8C (600-litre fermentor)	300	8.4	1.43	5.83	1.68

* All activities are in nmol glucose equivalents (DNSA) per min per ml of culture supernatant.
Both cultures were in the stationary phase (one month old).

The TP8A mixed culture was grown in a tightly sealed 500ml bottle on the normal basal medium including 0.3% YE and 1% Sigmacell 50. The TP8C mixed culture was grown under conditions very different from the normal. Owing to another worker's bacterium failing to grow, a 600 litre fermentor full of a different anaerobic medium was available with which to perform a test run of large scale anaerobic cellulose fermentation. The medium, adjusted to an intial pH of 7.2, consisted of 0.15% K_2HPO_4, 0.25% NaCl, 1% trypticase peptone, 0.0003% penicillin and 0.1% sulphur powder and 0.2% Whatman CC41 cellulose. Cysteine hydrochloride (0.5%) and a flow of N_2 gas across the surface of the liquid were used to maintain an anaerobic state. The medium was brought to the boil and then inoculated with 20 litres of TP8 culture as soon as it had returned to 75°C.

Growth of TP8 organisms in the fermentor was very slow

initially but once various air leaks had been located, good growth and cellulase production resulted. Gas production was sufficient to maintain a positive pressure and flushing with N_2 was unnecessary once growth was established. Having confirmed growth of these anaerobes to be practical on this large scale, a subculture was made and the fermentor drained. Microscopic examination showed that the rods of TP8 were very much more numerous than the cocci surviving from the original culture.

It is likely that the differing growth conditions in the 500ml and the 600 litre TP8 cultures favoured different cellulolytic component strains, thus accounting for the differences in the cellulase activities and stabilities. However it is possible that differences in the media may have produced such effects by influencing the stabilities (see Section 4.4.7) and the relative levels of production of the various cellulases from a single organism.

4.4.8.4 Isolation of cellulolytic TP8 strains. More conclusive evidence for the existence of more than one cellulolytic strain or species in the TP8 enrichment was obtained after the eventual successful growth and isolation of cellulolytic colonies on agar. This change of fortune accompanied a switch from the use of N_2 to CO_2 as a flushing and head-space gas in the anaerobic manipulations involved in producing the agar dilution series and in subsequent removal of individual colonies. Possibly CO_2 met some metabolic requirement (as in *Acetivibrio* (Patel *et al.*, 1980) and suspected in the case of *C. thermocellum* (Zertuche and Zall, 1982)) or else it simply provided a better anaerobic environment, being heavier than air. Cellulolytic isolates were derived from several different branches of a TP8 "family tree" (Fig. 4.5). Colonies of all these isolates produced distinct clearances of several millimetres in surrounding cellulose (MN300) agar.

All the TP8 isolates, when finally freeze-dried, had passed through at least 2 agar dilution series, the second of which was cellobiose agar, with intervening subculturing into cellobiose liquid medium. This passage through a soluble substrate helped reduce the possibility of co-isolating other

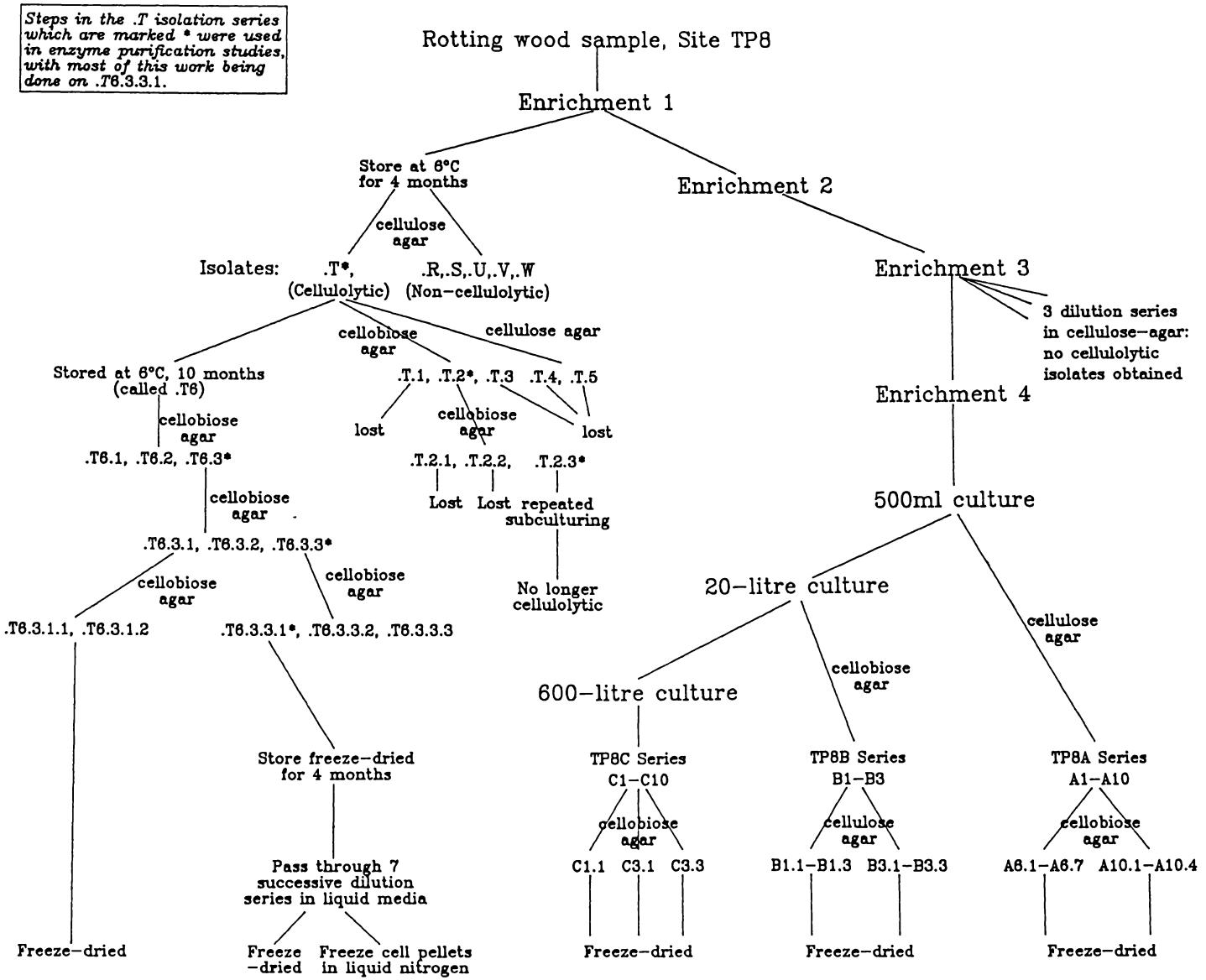


Figure 4.5 Isolation flow diagram for TP8 strains

bacteria (Hungate, 1950, McBee,1950, Lee and Blackburn, 1975) since several bacteria might otherwise carry through a dilution series adhered to a single cellulose particle and give rise to a single colony which would in fact be a co-culture.

4.4.8.5 Division of TP8 isolates into two types The TP8 isolates (See Fig. 4.5 for their origins) could be divided into two distinct groups or strains on the following basis:

(i) Reducing sugar accumulation was significantly greater in Type 1 strains after both 8 and 21 days' growth (Tables 4.9 and 4.10).

(ii) Avicelase activity, after 8 days' growth, was found only in the supernatants of Type 1 strains (Table 4.9). Nonetheless, colonies of both types were capable of clearing non-crystalline MN300 cellulose in surrounding agar.

(iii) CMCase levels (CMC saccharifying activities) were consistently higher in Type 2 strains at 8, 10 and 21 days (Tables 4.9 and 4.10).

(iv) Ratios of CMC plate-clearing activity to CMC saccharifying activity were higher in Type 1 strains, particularly in the younger cultures (Tables 4.10 and 4.11). A higher ratio is indicative of an increased ratio of endoglucanase to exoglucanase activity.

(v) CMCase stabilities at 85°C were lower for Type 1 strains (Table 4.10), with only 20-30% of their activity remaining after 1 hour at 85°C cf. 50-60% remaining of the Type 2 CMCases. After their initial 70-80% activity loss in the first hour, activities of Type 1 CMCases remained fairly constant for the next two hours, whereas those of Type 2 strains continued to fall. Possible reasons for the Type 1 pattern are discussed in Section 4.4.7.

Volatile end-product analysis by gas chromatography after growth on cellulose-containing medium revealed ethanol and acetate to be the major volatile end-products, these being formed in roughly equal proportions. There was no clear distinction between Types 1 and 2 on this basis, but a particular group of Type 2 isolates, TP8A.10.1, TP8A.10.2 and

Table 4.9: Reducing sugars accumulated, CMCase, avicelase, and CMC-plate-clearing activities in 8-day old culture supernatants of representative TP8 isolates

TP8 Type		TP8 Strain	Reducing Sugar Accumulated	Avicelase Activity*	CMCase Activity*	Plate Clearing Activity	Plate Clearing Activity / CMCase Activity
	Series	Isolation Numbers	mM	nmols.min^{-1}.ml^{-1}	nmols.min^{-1}.ml^{-1}	† Arbitrary units. ml^{-1}	
1	T	T.2.3	6.0	0.57	56	47	0.84
1	B	.1.1	4.5	0.51	73	65	0.89
1	B	.3	2.3	0.17	57	55	0.96
1	B	.3.2	3.8	0.25	67	N.D.	N.D.
2	A	.5	<1.6	0	84	14	0.17
2	A	.6.5	1.7	0	104	22	0.21
2	A	.10.3	1.7	0.01	74	16	0.22
2	C	.1.1	1.6	0	110	22	0.20

* Reducing sugars produced as glucose equivalents (DNSA)

† See Fig. 3.2

N.D. = Not done

Table 4.10: CMCase stability, specificity and reducing sugar accumulation in supernatants of the various series of TP8 isolates

Isolate Series	No. of Isolates	CMCase Stability			CMCase Specificity			Reducing Sugar Accumulation (mM)
		Initial Activity*	%CMCase remaining: After 1h at 85°C	After 3h at 85°C	Reducing sugar production*	Plate-clearing activity#	Plate-clearing R.S. production	
T	5	47±3†	23±3	18±3	46±3	25±2	0.56±0.05	7.7±0.8
A	3	167±3	55±1	39±1	122±4	16±1	0.13±0.01	2.3±0.2
B	5	68±5	19±1	18±2	61±3	28±3	0.46±0.05	7.3±0.8
C	1	196	48	38	154	17	0.11	3.4

\# arbitrary units (See Fig. 3.2)
* nmoles glucose equivalents. $min^{-1}.ml^{-1}$
† ± standard error of the mean

Stability measurements were made at day 10 of culture growth, whereas CMCase specificity and reducing sugar accumulation data was obtained from a different set of 21-day-old cultures.

TP8A.10.3 could be distinguished by a significantly higher ethanol:acetate ratio. This difference was not apparent at 9 days but was clearly evident after 15 days growth.

Morphologically, Types 1 and 2 were very similar, Gram negative rods. We have insufficient evidence to determine if they are different species or merely different strains. It is conceivable that several different strains might be represented even within each of the two "types". Techniques looking at DNA homology are probably likely to offer the most reliable guide.

Representatives from each of the T, A, B and C series of isolates (Fig. 4.5) were freeze-dried.

4.4.8.6 The final choice of an organism for biochemical studies, and further isolation steps required to obtain a reliable pure culture TP8T, being the first cellulolytic single colony isolate obtained from TP8, was used to produce cellulases in bulk for some of our preliminary biochemical studies. In order to be better assured of a pure culture, the organism was passed through two further agar dilution series (Fig. 4.5) with intervening growth in liquid cellobiose medium.

Isolate T.2.3 was selected because it produced marginally higher CMCase and avicelase activities than did the other isolates.

No non-cellulolytic isolates were obtained from the second and third agar isolation series, and all the colonies looked very similar in clear cellobiose agar.

The Type 2 isolates (Section 4.4.8.5) were produced in parallel isolation sequences (series A and C, Fig. 4.5) which ran concurrently with that of T.2.3. Although the Type 2 isolates were found to possess more stable CMCase activities than Type 1 (Table 4.10), their very low avicelase production rendered them unattractive subjects for biochemical studies which aimed to focus upon the avicelase complex. The potential for improving avicelase yields of Type 2 strains through genetic engineering was recognized, but the constraints of time and resources precluded this. Thus TP8.T.2.3 was selected to be the subject of continued biochemical studies.

The branch on the far left in Fig. 4.5, in which the TP8.T6 strains were produced, was a re-isolation programme made necessary by the complete loss of cellulolytic activity in TP8.T.2.3. This was discovered after several months of regular subculturing on basal medium plus 1% cellulose and 0.06% yeast extract. Our freeze-drier was just being commissioned at that stage. Fortunately the original TP8T enrichment had been stored at 6°C and gave rise to isolates which performed very similarly to TP8T.2.3.

After freeze-drying and reconstitution, these new isolates, T6.3.1.1, T6.3.1.2, T6.3.3.1, T6.3.3.2 and T6.3.3.3, were all found to be contaminated with a fatter, gram +ve rod, possibly a *Bacillus* or *Thermoanaerobium*, which would also grow at 75°C. The least contaminated, T6.3.3.1, was passed through seven successive dilution series in a variety of liquid media, with and without cellulose, until the contaminant was lost. Each transfer was made in early log phase, when cell numbers were normally $10^8-10^9 .ml^{-1}$. The resulting pure culture was freeze-dried in 15 separate vials.

The isolation procedure was complicated by problems in guaranteeing sterility of our media. We experienced intermittent problems with growth occurring in uninoculated media stored both at room temperature and at 60°C, despite autoclaving at 121°C for 30 minutes. These difficulties surfaced after a few months of working with extremely thermophilic anaerobes, and were probably due to contamination of the laboratory with extremely heat resistant spores. On one occasion Gram +ve contaminants were found "downstream" of apparently pure cultures in a dilution series. This required microscopic examination of each series and selection of the least contaminated as an inoculum for the next. No further additions to the strain number were made during the liquid culture dilution series since the numbers were only used to identify single colony isolates from agar, (apart from the "6", and the letters A, B and C which were used to identify parent cultures - see Fig. 4.5).

4.4.9 Hydrolysis of natural substrates by a concentrated culture supernatant of TP8.T6.3.3.1

The cell-free supernatant of a 9-day-old culture of TP8.T6.3.3.1, grown in basal medium supplemented with 0.2% trypticase peptone, 0.06% yeast extract and 0.2% Sigmacell 50 crystalline cellulose, was concentrated 1000-fold by ultrafiltration. This produced a 100-fold increase in the avicelase concentration, giving a final avicelase activity of approximately 120 nmoles.min^{-1}.ml^{-1} on the basis of a 17-hour incubation at 75°C. This concentrated supernatant was then applied to a range of natural substrates and to Sigmacell 50 crystalline cellulose also, and incubated as indicated in Table 4.11.

A degree of solubilisation was obtained with all of the substrates tested. *Eucalyptus* was least effectively hydrolysed, with the reducing sugar release accounting for only about 10% of the cellulose. Pretreatment of the *Eucalyptus* by steam explosion appeared to have no effect on the extent of hydrolysis. The most complete hydrolysis, accounting for 46% of the cellulose content, was achieved with the never-dried protein-extracted lucerne. This even exceeded the extent of hydrolysis of the purified crystalline cellulose (32%), and was almost double that achieved using the oven-dried, finely ground form of protein-extracted lucerne. This reduced susceptibility to cellulase activity as a consequence of drying was probably caused by a reduction in porosity of the substrate, which reduces the surface area accessible to the enzymes (Stone *et al.*, 1969).

The second addition of enzyme, following thorough washing of the residual substrates, did not produce as much hydrolysis of any substrate as had resulted from the first batch of enzyme (Table 4.11). This is to be expected, given the heterogenous nature of each substrate (microcrystalline cellulose included), since the more readily accessible material should be hydrolysed more rapidly than the more recalcitrant fractions.

It is possible that the values of Table 4.11 are an over-estimate of the extent of cellulose hydrolysis in the case of the natural substrates since they are based on the assumption that all of the reducing sugars released originate from the cellulose component. It is possible that hemicellulose and pectin hydrolysis was also contributing. Nevertheless it was evident that the

Substrate	Initial Dry Weight (mg)	Cellulose Content (% w/w)	Cellulose in substrate (mg)	First batch of enzyme: Glucose equivalents due to enzyme action (mg)	First batch of enzyme: % of total cellulose content	Second batch of enzyme: Glucose equivalents due to enzyme action (mg)	Second batch of enzyme: % of total original cellulose content	Overall % hydrolysis of cellulose content after two batches
Dried Protein-Extracted Lucerne	20	45[1]	9	1.62	16	0.92	9	25
Never-dried Protein - Extracted Lucerne	13	45[1]	5.9	1.94	30	1.03	16	46
Eucalyptus	20	51[2]	10	0.59	6	0.41	4	10
Steam-exploded Eucalyptus	20	51[2]	10	0.67	7	0.46	5	11
Pinus radiata	20	37[2]	7.4	0.83	11	0.42	6	17
Sigmacell 50	20	100	20	4.81	22	2.27	10	32

Table 4.11 The hydrolysis of various natural cellulosics by concentrated culture supernatant of TP8.T6.3.3.1

Substrates were incubated for 8 days at 75°C, followed by 1 day at 85 C, with 0.2 ml of ultrafilter-concentrated culture supernatant and the release of reducing sugars was measured by DNSA. After washing residual substrates, a further incubation with a repeat addition of the enzyme was performed, involving 11 days at 75°C followed by 1 day at 85°C.
Tubes containing the substrates plus buffer only served as blanks in each incubation. All substrates were in dry powdered form excepting one of the protein-extracted lucerne samples, which had a water content of 75% (w/w), and was not finely chopped. A fresh weight of 50 mg of this was applied, corresponding to 13 mg dry weight.

[1] Information provided by the supplier, Dr S. Vaughan, Ruakura Agricultural Research Centre, Hamilton.

[2] Sjostrom, 1981, p208.

concentrated cell-free supernatant was capable of hydrolysing all of the substrates tested to some extent, and there was no evidence of any water soluble inhibitors such as were found to affect *C. thermocellum* action on steam-exploded wheat and aspen (Saddler and Chan, 1984).

4.5 MORPHOLOGIES OF THE BACTERIA AND SUNDRY GROWTH CHARACTERISTICS

All of our isolates were Gram negative in the exponential phase and rod shaped, of similar dimensions to *C. thermocellum* (0.6 x 4 μm according to Ng et al., 1977). Most tended to form short chains of up to 4 cells long (Fig. 4.6). The cells grew smaller, particularly in width, during stationary phase, and most strains produced refractile bodies internally along their lengths which were most obvious under phase contrast. These bodies may have been spores, but were certainly quite different to the terminal spores of *C. thermocellum* which we saw, and didn't stain by Fleming's spore stain technique (Gurr, 1957).

Figure 4.6: TP8T6.3.3.1 cells from an actively growing cellobiose culture, after Gram-staining.

TP8.T6.3.3.1 exhibited pleomorphism under stress conditions, such as exposure to temperatures above 75°C, when it grew as long filaments with no evidence of septation. WAI21 isolates tended to form balls of cells, visible to the naked eye, which were probably due to entangling

of the particularly long thin filamentous cells seen on occasions in WAI21 cultures. These cells frequently were straight with a hooked end, like a walking stick.

The aerobic enrichment which yielded cellulases (TOK6) contained a mixture of long filamentous cells and shorter rods. Since the cellulolytic component of this culture didn't survive subculturing and the remnants were discarded, we do not know which cell-type was responsible for the CMCase production.

The colours of colonies in cellobiose agar ranged from white through cream to brown in most cases, but 3WUB produced both purple and white colonies. Cells from the different coloured colonies exhibited no obvious differences in morphologies under phase contrast, but those from the purple colonies formed the longest chains.

All the anaerobes were treated as though they were obligate anaerobes, and their possible particular gas requirements were not tested. Carbon dioxide is suspected of having a beneficial effect since instant success, following a succession of failures in culturing cellulolytic TP8 isolates, accompanied our switch from N_2 to CO_2 as the flushing gas in our anaerobic fish-tank. Poorly sealing lids of culture bottles were often the cause of growth failure. TP8 isolates were later found to exhibit considerable oxygen tolerance, as discussed in Section 5.4.

4.6 TAXONOMIC SPECULATION

A new genus may have to be established to accommodate these extremely thermophilic anaerobes. Since they don't form conventional spores, even when grown on solid medium, they are not strains of *Clostridium*. Further confirmation of this is their ability to grow well at 75°C, which is certainly above the growth limit of *C. thermocellum* (Hyun *et al.*, 1983) and probably also above that of *C. stercorarium*, a recently isolated cellulolytic species (Madden, 1983), which has a growth optimum of 65°C. Neither of these *Clostridia* ferment sucrose whereas at least one of our bacteria (TP8.T 6.3.3.1) will do so and accumulates glucose (Section 5.2). None of the other thermophilic cellulolytic bacteria (Section 1.5.2) bare much resemblance either morphologically or physiologically to our organisms. In fact, our organisms are in many ways more similar to certain mesophiles, such as *Acetivibrio cellulolyticus* gen. nov., sp. nov. (Patel *et al.*, 1980), or to strain B of a newly isolated marine anaerobic cellulolytic *Bacteroides* (Miyoshi, 1978) and to some newly isolated hemicellulolytic

strains of anaerobic extreme thermophiles which don't seem to fit into any recognized genus (Weimer *et al.*, 1984). The latter strains were not able to ferment cellulose.

Further taxonomy is necessary before our cellulolytic strains can be adequately categorised, but it is plain that none of the cellulolytic anaerobic bacteria studied in other publications are of the same species, and probably not of the same genus. Ljungdahl *et al.* (1981) established enrichment cultures that fermented cellulose from Icelandic hot springs. These included extreme thermophiles, capable of growth at 84°C, pH 8.6. To our knowledge no further characteristics of these organisms have been published.

4.7 CONCLUSIONS AND ADVICE FOR THOSE WHO MAY FOLLOW

With the benefit of hind-sight, several strategies appear particularly important for successfully obtaining and isolating cellulolytic anaerobes from hot pools. Firstly, "in-pool" enrichment is most useful, be it by artificial additions of cellulose or by simply taking advantage of the vegetation which falls naturally into many of our hot pools. By paying particular attention to sampling such material, a colleage on a second cellulase sampling trip obtained 30 CMCase-containing enrichments from 33 samples brought back, cf. 9 from 47 on our first trip.

Secondly a cellulose-agar dilution series should be produced from each sample as quickly as possible and grown concurrently with an enrichment in liquid medium. If colonies do grow in the agar dilution series, their cellulolytic capacity can be gauged by the presence and extent of clearing of the surrounding cellulose, and it may be possible to isolate the best bacteria without the risk of their being over-run by other bacteria in liquid enrichments and subcultures.

If perchance the agar series grows nothing of interest, due perhaps to low cell numbers in the inoculum, then another series can be established from the liquid enrichment, should tests on its supernatant reveal cellulolytic activity.

Alternative carbon sources to cellulose should be kept to a minimum in enrichment cultures so as to favour cellulolytic organisms. However, yeast extract at a level of at least 0.1%(w/v) seemed indispensable for reasonable growth. This dependence on yeast extract might be circumvented by supplementing the medium with additional vitamins to those supplied in the vitamin concentrate, and certain amino acids may also prove vital.

Once a cellulose-clearing colony has been removed from cellulose agar and grown on liquid medium, it should be freeze-dried while its viable cell numbers are high (i.e. in log or early stationary phase). This should be done even though further isolation procedures to ensure a pure culture might be planned, since to rely on subculturing for retention of these organisms is to court disaster. As a further precaution, liquid cultures should be removed from incubation into 4°C (while in the early stationary phase) and kept until the success of preservation by freeze-drying is confirmed by reconstitution.

CHAPTER FIVE:

FACTORS INFLUENCING CELL GROWTH AND CELLULASE PRODUCTION OF TP8T ISOLATES.

5.1 THE EFFECT OF YEAST EXTRACT CONCENTRATION

Purification of extracellular cellulases would obviously be simplified by not including yeast extract in the growth medium. The influence of the yeast extract concentration upon growth and production of CMCase and reducing sugars by TP8T is shown in Fig. 5.1. The basal medium contained the usual vitamin and trace element supplements, and 1% Sigmacell 50 provided the only carbon source apart from the yeast extract.

Optimal CMCase production occurred between 0.06 and 0.2% yeast extract at day 10, and after 20 days' growth the cultures containing 0.06% yeast extract had the most active CMCase. Note however that by this time CMCase in all but one of the cultures had fallen substantially from the day 10 levels, so the relative levels at day 20 are probably largely determined by the time elapsed since the death of the majority of the cells.

Growth and reducing sugar accumulation at day 7 were almost directly proportional to yeast extract concentration, up to 0.2%. 0.3% yeast extract seemed to be slightly inhibitory. The reducing sugar accumulation was evidence of avicelase activity over the preceding period. Therefore accumulation of reducing sugars in the absence of added yeast extract indicated that the latter was not essential for avicelase production. The relative levels of reducing sugar accumulated may not, however, have closely reflected the levels of avicelase activity in each of these cultures, since reducing sugar accumulation would have depended not only on the degree of cellulose hydrolysis but also on the extent of growth and the degree to which the bacteria were utilizing the reducing sugars for growth. Varying the yeast extract concentration is likely to have altered the latter dependence.

Carry over of yeast extract from the inoculum (0.3% yeast extract)

Figure 5.1: Effect of yeast extract concentration on CMCase level, reducing sugar accumulation and growth of TP8.T.

Culture medium containing 1% Sigmacell 50 crystalline cellulose and the yeast extract concentrations shown was inoculated from a culture originally containing 0.3% yeast extract at a 1:60 dilution. This could have allowed a yeast extract carry-over of no more than 0.005% (final concentration).

All values plotted are averages obtained from triplicate cultures.

Figure 5.1a

* = Growth at day 7 (using an arbitrary turbidity-based scale, described in Section 2.4).

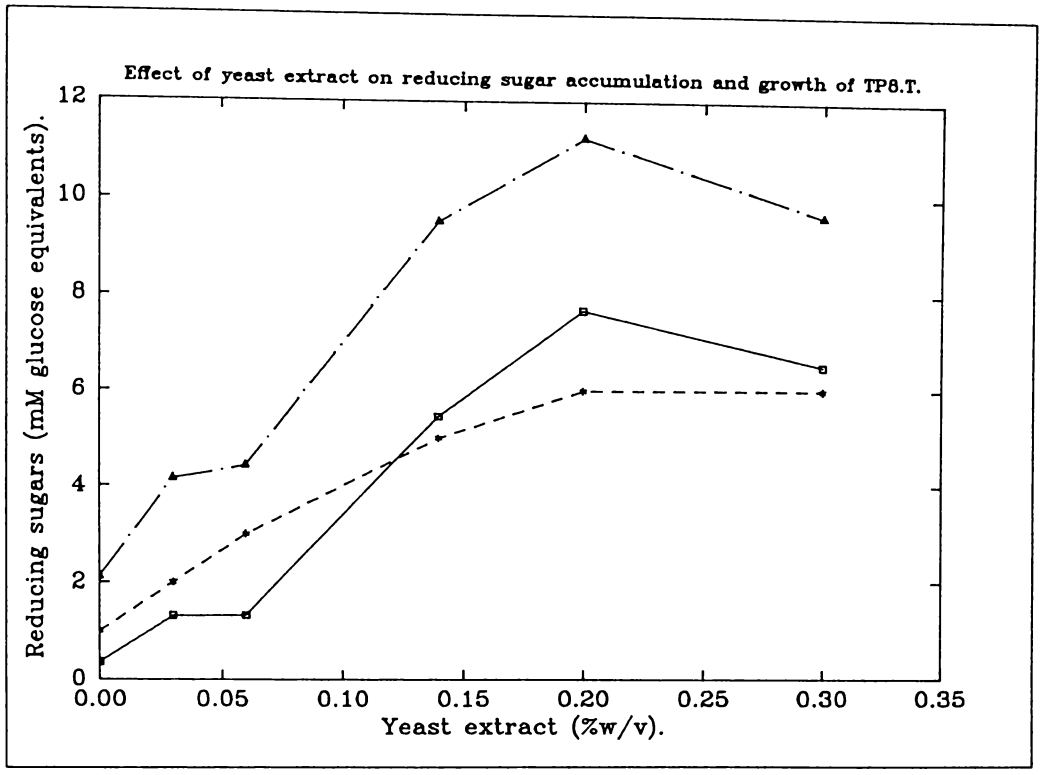
□ = Reducing sugar concentration in growth medium at day 10.

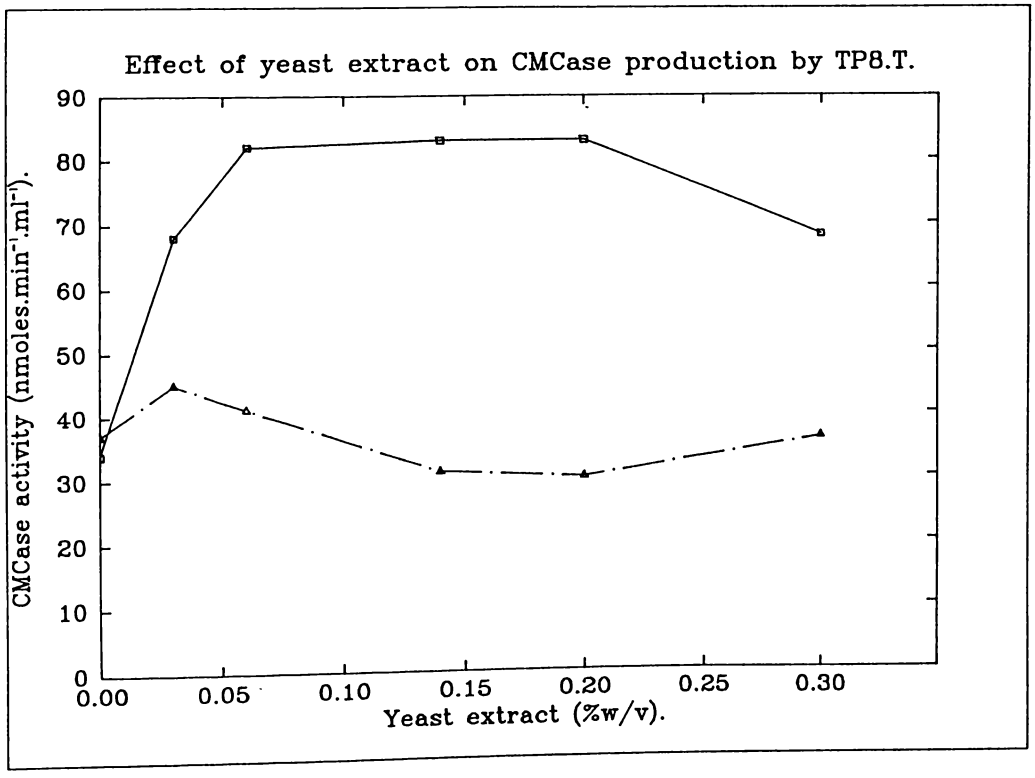
△ = Reducing sugar concentration in growth medium at day 20.

Figure 5.1b:

□ = CMCase activity at day 10

△ = CMCase activity at day 20


Effect of yeast extract on reducing sugar accumulation and growth of TP8.T.
Reducing sugars (mM glucose equivalents).
0
2
4
6
8
10
12
0.00
0.05
0.10
0.15
0.20
0.25
0.30
0.35
Yeast extract (%w/v).


Effect of yeast extract on CMCase production by TP8.T.
CMCase activity (nmoles.min-1.ml-1).
0
10
20
30
40
50
60
70
80
90
0.00
0.05
0.10
0.15
0.20
0.25
0.30
Yeast extract (%w/v).

could have resulted in, at most, a 0.005% yeast extract concentration in the medium intended to contain no yeast extract. To confirm that growth was possible at levels of yeast extract much lower than 0.005%, a subculture was produced from the "no yeast extract" medium by inoculating into more of the same medium. This subculture grew at a similar slow rate to the first.

Overall, the major effect of the yeast extract was to stimulate early growth, and the CMCase levels achieved by the time of stationary phase were largely independent of yeast extract concentration. A similar result was obtained for the TP10 isolates (Table 4.5).

5.2 THE EFFECTS OF ALTERNATIVE CARBON SOURCES

Eight 100ml bottles containing basal medium plus 0.06% yeast extract were individually supplemented with various carbon sources, (Table 5.1), a 1.5% inoculum of TP8.T6.3.3.1 was added to each, and the bottles were incubated at 75°C.

Various growth related parameters and cellulase activities were measured during the following 6 days (Table 5.1, Figs 5.2 and 5.3). Immediately before sampling, each bottle was shaken to create an homogenous suspension.

Growth, as indicated by visual assessment of unshaken turbidities, was most rapid in 0.2% glucose and 0.2% cellobiose (Table 5.1). Decreases in pH from an initial value of 7 were also most rapid in these cultures.

Plate clearing and CMCase activities after 1 day were clearly highest in the cultures containing cellobiose (Table 5.1 and Fig 5.2). Plate clearing activity in all cultures was partly cell associated at day 1, since larger zones of clearance were produced if wells were loaded with samples of the whole cultures rather than with cell-free supernatants.

Turbidity rankings at day 6 were almost the reverse of those of day 1. Turbidities of the most rapidly growing cultures remained fairly static after 2 days' growth until they began to decrease by 6 days. The cell pellets from centrifugation of the two 0.2% cellobiose and the 0.2% glucose cultures after 6 days' growth had become grey-black, probably due to an increased dead-cell component, whereas all the others remained creamy white.

The pH did not fall as low in the cultures which grew most rapidly and then stopped early as in others which grew more gradually, suggesting that pH was not the factor finally limiting growth in the

Table 5.1: Growth, pH, glucose, ethanol and acetate levels and avicelase and CMC-plate-clearing activities in cultures of TP8.T6.3.3.1 grown on various carbon sources

Carbon Source	After 1 day's growth					After 6 days' growth							
	Growth*	pH	Glucose (mM)†	CMC-Plate-Clearing (Units. ml^{-1})†† With cells	No cells	Growth*	pH	Glucose (mM)†	CMC-Plate-Clearing (Units. ml^{-1})†† With cells	No cells	Avicelase ($nmol.min^{-1}.ml^{-1}$)	Ethanol (Relative, arbitrary units)	Acetate
Cellobiose (0.2%)	4	5.5	<0.2	5.7	3.0	2	5.6	>1	3.7	4.6	0	83	87
Cellobiose (0.2%)	4	5.5	0.2	7.1	3.0	2	5.6	>1	3.7	4.6	N.D.	90	80
Cellobiose (0.1%)	3	6.1	<0.2	3.7	1.2	5	5.7	<0.2	2.4	3.0	0.08	101	102
Glucose (0.2%)	4	5.7	>1	1.0	0.2	1	5.0	>1	0	0	0	114	84
Sucrose (0.2%)	2	5.9	<0.2	0.7	0.2	4	5.4	~1	0	0.4	0	98	87
Trypticase Peptone (0.2%) + Sigmacell 50 (0.2%)	1	6.8	<0.2	0.5	0.2	7	5.0	0.5	7.1	7.1	0.97	120	45
Sigmacell 50 (0.2%)	1	6.8	<0.2	0.3	0.2	6	5.0	0.5	7.1	7.1	0.34	100	45
CMC# (0.2%)	0	7.0	<0.2	0	0	3	7.0	<0.2	1.2	1.0	0.06	0	67

\# Low viscosity soluble form (sodium salt, Sigma Product No. C8758)
* By visual assessment of turbidity (See Section 2.3)
† Using Clinistix test strips
†† Arbitrary units (See Figure 3.2)
N.D. = Not done

All cultures contained basal medium plus 0.06% yeast extract in addition to the various C-sources listed.

Figure 5.2 CMCase accumulation in the cell-free culture supernatant during growth of TP8.T6.3.3.1 on basal medium supplemented with various carbon sources.

• = 0.2% cellobiose (duplicate values shown).

+ = 0.1% cellobiose

○ = 0.2% sucrose

* = 0.2% glucose

△ = 0.2% CMC

□ = 0.2% Sigmacell 50

× = 0.2% Sigmacell 50 + 0.2% trypticase peptone

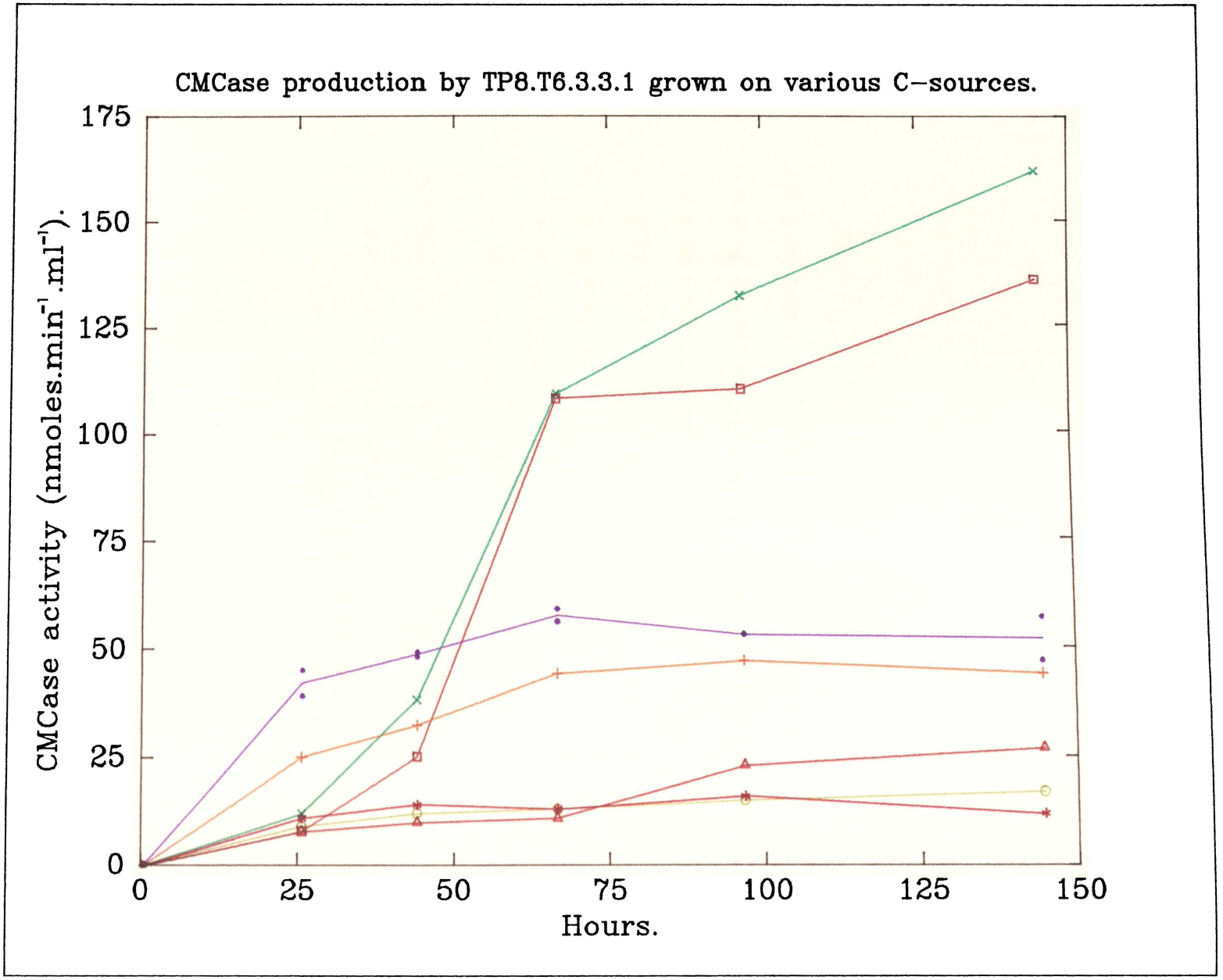

CMCase production by TP8.T6.3.3.1 grown on various C-sources.
CMCase activity (nmoles.min-1.ml-1).
175
150
125
100
75
50
25
0
0
25
50
75
100
125
150
Hours.

Figure 5.3 Reducing sugar accumulation in the cell-free culture supernatant during growth of TP8.T6.3.3.1 on basal medium supplemented with various carbon sources

• = 0.2% cellobiose (duplicate values shown).

+ = 0.1% cellobiose

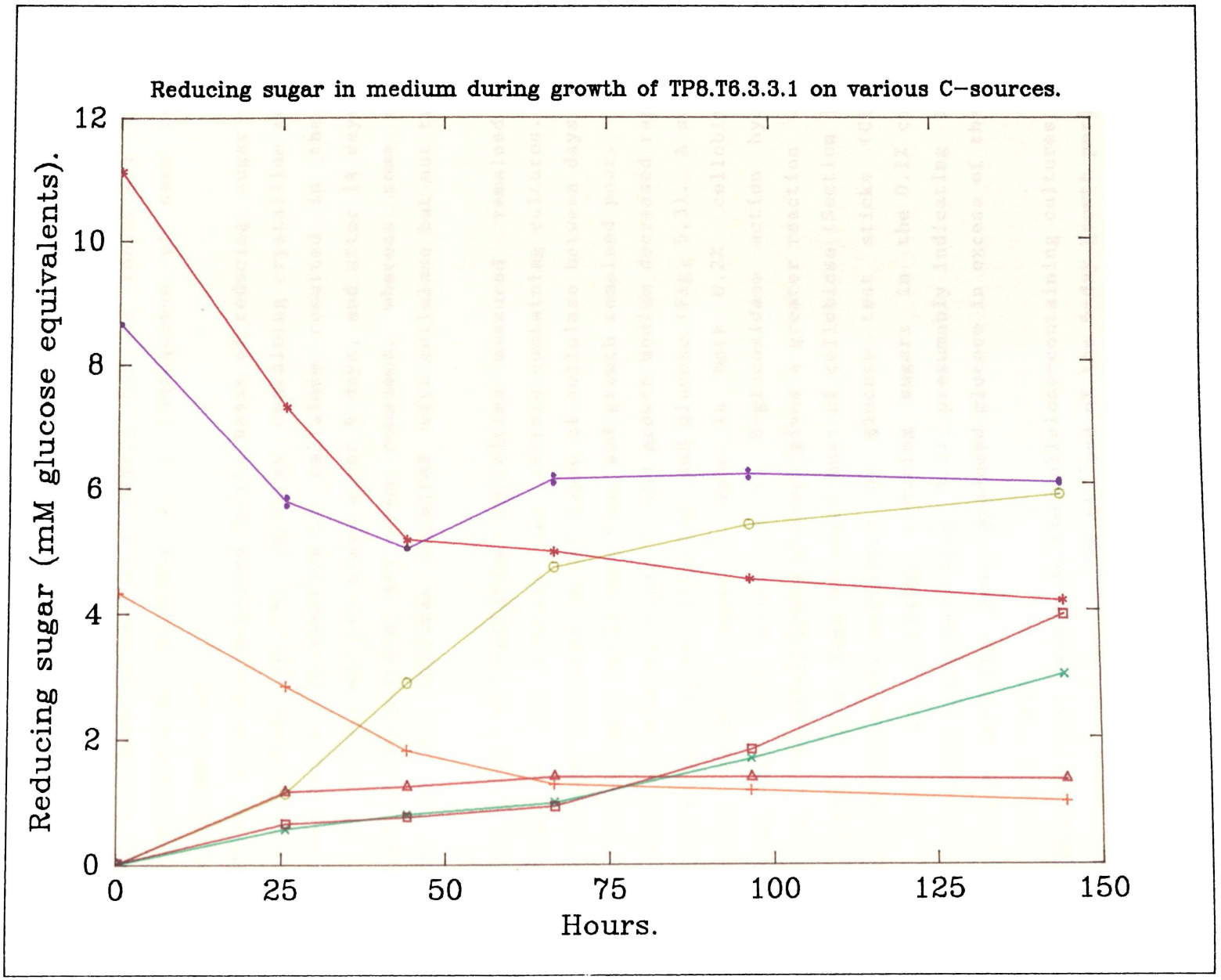
o = 0.2% sucrose

* = 0.2% glucose

△ = 0.2% CMC

□ = 0.2% Sigmacell 50

× = 0.2% Sigmacell 50 + 0.2% trypticase peptone


Reducing sugar in medium during growth of TP8.T6.3.3.1 on various C-sources.
Reducing sugar (mM glucose equivalents).
0
2
4
6
8
10
12
0
25
50
75
100
125
150
Hours.

fast-growing cultures.

CMC-plate-clearing activities at day 6 were highest in the two crystalline cellulose-containing cultures. By day 6, inclusion of cells in the wells resulted in similar or less plate clearance than if cell-free supernatant was used. In the case of the 6 day old sucrose culture, inclusion of the cells clearly had an inhibitory effect on CMC-plate-clearance, possibly as a consequence of some cell-lysis product accumulation.

CMCase activity (measured on the basis of reducing sugar release) was also highest in the cultures containing crystalline cellulose. Trypticase peptone in addition to cellulose resulted in the highest levels of CMCase and avicelase after 6 days, and after 14 days all the cellulose in the culture had been consumed, whereas some cellulose remained in the cultures supplied with cellulose but not trypticase peptone.

All types of cellulase activities measured remained barely detectable in the glucose and sucrose containing cultures. The CMC culture showed increases in all types of cellulase between days 1 and 6 but the levels were still very low, and growth remained poor.

Levels of reducing sugar in the growth medium decreased rapidly in the cultures growing on cellobiose and glucose (Fig. 5.3). A subsequent rise which occurred after 2 days in both 0.2% cellobiose-grown duplicates may have been due to ß-glucosidase action hydrolysing cellobiose to glucose, since glucose gives a greater reaction with the DNSA reagent than does an equal mass of cellobiose (Section 3.5.1.2). Glucose production was confirmed by glucose test sticks (Clinistix) (Table 5.1). In contrast, reducing sugars in the 0.1% cellobiose culture declined throughout (Fig. 5.3), presumably indicating that the ß-glucosidase activity never produced glucose in excess of the demands of the growing cells.

Reducing sugar levels of the cellulose-containing cultures began to increase more rapidly towards the end of the 6-day growth period. The final level achieved by the cellulose plus trypticase peptone culture was lower than that of the cellulose only culture, which was the reverse of their relative avicelase levels at day 6 (Fig. 5.2 and Table 5.1). This anomaly can probably be attributed to the greater cell numbers present in the trypticase-peptone-containing culture, which would have exerted a greater demand on the reducing sugars produced from cellulose.

The largest and most rapid increase in reducing sugars occurred

during growth on 0.2% sucrose (Fig. 5.3). Sucrose is not a reducing sugar itself, but can be split by invertase into fructose and glucose, which are both reducing sugars. By 6 days, over half of the sucrose supplied had been converted to reducing sugars found free in the medium. Of the 6mM total reducing sugar concentration, glucose was present at only 1mM concentration. It can thus be deduced that glucose was being used by the cells in preference to fructose.

Growth on CMC resulted in only a very slight increase in reducing sugars in the medium, indicating that the growth demands were keeping pace with CMC hydrolysis to reducing sugars.

Ethanol was produced on all carbon sources apart from CMC, with the highest levels accumulating in the glucose and cellulose plus trypticase peptone cultures (Table 5.1). The latter also contained the highest ratio of ethanol to acetate. Growth on CMC was remarkable in that it produced acetate only, and in greater quantities than did growth on cellulose, despite the relatively poor growth and CMCase levels in the CMC culture.

In conclusion, although growth on the soluble carbon sources tested, was rapid (with the exception of CMC), it did not result in levels of cellulase activity which were as high as those finally attained after slower growth on crystalline cellulose. Trypticase peptone at 0.2% was shown to be a useful promoter of both growth and cellulase production.

5.3 THE EFFECTS OF GROWTH TEMPERATURE

TP8T would not grow at 80°C and a sharp cut-off of growth occurred somewhere in the 75-80°C range. Although growth rates at 70°C and 75°C appeared similar, on the basis of visual observations of the turbidity increase of unshaken cultures, growth continued for longer periods without flocculation at 70°C than at 75°C. CMCase and reducing sugars reached higher levels in the 70°C cultures than at 75°C (220% and 170% of the 75°C levels respectively).

The optimum for growth may be below 70°C, since no lower temperatures were tried. In the interests of maintaining selective pressures favouring thermophilic mutations and to disadvantage any less thermophilic contaminants, TP8T and its lineage of isolates were grown at 75°C (except agar cultures, which were grown at 70°C). When enzyme production on a large scale was the aim, the growth temperature of the fermentor was set to 72-73°C, since if it drifted beyond 75°C at any stage the culture growth would be dramatically set back.

5.4 THE INFLUENCE OF CYSTEINE-HYDROCHLORIDE (A REDUCTANT)

Two 500ml bottles, both containing basal medium, 0.06% yeast extract, 0.2% trypticase peptone and 0.2% Sigmacell 50, but differing in that only one contained 0.1% cysteine - HCl while the other contained no reductant, were inoculated with 12ml of TP8.T6.3.3.1 culture and incubated at 75°C. Both bottles of media were warmed to 75°C prior to inoculation.

Initially the resazurin redox indicator was red only in the bottle lacking cysteine-HCl. Both headspaces were gassed with CO_2 and incubated without shaking at 75°C.

After a few hours the resazurin became colourless in the bottle without cysteine-HCl, indicating that the bacteria had brought about reduction of the medium.

After 3 days growth, both cultures contained very similar looking Gram-ve cells, and possessed similar CMC-plate-clearing activities.

Cell growth and gas production were more vigorous in the absence of cysteine-HCl until after one month all of the cellulose in this culture had been consumed. At this point the pH was 4.7 and the medium contained glucose at 0.5mM. The other culture at one month still contained some cellulose. Its pH was 4.4 and its glucose level was below 0.2mM. Thus it was concluded that addition of cysteine-HCl was unnecessary and inhibitory to a degree.

TP8.T6.3.3.1 was only able to cope with restricted quantities of oxygen. No growth would occur if bottles had poorly sealing lids, and the resazurin would remain purple. It was oxygen-tolerant however, since spun-down cells remained viable after exposure to air for several hours as a dry paste, followed by freezing in air-filled containers in liquid nitrogen.

5.5 THE INFLUENCE OF AGITATION DURING GROWTH

Agitation during growth, sufficient to prevent the cellulose from settling out of suspension, increased the rate of growth and cellulose degradation, provided sufficient time was allowed for the medium to become reduced before shaking commenced. If shaking commenced immediately after inoculation, dispersal of the headspace gasses throughout the medium resulted in levels of dissolved oxygen that were inhibitory to growth. However, if growth of the inoculum was allowed to first become established by not shaking the bottle, the bacteria would eventually "mop up" the oxygen in the headspace, so that subsequent agitation would then hasten growth.

CHAPTER SIX

PURIFICATION AND PARTIAL CHARACTERISATION OF COMPONENTS OF THE CELLULASE COMPLEX OF TP8.T6.3.3.1

6.1 INTRODUCTION

The ability to significantly hydrolyse crystalline cellulose, or "avicelase" activity, was a capacity which had served to distinguish the TP8T strains from most of the other isolates of the screening programme. This was also an attribute likely to be of great importance in any industrial application, so we set our sights on purifying and characterising the "avicelase" component(s) of the cellulase complex.

The literature on bacterial cellulases gave no indication as to whether we might have expected to find a synergistic team of enzymes bearing joint responsibility for the avicelase activity, or whether this capacity might reside in a single enzyme. In fact very little had been published on the purification of components of cellulase complexes of bacteria, whereas fungal cellulases were comparatively well understood. Therefore our initial attempts to fractionate our cellulase complex utilized some conventional techniques which had been widely employed with success in fungal cellulase fractionation.

It eventually became apparent that these conventional techniques alone were insufficient to result in purification of single protein components from the complex, whereas considerably better progress was possible using preparative SDS electrophoresis and isoelectric focussing.

An evaluation of the various methods of concentration and fractionation employed in several trial purification sequences is outlined below. The most successful purification sequence will then be described in detail, followed by a demonstration of synergism between partially purified components of the complex.

6.2 EVALUATION OF THE METHODS OF CONCENTRATION

6.2.1 Ammonium sulphate precipitation and ultrafiltration

The efficiency of ammonium sulphate precipitation alone as a concentration method was not tested, owing to the interfering effects of ammonium sulphate on the enzyme activities and on the DNSA reaction.

A small scale trial combination of ammonium sulphate-induced precipitation (85% saturated) followed by redissolving, washing and reconcentrating on a 10,000 M.W. cut-off ultrafilter (PT Series, Millipore) gave a yield of 60% for CMCase and 100% for avicelase activity. However, when these procedures were scaled up in an attempt to recover the enzymes from 80 litres of culture supernatant, the resulting yields were very much lower, i.e. ß-glucosidase 4%, CMCase 30%, CMC-plate-clearing activity 4%, avicelase 2%. No significant improvement in these yields was achieved by further washing of the insoluble material which remained, and no activity was found in the ultrafiltrate. The large scale concentration had differed from the small scale trial in that the latter employed crude (fertilizer grade) ammonium sulphate whereas the former used lab. grade. It was possible that the majority of the activities of interest were permanently inactivated or rendered insoluble by some impurity in the crude ammonium sulphate.

6.2.2 Ultrafiltration

8ml collodion bags (Sartori us), made of cellulose nitrate and with a 12,000 M.W. cut-off, proved extremely effective for small scale concentrations. Provided concentration was not taken to the point at which the protein began to precipitate, yields were very high (80-100%) for all of the types of cellulolytic activities measured and also for ß-glucosidase.

Large scale ultrafiltration to concentrate the enzymes in 100 litres of cell-free supernatant down to 1 litre using a 50 square foot 10,000 M.W. cut-off Millipore polysulfone membrane in a continuous flow cassette system resulted in the following yields: ß-glucosidase 120%, CMCase 70%, CMC-plate-clearing activity 20% and avicelase 20%. A further 10-fold concentration of this material in a 1-litre ultrafilter over a 10,000 M.W. cut-off membrane (Millipore P.T. series) gave a 30% yield of avicelase activity, thus resulting in an overall avicelase yield of 7% for the two

ultrafiltration steps. It is possible that precipitation which occurred during both stages of the ultrafiltration accounted for a large part of this avicelase loss.

6.2.3 Rotary evaporation

This technique proved useful for concentrating volumes of 1-2 litres or less. It was only used when the ionic strength was low since, unlike ultrafiltration, the salts were concentrated as well as the protein. A 20-fold concentration at 60°C, pH5.6 resulted in yields of 90% for activity towards cellulose azure and 75% for CMC-plate-clearing activity. On a separate occasion, a 10-fold concentration at 42°C, pH 5 gave CMCase and avicelase yields of 75%.

6.2.4 Adsorption to cellulose

6.2.4.1 Adsorption to different celluloses Adsorption of avicelase, CMCase and ß-glucosidase activities to three different forms of powdered cellulose is shown in Table 6.1. The two types of crystalline cellulose (Sigmacell 50 and Whatman CC-31) exhibited similar adsorptions of the various activities measured, with ß-glucosidase being adsorbed to a much lesser extent that were the other activities. MN300, a non-crystalline powdered cellulose, adsorbed significantly more ß-glucosidase and less avicelase than did the crystalline forms. Sigmacell 50 was selected for further study.

6.2.4.2 Influence of pH and ionic strength on binding of the various activities to Sigmacell 50 Binding of avicelase to Sigmacell 50 occurred to the same extent in the presence of 5,20 or 100mM sodium citrate buffer pH6, but binding of CMCase activity was increased from 75% to 90% by increasing the molarity of the buffer from 5 to 100mM. The binding of CMC-plate-clearing activity was also increased by increased ionic strength, although the change was not quantified.

Avicelase, CMC-ase and CMC-plate-clearing activities were not significantly released from Sigmacell 50 by either a decrease of the eluent pH from 6 to 4 or an increase to pH 7.8. Release of up to 80% of bound avicelase, and to a less complete release of CMCase and CMC-plate-clearing activity, was however induced by washing the cellulose with distilled water. Heating the cellulose while washing with water further enhanced the release of these activities.

Table 6.1: Adsorption of cellulase components onto various forms of cellulose

Cellulose Type	Residual unbound activity (% of control)			
	Avicelase	CMCase	CMC-plate clearing	β-Glucosidase
Sigmacell 50	15	25	40	90
MN300	30	30	60	35
Whatman CC-31	15	35	60	95

To 4 ml of a 10% (w/v) slurry of each cellulose type in 0.1M sodium citrate buffer pH 6.0 was added 0.2 ml of ultrafilter-concentrated culture supernatant. A control comprised 4 ml of the buffer without cellulose and 0.2 ml of the concentrated supernatant. After incubation, with frequent agitation, at 2-5°C, the tubes were centrifuged and their supernatants assayed for the four cellulolytic activities listed, which are expressed as a percentage of each activity in the control.

Reese (1982) concluded that this weakening effect of high temperature on the cellulose - cellulase binding forces, considered in the light of the bond-weakening effect that was also produced by lowered ionic strength, suggested that hydrogen bonding rather than hydrophobic interactions was most important in binding cellulases to cellulose.

6.2.4.3 Direct binding of culture supernatant cellulases onto crystalline cellulose as an initial concentration and purification step A small scale trial was performed to investigate the feasibility of directly binding cellulases from a cell-free culture supernatant onto crystalline

cellulose (Fig. 6.1). For each type of activity applied in the cell-free supernatant, the percentage which passed straight through the column without binding is shown in Table 6.2. ß-glucosidase was the only one of the activities measured which showed no significant binding to the cellulose. Each of the step-wise reductions in the ionic strength of the eluent caused the release of a peak of protein containing cellulolytic activity. The majority of the avicelase and CMCase was nonetheless eluted subsequently in fractions 27 to 33 when the column temperature was gradually elevated to 70°C while elution with water continued. An 8-fold concentration of these activities was achieved by use of this small cellulose column.

A degree of separation of endoglucanase and exoglucanase activities was also produced, as can be seen from the changes in relative CMCase and CMC-plate-clearing activities across the elution profile (Fig. 6.1 and Table 6.2). Only 0.25% of the applied ß-glucosidase activity was bound to the column. Glucose comprised between 10 and 20% of the soluble carbohydrates released in the avicelase assay of each fraction, apart from fraction 1 and the preceding unbound material, in which ß-glucosidase presence meant that a larger proportion of the hydrolysate was glucose (data not shown).

A substantial purification was achieved for cellulolytic activities which were bound to the column, as is evident from comparison of the relatively low A_{280} levels of the subsequently eluted cellulase peaks with the preceding large A_{280} peak of the unbound material.

The yields of the various activities from this column are given in Table 6.2. Although the avicelase yield was the lowest (40%), part of the activity loss could probably be attributed to the separation of the avicelase from other synergistic components of the complex (Section 6.6). In any case, a 40% yield was an improvement on that obtained by the use of ultra-filtration as a means of preliminary concentration (Section 6.2.2) and was 20 times better than the yield after the combined use of ammonium sulphate precipitation and ultrafiltration (Section 6.2.1). In addition, the concentration on cellulose was selective and so

Figure 6.1 Elution of cellulases from cellulose after adsorption directly from culture supernatant

□ = CMC-ase activity

+ = CMC-plate-clearing activity

△ = avicelase activity

☆ = total protein (A_{280nm})

100ml of culture supernatant was cooled to 2°C and passed through a 4mm diameter column containing 0.2g of crystalline cellulose (Sigmacell 50), also at 2°C. The column was then washed as follows:

Fctns	Eluting conditions.
1-5	200mM sodium acetate buffer pH5.6 at 2°C.
6-10	2mM sodium acetate buffer pH5.6 at 2°C.
11-15	0.2mM sodium acetate buffer pH5.6 at 2°C.
16-24	distilled water at 2°C.
25-30	distilled water while temperature increased to 70°C.
31-47	distilled water at 70°C.

Fraction volume = 2ml.

Flow rate during loading was 38ml.h^{-1}, reduced to 20ml.h^{-1} from fraction 6 onwards.

Column dimensions: 4mm i.d. x 6cm

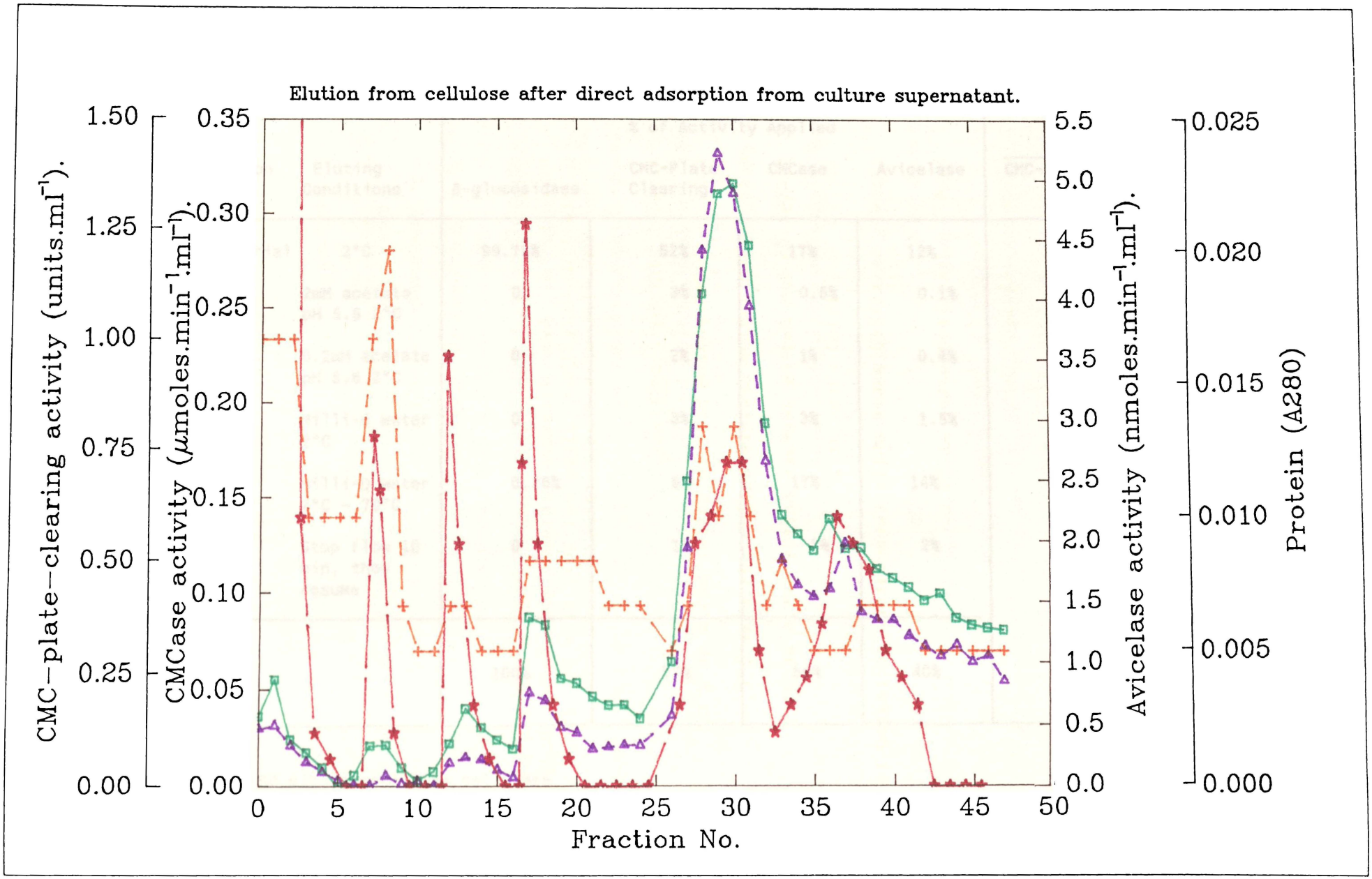

Elution from cellulose after direct adsorption from culture supernatant.
CMC-plate-clearing activity (units.ml-1).
CMCase activity (μmoles.min-1.ml-1).
Avicelase activity (nmoles.min-1.ml-1).
Protein (A280)
Fraction No.
1.50
1.25
1.00
0.75
0.50
0.25
0.00
0.35
0.30
0.25
0.20
0.15
0.10
0.05
0.00
5.5
5.0
4.5
4.0
3.5
3.0
2.5
2.0
1.5
1.0
0.5
0.0
0.025
0.020
0.015
0.010
0.005
0.000
0
5
10
15
20
25
30
35
40
45
50

Table 6.2: Overall yields and distributions of cellulolytic components following the direct application of a crude cell-free culture supernatant to a cellulose* column

Peak	Fraction Nos.	Eluting Conditions	% of Activity Applied				CMCase† / CMC-Plate-Clearing
			β-glucosidase	CMC-Plate Clearing	CMCase	Avicelase	
Unbound material		2°C	99.75%	52%	17%	12%	37
I	7-9	2mM acetate pH 5.6 2°C	0	3%	0.5%	0.1%	21
II	12-15	0.2mM acetate pH 5.6 2°C	0	2%	1%	0.4%	85
III	17-21	Milli-Q water 2°C	0	3%	3%	1.5%	131
IV	27-33	Milli-Q water 2°C → 70°C	0.25%	5%	17%	14%	408
V	36-37	Stop flow 10 min, then resume	0	1%	3%	2%	437
Column yield (as % of activity applied)			100%	77%	56%	40%	

* Sigmacell 50 microcrystalline cellulose
† The cell-free supernatant applied gave a value of 112 for this ratio

Details of the methods and materials employed accompany Fig. 6.1

doubled as a very useful purification step (further discussed in Section 6.3.1).

Overall, affinity concentration of cellulases on cellulose seemed the most effective, simplest and cheapest means of "harvesting" cellulases from the culture supernatant.

6.3 EVALUATION OF METHODS OF FRACTIONATION

6.3.1 Sequential elution from cellulose

Biospecific adsorption onto cellulose, followed by sequential elution of various components of the complex, had been employed in purification of fungal cellulases (Toyama, 1969, Halliwell and Griffin, 1973, Weber *et al.*, 1980, Nummi *et al.*, 1981b). We therefore tried using adsorption to crystalline cellulose as a means of fractionating the cellulase complex, hoping that the avicelase component(s) would show greater affinity for the cellulose than would other components of the complex.

A variety of elution regimes involving various combinations of heat treatment, reduction of the ionic strength of the eluent and elution with cellobiose and CMC solutions were tested as means of obtaining sequential elution of components of the cellulase complex. As the technique was scaled up to involve the use of larger columns, the efficiency of the cellulase recoveries decreased. This was largely due to blockage of column outlets by very fine cellulose particles and to channelling which prevented uniform washing of the cellulose.

One of the most successful medium-scale cellulase fractionations is shown in Fig. 6.2. Distribution of the activities and protein between the various peaks and also their overall yields are summarised in Table 6.3. 95% of the ß-glucosidase activity and 55% of the total protein applied didn't bind to the cellulose, whereas only 4% of the avicelase activity passed through unbound in Peak I.

The second major peak of protein and activity (Peak II) emerged in response to heating the column to 75°C. This peak displayed the highest CMCase:avicelase ratio and the lowest CMCase:CMC-plate-clearing ratio (Table 6.3). This combination was consistent with the Peak II cellulases containing a greater proportion of centrally or randomly-acting chain cleaving endoglucanases than the other peaks.

Peak III, which was released as the ionic strength of the

Figure 6.2 Fractionation of crude ultrafilter-concentrated cell-free supernatant of TP8.T6.3.3.1 on a column of Sigmacell-50 microcrystalline cellulose.

□ = CMC-ase activity

+ = CMC-plate-clearing activity

○ = ß-glucosidase activity

△ = avicelase activity

☆ = total protein (A_{280nm})

Fctn.	Eluting conditions.
1-4	Loaded 30ml concentrated culture supernatant at 2°C.
5-13	Washed with 20mM sodium citrate pH 6, at 2°C.
14-25	Linear gradient from 20mM citrate pH6 - MilliQ water. Simultaneously began warming column to 75°C.
26-58	Washed with MilliQ water at 75°C.

Flow rate was highly temperature-dependent, being $10ml.h^{-1}$ initially and doubling when the temperature was raised, until fine particles greatly reduced the flow from fraction 27 onwards.

Column dimensions: 1.6cm by 10cm.

Packing: 9g Sigmacell-50 (previously "defined").

Pre-equilibration: 20mM sodium citrate buffer pH 6.0 at 2°C

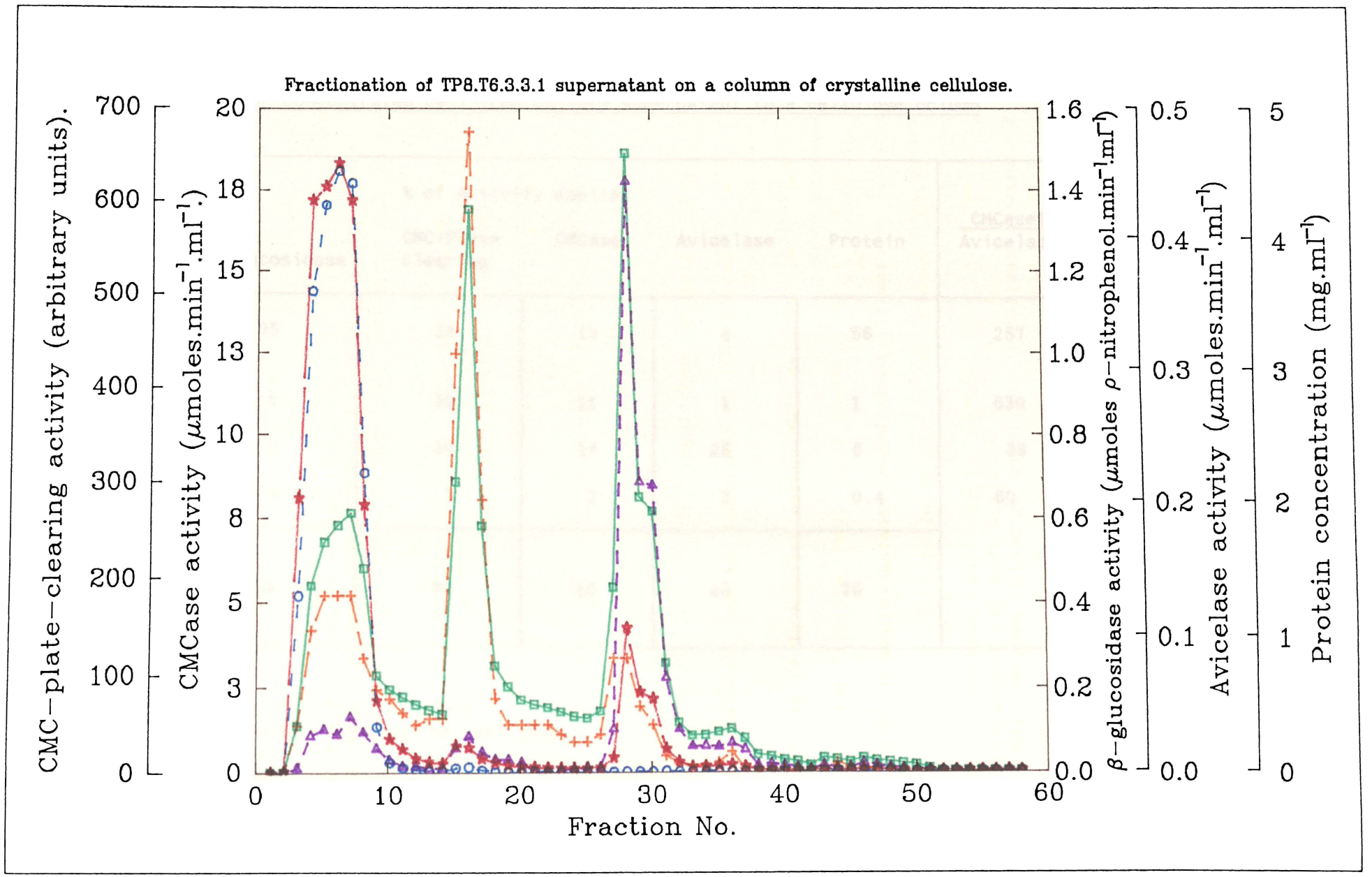

Fractionation of TP8.T6.3.3.1 supernatant on a column of crystalline cellulose.
CMC-plate-clearing activity (arbitrary units).
CMCase activity (μmoles.min⁻¹.ml⁻¹.)
β-glucosidase activity (μmoles ρ-nitrophenol.min⁻¹.ml⁻¹)
Avicelase activity (μmoles.min⁻¹.ml⁻¹)
Protein concentration (mg.ml⁻¹)
Fraction No.
0
100
200
300
400
500
600
700
3
5
8
10
13
15
18
20
0.0
0.2
0.4
0.6
0.8
1.0
1.2
1.4
1.6
0.1
0.2
0.3
0.4
0.5
1
2
3
4
5
0
10
20
30
40
50
60

Table 6.3: Overall yields and distributions of various cellulolytic activities and protein following application of ultrafilter-concentrated cell-free culture supernatant to a cellulose column

Peak	Fraction Nos.	% of Activity Applied: β-glucosidase	CMC-Plate Clearing	CMCase	Avicelase	Protein	CMCase† / Avicelase	CMCase† / CMC-Plate-Clearing
I (unbound)	3-9	95	24	13	4	56	257	40
II	15-17	0	35	11	1	1	639	25
III	27-31	0	10	14	25	6	38	119
IV	34-37	0	1	2	2	0.4	60	91
Column yield (as % of activity applied)		95	90	50	40	70		

* Sigmacell 50 microcrystalline cellulose
† Calculated for the middle fraction of each peak

Details of the experimental methods and materials accompany Fig. 6.2

eluent was reduced to approach that of Milli-Q water while the column temperature was maintained at 75°C, contained cellulases which contrasted markedly with those of Peak II in having the lowest CMCase:avicelase ratio and the highest CMC:CMC-plate-clearing ratio (Table 6.3). The high latter ratio clearly indicated that Peak III cellulases were the most exo-acting, being relatively more effective in producing reducing sugars from CMC than in rapidly shortening the average CMC chain length. The fact that Peak III exhibited the lowest CMCase : avicelase ratio was probably due to its having the highest proportion of enzymes which could catalyse the cleavage of interpolymer H-bonds involved in maintaining the crystal structure. This hypothesis was supported by the milky turbidity which occurred in the fractions of Peaks III and IV. This turbidity, which was most intense in the most active fractions of Peak III, was shown to contain extremely fine particles. Filtration through a 0.2μm polycarbonate filter had little clarifying effect and even centrifugation at 44,000g for 15 minutes would not completely remove the particles from suspension.

Reese (1982) reported that a similar "opalescence" accompanied the release of *Trichoderma* cellobiohydrolase from cellulose when eluted with water. He attributed this to cellulosic material being rendered partially soluble at low ionic strength by bound protein. Assuming that the turbidity had the same cause in our case suggested that some component of Peak III was, by its attachment to an external glucose polymer of a cellulose crystal, weakening that polymer's H-bonds with its neighbours to a degree which caused them to break once the ionic strength fell below a certain level. It would have been interesting to investigate the effect of ionic strength on avicelase activity of this complex. Low ionic strengths may well enhance avicelase activity.

The release of fine particles led to a decreased flow rate and eventual blockage of the outlet net, which had to be removed and washed during the course of collecting fractions 27 and 43.

The degree of purification achieved by the cellulose column for each of the four types of activity measured is shown on Table 6.4. CMC-plate clearing activity appeared to have been purified to the greatest extent (37-fold) and was retrieved from the column with a yield of 90%. Synergisms with respect to avicelase activity

were shown to exist between Peaks I, II and III (Section 6.6) so the actual yields and degrees of purification of the component enzymes would have been higher than the figures for avicelase activity shown in Tables 6.3 and 6.4 suggest.

Table 6.4: Purification of the component activities of the cellulase complex achieved by passage through the cellulose column of Fig. 6.2.

Activity Type	Specific activity* In concentrate applied	Specific activity* Highest from cellulose column	Fraction Involved	Peak No.	Purification factor
β-glucosidase	0.176	0.358	8	I	2
CMC-plate -clearing	0.101	3.69	16	II	37
CMCase	7.38	92.6	16	II	13
Avicelase	0.110	0.528	33	III	5

* Units of specific activity:
β-glucosidase = μmoles ρ-nitrophenol. min^{-1}.mg $protein^{-1}$
CMC-plate-clearing = arbitrary units.mg $protein^{-1}$
CMCase = μmol glucose equiv. min^{-1}.mg $protein^{-1}$
Avicelase = μmol glucose equiv. min^{-1}.mg $protein^{-1}$

Details of the experimental methods and materials accompany Fig. 6.2

Non-denaturing electrophoresis, employing the Laemmli (1970) system but without the SDS, (Fig. 6.3) revealed the unbound Peak I material (Lane 1 and 8) to be very similar to the concentrate applied (Lanes 4 and 5) except in that the Peak I pattern lacked, or exhibited lower levels of, some of the slowest migrating bands found near the top of the concentrate band pattern. As expected, these slow-moving bands were found to be major components of the Peak II and Peak III patterns. The latter peaks produced band patterns which were quite different from one another, with poor mobility being a common feature (Fig. 6.3, Lanes 6 and 7). A major component of each had not even entered the separating gel. It was quite apparent from the simplified band patterns of Lanes 6 and 7 cf. Lane 1, the material applied, that the cellulose column

separation step had greatly purified the cellulase components in Peaks II and III. This was borne out by the specific activity data of Table 6.4.

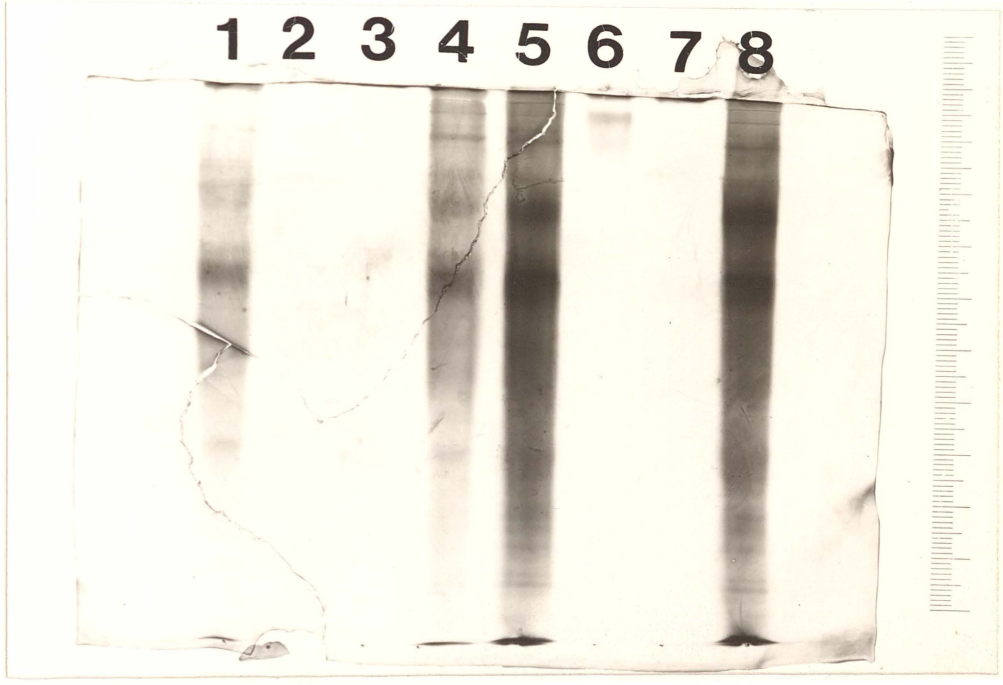


Figure 6.3 Non-denaturing electrophoresis of the three main peaks from the cellulose column of Fig. 6.2 and of the concentrate applied.

Lane 1	10µl Fctn.6 (i.e.Peak I)
Lane 2	10µl Fctn.15 (i.e.Peak II)
Lane 3	10µl Fctn.28 (i.e.Peak III)
Lane 4	10µl concentrate as applied to the column
Lane 5	40µl concentrate as applied to the column
Lane 6	40µl Fctn.28 (i.e.Peak III)
Lane 7	40µl Fctn.15 (i.e.Peak II)
Lane 8	40µl Fctn.6 (i.e.Peak I)

Saturated sucrose comprised 10% of the volume of each sample loaded.

The system used was that of Laemmli (1970) except that SDS was not included.

The separating gel was 7.5%(w/v) acrylamide.

Since Ait *et al.* (1979b) and Tong (1980) had found that SDS electrophoresis resulted in fragmentation of cellulases which moved as single bands under a non-denaturing system, Peaks II and III were also run under the normal Laemmli-SDS conditions (Fig. 6.4). Both peaks produced multiple band patterns which covered a range of

Figure 6.4 SDS-PAGE of peaks II and III from the cellulose column of Fig. 6.2.

Lane 1 35µl Fctn 15 (i.e. Peak II)

Lane 2 35µl Fctn 16 (i.e. Peak II)

Lane 3 35µl Fctn 28 (i.e. Peak III)

All samples were pretreated as described in Section 2.9.3.
The separating gel contained 10%(w/v) acrylamide.

Figure 6.5 SDS-PAGE of Peak III of the cellulose column of Fig. 6.2 and of the two peaks resulting from its subsequent fractionation on an Ultrogel AcA44 column.

Lane 1 35µl Fctn.28 (Peak III of cellulose column, Fig.6.2).

Lane 2 35µl of first of two peaks produced by fractionation of Peak III on Ultrogel AcA44.

Lane 3 35µl of second of two peaks produced by fractionation of Peak III on Ultrogel AcA44.

All samples were pretreated as described in Section 2.9.3.

The separating gel contained 10%(w/v) acrylamide.

Figure 6.6 SDS-PAGE of the peaks from cellulose columns.

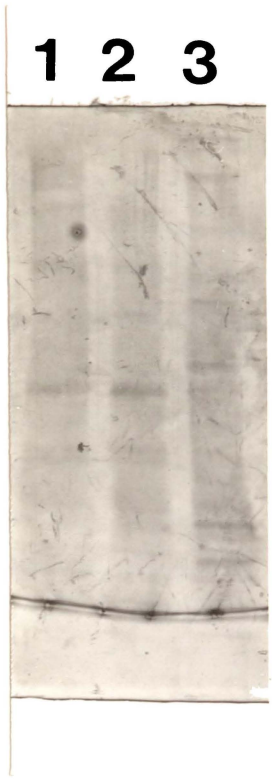
Lane 1 50µl Peak III from the cellulose column of Fig.6.2.

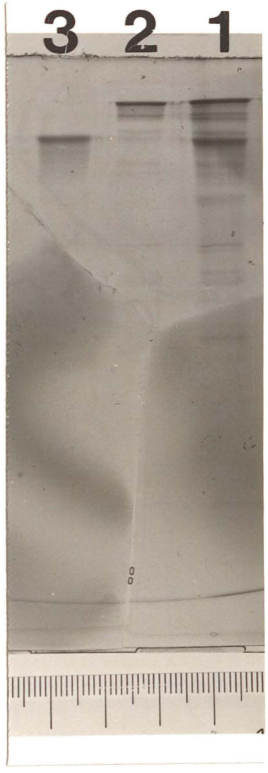
Lane 2 50µl of a water-eluted peak obtained by rerunning Peak III above on a second cellulose column.

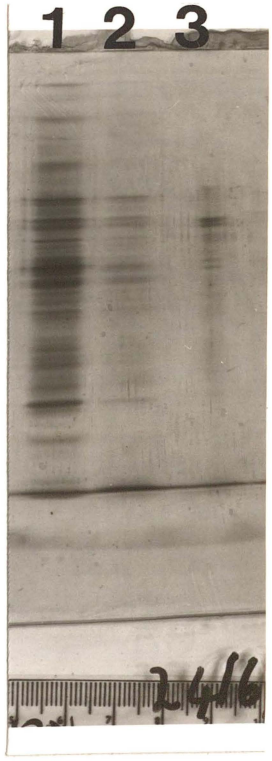
Lane 3 50µl of a heat-eluted peak obtained by rerunning Peak III above on a second cellulose column.

All samples were pretreated as described in Section 2.9.3.

The separating gel contained 10%w/v acrylamide.


1 2 3


3 2 1


1 2 3

mobilities corresponding to molecular weights of between ca. 15,000 and 145,000. Peak II appeared to contain a major component with a molecular weight of 34,000. Peak III (Fig. 6.4, Lane 3) produced several bands of fairly equal intensity. Better silver stains of Peak III appear in Figs. 6.5 (Lane 1) and 6.6 (Lane 1), which emphasise the extreme complexity remaining, despite the use of a biospecific affinity purification step (i.e. the cellulose column). Note that the gel of Fig. 6.5 was stained with Coomassie Brilliant Blue R250 (Sigma) whereas that of Fig. 6.6 was silver stained. The Coomassie stain emphasised the high molecular weight (slow-moving) bands whereas the silver stain gave quite a different impression of relative protein levels in these bands.

Peak III, to which sodium acetate pH5.6 buffer had been added to give a final concentration of 50mM, was rerun on a second cellulose column and treated with the same regime of heat and declining ionic strength as applied to the first column. This time all of the activity and protein was bound, as expected and only a very minor component (Fig. 6.6, Lane 3) eluted when the column was heated prior to the reduction of ionic strength of the eluent. The bulk of the protein was eluted by conditions similar to those under which Peak III had emerged from the previous column (i.e. by the combined effects of heat and a very low ionic strength). This major peak from the second cellulose column (Fig. 6.6, Lane 2) closely resembled the original Peak III material (Fig. 6.6, Lane 1).

The minor heat-released peak of the rerun did not contain CMC-plate-clearing activity, in contrast with the material released at the equivalent point from the previous cellulose column. Further, its SDS-PAGE band pattern did not resemble that of the heat-released peak of the previous column (Fig. 6.6 Lane 3 cf. Fig. 6.4 Lanes 1 and 2. In summary, apart from the unexplained appearance of the small heat-released peak, the behaviour of the water-eluted peak upon rerunning on a second cellulose column was reproducible.

Weber *et al.* (1980) reported that cross-linked cellulose provided a more useful affinity matrix for cellulases from *Trichoderma* than did untreated crystalline cellulose. They found that exoglucanases (ß-(1-4)-D-glucan cellobiohydrolase and ß-glucosidase) passed through the cross-linked cellulose column in

the equilibrating buffer, whereas the endoglucanases became bound under the same conditions. Stepwise elution with increasing concentrations of CMC led to the fractionation of five different endo-ß(1-4)-glucanases. They were unable to compare these endoglucanases successfully on gel electrophoresis and attributed this to the large amount of carbohydrate associated with each.

We made some cross-linked cellulose by the same method as used by Weber *et al.* (1980), involving epichlorohydrin, but substituting Sigmacell 50 for the Whatman crystalline cellulose as the raw material. The ultrafilter-concentrated supernatant, washed in 0.1M sodium citrate buffer pH5.6, was applied to a small column filled with the cross-linked cellulose, equilibrated in 30mM sodium acetate buffer pH 5.6. Negligible binding resulted for any of the various cellulolytic activities measured and the band pattern produced after SDS-PAGE was not noticeably altered by passage through the column (Fig. 6.7 Lane 1 cf. Lane 2). The very small amount of each activity which did bind was released more effectively by a final water wash than by the range of CMC concentrations applied first, that reproduced the elution conditions of Weber *et al.* (1980).

The unbound cellulases from the cross-linked cellulose column proved to be unexpectedly difficult to subsequently bind to unmodified crystalline cellulose, with 65% of the avicelase activity failing to bind to a column of Sigmacell 50. It is possible that the small amount of protein and activity which did bind to the cross-linked cellulose might have included a component of the complex which was vital for binding the complex to unmodified crystalline cellulose. Two such components have been shown to exist in the *C. thermocellum* cellulase complex (Lamed *et al.*, 1983 and Ljungdahl *et al.*, 1983).

Binding to Sigmacell 50 by cellulases which had not bound to cross-linked cellulose resulted nevertheless in a distinct change in the band pattern produced by SDS-PAGE (Fig. 6.7 Lane 2 cf. Lane 3) despite the fact that only 35% binding of the avicelase was achieved. The least mobile band on SDS-PAGE had been completely bound. Lanes 4, 5 and 6 of Fig. 6.7 show the SDS-PAGE band patterns of the subsequently-eluted active material obtained by heating the column to 75°C, eluting with 0.05% (w/v) CMC and finally with distilled water. The water-eluted material (Lane 6)

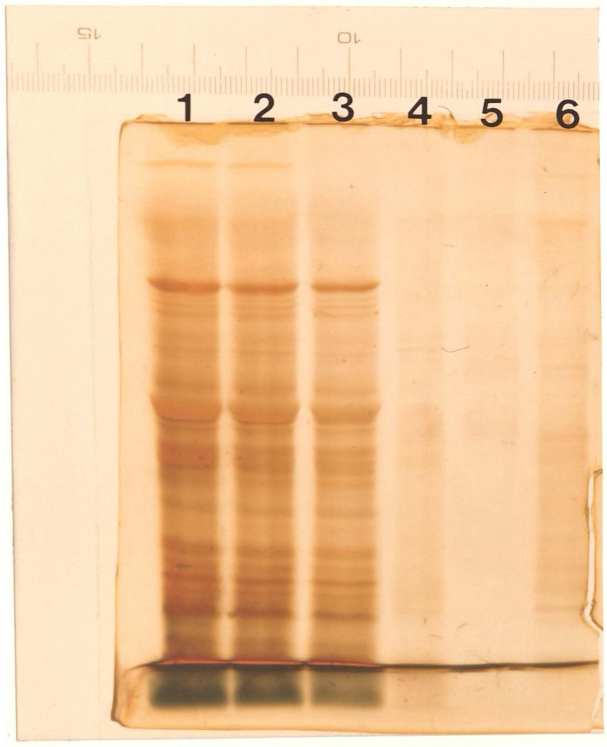


Figure 6.7 SDS-PAGE of various fractions from cross-linked and unmodified cellulose columns.

Lane 1 Crude ultrafilter-concentrated culture supernatant.

Lane 2 Components of the culture supernatant which did not bind to the cross-linked cellulose column.

Lane 3 Components of the culture supernatant which did not bind to either the cross-linked cellulose column or to an unmodified cellulose column.

Lane 4 Heat-eluted peak which had bound to unmodified cellulose.

Lane 5 CMC-eluted peak which had bound to unmodified cellulose.

Lane 6 Water-eluted peak which had bound to unmodified cellulose.

All samples were pretreated as described in Section 2.9.3.
The separating gel contained 7.5% acrylamide(w/v).

contained the above-mentioned least-mobile band along with innumerable other bands. The heat and CMC-eluted peaks also produced complex band-patterns, with some bands in common while others served to distinguish between them.

6.3.2. Immobilized cellobiose

No significant binding of any type of cellulolytic or ß-glucosidase activity was observed in experiments employing a small column of immobilized cellobiose (Pierce Selectin 13). We attempted to bind the cellulolytic activity to the Selectin gel with both the gel and the sample equilibrated in 5mM sodium acetate buffer, pH 5.6. The ß-glucosidase binding test was performed at pH 6.0 in 0.1M sodium citrate buffer. We are not aware of any reported application of this material to cellulase purification in the literature.

6.3.3. Ion exchange chromatography

The results of a small-scale batch-wise adsorption of CMCase, avicelase and ß-glucosidase onto a variety of anion exchangers are shown in Table 6.5. All four types of exchanger adsorbed virtually all the ß-glucosidase activity, but DEAE-cellulose was clearly the most effective with regards adsorption of avicelase and CMCase. It seemed likely that these enzymes had an affinity for DEAE cellulose which extended beyond the charge interactions normally involved in binding proteins to ion exchangers. This additional affinity was recognized and used to advantage by Marshall (1972, 1973a, b, c) in purifying snail cellulases. However when we attempted to use a DEAE-cellulose column for further fractionating the water-eluted cellulose peak obtained after binding to a cellulose column, the yields of avicelase and CMCase were only 10-20%, due to the majority of these activities being apparently irreversibly bound. No further activity was liberated by the high salt or low pH treatments which Marshall found appropriate.

ß-glucosidase and cellulolytic activities which did not bind to crystalline cellulose behaved in a more predictable manner than did avicelase and CMCase on DEAE-cellulose (Section 6.5.4), and eluted at various points on a simple salt gradient.

A column of DEAE-Sepharose CL-6B was found to completely bind the various cellulolytic activities contained in the water-eluted peak from a cellulose column. The pH was adequately maintained at pH 7.6 during binding, which may account for the better binding

compared with that of the small-scale trial, (Table 6.5). Subsequent reduction of the pH to 4 would result in a sudden release of all the bound avicelase and CMCase activities in a single peak, with no fractionation being achieved. Elution with pH or ionic strength gradients was not effective in fractionating the complex into more than one distinct protein peak.

Table 6.5: Binding of avicelase, β-glucosidase and CMCase activities to various anion exchangers

	Unbound activity (% of control)		
Anion Exchanger	Avicelase	CMCase	β-Glucosidase
QAE-Sephadex A-50	120	80	6
DEAE Sephadex A-50	97	79	6
DEAE Sepharose-CL-6B	89	71	5
DEAE Cellulose	28	35	6

0.25 g of each anion exchanger was equilibrated in 0.005M Tris/HCl buffer pH 7.6 in a final volume of 5 ml and 0.2 ml of ultrafilter-concentrated culture supernatant in 0.1M sodium citrate buffer pH 5.7 was added to each*.

Activities in the supernatant were measured after 1 h at 20°C, with frequent shaking, and expressed as percentages of the activities of the control.

As a control, the same volume of enzyme concentrate was added to a tube containing 0.005M Tris/HCl buffer pH 7.6 only.

* It is likely that the pH5.7 buffer in the concentrated supernatant would have reduced the pH of each mixture to less than pH 7.6 during the period of the binding test.

CM-Sepharose CL-6B equilibrated in 5mM sodium acetate buffer pH 4.7, was also found to completely bind the cellulolytic activities in the water-eluted active fractions from a cellulose column, while some noncellulolytic protein passed through unbound,

thus effecting a degree of purification (Fig. 6.8). The majority (70-100%) of each of the types of cellulolytic activity measured was released by eluting with a 5 - 100mM gradient of sodium acetate buffer pH 4.7 (Fig. 6.8). Although no clear fractionation of the various types of activity was achieved, SDS-PAGE revealed that a shoulder (Fig. 6.9, Lane 2) differed substantially from the main part of the protein and activity peak (Lane 4), so it appeared that the complex had been divided into at least two subcomplexes by the CM-Sepharose step.

A greater degree of separation of cellulolytic and non-cellulolytic protein by a CM-Sepharose CL-6B column was achieved by applying the heat-released material from a cellulose column (Fig. 6.10). The bulk of the protein emerged in fractions 41-45 (Lane 7, Fig. 6.11) and was thus separated from the majority of the cellulolytic activity which emerged later in the 5 - 100mM gradient of sodium acetate buffer pH 4.7 (Fig. 6.10). Continued elution with the 100mM buffer and subsequently with 0.2M sodium acetate buffer pH 5.6 produced several protein peaks which gave differing relative responses under the three different cellulase assays employed (Fig. 6.10). The later-eluting peaks evidently had a lower proportion of endoglucanase activity than the early peaks, based on the ratios of CMC-plate-clearing activity to release of both dye and glucose from cellulose azure. These various peaks displayed some differences in band patterns produced by SDS-PAGE (Fig. 6.11 Lanes 1-6), but clearly there were numerous proteins present in each.

It was concluded that ion exchange chromatography offered a means of fractionating the cellulase complex into several sub-complexes but no further sub-division into individual proteins appeared possible by this method.

Figure 6.8 Cation exchange chromatography on CM-Sepharose CL-6B of cellulolytic fractions in the water-eluted peak from a cellulose column.

● ="Cellulose-azurase" activity (dye release from cellulose azure)

△ = avicelase activity

\+ = CMC-plate-clearing activity

✡ = protein (A_{280nm})

The column (1.6cm by 10cm) was pre-equilibrated in 5mM sodium acetate buffer, pH 4.7.

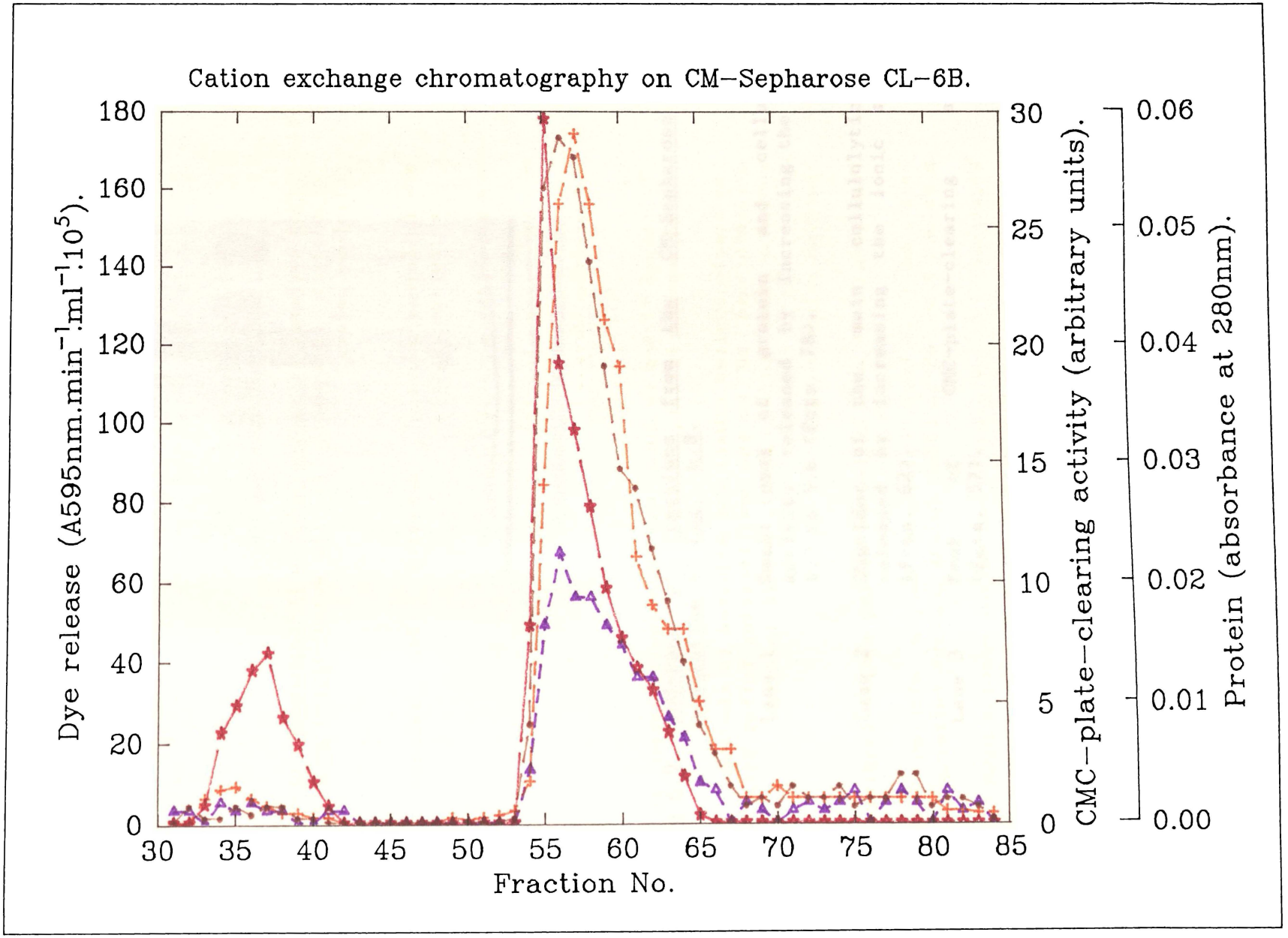
The sample applied (20ml) was adjusted to the same ionic strength and pH as the equilibrating buffer with 500mM sodium acetate buffer, pH4.7, before loading.

Fraction volume = 5ml.

Fractions 31-34 were collected during sample loading.

Fractions 35-70 were collected during elution with a gradient from 5mM to 100mM sodium acetate buffer,pH 4.7.

Fractions 71-85 were collected during elution with 100mM sodium acetate buffer, pH 5.6.


Cation exchange chromatography on CM-Sepharose CL-6B.
Dye release (A595nm.min^{-1}.ml^{-1}.10^5).
CMC-plate-clearing activity (arbitrary units).
Protein (absorbance at 280nm).
Fraction No.
0
20
40
60
80
100
120
140
160
180
0
5
10
15
20
25
30
0.00
0.01
0.02
0.03
0.04
0.05
0.06
30
35
40
45
50
55
60
65
70
75
80
85

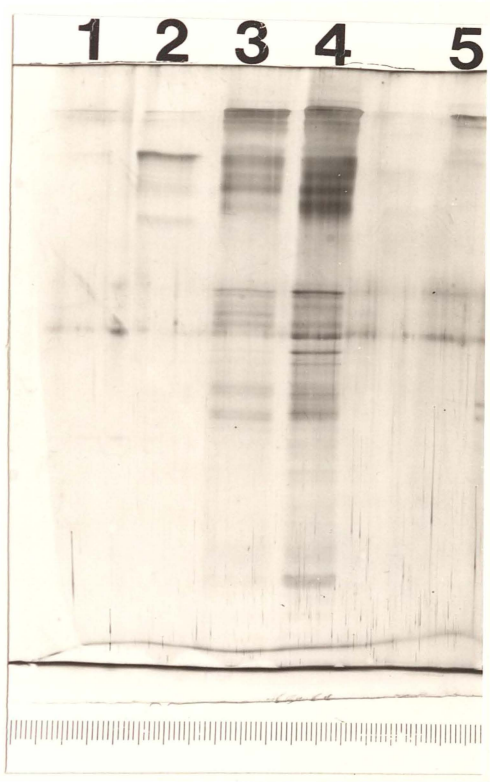


Figure 6.9 SDS-PAGE of fractions from the CM-Sepharose CL-6B separation of Fig. 6.8.

Lane 1 Small peak of protein and cellulolytic activity released by increasing the pH from 4.7 to 5.6 (Fctn. 78).

Lane 2 Shoulder of the main cellulolytic peak released by increasing the ionic strength (Fctn. 62).

Lane 3 Peak of CMC-plate-clearing activity (Fctn. 57).

Lane 4 Peak of protein release (Fctn. 55).

Lane 5 Small peak of unbound material (Fctn. 37).

All samples were pretreated as described in Section 2.9.3.

The separating gel contained 10%(w/v) acrylamide.

Figure 6.10 Cation exchange chromatography on CM-Sepharose CL-6B of the cellulolytic fractions collected from a P-10 desalting column.

• ="Cellulose-azurase" (dye release from cellulose azure)

* = glucose release from cellulose azure

+ = CMC-plate-clearing activity

☆ = total protein (A_{280nm})

The column (1.6 by 10cm) was equilibrated in 5mM sodium acetate buffer, pH 4.7.

The sample applied (190ml) contained material which had been released from a cellulose column by heating to 75°C and which then passed through a P-10 gel permeation column in the excluded peak, dissolved in the above buffer.

Fractions 1-18	were collected during sample loading.
Fractions 19-23	were collected during the wash of bound material with 5mM sodium acetate buffer pH4.7.
Fractions 24-54	were collected during elution with a 5mM to a 100mM gradient of sodium acetate buffer pH 4.7.
Fractions 55-67	were collected during the further wash with 100mM sodium acetate buffer pH4.7.
Fractions 68-77	were collected during the wash with 200mM sodium acetate buffer pH5.6.
Fractions 78-93	were collected during the wash with 500mM Tris-HCl buffer pH7.6.

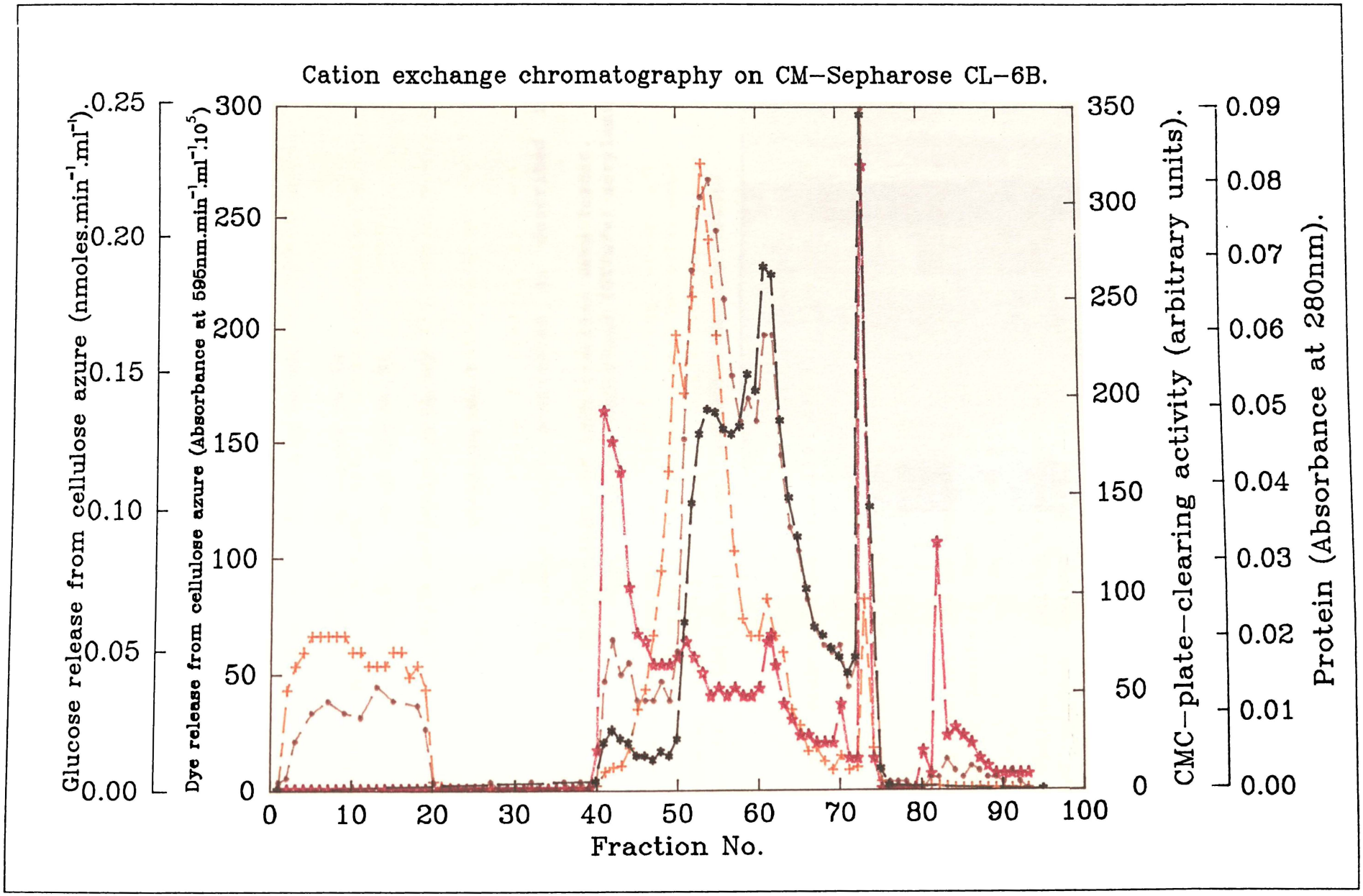

Cation exchange chromatography on CM–Sepharose CL–6B.
Glucose release from cellulose azure (nmoles.min-1.ml-1).
Dye release from cellulose azure (Absorbance at 595nm.min-1.ml-1.10-5)
CMC–plate–clearing activity (arbitrary units).
Protein (Absorbance at 280nm).
Fraction No.
0.25
0.20
0.15
0.10
0.05
0.00
300
250
200
150
100
50
0
350
300
250
200
150
100
50
0
0.09
0.08
0.07
0.06
0.05
0.04
0.03
0.02
0.01
0.00
0
10
20
30
40
50
60
70
80
90
100

Figure 6.11 SDS-PAGE of fractions from the CM-Sepharose CL-6B column of Fig.6.10.

Lane 1 Fraction No.82

Lane 2 Fraction No.73

Lane 3 Fraction No.70

Lane 4 Fraction No.61

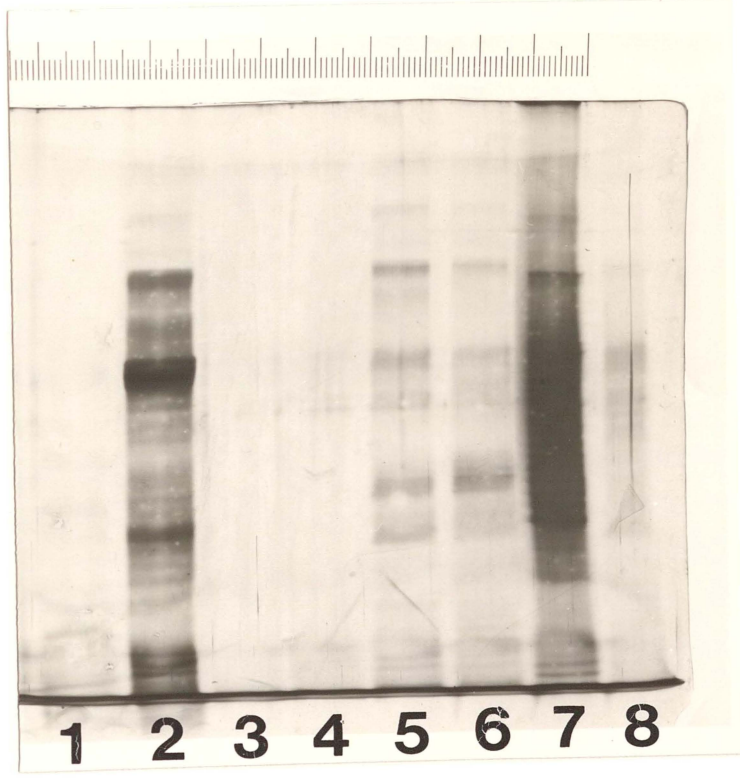
Lane 5 Fraction No.54

Lane 6 Fraction No.51

Lane 7 Fraction No.42

Lane 8 Fraction No.7

All samples were pretreated as described in Section 2.9.3.
Equal volumes of each fraction were loaded.
The separating gel contained 10%(w/v) acrylamide.



6.3.4 Gel permeation chromatography

6.3.4.1 Testing various gel filtration media for binding of avicelase Sepharose CL-6B, Sephacryl S-200, Sephadex G-100 (Pharmacia), Biogel P-300 (BioRad) and Ultrogel AcA44 (LKB) were tested in small syringe-columns for their capacities to retain avicelase activity from crude ultrafilter-concentrated culture supernatant. Each had been equilibrated in and was eluted with 0.05M sodium acetate buffer pH 5.0.

Only Sephadex G-100 was found to bind the avicelase activity. After eluting with 2 column volumes of buffer, only 45% of the applied avicelase had been recovered from the Sephadex G-100. The resemblance of Sephadex, a dextran gel, to cellulose probably explains why it bound the avicelase. The production of reducing sugars when Sephadex was incubated with the concentrated culture supernatant showed that it was binding to the active sites of the enzymes.

6.3.4.2 Sephadex G-100 The affinity of certain components of the cellulase complex was found to assist in its fractionation on Sephadex G-100 (Fig. 6.12). The protein peak which was centred on fraction 95 was clearly retarded by some degree of binding to the column since its elution occurred at a point otherwise indicative of an extremely low molecular weight (<2000). The cellulolytic characteristics of the retarded peak were distinctive in that it lacked avicelase activity and it had a higher proportion of endoglucanase activity than did the material which eluted earlier, as shown by the ratios of CMCase : CMC-plate-clearing activities (Fig. 6.12).

ß-glucosidase eluted as a single sharp peak of activity, at a point shown to correlate with a M.W. of 55,000 by calibration of the column with standards, but was entirely within the confines of a series of overlapping peaks with CMC-plate-clearing, CMCase and avicelase activities, that eluted at points corresponding to M.W.s covering the range 20,000 to 60,000.

Substantial separation of cellulases from inactive protein was achieved on the G-100 column (Fig. 6.12). The total protein yield was 80%. Yields of the various activities were: avicelase 120%, CMC-plate-clearing activity 90%, CMCase 60%, ß-glucosidase 426%. Evidently the ß-glucosidase activity had been separated from some inhibitor present in the concentrate applied to the column

Figure 6.12 Gel permeation chromatography on Sephadex G-100.

□ = CMCase activity

△ = avicelase activity

○ = ß-glucosidase activity

+ = CMC-plate-clearing activity

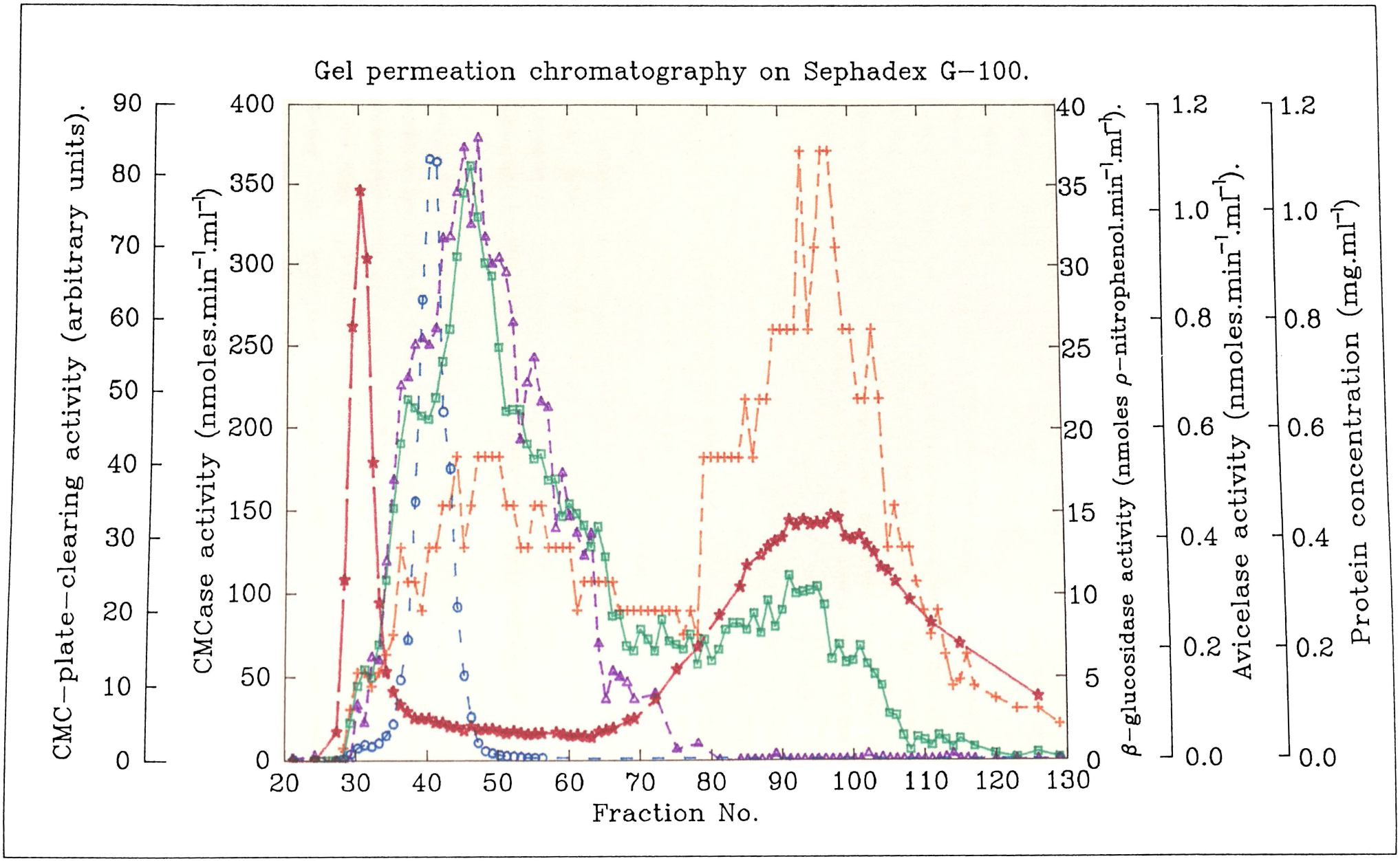
★ = total protein

Sample applied: Proteins of TP8.T6.3.3.1 culture supernatant which had been concentrated by ammonium sulphate precipitation, then redissolved, washed and further concentrated on a PM10 ultrafilter in 50mM sodium acetate buffer pH5.0.

Volume applied to column = 10ml.

Eluent = 50mM sodium acetate pH5.0.

Column dimensions = 2.5cm x 80 cm.


Gel permeation chromatography on Sephadex G–100.
CMC–plate–clearing activity (arbitrary units).
CMCase activity (nmoles.min^{-1}.ml^{-1})
β–glucosidase activity (nmoles ρ–nitrophenol.min^{-1}.ml^{-1}).
Avicelase activity (nmoles.min^{-1}.ml^{-1}).
Protein concentration (mg.ml^{-1})
Fraction No.
0
10
20
30
40
50
60
70
80
90
0
50
100
150
200
250
300
350
400
0
5
10
15
20
25
30
35
40
0.0
0.2
0.4
0.6
0.8
1.0
1.2
0.0
0.2
0.4
0.6
0.8
1.0
1.2
20
30
40
50
60
70
80
90
100
110
120
130

(redissolved ammonium sulphate precipitate of culture supernatant, washed by ultrafiltration).

The avicelase activity eluted more readily than expected from the G-100 column of Fig. 6.12, given the binding which was observed in the small scale trial. This may be attributable to the differing methods used in concentrating the material loaded (ammonium sulphate precipitation followed by desalting by ultrafiltration c.f. ultrafiltration alone). This was supported by later findings that cellulases which had been concentrated on a cellulose column and eluted with water would bind so strongly to a column of G-100 that very poor yields and no useful fractionation resulted.

6.3.4.3 Biogel P-300 Our cellulase complex passed through a 1.6cm x 30cm column of Biogel P-300 as a single broad peak without any substantial fractionation of the complex, since all of the different types of activity measured were superimposed. There was no evidence of binding to the column matrix, (polyacrylamide beads).

6.3.4.4 Ultrogel AcA44 A 2.5cm x 85cm column of ultrogel AcA44 split the water-eluted peak from a cellulose column into two slightly overlapping peaks of protein.

SDS-PAGE (Fig. 6.5) and IEF (not shown) of the two peaks (Fig. 6.5, Lanes 2 and 3) revealed that each peak contained only one of the two dominant proteins present in the material applied (Lane 1 Fig. 6.5), along with several other more mobile proteins in lesser quantities. It is possible that these minor proteins were fragments of the dominant protein in each peak, since they were all smaller than the dominant protein. Alternatively they may have been components of a genuine complex, being quite different proteins which were only separated from one another by SDS-PAGE.

Five peaks of protein and activity resulted from another Ultrogel separation of the water-eluted cellulases from the 600 litre culture (fully described in Section 6.4.6). The separation of these peaks was superior to that obtained by HPLC gel permeation chromatography on a TSK 3000SWG column.

Inclusion of 0.1% SDS in the Ultrogel AcA44 column eluent did not produce further fractionation of the sub-complexes.

6.3.4.5 TSK 3000SWG A sample of the material which had produced the five peaks of protein and activity following separation on Ultrogel AcA44 (Section 6.4.6) was applied to an HPLC gel permeation column (TSK 3000SWG). Elution with 0.05M sodium acetate buffer pH5.6 (as used for the Ultrogel run) produced five protein peaks, as had the Ultrogel separation, but the relative protein levels in the peaks were completely different. Peak separation was not as good as that achieved on Ultrogel.

Substitution of distilled water as the eluent of the TSK 3000SWG column, reloaded with the same cellulase complex as above, resulted in a completely different A_{280} profile. A single large peak was dominant, which eluted much earlier than any peak from the acetate-eluted run, and which contained virtually all of the CMC-plate-clearing activity. The reduced ionic strength presumably caused the proteins to aggregate and thus elute earlier. Nonetheless, this major peak was clearly binding to the column matrix since it was known to contain proteins with M.Ws in excess of 100,000 (Fig. 6.5, Lane 1) and yet it eluted later than did bovine serum albumin (M.W. 66,000) run under the same conditions.

It is possible that eluents of much higher ionic strengths or dissociating agents such as detergents or urea might have improved the separation attainable on the TSK column.

6.3.5 Electrophoretic separation

6.3.5.1 A comparison of mobilities on several electrophoresis systems

The water-eluted cellulase complex obtained from the cellulose column fractionation had very poor mobility on all the non-denaturing electrophoresis systems tested. Using the Laemmli (1970) system, but without SDS, a large portion of the protein of the complex didn't enter the separating gel, and that which did penetrate moved only a few millimetres into the gel (Fig. 6.3, Lane 6). The system of Reisfeld *et al.* (1962) gave similar very poor mobilities, with the bands being too close together to be readily distinguished (Fig. 6.13 Lane 1). The non-denaturing alkaline electrophoresis system of Davis (1964) produced similar results (not shown). In contrast, the SDS-PAGE system of Laemmli (1970) produced 30-40 bands of protein from this same material, with a range of mobilities corresponding to molecular weights between 15,000 and 140,000

(Fig. 6.6, Lane 1). No proteins were left at the gel interface, so it seemed that the SDS-mercaptoethanol pretreatment had markedly improved the mobility of these proteins in the 10% (w/v) acrylamide gels.

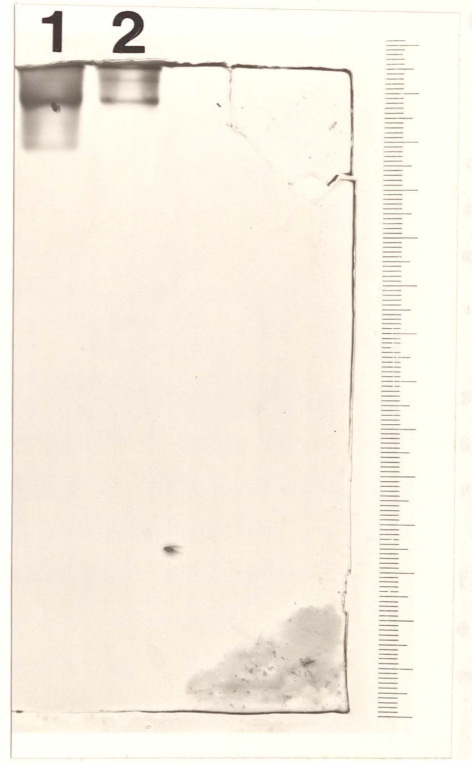


Figure 6.13 Non-denaturing electrophoresis using the Reisfeld system.

Lane 1 Peak III (water-eluted) from the cellulose column of Fig. 6.2.

Lane 2 Bulked cellulolytic fractions from an Ultrogel separation of Peak III above.

The PAGE system of Reisfeld et al (1962) was used, with a 10% (w/v) acrylamide separating gel.

6.3.5.2 The effects of various SDS-PAGE sample pretreatments on the level and mobilities of cellulase activities In order to discover whether or not the cellulases from our organism would retain their activities after SDS-PAGE, a 0.4ml sample of concentrated supernatant was mixed with 0.4ml of the normal sample buffer (Laemmli, 1970) containing 2% (w/v) SDS and 5% (v/v) mercaptoethanol, warmed to 75°C for 15 minutes and run on SDS-PAGE. The gel was then sliced up and the proteins were washed from the slices in 0.1M sodium phosphate buffer pH 7.6

(Dawson *et al.*, 1974) and assayed for avicelase and CMC-plate-clearing activities. The yields of avicelase and CMC-plate-clearing activities were 1.3% and 4% respectively after washing the sectioned gel, as described in Section 3.5.4.5. Electrophoretic elution by the method of Suzuki *et al.*, (1973) did not result in improved recovery of these activities and neither did washing the eluted proteins in a collodion bag. Evidently they had been irreversibly denatured by some component(s) of the Laemmli system.

It was later found that pretreatment with 5% (v/v) mercaptoethanol, even at room temperature, would reduce CMCase activity by 90%. SDS was also strongly inhibitory to both CMCase and avicelase activities when present at 0.1% (w/v) in the assay incubations. However, pre-exposure to 0.1% (w/v) SDS had no effect on avicelase activity if the SDS was subsequently diluted 10-fold in the assay incubation. Ammonium persulphate at 0.05%(w/v) was found to irreversibly eliminate avicelase activity. None of the other components of the Laemmli (1970) SDS-PAGE system produced any loss of CMCase or avicelase activity, i.e. TEMED, Tris/HCl buffer, bromophenol blue.

The effects of a simplified sample pretreatment compared with the normal Laemmli (1970) pretreatment on the separation obtained by SDS-PAGE are shown in Fig. 6.14. The simplified pretreatment (Lane 1) was aimed at minimizing the activity loss by complete omission of the mercaptoethanol and the heat treatment, and by lowering the concentration of SDS. A zymogram overlay revealed CMC-plate-clearing activity in both lanes, but bands of clearance were much stronger in Lane 1 than Lane 2. Positions of these bands are marked on the densitometer scan of the silver stain (Fig. 6.14). The most obvious effect of this simplification on the distribution of active bands was the loss of the two most mobile bands of CMC-plate-clearing activity. These bands (Fig. 6.14, Lane 2 only) exhibited mobilities which suggested molecular weights of 35,000 and 30,000, so were possibly the result of cleavage of larger enzymes. Mobilities of the four other discrete bands of CMC-plate-clearing activity, and of the area of continuous clearance in the upper third of the zymogram, were

Figure 6.14 A comparison of the separation and mobilities of component proteins in TP8.T6.3.3.1 culture supernatant obtained by using the Laemmli SDS-PAGE system after two different pretreatments.

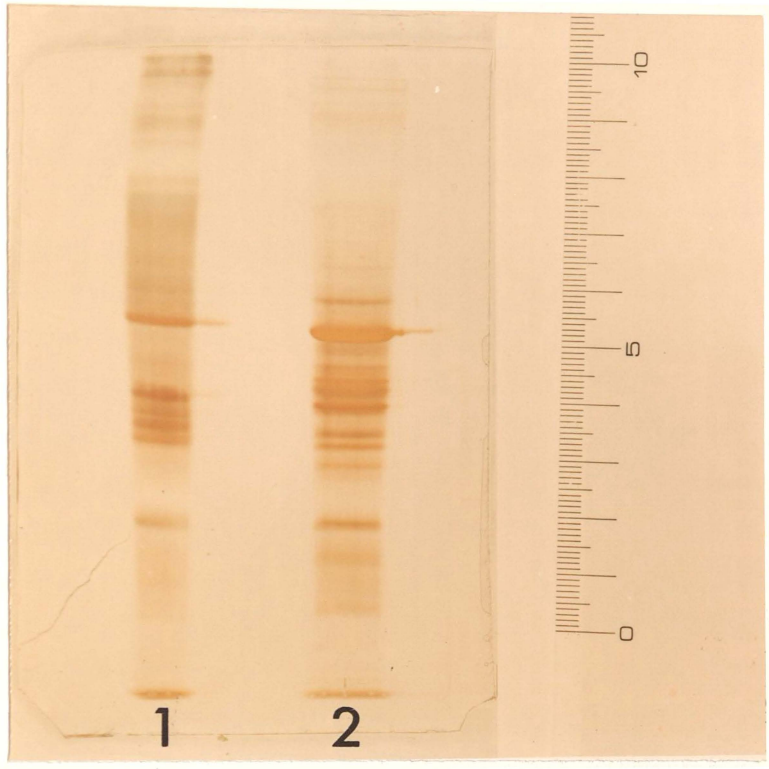
Lane 1 No mercaptoethanol, low SDS.
10µl of ultra-filter-concentrated and washed culture supernatant plus 10µl of sample buffer "a" (see below).

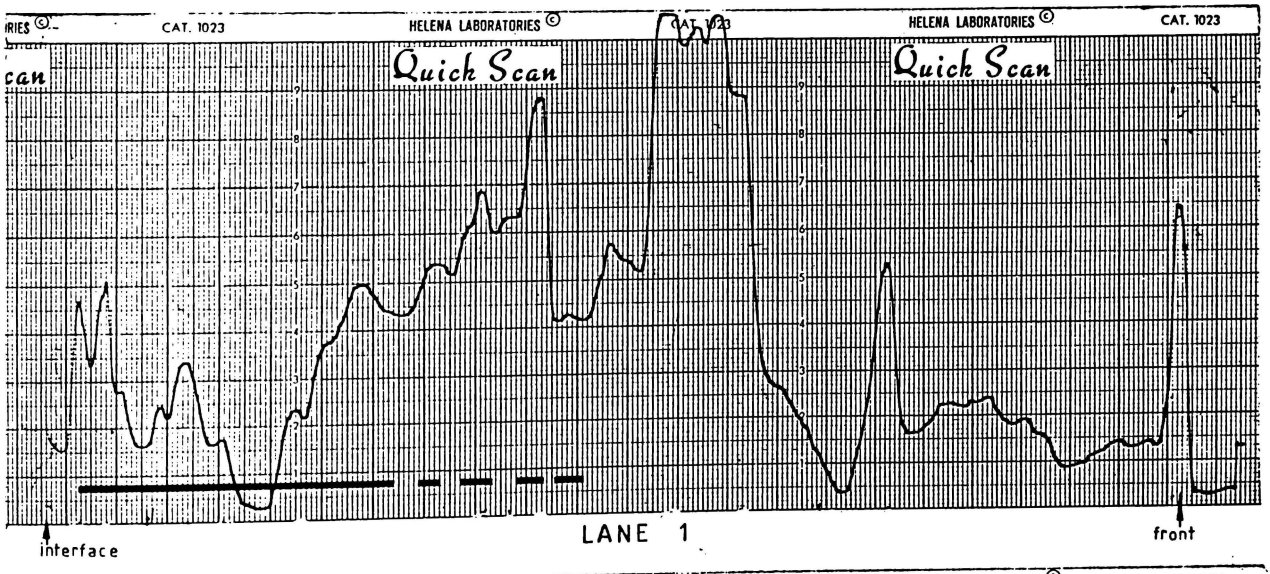
Lane 2 Normal sample pretreatment for the Laemmli system.
10µl of ultrafilter-concentrated and washed culture supernatant plus 10µl sample buffer "b" (see below), heated to 70°C for 5min.

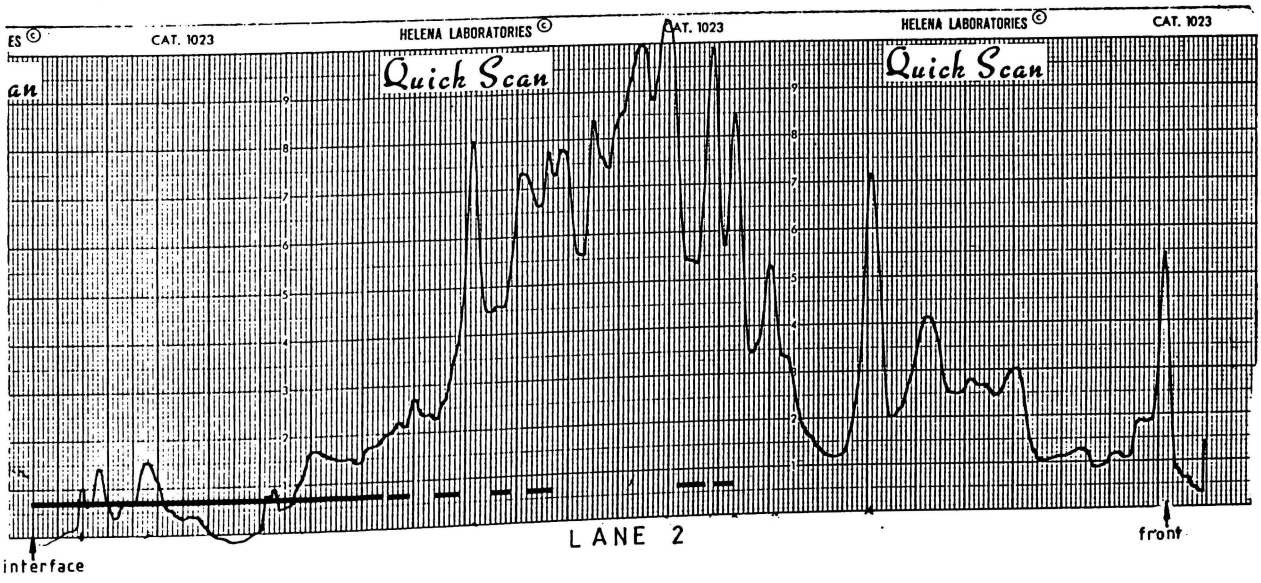
Sample buffer "a": 5µl 10%(w/v)SDS
200µl 0.25M Tris-HCl buffer pH6.8
20µl 0.1%(w/v) bromophenol blue

Sample buffer "b": 20µl 10%(w/v)SDS
20µl 0.25M Tris-HCl buffer pH6.8
5µl mercaptoethanol
5µl 0.1%(w/v) bromophenol blue

A CMC-agarose replica gel was prepared before the PAGE gel was silver-stained. Bands of clearance (CMC-plate-clearing activity) are depicted as horizontal lines on the densitometer traces of the silver stained proteins.


10
5
0
1
2


CAT. 1023
HELENA LABORATORIES
Quick Scan
interface
LANE 1
front


CAT. 1023
HELENA LABORATORIES
Quick Scan
interface
LANE 2
front

very similar following either pretreatment (Fig. 6.14). The pattern of total protein distribution was quite markedly influenced by the pretreatment regime (Fig. 6.14), with the mercaptoethanol pretreatment resulting in more bands in the middle and lower half of the gel at the expense of larger less mobile proteins.

Although omission of the mercaptoethanol pretreatment prior to SDS electrophoresis clearly did result in altered mobilities for many of the proteins of the crude supernatant concentrate, mobilities and separations of the proteins of the complex were still much better than those achieved by the "non-denaturing" electrophoresis systems tried earlier.

It was concluded that in order to minimise the deactivation of cellulases subjected to SDS-PAGE, the sample buffer should not include mercaptoethanol, the SDS concentration of the sample buffer should be lowered to produce a concentration of 0.1% (w/v) in the final mixture and the gel should be pre-run for 2-3 minutes in order to remove residual ammonium persulphate from the walls of the sample wells. Prolonged prerunning would have negated the band sharpening conferred by the discontinuous buffer system.

6.3.5.3 A trial preparative SDS-PAGE separation of cellulase components A preparative SDS-PAGE separation of a 0.2ml sample of crude ultrafiltered concentrate of cell-free supernatant was attempted using a 1.5mm-thick SDS gel. No pretreatment with either mercaptoethanol or SDS was employed, but SDS was present in the electrode buffer at the normal concentration (0.1% w/v).

Without making any form of replica first, the preparative gel was divided into 2 and 4mm slices, cut parallel with the front. Gel slice washings were then assayed for ß-glucosidase, CMCase, CMC-plate-clearing and avicelase activities (Fig. 6.15). The yields for these four types of activity from the preparative gel were 15%, 55%, 55% and 30% respectively.All activities, apart from the final ß-glucosidase peak, were confined to the top 75mm of the gel.

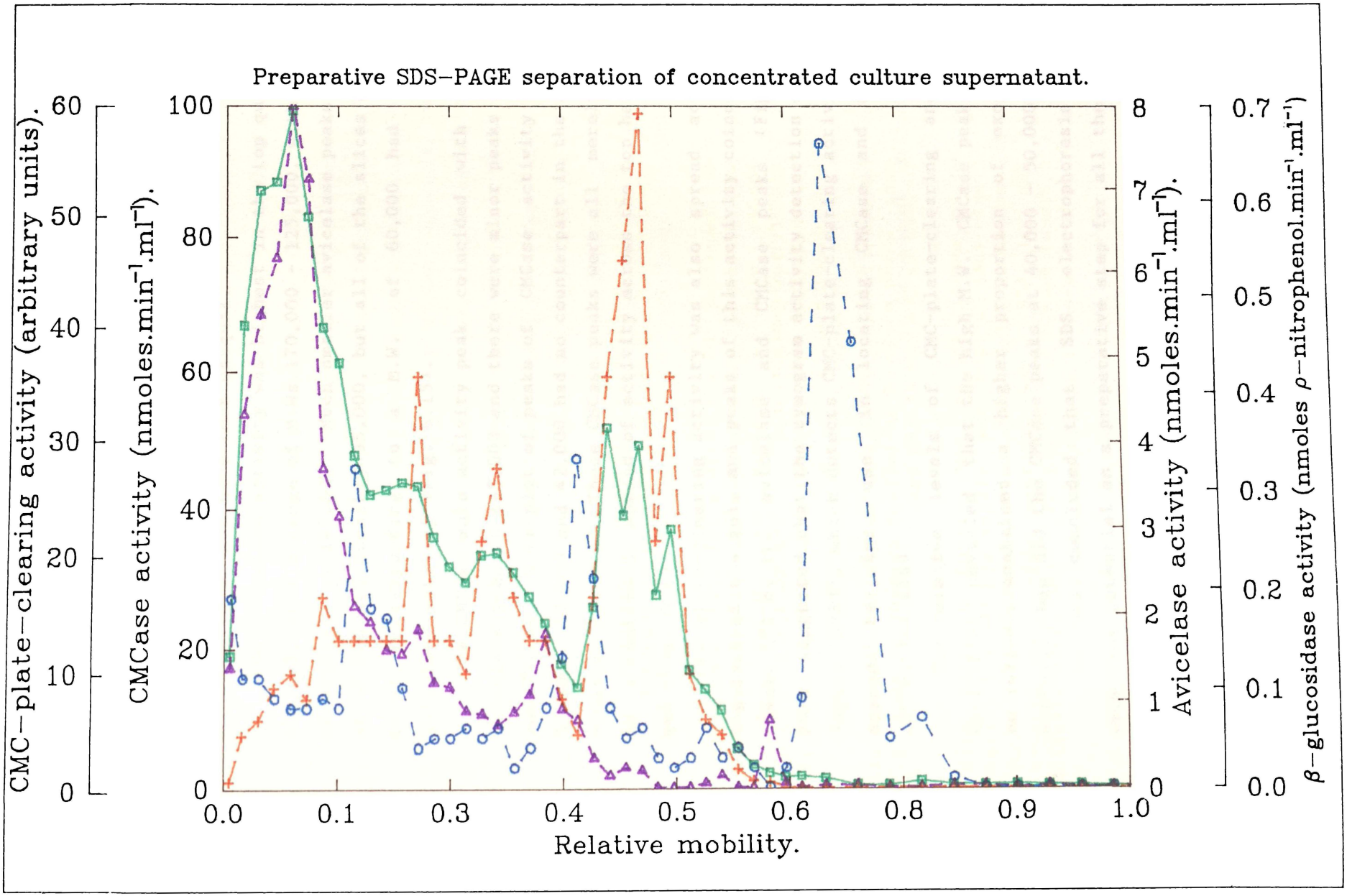
The ß-glucosidase activity occurred as three distinct bands, corresponding to M.Ws of ca. 25,000, 60,000 and 120,000. These values were suggestive of a monomer, dimer,

Figure 6.15 A trial preparative SDS-PAGE separation of crude concentrated TP8.T6.3.3.1 culture supernatant.

A 1.5mm-thick SDS gel (with a 10%(w/v) separating gel) was prepared, using the 5cm-wide preparative comb, and loaded with a mixture of 0.2ml of crude ultrafilter-concentrated TP8.T6.3.3.1 cell-free supernatant, 0.02ml glycerol and 0.02ml of 0.1%(w/v) bromophenol blue.

Constant voltage (200V) was maintained throughout the run, which produced an initial current of 40mA, falling to 20mA by the end of the run.

After running, the gel was sliced parallel with the front into 2 and 4mm slices and the gel strips were immersed in 2ml of 50mM sodium acetate buffer pH5.6 for 24h at 4°C. The washings were then assayed for ß-glucosidase(○), CMCase(□), CMC-plate-clearing(+) and avicelase(△) activities.


Preparative SDS–PAGE separation of concentrated culture supernatant.
CMC–plate–clearing activity (arbitrary units).
CMCase activity (nmoles.min^{-1}.ml^{-1}).
Avicelase activity (nmoles.min^{-1}.ml^{-1}).
β–glucosidase activity (nmoles ρ–nitrophenol.min^{-1}.ml^{-1})
Relative mobility.
0
10
20
30
40
50
60
0
20
40
60
80
100
0
1
2
3
4
5
6
7
8
0.0
0.1
0.3
0.4
0.5
0.6
0.8
0.9
1.0
0.0
0.1
0.2
0.3
0.4
0.5
0.6
0.7

tetramer relationship. The most mobile of them contained the greatest activity, but since the total yield of ß-glucosidase activity from the entire gel was only 15% of that applied, little significance should be placed on the relative peak activities obtained since the yields of the individual ß-glucosidases may have been unequal.

The avicelase activity was highest in the top quarter of the gel, over a range of M.Ws 170,000 - 125,000, with the peak of activity at 145,000. Much smaller avicelase peaks occurred at 65,000 and possibly 95,000, but all of the slices down to a point corresponding to a M.W. of 60,000 had definite avicelase activity (Fig. 6.15).

The CMCase main activity peak coincided with the main avicelase peak at 145,000 and there were minor peaks at 95,000 and 75,000. A triplet of peaks of CMCase activity at M.Ws 52,000, 47,000 and 42,000 had no counterpart in the avicelase activity profile. These CMCase peaks were all merely apices on a continuous spread of activity across the top half of the gel (Fig. 6.15).

CMC-plate-clearing activity was also spread across the top half of the gel, and peaks of this activity coincided with almost all of the avicelase and CMCase peaks (Fig. 6.15). This suggested that the zymogram activity detection system of Beguin (1983), which detects CMC-plate-clearing activity, was appropriate for use in locating CMCase and avicelase activities also.

The relative levels of CMC-plate-clearing and CMCase activities revealed that the high M.W. CMCase peak, centred on 145,000, contained a higher proportion of exoglucanase activity than did the CMCase peaks at 40,000 - 50,000.

It was concluded that SDS electrophoresis showed excellent potential as a preparative step for all the types of activity studied, except perhaps for ß-glucosidase, which suffered the greatest activity loss. The yields of the other activities were not as high as those obtained earlier by column chromatography. This factor, combined with the extreme complexity of the complete complex which gave rise to many very closely spaced bands, meant that preparative SDS electrophoresis would be better suited to the latter stages of

a purification sequence.

6.3.6 Isoelectric focussing

Analytical IEF was found to fractionate subcomplexes which behaved as single peaks on conventional gel permeation and ion exchange chromatrography, (Section 6.4.6.2).

Preparative flat-bed iEF normally employs comparatively thick gel slurries of beaded dextrans - e.g. Sephadex. Since the affinity of our cellulases for dextrans (Section 6.3.4.1) was likely to hinder both focussing and subsequent enzyme recovery, for preparative IEF we used the same 0.3mm-thick polyacrylamide system which had successfully separated these enzymes on an analytical scale. The system required much less of the expensive ampholines than the normal gel-slurry method, and could be simply replicated using the CMC/polyacrylamide zymogram method.

We didn't perform any quantitatively assessed trial of this preparative IEF system prior to its application in the following purification sequence (Section 6.4.7). Specific conclusions are discussed there, but overall the system was not as successful as SDS-PAGE for preparative separations, with lower yields and much poorer reproducibility.

6.4 THE PURIFICATION SEQUENCE WHICH PRODUCED THE GREATEST FRACTIONATION OF THE CELLULASE COMPLEX

6.4.1 Background

Several earlier purification sequences which were aimed at purification of avicelase activity, using relatively conventional column separation techniques, had to be terminated without having achieved homogeneity for any avicelase component of the complex since the avicelase activities had dwindled to below levels practical for study. Poor yields of avicelase in particular were associated with most steps, but especially with the initial concentration. Conventional gel permeation and ion exchange chromatography would fractionate the cellulase complex into 4 or 5 subcomplexes, but these resisted further separation despite the use of different ion exchangers, gel filtration media and conditions etc. as described in Section 6.3. When finally the applicability of preparative SDS-PAGE and IEF as means of splitting these subcomplexes was realized, a fresh batch of TP8.T6.3.3.1 cellulases was concentrated from 600 litres of culture supernatant, divided up into simpler sub-complexes by a minimum of column chromatography,

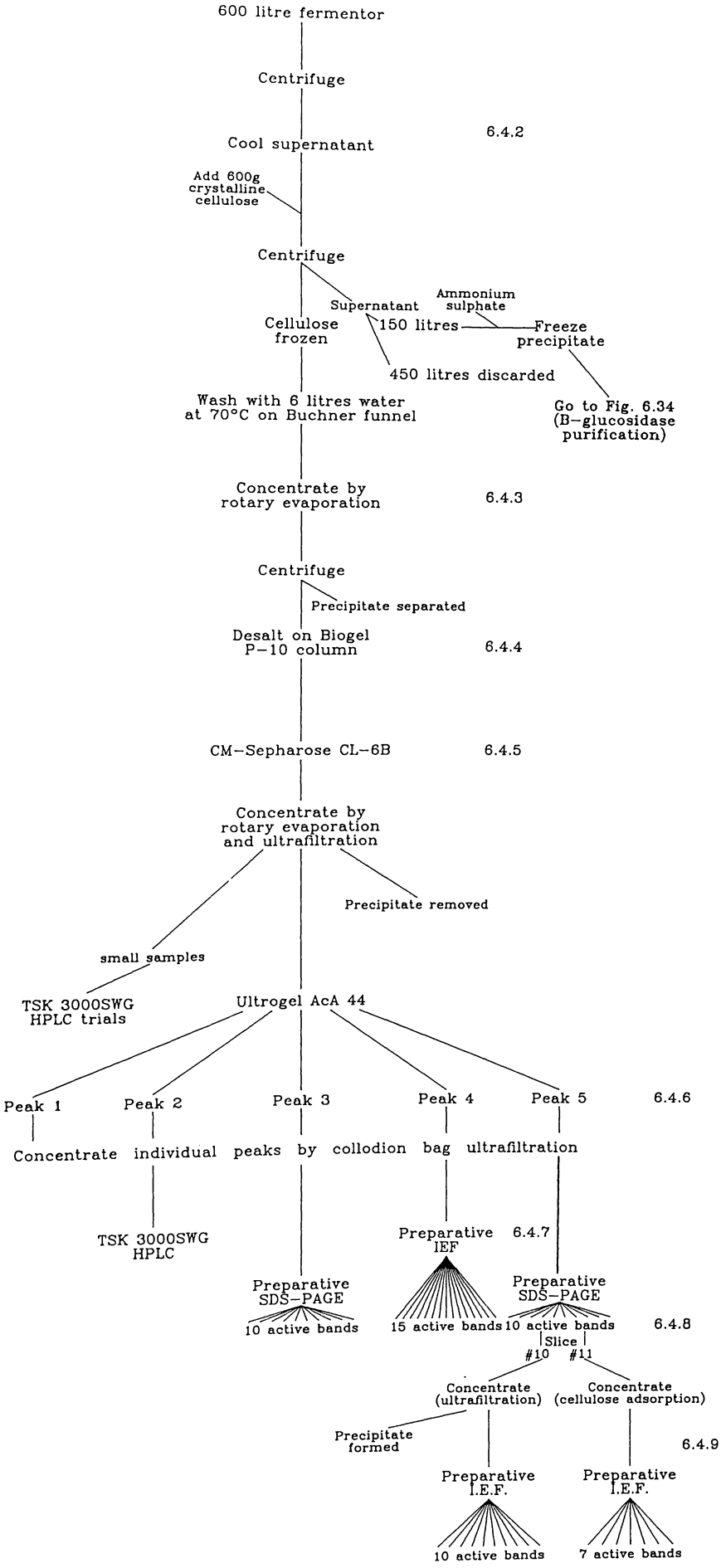


Fig. 6.16 Purification of cellulases from the 600-litre culture of TP8.T6.3.3.1

and then these subcomplexes were further subdivided by SDS-PAGE and IEF. The final cellulase purification sequence is described in detail below, and is also summarised as a flow chart in Fig. 6.16.

6.4.2 Production and harvest of cellulases in a 600 litre culture

Six hundred litres of sterile medium was prepared in a stainless steel fermentor as described in Section 2.1.1, and inoculated with 20 litres of 2-day-old culture of TP8.T6.3.3.1.

Samples were removed from the fermentor before and during growth, and the level of CMCase in the cell-free supernatant of each was immediately determined (Fig. 6.17). This assay was chosen to monitor cellulase production, rather than an avicelase assay, because the results were available within 35 minutes of sampling whereas any avicelase assay method would have required incubation for several hours, given the levels of activity expected. The aim was to centrifuge out the cells as soon as the CMCase activity exceeded 100 $nmol.min^{-1}.ml^{-1}$, in order to obtain a cell-free supernatant which was not greatly contaminated by intracellular material stemming from cell lysis.

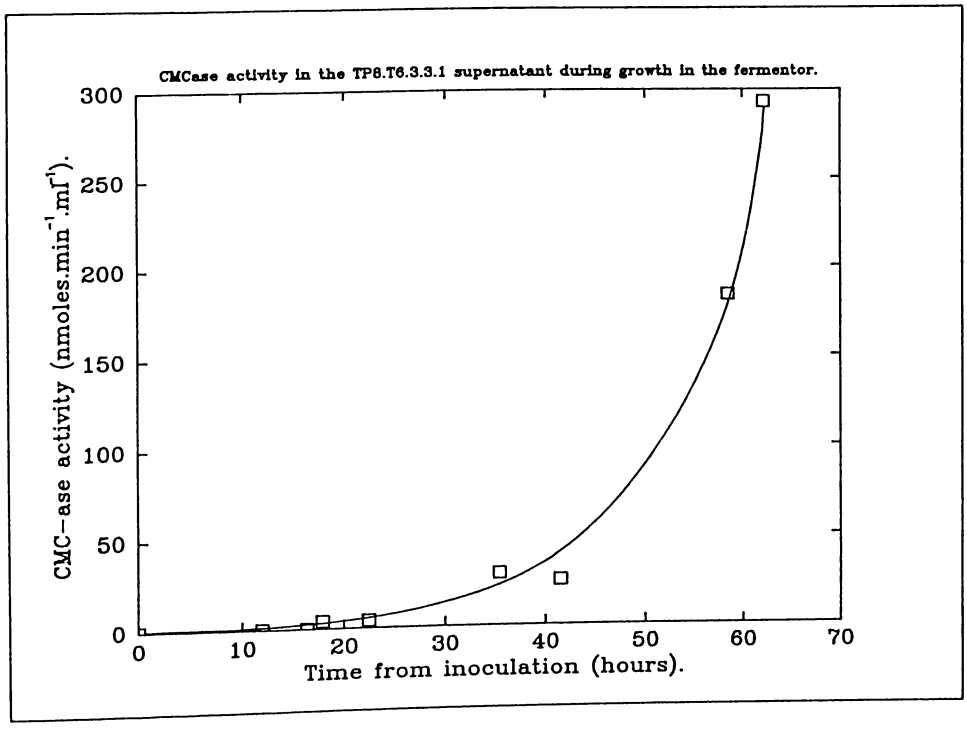


Figure 6.17 CMCase activity in cell-free supernatant during growth of TP8.T6.3.3.1 in the 600-litre fermentor.

Cells from each sample were Gram-stained and examined

microscopically. No contaminants were found.

Centrifugation to remove the cells and residual cellulose from the culture was begun at 60 hours. The level of CMCase in the culture continued to rise rapidly during the course of the centrifugation (Fig. 6.17). At the time of harvest, Gram-stained cell smears revealed very few obviously lysed cells.

The supernatant was returned to the fermentor, and cooled by running tap water through the external water jacket. Cooling was intended to enhance the binding of the cellulases to crystalline cellulose (600g of Sigmacell 50), which was added once the supernatant had reached 38°C, and was left stirring for 150 minutes while the temperature continued to fall to 28°C. The cellulose was then harvested by centrifugation and stored at -20°C.

80% of the CMCase activity and 75% of the activity towards cellulose azure were removed from the supernatant by adding the cellulose.

No further reduction in the residual level of CMCase in the supernatant resulted from addition of wood-pulp or further Sigmacell 50, nor from filtering the supernatant several times through Whatman No. 1 filter paper.

Proteins remaining in a 150 litre portion of the final supernatant were precipitated through the addition of 80kg of crude (fertilizer grade) ammonium sulphate, (which resulted in an 80% saturated solution). The remaining 450 litres of supernatant was discarded, as was the supernatant obtained following centrifugation of the ammonium sulphate precipitate. The latter precipitate was stored at -20°C for 5 months before the ß-glucosidase activity was recovered from it (Section 6.5).

6.4.3 Recovery of cellulases from cellulose

In order to later recover the cellulose-bound enzymes, the cellulose was thawed, broken up into small lumps and placed on Whatman No.1 filter paper in a 24cm diameter Buchner funnel. Two litres of Milli-Q water at 70°C was added. The resulting thick slurry was stirred gently during vacuum filtration to break up the remaining lumps and to ensure complete wetting of the cellulose. The liquid ran through completely within 10 minutes and was then replaced with a further 4 litres of Milli-Q water, also at 70°C. However the flow then slowed dramatically. Gentle stirring with a round-tipped glass rod across the surface of the filter paper

didn't improve the flow rate. It took a further 6 hours for all of the 4 litres to pass through the filter. By this time the temperature of the mixture had fallen from 75°C to room temperature. The cellulose was then refrozen at -20°C. The filtrate was opalescent, presumably due to the creation of "fines", which were probably also responsible for clogging the filter paper pores. A similar release of "fines" had also occurred during cellulase elution from cellulose columns when the ionic strength of the eluent fell below a certain level.

Rotary evaporation at 60°C was used to separately concentrate the two batches of cellulose washings down to ca. 40ml. These concentrates were then clarified by centrifugation in a swing-out head at 27,000*g* for 15 minutes.

Further elution of cellulases from the cellulose with water was not practical using the Buchner vacuum filtration system due to the almost immediate blockage of the pores in any of a range of filter papers types tried. An alternative washing system using a 7cm diameter column with Whatman GFA glass fibre filters at either end was then employed. These filters could be readily replaced when blocked. The column was maintained at 70°C to hasten the cellulase release while water was pumped through under pressure. Frequent blockages of the outlet filter, an extremely slow flow rate and channelling in the cellulose were problems which prevented the wash from being completed. The cellulose was then refrozen at -20°C.

It is possible that freezing the wet cellulose following its removal from the fermentor exacerbated its poor flow properties or altered the way in which the enzymes bound to the cellulose. Certainly it proved much more difficult to recover the bound cellulases on a large scale from 600g of cellulose than it had been previously from 5-10g of cellulose in small columns. In fact only around 2% of the activity towards cellulose azure which had been harvested from the fermentor was recovered by the first two washes on the Buchner funnel. This material alone was purified further in the sequence outlined in Fig. 6.16.

The question arises as to whether or not this small portion was representative of the avicelase component of the cellulase complex secreted by this organism. We found that (i) the avicelases which it yielded encompassed the entire molecular weight

range of such activity found in concentrates of earlier cultures, and (ii) further avicelase activity retrieved by the column wash of the cellulose described above released a very similar spectrum of cellulases, as determined by fractionation on Ultrogel and SDS-PAGE. For these reasons we feel that the further purification of such a small percentage of the starting material was nevertheless valid.

6.4.4 Biogel P-10 desalting column chromatography

The clarified concentrates of the two batches of washings from the Buchner funnel were applied separately to a 2.5cm x 95cm Biogel P-10 desalting column (Fig. 6.18a, b). P-10 gel is composed of acrylamide beads with an exclusion limit of 20,000 daltons. The cellulases were separated into two groups. The first, the void volume peak, contained 75% and 65% of the CMC-plate-clearing and cellulose azure dye release activities respectively which were recovered from the gel, but only a small proportion of the total protein (Fig. 6.18 a, b). The remaining activity and the majority of the protein eluted later as several broad overlapping peaks. Either this latter material was comprised of low (<20,000 M.W.) molecules or else the low ionic strength of the eluent (5mM sodium acetate buffer, pH4.7) may have allowed the P-10 gel to behave like an ion-exchanger, selectively retarding substances on a charge basis.

The yields of CMC-plate-clearing activity and activity towards cellulose azure from the P-10 columns were 160% and 120%, suggesting that these activities were under some form of inhibition in the concentrates applied.

6.4.5 CM-Sepharose-CL-6B cation exchange chromatography

The void volume peaks (fractions 18-26) from both P10 runs were pooled and loaded onto a 1.6cm x 10cm CM-Sepharose-CL-6B column which was equilibrated in 5mM sodium acetate buffer pH 4.7. 70% of the CMC-plate-clearing activity, 60% of the activity towards cellulose azure and 90% of the protein applied eluted in a single broad peak during the first gradient employed (i.e. 5mM - 100mM sodium acetate buffer pH 4.7). No further significant release of cellulase activity or protein resulted from increases in eluent ionic strength and pH, which culminated in 2M NaCl in 455mM Tris/HCl buffer, pH 7.6.

Since negligible fractionation was achieved by the

Figure 6.18 Gel permeation chromatography on Biogel P-10 polyacrylamide.

Fig. 6.18a Sample applied = 43ml of concentrate from the hot-water wash of the cellulose (Section 6.4.3).

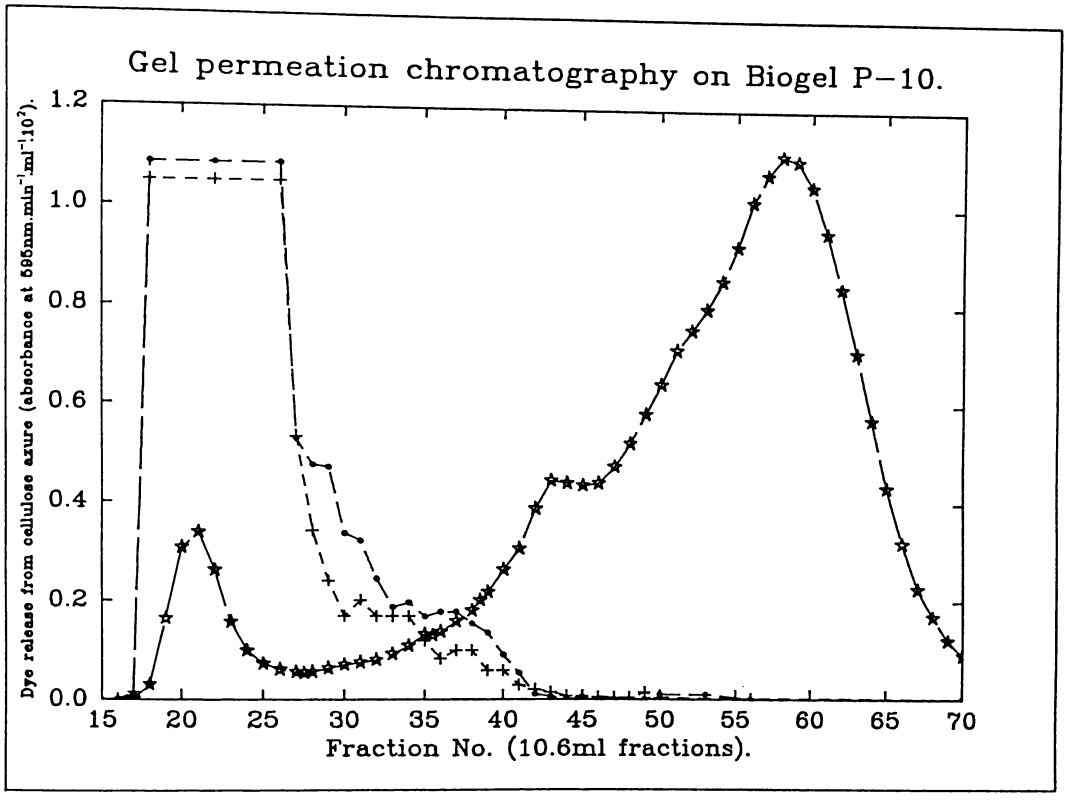
Fig. 6.18b Sample applied = 36 ml of concentrate from the cold-water wash of the cellulose (Section 6.4.3).

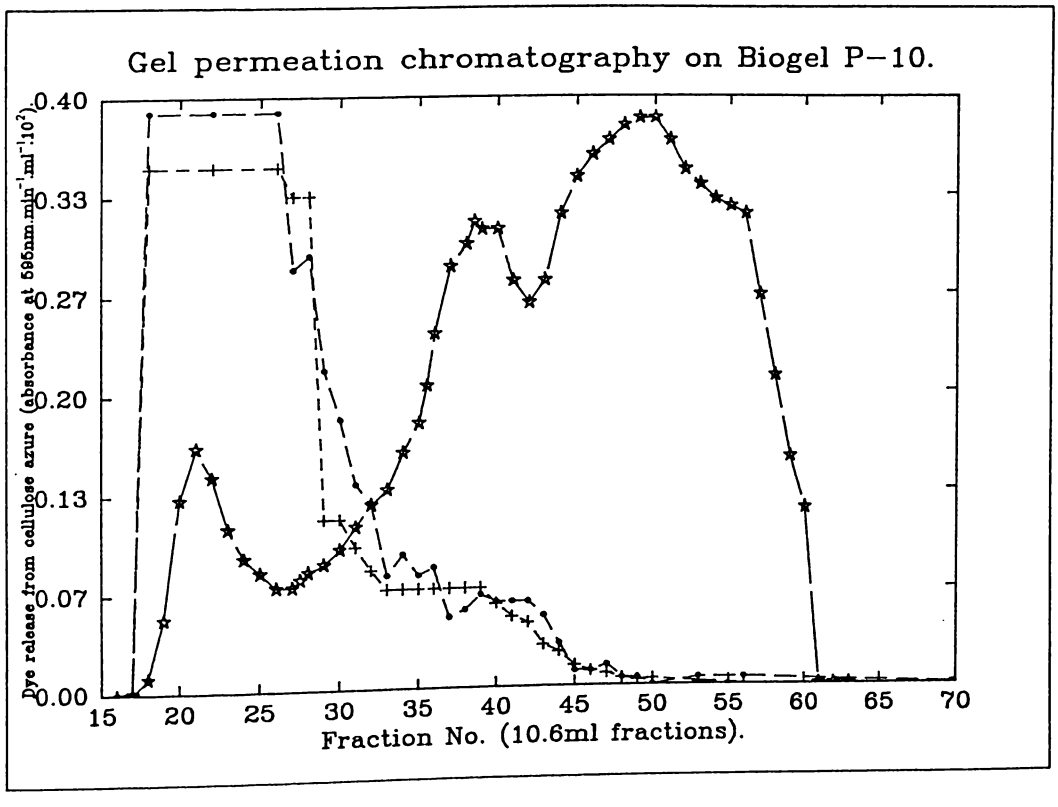
Running conditions for both 6.18a and 6.18b:
Fraction volume = 10.6ml
Eluent = 5mM sodium acetate pH 4.7
Column dimensions = 2.5cm x 90cm
Flow rate = 120ml.h^{-1}

● ="Cellulose azurase"

+ = CMC-plate-clearing activity

✡ = Protein (A_{280nm})


Gel permeation chromatography on Biogel P–10.
Dye release from cellulose azure (absorbance at 595nm.min^{-1}.ml^{-1}.10^{2}).
1.2
1.0
0.8
0.6
0.4
0.2
0.0
15 20 25 30 35 40 45 50 55 60 65 70
Fraction No. (10.6ml fractions).


Gel permeation chromatography on Biogel P–10.
Dye release from cellulose azure (absorbance at 595nm.min^{-1}.ml^{-1}.10^{2}).
0.40
0.33
0.27
0.20
0.13
0.07
0.00
15 20 25 30 35 40 45 50 55 60 65 70
Fraction No. (10.6ml fractions).

CM-Sepharose-CL-6B column, all the active fractions were bulked, adjusted to pH 6.5 with 500mM Tris/HCl pH 7.6, and concentrated by rotary evaporation followed by collodion bag ultrafitration in preparation for the Ultrogel step.

6.4.6 Gel permeation chromatography on Ultrogel AcA44 and TSK 3000SWG

The Ultrogel AcA44 column fractionated the cellulase complex into 5 overlapping protein peaks (Fig. 6.19), all of which contained cellulolytic activity. Peak 5 was distinctive in containing the lowest protein concentration while exhibiting the highest concentrations of most of the types of cellulolytic activity (Fig. 6.19).

The five peaks were clearly different in their relative activities towards various substrates (Fig. 6.19). The first two peaks produced very little CMC-plate-clearing while they possessed definite activity towards dyed Sigmacell 50 and cellulose azure.

HPLC analysis on the Waters Sugar-PAK 1 ion exchange column showed the end-products of hydrolysis of Sigmacell 50 by Peak 1 to be 60% cellobiose, 20% glucose with an unidentified product with a slightly longer retention time, possibly cellotriose, comprising the remaining 20%.

Later-eluting peaks, particularly peak 5, exhibited higher ratios of CMC-plate-clearing to dyed Sigmacell 50 activity, (Fig. 6.19) indicating that they contained a larger proportion of endoglucanase activity relative to total cellulolytic activity. If cellulose azure was substituted for dyed Sigmacell 50 as the substrate, the relative magnitudes of the activities of peaks 4 and 5 were reversed and the activities in peaks 1 and 2 were diminished relative to the other peaks. In view of the differing ratios of endoglucanase to total cellulolytic activities in these peaks (discussed above), the endo-acting component appeared to have more significance in producing activity towards cellulose azure than towards dyed Sigmacell 50.

Overall yields for the various activities from the Ultrogel column were: 60% for activity towards dyed Sigmacell 50 (by measuring both dye and soluble carbohydrate release), 120% for activity towards cellulose azure and 97% for CMC-plate-clearing activity.

The five peaks were individually washed and concentrated down

Figure 6.19 Gel permeation chromatography on Ultrogel AcA44.

Sample applied = 2.8ml of pooled and concentrated cellulolytic fractions (18-50) from the CM-Sepharose column (Section 6.4.5).

△ = Avicelase activity, based on dye release from dyed Sigmacell 50.

× = Avicelase activity, based on total soluble carbohydrate released from dyed cellulose.

● ="Cellulose azurase"

\+ = CMC-plate-clearing activity

✡ = Protein (A_{280nm})

Sample applied = 2.8ml of pooled and concentrated cellulolytic fractions (18-50) from the CM-Sepharose column (Section 6.4.5).

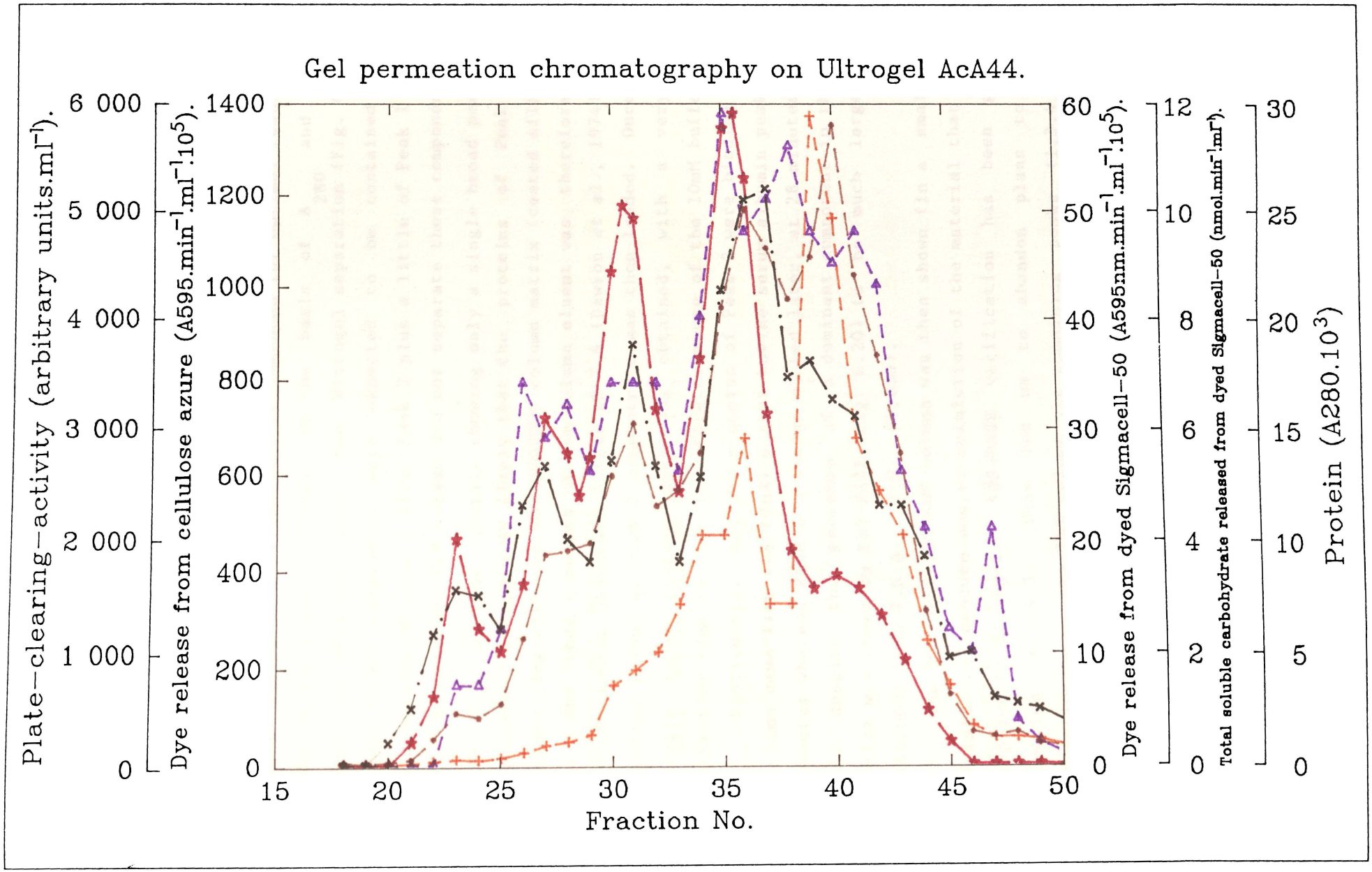
Column dimensions = 2.6 x 85cm.

Eluent = 50mM sodium acetate pH 5.6.

Flow rate = 0.5ml.min^{-1}

Fraction volume = 7ml

For molecular weight calibration, see Fig. 2.3


Gel permeation chromatography on Ultrogel AcA44.
Plate-clearing-activity (arbitrary units.ml-1).
Dye release from cellulose azure (A595.min-1.ml-1.10^5).
Dye release from dyed Sigmacell-50 (A595nm.min-1.ml-1.10^5).
Total soluble carbohydrate released from dyed Sigmacell-50 (nmol.min-1.ml-1).
Protein (A280.10^3)
Fraction No.
0
1 000
2 000
3 000
4 000
5 000
6 000
200
400
600
800
1000
1200
1400
10
20
30
40
50
60
2
4
6
8
10
12
5
10
15
20
25
30
15
20
25
30
35
40
45
50

to 0.4ml in 0.1M sodium phosphate buffer (Dawson *et al.*, 1974) by collodion bag ultrafiltration.

Peak 2 concentrate was subjected, in two batches, to HPLC gel filtration through a TSK column (3000SWG). One 0.2ml batch was eluted with 0.01M Tris/acetate buffer pH 7.0 at the flow rate of 0.5ml.minute^{-1}, i.e. the rate recommended for optimal resolution by the column's manufacturers. On the basis of A_{280} and activity profiles obtained after the Ultrogel separation (Fig. 6.19), at least three components were expected to be contained in the concentrate of peak 2 (i.e. Peak 2 plus a little of Peak 1 and Peak 3). However the TSK column did not separate these components, with the A_{280} elution profile showing only a single broad peak with a shoulder. It appeared likely that the proteins of Peak 2 were interacting with the TSK 3000SWG column matrix (coated silica).

The ionic strength of the column eluent was therefore raised (0.1M sodium phosphate buffer pH 7.6 (Dawson *et al.*, 1974)), and a further 0.14ml of Peak 2 concentrate was then loaded. Once again a single broad protein peak was obtained, with a very similar retention time to that resulting from use of the 10mM buffer.

Confirmation that the proteins of Peak 2 were binding to the column came from the finding that bovine serum albumin peaked at 21 minutes whereas Peak 2 proteins eluted later, at 28 minutes. This was despite the presence of a dominant component in the latter which was shown by SDS-PAGE (Fig. 6.20) to be much larger (M.W. 145,000) than B.S.A. (M.W. 66,000).

The TSK HPLC 3000SWG column was then shown (in a small scale trial) to produce poorer resolution of the material that had been applied to Ultrogel. SDS-PAGE verification has been given in Section 6.3.4.5. This led us to abandon plans to further fractionate on the TSK column the remaining peaks (1,3,4 and 5) from the Ultrogel column.

6.4.6.1 SDS-PAGE analysis of peaks from the Ultrogel separation SDS-PAGE, without any mercaptoethanol pretreatment (Fig. 6.20) showed that all five peaks contained several components, although Peaks 1 and 2 each produced one very dominant band (M.Ws 150,000 and 145,000 respectively). The dominant bands arising from Peaks 3, 4 and 5 followed the downward progression in molecular weight, as expected on the basis of their relative retention times on Ultrogel. Most

Figure 6.20 SDS-PAGE of the protein applied to the Ultrogel column of Fig. 6.19 and of the 5 resulting peaks of cellulolytic activity.

Lane 1 Material applied to the Ultrogel column.

Lane 2 Peak 1 from the Ultrogel column.

Lane 3 Peak 2 from the Ultrogel column.

Lane 4 Peak 3 from the Ultrogel column.

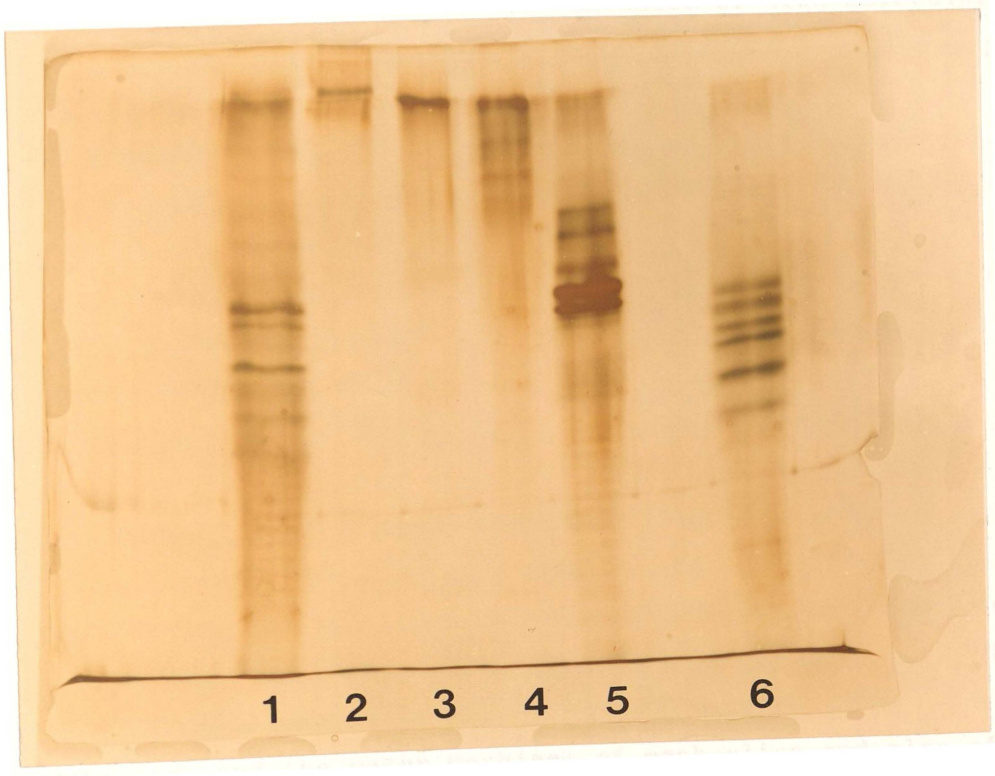
Lane 5 Peak 4 from the Ultrogel column.

Lane 6 Peak 5 from the Ultrogel column.

Sample buffer: 0.7ml electrode buffer (Laemmli,1970)
0.2ml glycerol
0.1ml 0.1%(w/v) bromophenol blue.

5µl of each sample was mixed with 6µl of sample buffer and 10µl of the mixture was applied to the well.

Acrylamide in the separating gel = 7.5%(w/v).



peaks, especially Peak 4, gave rise to bands which encompassed a wide range of molecular weights, extending well below and above the range expected from their retention times on Ultrogel. Disaggregation by SDS could well have produced the lower molecular weight material. The presence of material with an apparent M.W. of 145,000 in all the peaks was more difficult to explain. It is possible that attached carbohydrate groups might have been responsible for reducing molecular mobilities during SDS-PAGE (Segrest and Jackson, 1972). We didn't attempt to prove whether or not any of our enzymes were glycoproteins, since insufficient quantities were available when they were finally separated.

6.4.6.2 IEF analysis of peaks from the Ultrogel separation

Isoelectric focussing confirmed that all of the Ultrogel peaks contained multiple proteins. The numerous bands produced by each peak all exhibited pIs within the range 4.5 - 5.5. Although each peak produced a distinctive pattern of relative staining intensities of the various bands, it was possible that many of the proteins were common to several of the peaks.

Complete focussing by IEF was never achieved for Peak 1. This was probably due to the predominance of high molecular weight components which would have been retarded by sieving effects of the polyacrylamide.

A CMC/polyacrylamide zymogram showed activity towards CMC was associated with all the areas of protein staining, on the IEF gel (not shown).

The dominant proteins of Peak 4 were separated and focussed particularly well so this peak was selected for fractionation by preparative IEF.

6.4.7 Preparative IEF to fractionate Peak 4 of the Ultrogel separation

Peak 4 from the Ultrogel separation was concentrated by collodion bag ultrafiltration and applied to a pH 3-7 IEF gel. Specific application and running conditions accompany the zymogram (Fig. 6.21), which was prepared from the focussed IEF gel and used as a template in excising the twelve or so active bands. The total gel was sectioned, and the water washings of each slice and also of the applicator and electrode strips were tested for various types of cellulolytic activity (Table 6.6). The highest levels of each

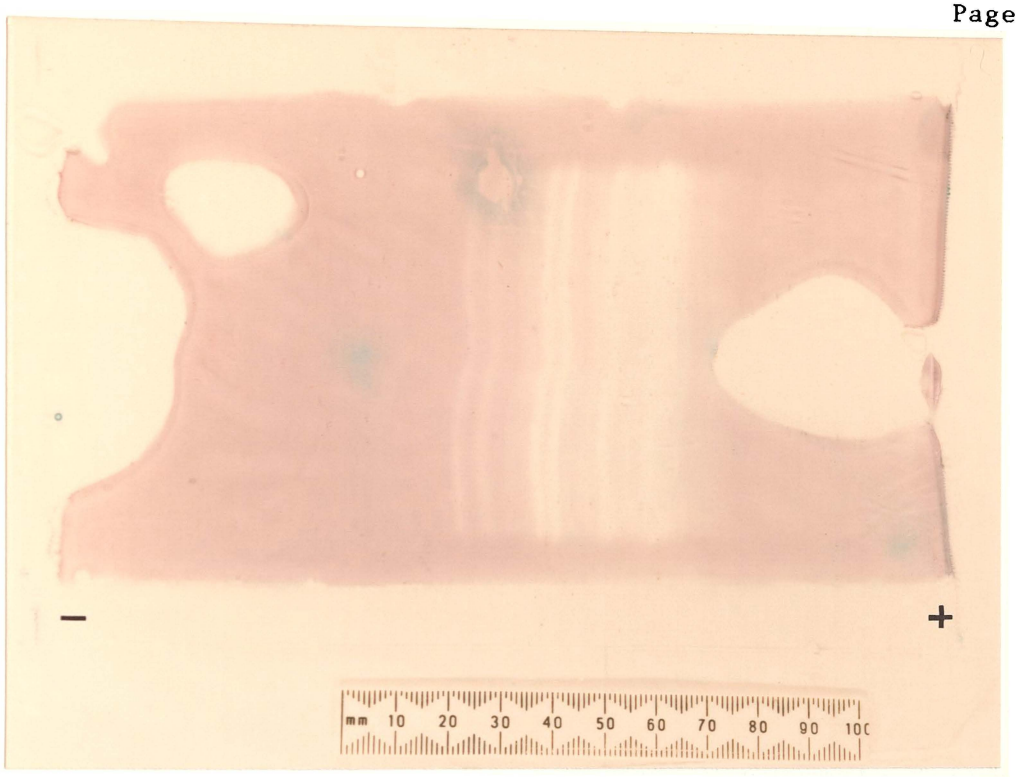


Figure 6.21 A CMC/polyacrylamide zymogram produced after the preparative IEF separation of Peak 4 from the Ultrogel column.

The original gel measured 17.5cm x 9.5cm x 0.3mm, and incorporated pH 3-5, 4-6 and 5-7 Ampholines (LKB) in a 1:2:1 ratio, producing a final combined ampholine content of 2.6%(w/v). Urea was incorporated (concentration in gel = 5M). Gel production methods are described in Section 2.10.2 and 2.10.3.

Anode solution:	0.1M glutamic acid in 0.5M orthophosphoric acid.
Cathode solution:	0.1M ß-alanine.
Sample loading:	0.35ml of Peak 4 concentrate +20µl pH4-6 Ampholine (40%w/v) +0.144g urea. Sample mixture was applied to a glass fibre wick (7cm x 4mm x 2mm) positioned 4cm from the cathode.
Running conditions:	Current limit = 3mA. Wattage limit = 4W Voltage limit = 2000V

After 4 hours a zymogram showed focussing to be incomplete, so the voltage was reduced to 2000V for 15 minutes the next day. Total running time was 17.5h, of which 8.5h were at 2000V.
Excised strips of gel, the applicator and electrode strips were washed in 7.5ml distilled water at 4°C for 4h.

Table 6.6: Activity and molecular weight data for the fractions obtained by preparative I.E.F. over the pH range 4-6 of Peak 4 from the Ultrogel column separation

NOTES:

Units of cellulose azurase, TNP-CMCase and avicelase (using dyed Sigmacell 50) are arbitrarily defined changes in absorbance per minute, as described in Section 3.

A unit of CMC-plate-clearing activity is relative to an arbitrarily selected standard activity (See Fig. 3.2).

Note that all of the above activities are expressed as units per ml of the solution used to wash each gel strip (7.5 ml Milli-Q-water).

Molecular weights of the dominant protein bands from slices 5 and 10 were obtained by comparison with the molecular weight marker proteins run on the same SDS-PAGE system (Fig. 6.22).

Yields of each type of activity from this preparative IEF gel were:

cellulose azurase	=	4%
TNP-CMCase	=	34%
Avicelase	=	31%
CMC-plate-clearing	=	6%

Table 6.6: Activity and molecular weight data for the fractions obtained by preparative I.E.F. over the pH range 4-6 of Peak 4 from the Ultrogel column separation

Slice No.	Cellulose-Azurase (Units. ml^{-1})	TNP-CMCase (Units. ml^{-1})	Avicelase (Units. ml^{-1})	CMC-plate-clearing (Units.ml^{-1})	CMC-plate-clearing / TNP-CMCase	CMC-plate-clearing / Avicelase	TNP-CMCase / Avicelase	CMC-plate-clearing / Cellulose azurase	Band on zymogram?	Molecular weight (10^{-3} daltons)
-9 (At Cathode) to -2	All 0	All 0	All 0	All 0					No	
-1	0	6	0	3					No	
0	0	0	0	7					No	
1	0	8	0	10	1.3				Yes	
16	7	1	3	12	12	4	0.3	2	Yes	
2	0	19	0	10	0.5	∞	∞	∞	Yes	
2b	0	3	1	7	2	7	3	∞	Yes	
3	0	22	0	19	0.9	∞	∞	∞	Yes	
3b	0	24	3	19	0.8	6	8	∞	Yes	
4	1	25	4	25	0.7	6	9	25	Yes	
5	15	209	11	88	0.4	8	19	6	Yes	66
6	4	16	1	25	1.6	25	16	6	Yes	
6b	1	33	1	14	0.4	14	33	14	Yes	
7	9	81	3	19	0.2	6	27	2	Yes	
8	9	134	5	25	0.2	5	27	3	Yes	
9	15	127	5	35	0.3	7	25	2	Yes	
10	101	837	23	88	0.1	4	36	1	Yes	88
11	24	300	8	47	0.2	6	38	2	Yes	
12	1	22	1	12	0.6	12	22	12	No	
13	0	0	0	7					No	
14	0	0	1	5					No	
15	0	0	0	4					No	
16	3	0	0	4					No	
17	0	0	0	4					No	
18	0	0	0	4					No	
19	0	0	0	4					No	
20	0	0	0	4					No	
21	0	0	0	4					No	
22	0	0	0	2					No	
23 at anode)	0	0	0	0					No	

of the types of activity measured were obtained from slices 5, 8, 9, 10 and 11. Only traces of cellulolytic activity were recovered from outside the section of the gel which gave rise to the bands of clearance on the zymogram, (Fig. 6.21) and no activity was obtained from either electrode. The yields of this preparative IEF step wire unexpectedly low (Table 6.6). A preliminary test had shown that TNP-CMCase activity was not altered by the levels of urea and ampholines which were added to the sample loaded. Despite this, only 34% of the TNP-CMCase activity applied was recovered in the gel washings and other activities exhibited even poorer yields. It is possible that 4 hours soaking was insufficient time to completely wash these enzymes from their slices of gel. Less than 10% of each type of activity recovered was recovered from the applicator strip.

6.4.7.1 Activities of the IEF gel slice washings towards various substrates Differences were evident in the ratios of the levels of the various activities obtained from different gel slices (Table 6.6). The Ultrogel Peak 4 had evidently contained a range of enzymes with different properties. For example, slice 5 washings had a clearly higher ratio of CMC-plate-clearing activity : avicelase (i.e. indicative of an endo-acting mode of action) than another major band of activity from slice 10. Little significance should be attached however to the activity ratios arising from those slices which had very low levels of activity, due to their high degrees of uncertainty.

6.4.7.2 SDS-PAGE and IEF analysis of the most active slice washings Washings from slices 5, 9 and 10, along with the Peak 4 concentrate as applied to the IEF gel, were compared on by SDS-PAGE (Fig. 6.22). Although it was clear that substantial progress had been made in fractionating the complex mixture of proteins evident in the Peak 4 material applied, none of the slices gave rise to a single band. Slice 5 came closest, producing only two bands with very similar molecular weights of around 65,000.

Upon rerunning washings of slices 5 and 10 on IEF, but over a narrower range (pH 4.5 - 5.0), neither produced single bands (Fig. 6.23). Slice 5 produced 5 closely spaces main bands with two fainter bands further towards the anode. Slice

Figure 6.22 SDS-PAGE of the most active cellulolytic fractions from the preparative IEF separation.

Lane 1 40µl of slice 10 washings.

Lane 2 40µl of slice 9 washings.

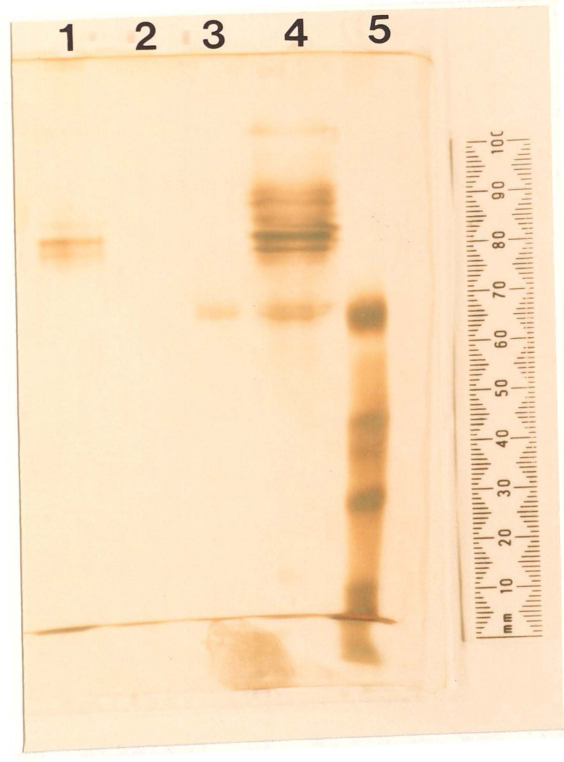
Lane 3 40µl of slice 5 washings.

Lane 4 4µl of the material applied to the IEF gel (i.e. Peak 4 concentrate, urea and ampholines).

Lane 5 SDS marker proteins (Sigma).
BSA (MW=66,000)
Ovalbumin (MW=45,000)
Glyceraldehyde-6-phosphate (MW=36,000)
Carbonic anhydrase (MW=29,000)
Trypsinogen (MW=24,000)
Trypsin inhibitor (MW=20,000)

Each sample was mixed in a 4:1 ratio with sample buffer comprising:
0.5ml 10%(w/v) SDS
0.5ml 0.25M Tris/HCl buffer pH 6.8
1ml glycerol

The separating gel contained 7.5%(w/v) acrylamide.



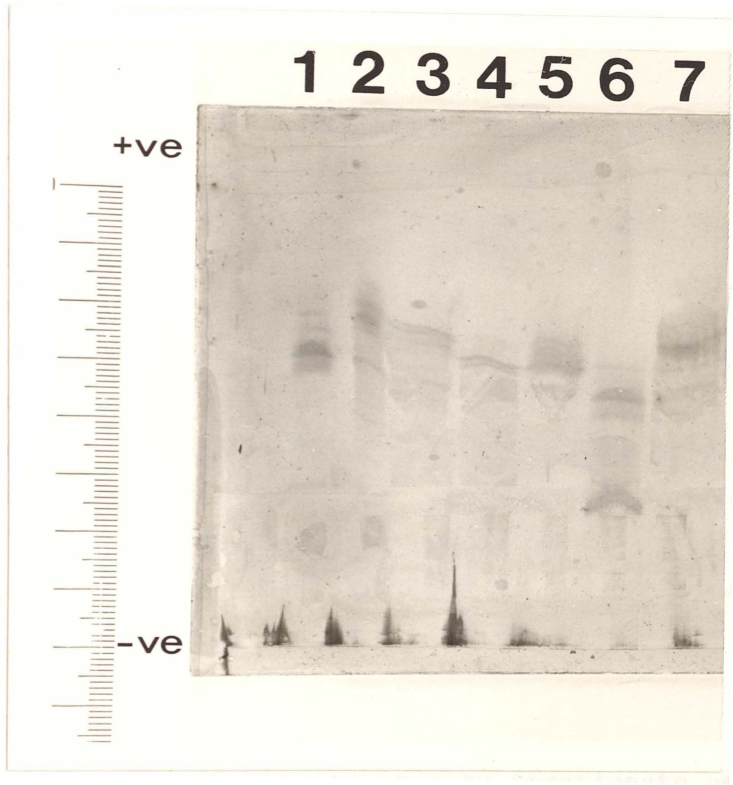


Figure 6.23 Narrow-range IEF of cellulolytic proteins from the preparative IEF separation of Ultrogel Peak 4 and from preparative SDS-PAGE separation of Ultrogel Peaks 3 and 5.

Lane 1 Slice 5 washings from Peak 4 prep. IEF.

Lane 2 Slice 10 washings from Peak 4 prep. IEF.

Lane 3 Slice 10 washings from Peak 5 prep. SDS-PAGE.

Lane 4 Slice 11 washings from Peak 5 prep. SDS-PAGE.

Lane 5 Slice 13 washings from Peak 5 prep. SDS-PAGE.

Lane 6 Slice 10 washings from Peak 3 prep. SDS-PAGE.

Lane 7 Slice 18 washings from Peak 3 prep. SDS-PAGE.

The gel pH gradient of 4.5-5.0 was created with Servalytes (2%w/v).

10 produced 3 main bands focussed at a lower pI than the main bands from slice 5, and two minor bands with higher pIs.

The observed series of bands from slices 5 and 10 in Fig. 6.23 covered overlapping ranges of pI. This was unexpected considering that their source slices were well separated (by at least 1.5cm) on the original IEF gel, which covered a broader pH range than the gel of Fig. 6.23. It thus appears probable that some of the bands produced in rerunning the washings of slices 5 and 10 on the narrower range IEF gel were artefacts of the system, possibly a result of the switch from the use of LKB Ampholines to Servalytes to produce the pH gradient in the second gel (Bredenkamp and Joubert, 1982, Basset *et al.*, 1983). This did not however alter the conclusion, drawn from the SDS-PAGE gel of Fig. 6.22 and the assays of Table 6.6, that the preparative IEF step had clearly split the Peak 4 concentrate into genuinely different cellulolytic components.

6.4.8 Preparative SDS electrophoresis to fractionate peaks 3 and 5 of the Ultrogel separation

A preliminary small-scale separation of crude supernatant concentrate had demonstrated that recoveries of activity of 30-55% were possible by preparative SDS-PAGE (Section 6.3.5.3), so that technique was applied to fractionate Peaks 3 and 5 from the Ultrogel separation. Discontinuous 1.5mm-thick SDS-PAGE gels with 10% (w/v) acrylamide separating gels, were prepared (Laemmli, 1970). Wells (5cm wide) were loaded with 0.37ml of a mixture of sample buffer and sample, (described in Figs. 6.24 and 6.25). After running, CMC/polyacrylamide zymograms (Figs 6.24 and 6.25) were produced from each and then used as templates to facilitate individual slicing of the cellulolytic bands from the gels (described in Sections 3.5.4.3 and 3.5.4.4). The closeness of the 10 or so active bands, particularly those arising from Peak 3 (Fig. 6.25) made it very difficult to excise them separately. A lower percentage acrylamide (e.g. 7.5%) would have spread the bands and made their separation easier.

Figure 6.24 Polyacrylamide/CMC zymogram produced from the preparative SDS-PAGE separation of Ultrogel Peak 5.

Sample buffer and sample were mixed 1:4 to produce a final volume loaded of 0.37ml.

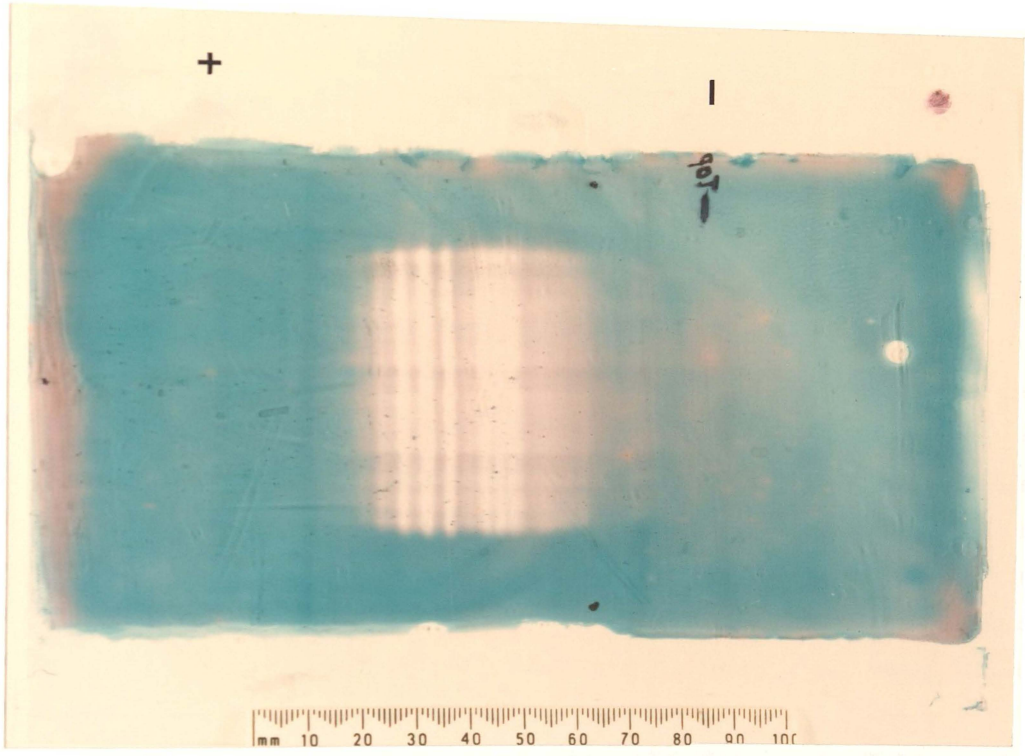
Sample buffer comprised 0.5% SDS(w/v), 50% glycerol(v/v) and 0.005% bromothymol blue in 0.25M Tris/HCl buffer pH6.8.

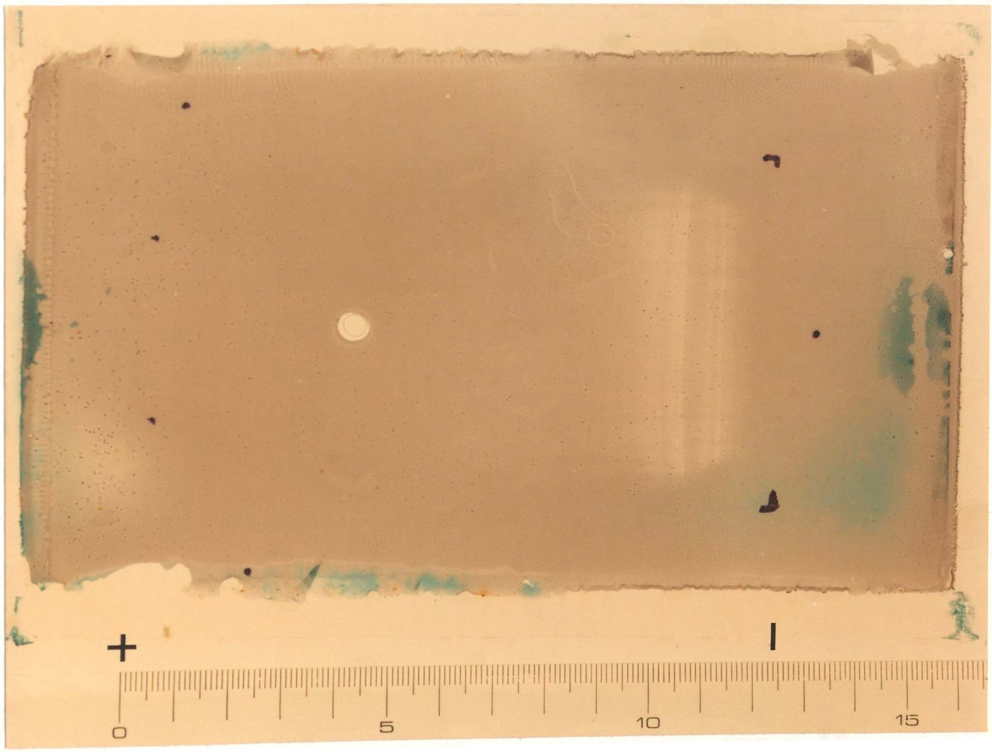
Running conditions = 45mA for 2h.

Figure 6.25 Polyacrylamide/CMC zymogram produced from the preparative SDS-PAGE separation of Ultrogel Peak 3.

Sample pretreatment, loading and final volume were as for Fig. 6.24.

Running conditions = 45-50mA for 2.5h.


+
I
mm 10 20 30 40 50 60 70 80 90 100


+
I
0 5 10 15

6.4.8.1 Activities of the SDS-PAGE gel slice washings towards various substrates The avicelase, TNP-CMCase and CMC-plate-clearing activities released into the gel slice washings and the ratios of these various activities are shown in Tables 6.7 and 6.8. These ratios revealed that avicelase comprised an increasing proportion of the total activities with increasing molecular weight. (This applied not only within each group of enzymes separated from an Ultrogel peak but also to the Ultrogel peaks *per se*).

A trio of active bands from Peak 5 which had stood out most clearly and exhibited the best separation in the zymogram (Fig. 6.24) together accounted for 95% of the total TNP-CMCase recovered from the gel (slices 9, 10 and 11, Table 6.7). The comparatively high ratios of CMC-plate-clearing activity to avicelase activity in these slices also suggested the presence of a greater endoglucanase component than in most other slices.

There is little suggestion from the data of Tables 6.7 and 6.8 that the two SDS-PAGE separations of Ultrogel Peaks 3 and 5 produced any bands of activity in common. Some active bands from the two gels shared similar molecular weights, but the ratios of their activities on the various substrates did not match up well.

Yields of the various activities obtained from the SDS-PAGE fractionation of Peaks 3 and 5 (Tables 6.7 and 6.8) were substantially better than those from the IEF fractionation of Peak 4 (Table 6.6). In fact, the TNP-CMCase activity of Peak 3 appeared to double as a consequence of the SDS-PAGE separation. The vast majority of each type of activity measured was found within the confines of the 10-12 bands of clearance on each. Nonetheless, unmistakable CMC-plate-clearing activity was found in all sections of the gels apart from those corresponding to molecular weights below 20,000.

6.4.8.2 SDS-PAGE reruns of the most active slices from preparative SDS-PAGE It was of interest to discover whether or not the separated enzymes from preparative SDS-PAGE of Peaks 3 and 5 would retain their integrities and relative mobilities upon rerunning on SDS-PAGE. This rerunning would also allow

Table 6.7: Activity and molecular weight data for the fractions obtained by preparative SDS-PAGE of Peak 5 from the Ultrogel column separation

NOTES:

Units of TNP-CMCase and avicelase (using dyed Sigmacell 50) are arbitrarily defined changes in absorbance as described in Section 3.

A unit of CMC-plate-clearing activity is relative to an arbitrarily selected standard activity (See Fig. 3.2).

Note that all of the above activities are expressed as units per ml of the solution used to wash each gel strip (5 ml of 0.25M sodium acetate buffer, pH 5.7).

Molecular weights were calculated on the basis of mobilities upon rerunning on a 10% (w/v) acrylamide SDS-PAGE gel (Fig. 6.26) and also using the locations of the slices on the original gel, with reference to molecular weight standards

Yields of each type of activity from this preparative SDS-PAGE were:

TNP-CMCase	=	50%
Avicelase	=	50%
CMC-plate-clearing	=	50%

Table 6.7: Activity and molecular weight data for the fractions obtained by preparative SDS-PAGE of Peak 5 from the Ultrogel column separation

Slice No.	TNP-CMCase (Units. ml^{-1})	Avicelase (Units. ml^{-1})	CMC-plate -clearing (Units. ml^{-1})	CMC-plate-clearing / TNP-CMCase	CMC-plate-clearing / Avicelase	TNP-CMCase / Avicelase	Band on zymogram?	Molecular weight (10^{-3} daltons)
1	27	5	0	0	0	5	No	
2	14	5	0	0	0	3	No	
3	7	4	0	0	0	2	No	
4	24	3	7	0.3	2	8	No	20
5	0	2	12	-	6	0	No	
6	0	1	70	-	70	0	No	
7	0	2	119	-	60	0	No	30
8	28	3	412	15	140	9	Yes	33
9	2980	11	1700	0.6	160	270	Yes	36
10	6750	14	2890	0.4	210	480	Yes	41 (major) +39 (minor)
11	3330	10	2030	0.6	200	330	Yes	47
12	79	4	246	4.4	87	20	Yes	53 (major) +41 (minor)
13	50	11	1000	20	91	5	Yes	58
14	37	12	838	23	70	3	Yes	62
15	43	12	838	20	70	4	Yes	69
16	25	3	70	2.8	23	8	Yes	
17	19	7	170	8.9	24	3	Yes	80
18	22	4	143	6.5	36	6	Yes	88
19	54	3	49	0.9	16	18	Yes	94
20	21	8	59	2.8	7	3	Yes	98
21	40	1	29	0.7	29	40	No	104
22	18	1	24	1.3	24	18	No	
23	46	1	24	0.5	24	46	No	120
24	0	1	17	-	17	0	No	
25	0	2	10	-	5	0	No	
26	0	1	6	-	6	0	No	
27	14	1	5	0.4	5	14	No	
28	40	0	3	0.1	-	-	No	160
29	23	0	6	0.3	-	-	No	

Table 6.8: Activity and molecular weight data for the fractions obtained by preparative SDS-PAGE of Peak 3 from the Ultrogel column separation

NOTES:

Units of TNP-CMCase and avicelase (using dyed Sigmacell 50) are arbitrarily defined changes in absorbance as described in Section 3.

A unit of CMC-plate-clearing activity is relative to an arbitrarily selected standard activity (See Fig. 3.2).

Note that all of the above activities are expressed as units per ml of the solution used to wash each gel strip (5 ml of 0.25M sodium acetate buffer, pH 5.7).

Molecular weights were calculated on the basis of mobilities upon rerunning on a 7½% (w/v) acrylamide SDS-PAGE gel (Fig. 6.27) with reference to molecular weight standards.

Yields of each type of activity from this preparative SDS-PAGE were:

TNP-CMCase	=	210%
Avicelase	=	98%
CMC-plate-clearing	=	96%

Table 6.8: Activity and molecular weight data for the fractions obtained by preparative SDS-PAGE of Peak 3 from the Ultrogel column separation

Slice No.	TNP-CMCase (Units. ml^{-1})	Avicelase (Units. ml^{-1})	CMC-plate -clearing (Units. ml^{-1})	CMC-plate-clearing / TNP-CMCase	CMC-plate-clearing / Avicelase	TNP-CMCase / Avicelase	Band on zymogram?	Molecular weight (10^{-3} daltons)
1	6	2	3.5	0.58	2.2	3.8	No	
2	15	1	3.5	0.23	4.4	19	No	
3	22	4	3.5	0.16	0.9	5.6	No	
4	13	6	10	0.77	1.6	2.1	No	
5	27	1	20	0.74	25	34	No	
6	43	2	35	0.81	15	19	Yes	128
7	45	10	29	0.64	2.9	4.5	Yes	125
8	13	2	5.9	0.45	2.6	5.7	No	
9	87	8	59	0.68	7.6	11	Yes	122 (+ 55)
10	222	12	119	0.54	9.6	18	Yes	120 (+ 55)
11	201	9	143	0.71	15	22	Yes	117 (+ 54)
12	257	6	119	0.46	19	42	Yes	106 (+ 54)
13	211	5	100	0.47	21	45	Yes	103 to 114
14	183	4	84	0.46	22	47	Yes	102 to 112
15	359	5	119	0.33	22	67	Yes	98
16	457	6	346	0.76	56	74	Yes	96
17	408	6	289	0.71	47	66	Yes	92 + 90
18	543	6	492	0.91	79	88	Yes	90 + 86
19	399	7	242	0.61	35	57	Yes	82
20	325	5	289	0.89	62	69	Yes	79
21	115	3	119	1.03	38	37	No	74
22	57	2	84	1.47	37	25	No	
23	38	4	41	1.08	11	9.7	No	
24	34	0	17	0.50	∞	∞	No	
25	34	0	14	0.41	∞	∞	No	
26	43	0	8.4	0.20	∞	∞	No	
27	27	0	5.9	0.22	∞	∞	No	
28	41	0	5.0	0.12	∞	∞	No	
29	29	0	2.4	0.08	∞	∞	No	
30-37	All 40-60	0	All <5	All <0.1	∞	∞	No	

us to ascertain the degree to which gel slicing had been successful in separating the bands of protein.

Samples of the most active slice washings from the Peak 5 SDS prep. gel (Slices 8 - 15) were rerun on a 10% acrylamide SDS gel (Fig. 6.26). The original SDS-PAGE band pattern produced by Peak 5 is displayed in Lane 6, Fig. 6.20, for comparison. Single protein bands were produced by washings of gel slices 8, 9, 11, 13 and 14, whereas slices 10, 12 and 15 each included a second band, much fainter than the main band. The two bands from slices 10 and 15 were close enough together to suggest that they had simply not been separated in the slicing of the gel. However the minor band of slice 12 was too distant from the main band for this to be likely, having the same Rf as the main band of slice 10. Also, there was no trace in-between them of the protein that had been centred in the intervening slice 11. Therefore it seemed most likely that the main protein in slice 12 was exhibiting a tendency to break down into a smaller molecule, possibly identical with the main protein of slice 10.

The Rfs of the main protein bands on this second gel were very similar to those of the strips on the original from which they came. Clearly they were able to maintain their integrities in the presence of 0.1% SDS, with the possible exception of the slice 12 protein, as mentioned above. It therefore seemed most unlikely that this series of enzymes could merely have been artefacts produced by the separation system.

SDS-PAGE reruns of the most active slice washings from the preparative SDS-PAGE of Peak 3 are shown in Fig. 6.27. No mercaptoethanol pretreatment was employed. Note the appearance of new bands with M.Ws around 55,000 after rerunning washings of slices from the upper portions of the original gel (Lanes 14-17). These slice washings still contained a dominant high molecular weight band as expected (i.e. M.W.90,000 - 120,000) but evidently this had a tendancy to partially break down upon rerunning in 0.1% SDS, resulting in products of approximately half the size of the parent molecules.

Figure 6.26 SDS-PAGE of proteins from the most actively cellulolytic slices of the Peak 5 SDS-PAGE prep. gel.

Lane 1 Slice 8 washings

Lane 2 Slice 9 washings

Lane 3 Slice 10 washings

Lane 4 Slice 11 washings

Lane 5 Slice 12 washings

Lane 6 Slice 13 washings

Lane 7 Slice 14 washings

Lane 8 Slice 15 washings

Sample buffer comprised 0.5% SDS(w/v), 50% glycerol(v/v) and 0.005% bromothymol blue in 0.25M Tris/HCl buffer pH6.8.

Ratio of sample buffer:sample = 7:30.

Equal volumes (30µl) were applied to each well.

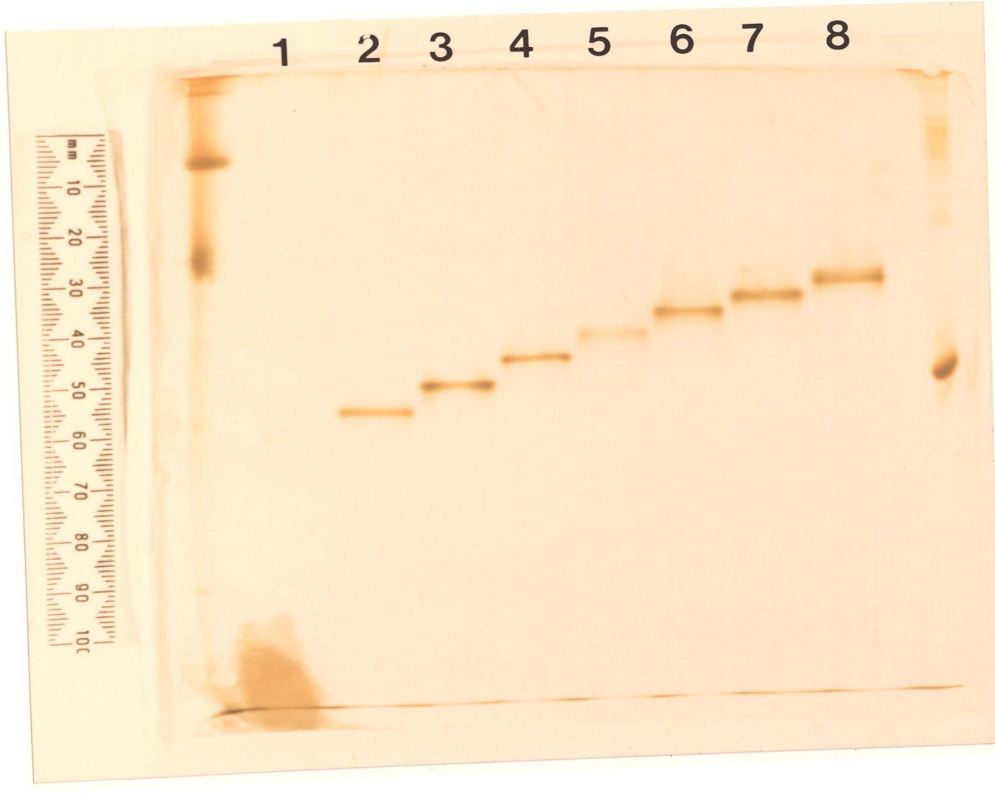
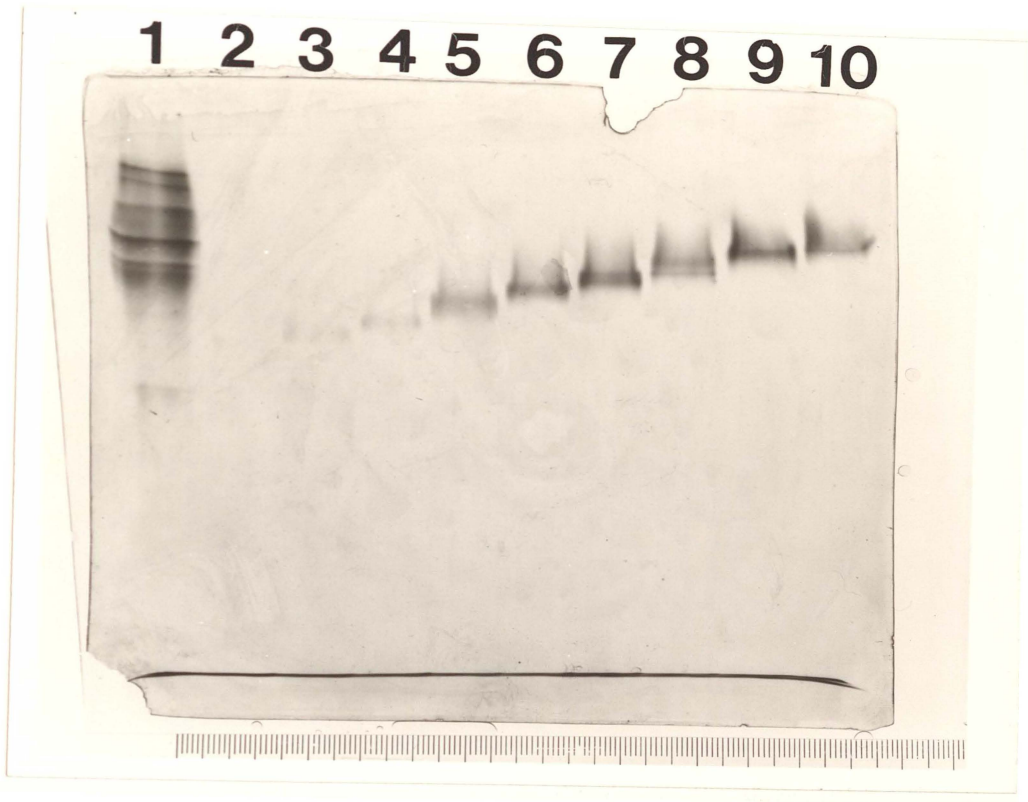


Figure 6.27 SDS-PAGE reruns of proteins from the most actively cellulolytic slices of the Peak 3 SDS-PAGE prep. gel.

Lane 1	Peak 3 concentrate
Lane 2	Slice 23 washings
Lane 3	Slice 22 washings
Lane 4	Slice 21 washings
Lane 5	Slice 20 washings
Lane 6	Slice 19 washings
Lane 7	Slice 18 washings
Lane 8	Slice 17 washings
Lane 9	Slice 16 washings
Lane 10	Slice 15 washings
Lane 11	Peak 3 concentrate
Lane 12	Slice 14 washings
Lane 13	Slice 13 washings
Lane 14	Slice 12 washings
Lane 15	Slice 11 washings
Lane 16	Slice 10 washings
Lane 17	Slice 9 washings
Lane 18	Slice 8 washings
Lane 19	Slice 7 washings
Lane 20	Slice 6 washings

A 7.5%(w/v) acrylamide separating gel was used.

No sample pretreatment was employed other than the addition of glycerol to produce a 10%(v/v) solution.


1 2 3 4 5 6 7 8 9 10

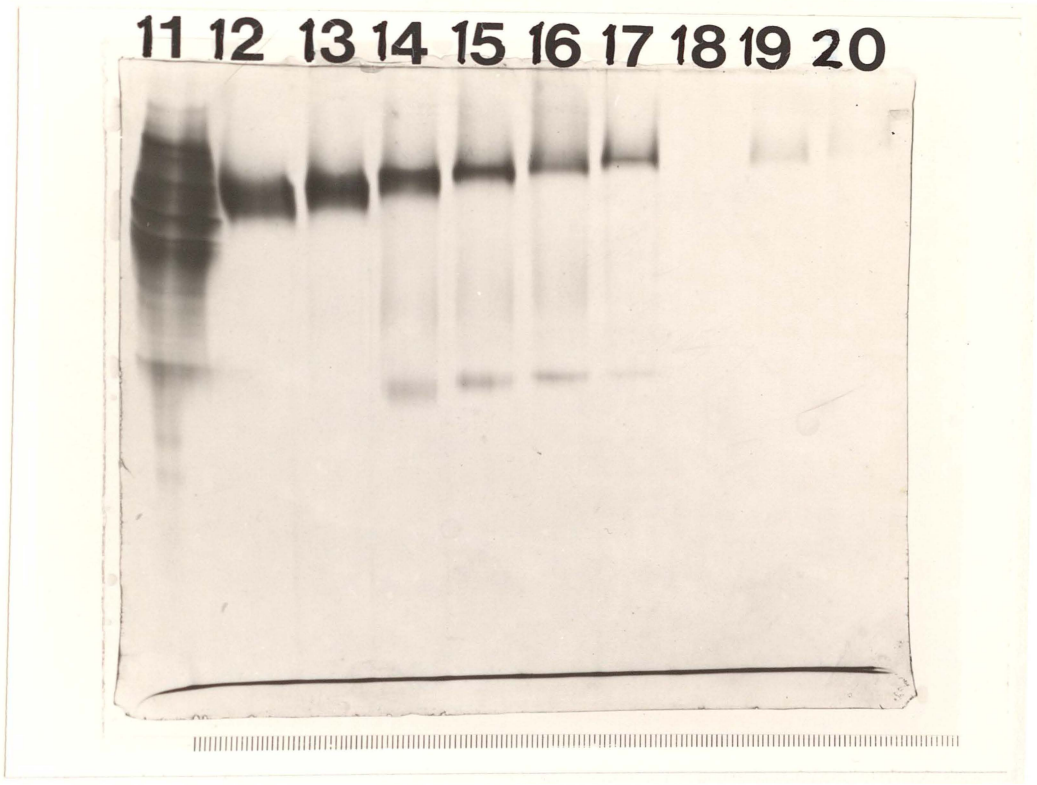

11 12 13 14 15 16 17 18 19 20

Figure 6.28 IEF of Peak 5 and of gel slice washings from the preparative SDS-PAGE separation of Peak 5.

Lane 1 1µl Peak 5 concentrate

Lane 2 10µl slice 8 washings

Lane 3 10µl slice 9 washings

Lane 4 10µl slice 10 washings

Lane 5 10µl slice 11 washings

Lane 6 10µl slice 12 washings

Lane 7 10µl slice 13 washings

Lane 8 10µl slice 14 washings

Lane 9 10µl slice 15 washings

pH range = 4-6

Urea concentration in gel = 5M.

The lighter cellulases from Peak 3 (i.e. M.W.74,000 - 100,000) did not yield any unexpected additional bands upon rerunning on SDS electrophoresis which couldn't simply be attributed to inadequate band separation during slicing of the original gel (Fig. 6.27, Lanes 2-13). Some slices appeared to contain only single proteins while others produced two, or possibly more, closely spaced bands.

6.4.8.3 I.E.F. analysis of the most active bands from preparative SDS-PAGE None of the most active gel slice washings from the preparative SDS-PAGE separations of Ultrogel Peaks 3 and 5 gave rise to single bands upon IEF. However, most produced only one or two dominant bands, with these and other minor bands being grouped closely within a 0.1 pH unit range (Figs. 6.23, 6.28, 6.29). All protein bands were found to focus inside the pH range 4.5 - 5.0. The zymogram (Fig. 6.30) showed the bands arising from the gel slices of the Peak 3 separation more clearly than did the silver stain of the original (Fig. 6.29), and it was clear that almost every band of protein had some CMC-plate-clearing activity.

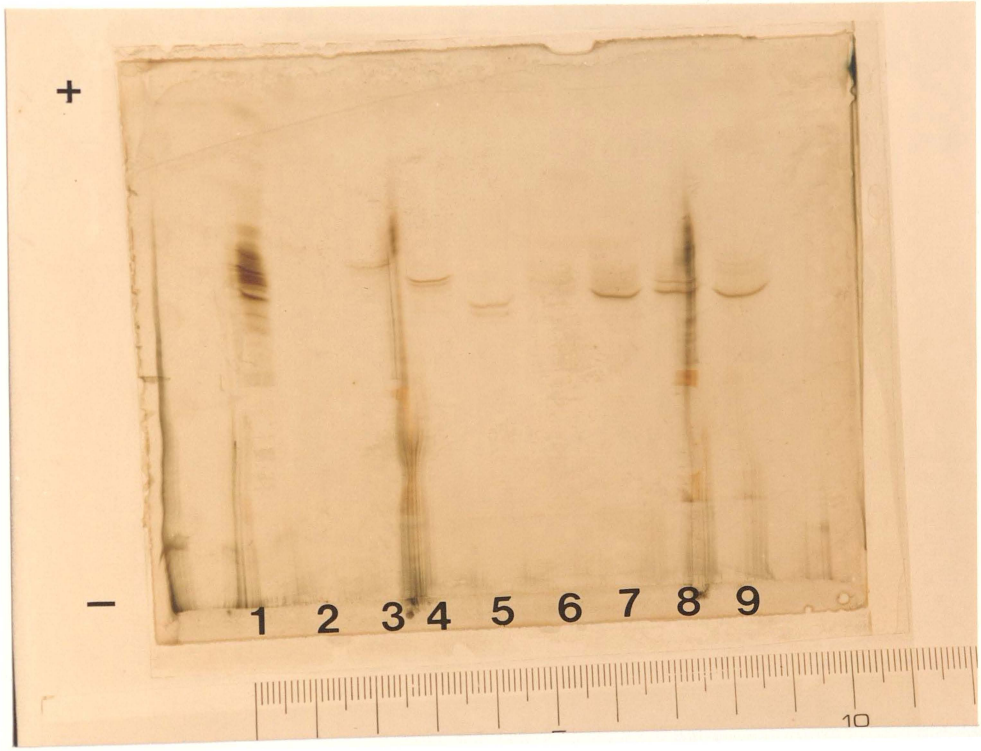


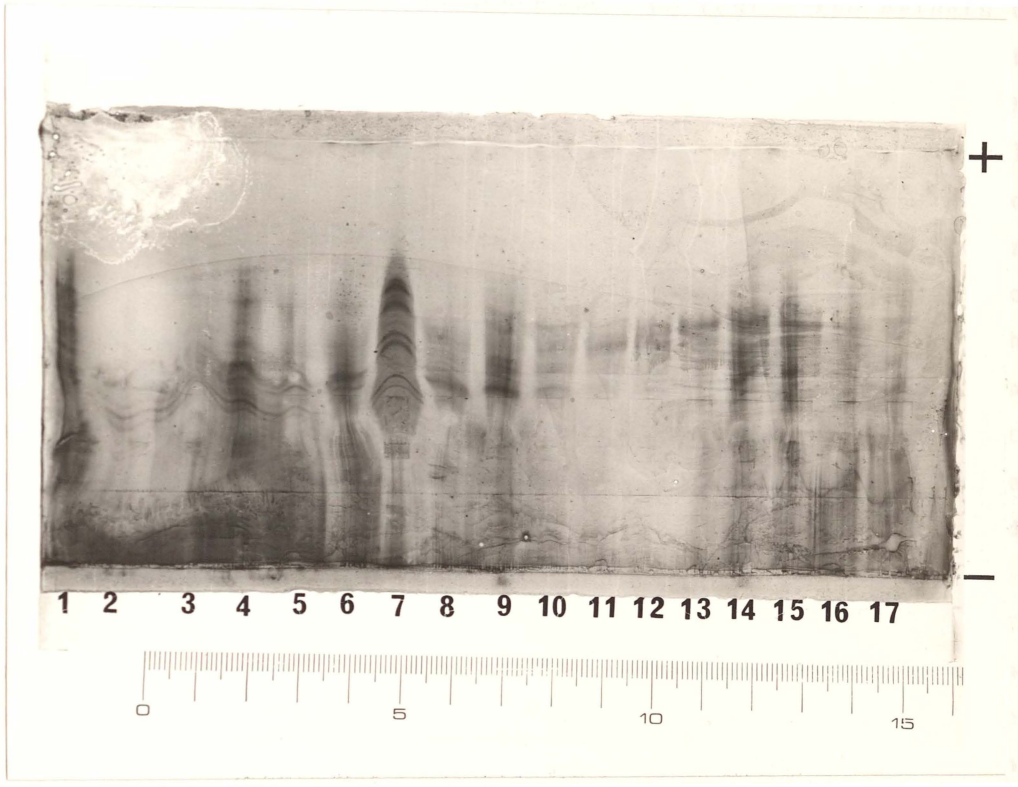
Figure 6.29 IEF of Peak 3 and of gel slice washings from the preparative SDS-PAGE of Peak 3.

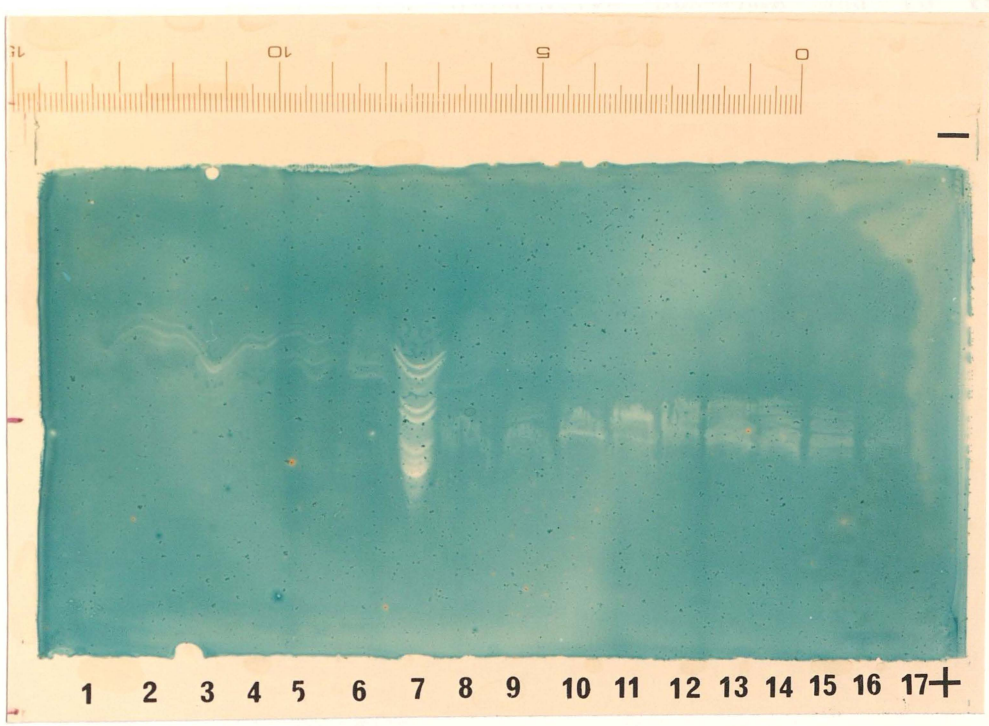
Lane 1	10µl slice 4 washings
Lane 2	10µl slice 7 washings
Lane 3	10µl slice 9 washings
Lane 4	10µl slice 10 washings
Lane 5	10µl slice 11 washings
Lane 6	10µl slice 12 washings
Lane 7	1µl Peak 3 concentrate
Lane 8	10µl slice 13 washings
Lane 9	10µl slice 14 washings
Lane 10	10µl slice 15 washings
Lane 11	10µl slice 16 washings
Lane 12	10µl slice 17 washings
Lane 13	10µl slice 18 washings
Lane 14	10µl slice 19 washings
Lane 15	10µl slice 20 washings
Lane 16	10µl slice 21 washings
Lane 17	10µl slice 22 washings

pH range = 4-6

Urea concentration in gel = 5M.

Figure 6.30 Polyacrylamide/CMC zymogram of the IEF gel of Fig.6.29.


+
−
1 2 3 4 5 6 7 8 9 10 11 12 13 14 15 16 17
0
5
10
15


−
1 2 3 4 5 6 7 8 9 10 11 12 13 14 15 16 17+

The question arose as to how many of these multiple IEF bands could have been artefacts of the system, especially since in some cases they were obtained from material which had produced a single band on SDS-PAGE. We tested the effects of the point of application on the IEF patterns produced by material from three of the most active slices from the preparative SDS-PAGE gels (Fig. 6.31). Although sample application near the anode resulted in considerable distortion of the ampholine bands, which in turn displaced each entire pattern of protein bands towards the anode, the number of bands and their relative intensities was not affected by the point of application. This suggested that the band multiplicity was not an artifact caused by interactions with particular ampholines. Note however that the gel had not been prefocussed prior to sample application, which would have made this experiment a more thorough test for binding artefacts.

Still a better test for artefacts would have been to refocus individual bands excised from an IEF gel on a second identical IEF gel in order to discover whether they refocussed as single bands or as a group of bands resembling that of the previous gel. Another alternative method, which we tried, was to use IEF as a preparative technique in separating material from single slices of the preparative SDS-PAGE and to then endeavour to detect differences in the substrate specificities among the bands of activity resulting (Section 6.4.9).

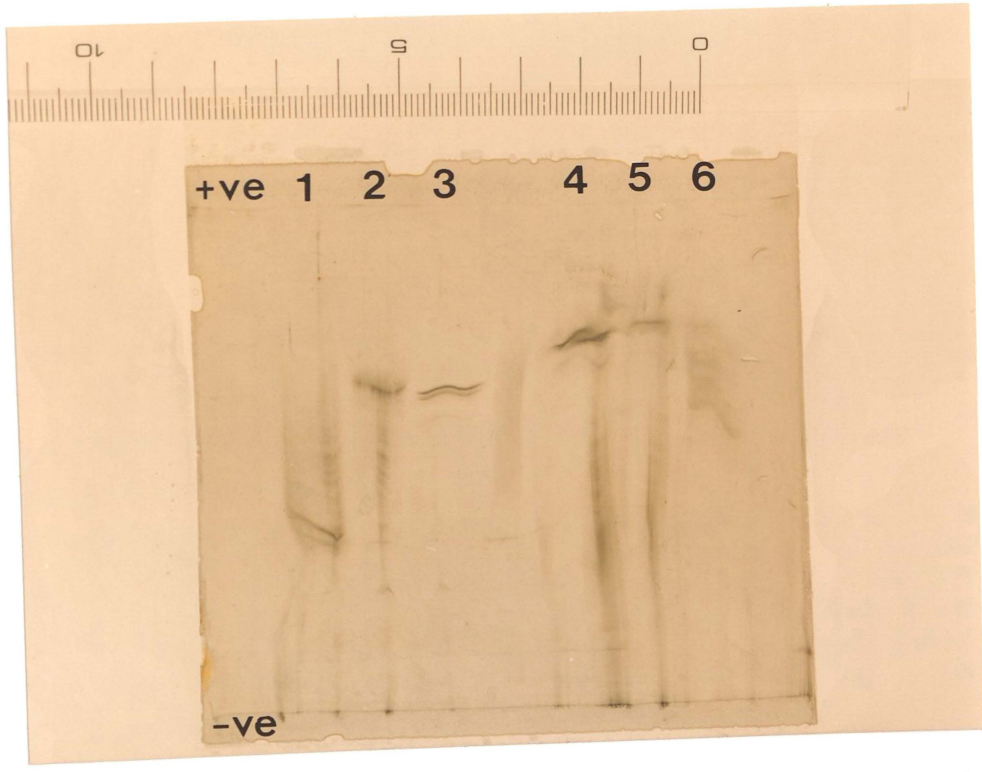
6.4.9 Preparative narrow-range isoelectric focussing to further fractionate band 10 from SDS-PAGE of Peak 5

Cellulases in the washings of slice 10 from the Peak 5 SDS-PAGE prep. gel were concentrated and washed in a collodion bag in order to reduce the volume and ionic strength in preparation for IEF. During the final stages of concentration a precipitate formed, probably as a result of the low ionic strength. This did not redissolve when the ionic strength and pH were increased through the addition of 0.1M phosphate buffer, pH 7.6 (Dawson *et al.*, 1974), nor during subsequent washing and reconcentration in 3M urea, which returned to ionic strength to a low level suitable for IEF. The precipitate was therefore removed by centrifugation, leaving 0.65ml of concentrate.

The pH 4.5 - 5.0 gel was prepared as in Section 2.10.2, but

Figure 6.31 The influence of point of sample application on the IEF separation of proteins from single bands cut from SDS-PAGE gels.

Lanes 1-3:	Samples applied 2cm from cathode (i.e. as normal).
Lanes 4-6:	Samples applied 2cm from anode.
Lanes 1 and 6:	Washings of slice 10 from the SDS-PAGE preparative separation of Ultrogel Peak 3.
Lanes 2 and 5:	Washings of slice 18 from the SDS-PAGE preparative separation of Ultrogel Peak 3.
Lanes 3 and 4:	Washings of slice 10 from the SDS-PAGE preparative separation of Ultrogel Peak 5.



without urea. This narrow pH range, spread across a 20cm wide gel, meant that proteins with pIs differing by only 0.01 units would have been expected to focus with a 4mm separation. Sample application and running conditions accompany the activity zymogram (Fig. 6.32), which was used as a template in attempting to excise separately the 10 or so active bands from the IEF gel. Separating the active bands proved extremely difficult since the bands were not straight, and at one point were very compressed (5 or 6 bands within 8mm). The entire remaining gel was sliced and the slices

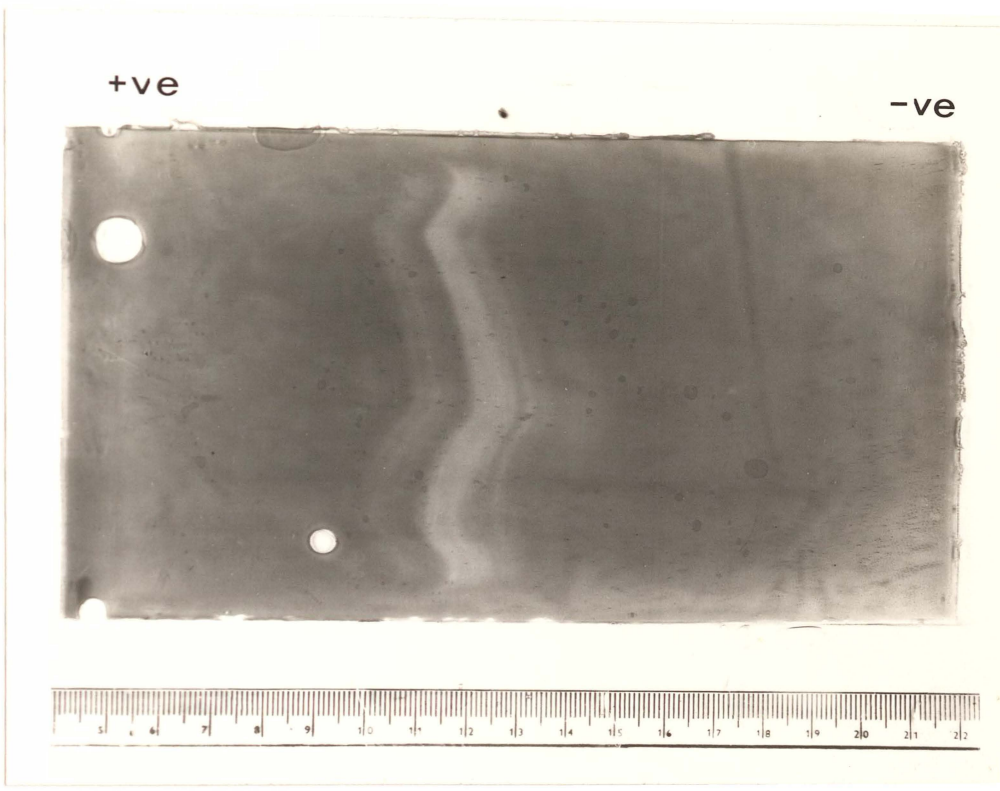


Figure 6.32 A CMC/polyacrylamide zymogram of the preparative IEF separation of washings of slice 10 from the Peak 5 SDS-PAGE prep. gel.

Anode solution: 0.33%(w/v) L-aspartic acid and 0.37%(w/v) L-glutamic acid.

Cathode solution: 2%(w/v) glycine.

Sample loading: 0.6ml concentrated slice 10 washings (centrifuged) + 0.02ml Servalyte pH4.5-5.0 (40%w/v ampholines) were applied to a glass fibre wick (8.5cm x 5mm x 2mm) lying across gel 1.7cm from cathode.

Running conditions: Current limit 1.8mA
Wattage limit 3W
Voltage limit 2000V
After 8h, voltage limit was reduced to 800V for 9 hours, then returned to 2000V for 1 final hour.

Dimensions of the original IEF gel = 21cm x 9.5cm x 0.3mm.

The final concentration of Servalyte pH 4.5-5.0 ampholine in the original IEF gel = 2%.

were washed overnight in 8ml of 0.05M sodium acetate buffer pH 5.7 at 4°C, as were the sample applicator wick and both electrodes.

CMC-plate-clearing activity was confined to the 12 slices taken from the area of gel corresponding to the banded section of the zymogram. The yield of CMC-plate-clearing activity for the preliminary concentration and preparative IEF steps combined was 45%, 65% of which was contained in three slices in the centre of the active area.

TNP-CMCase activity did not peak in any slices in particular but instead was distributed across most of the central zone of the gel. The yield of TNP-CMCase for the IEF separation alone was 30 - 45%. However, only 20% of the TNP-CMCase from slice 10 had survived the preceding collodion bag concentration step, the remainder presumably being precipitated out. Thus the overall TNP-CMCase yield for the two steps was just 10%. This lower overall yield for TNP-CMCase compared with CMC-plate-clearing activity suggested that these two activities were associated with different components from slice 10 of the SDS-PAGE prep. gel.

The 10 or so bands of CMC-plate-clearing activity did not possess adequate levels of any other type of cellulolytic activity to allow meaningful comparisons of the ratios of the various activities and to thus determine the extent of dissimilarities between the bands. Rerunning of two of the strongest bands of CMC-plate-clearing activity on a second pH 4.5 - 5.0 gel gave inconclusive silver staining due to insufficient protein.

6.4.10 Preparative narrow-range IEF to further fractionate band 11 from SDS-PAGE of Peak 5

Precipitation of protein during concentration, either by rotary evaporation or ultrafiltration in a collodion bag, had been a recurrent cause of losses at almost every stage in the cellulase purification scheme (Fig. 6.16). Therefore in an attempt to more successfully concentrate proteins of slice 11 from the SDS-PAGE separation of Peak 5, in preparation for IEF, an alternative method was tested which employed concentration onto cellulose. This cellulose was then applied directly to the IEF gel. Details of the sample pretreatment and running conditions for the pH 4.5 - 5.0 IEF gel accompany the zymogram (Fig. 6.33).

The efficiency of the preliminary cellulose adsorption varied for the different types of activity assayed. 40% of the TNP-CMCase

Figure 6.33 An agarose/CMC zymogram from the preparative pH4.5-5.0 IEF separation of washings of slice 11 from the SDS-PAGE of Peak 5.

Running conditions: Current limit = 2mA
Wattage limit = 3W
Voltage limit = 1800V

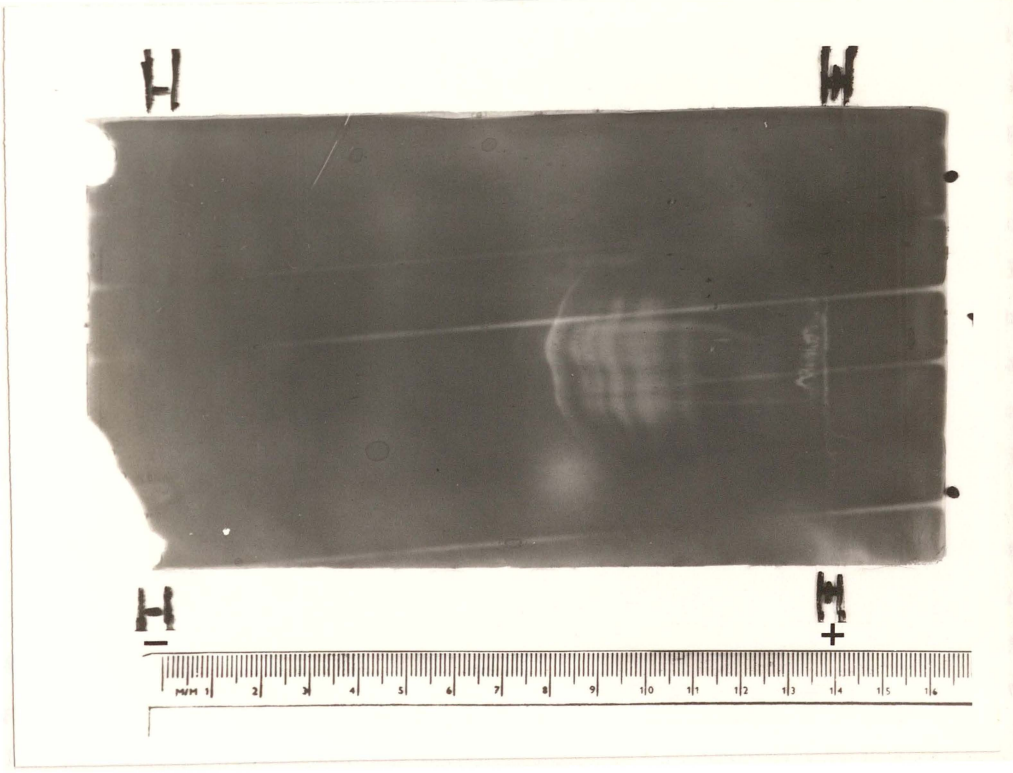
After running for 6 hours, the sample wick bearing the extruded cellulose was removed and then focussing continued for a further 4 hours.

Sample preparation: Slice 11 washings were mixed with 0.2g Sigmacell 50 crystalline cellulose for 3 hours at 4°C. The cellulose slurry was then washed in a 1ml syringe with 2mM sodium citrate buffer pH 5.6 and allowed to run dry before being extruded onto a glass fibre wick positioned 3cm from the cathode and wetted with a mixture comprising 0.4ml saturated urea, 0.05ml glycerol and 0.02ml pH4.5-5.0 Servalyte ampholines (40%w/v).

Original IEF gel dimensions = 15cm x 10cm x 0.3mm.

Anode solution: 0.33%(w/v) L-aspartic acid and 0.37%(w/v) L-glutamic acid.

Cathode solution: 2%(w/v) glycine.



activity of slice 11 washings was retained on the cellulose whereas the CMC-plate-clearing activity was not detectably lowered by passage through the cellulose column. (This clearly indicated that the two types of activity were not associated with the same proteins).

The zymogram of the focussed gel (Fig. 6.33), which revealed around 7 bands of clearance, was used as a template in slicing up the IEF gel.

All the gel slice washings were assayed for TNP-CMCase and CMC-plate-clearing activities. TNP-CMCase was released from only one section of gel, a large unsliced piece from between the cathode and the zone of the zymogram bands. This section released 40% of the TNP-CMCase recovered, with the remainder being found in washings of the applicator strip. The TNP-CMCase yield from this IEF separation was 140%.

CMC-plate-clearing activity was found only in the applicator strip washings and was not detectable in any of the gel slice washings, although the existence of at least 7 distinct bands of this type of activity had been revealed by the CMC clearance on the zymogram (Fig. 6.33). Presumably the levels were simply too low in the gel washings to be detectable. Very low levels of CMC-plate-clearing activity were to be expected since virtually none of this type of activity bound to the cellulose during the initial concentration step.

The question remains as to just how different from one another were the 7 or so distinct bands of CMC-plate-clearing activity obtained from slice 11 (Fig. 6.33). Had there been larger quantities of protein present in each band, it would have been interesting to rerun some of the bands individually on an identical narrow range IEF gel to check for further band splitting. Overall, the technique of concentrating cellulases on a cellulose column, followed by the use of polyacrylamide IEF to remove and separate the enzymes, was moderately successful with respect to TNP-CMCase yield but was clearly inappropriate for purifying CMC-plate-clearing activity, since the latter was hardly bound to the cellulose column. As an additional complication, 40% of the TNP-CMCase recovered did not migrate out of the extruded cellulose during the IEF. This might have been remedied by addition of a larger volume of ampholines to the cellulose, or by placing the cellulose directly onto the gel.

6.5 PARTIAL PURIFICATION OF A ß-GLUCOSIDASE FROM TP8.T6.3.3.1

6.5.1 Introduction

Although study of the ß-glucosidases produced by this organism fell outside the main aims of this thesis, it seemed appropriate to at least separate a ß-glucosidase from all cellulolytic activities in order that its possible enhancement of avicelase activity could be assessed without any uncertainty due to possible synergistic involvement of contaminating cellulases. The sequence of steps employed to achieve a cellulase-free ß-glucosidase is displayed in Fig. 6.34.

We had previously identified three ß-glucosidases in a small scale preparative SDS-PAGE separation of crude concentrated supernatant (Fig. 6.15), with molecular weights of about 25,000, 60,000 and 120,000 indicating a possible monomer, dimer, tetramer interrelationship. However, since a large component of ß-glucosidase activity was cell-associated (Table 4.3), it was likely that culture age and the extent of cell lysis would have a marked influence on the number of different ß-glucosidases present in the culture supernatant.

6.5.2 Concentration by ammonium sulphate precipitation

ß-glucosidases were not expected to bind to the crystalline cellulose which was added to harvest the cellulases from the supernatant of the 60-hour-old 600 litre culture of TP8.T6.3.3.1 (Section 6.4.2). Therefore proteins remaining in a 150 litre sample of the supernatant after the cellulose adsorption step and expected to contain ß-glucosidases were precipitated out with ammonium sulphate (80% of saturation) and collected by centrifugation.

6.5.3 Redissolving and dialysis

A portion of the precipitated protein was removed from -20°C storage after 5 months, thawed, redissolved, filtered and dialysed against tap water until its conductivity had fallen to about 600µS.

6.5.4 Ion exchange chromatography on DEAE-cellulose

The dialysis residue was loaded onto a DEAE-cellulose column (microcrystalline, Sigma). This particular anion exchanger was chosen because we had previously found exceptional difficulty in recovering bound cellulases from it whereas ß-glucosidase activity had been more readily released (Section 6.3.3).

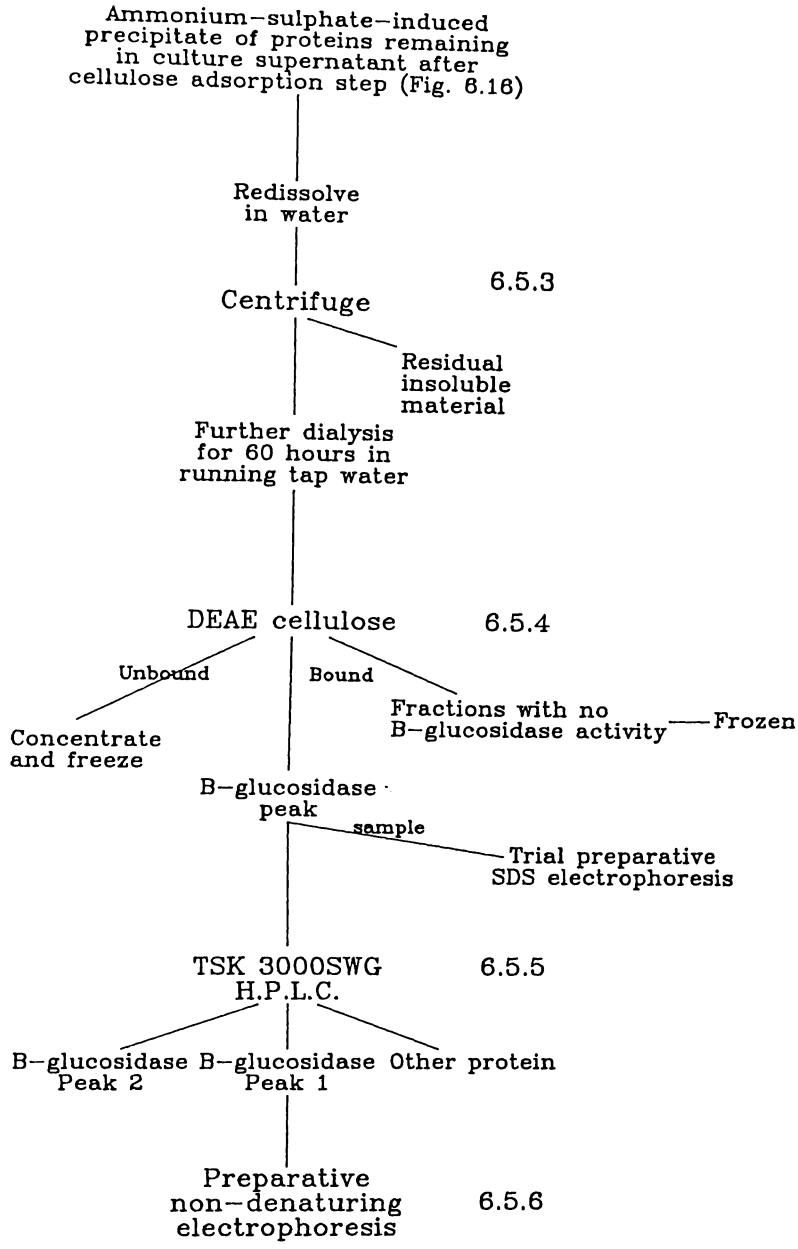


Fig. 6.34 Partial purification of B-glucosidases from the 600-litre culture of TP8.T6.3.3.1

Elution of the bound ß-glucosidases occurred during a sodium chloride gradient, with a 140% yield of activity being contained in two incompletely separated peaks that were released before the majority of the protein and CMC-plate clearing activity (Fig. 6.35).

6.5.5 HPLC gel permeation chromatography

The ß-glucosidase fractions from the DEAE-cellulose column were concentrated by collodion bag ultrafiltration and subjected to HPLC gel permeation chromatography. The ß-glucosidase activity emerged as two overlapping peaks, separated from most of the CMC-plate-clearing activity and total protein (Fig. 6.36). The yield of ß-glucosidase from the column was 130%.

6.5.5.1 Analytical electrophoresis of the ß-glucosidase peaks from the HPLC separation The nondenaturing PAGE System of Patchett (Section 2.9.2), which incorporates p-nitrophenol glucopyranoside in the gel, was used to study the two peaks of ß-glucosidase activity resulting from the HPLC separation of Fig. 6.36. The positions of ß-glucosidase bands, simply revealed by incubating the gel at 75°C for 10 minutes, were marked and then the gel was silver-stained (Fig. 6.37).

Two distinct ß-glucosidase bands, with Rfs 0.26 and 0.39, were produced by the material which had been applied to the HPLC column (Fig. 6.37, Lane 1). These two ß-glucosidases were separated by the HPLC column, with the earlier-eluting, more active HPLC peak (Lane 4, Fig. 6.37) containing only the slower-migrating band of activity while the minor ß-glucosidase peak contained just the faster-migrating band of activity (Lanes 2 and 3, Fig. 6.37). Other proteins apart from the ß-glucosidase were revealed in each HPLC peak when the gel was silver stained (Fig. 6.37).

6.5.6 Preparative non-denaturing electrophoresis

A preparative electrophoretic separation was performed on the combined fractions 34 and 35 from the HPLC fractionation, after concentrating them to 0.9 ml in a collodion bag. The same non-denaturing system was employed as in Section 6.5.5.1. A single ß-glucosidase-containing band was obtained, and, when washed out into 0.1M sodium citrate buffer pH6.0, accounted for 60% of the ß-glucosidase activity which had been applied to the gel. It released no detectable CMCase, TNP-CMCase and avicelase, nor any CMC-plate-clearing activity or activity towards cellulose azure.

Figure 6.35 Ion exchange chromatography on DEAE-cellulose of the ammonium sulphate-precipitated portion of the TP8.T6.3.3.1 cell-free supernatant proteins remaining after the cellulose adsorption step.

+ = CMC-plate-clearing activity

○ = ß-glucosidase activity

✱ = total protein

•——• = NaCl concn.

Material loaded: 900ml solution of protein redissolved from the ammonium sulphate precipitate, dialysed against tap water, clarified by centrifugation and filtration and adjusted to pH 7.6 by the addition of 5ml of 0.1M Tris-HCl buffer.

Fctn.	Eluting Conditions.
1-11	5mM Tris/HCl buffer pH7.6
12-65	NaCl gradient (0M to 1M), still in 5mM Tris/HCl buffer pH7.6.
66-73	1M NaCl in 5mM Tris/HCl buffer pH7.6.
74-105	2M NaCl in 5mM Tris/HCl buffer pH7.6 (flow stopped during collection of Fctn. 100).
106-120	0.1%(v/v) Triton X-100 in 5mM Tris/HCl buffer pH7.6.

Flow rate throughout loading and elution = $100ml.h^{-1}$.

Fraction volume = 3ml for 1-75, 6ml for 76-105.
Column dimensions = 1.6 x 10cm.

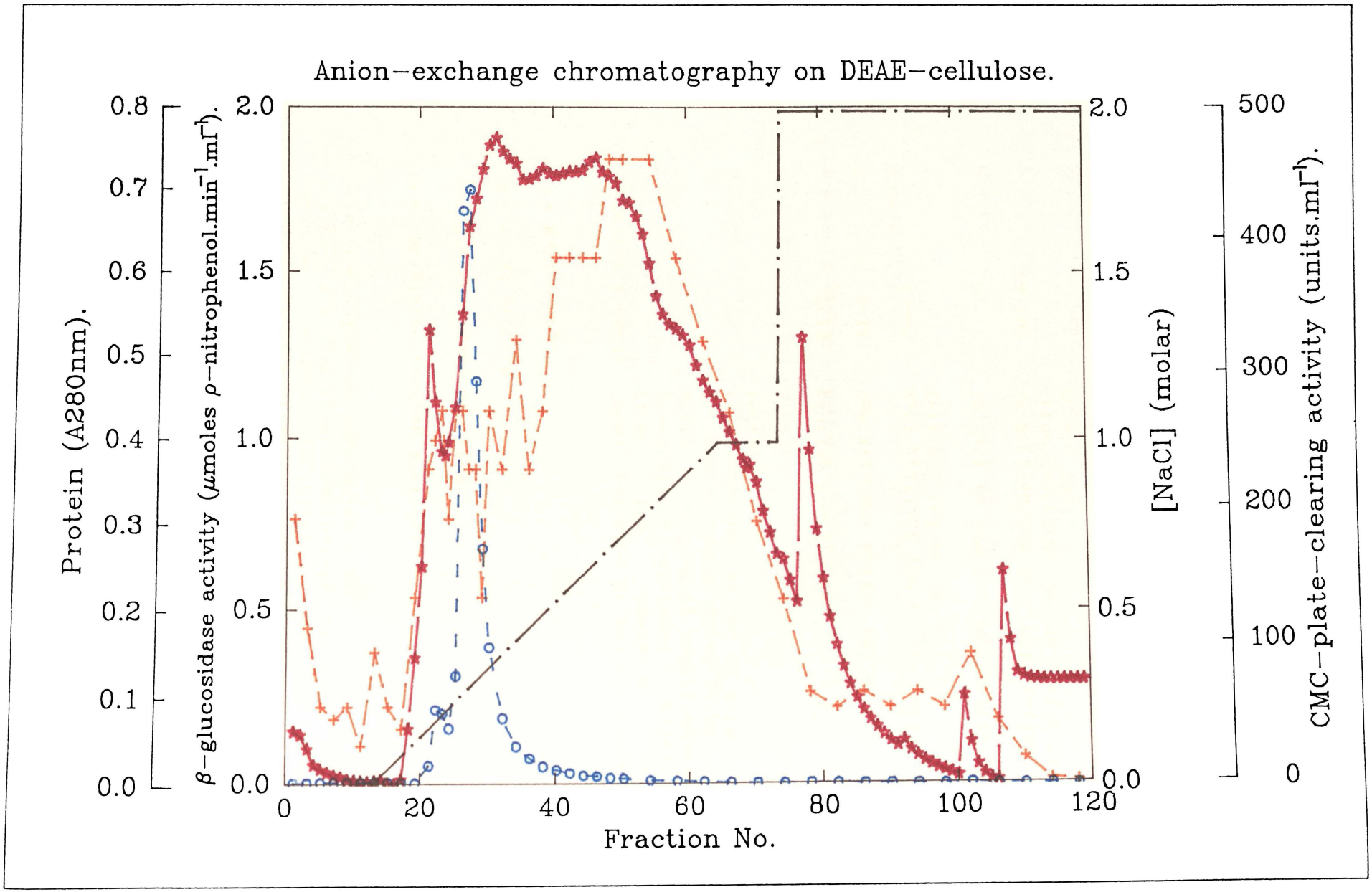

Anion–exchange chromatography on DEAE–cellulose.
Protein (A280nm).
β–glucosidase activity (μmoles ρ–nitrophenol.min⁻¹.ml⁻¹).
[NaCl] (molar)
CMC–plate–clearing activity (units.ml⁻¹).
Fraction No.
0.0
0.1
0.2
0.3
0.4
0.5
0.6
0.7
0.8
0.0
0.5
1.0
1.5
2.0
0.0
0.5
1.0
1.5
2.0
0
100
200
300
400
500
0
20
40
60
80
100
120

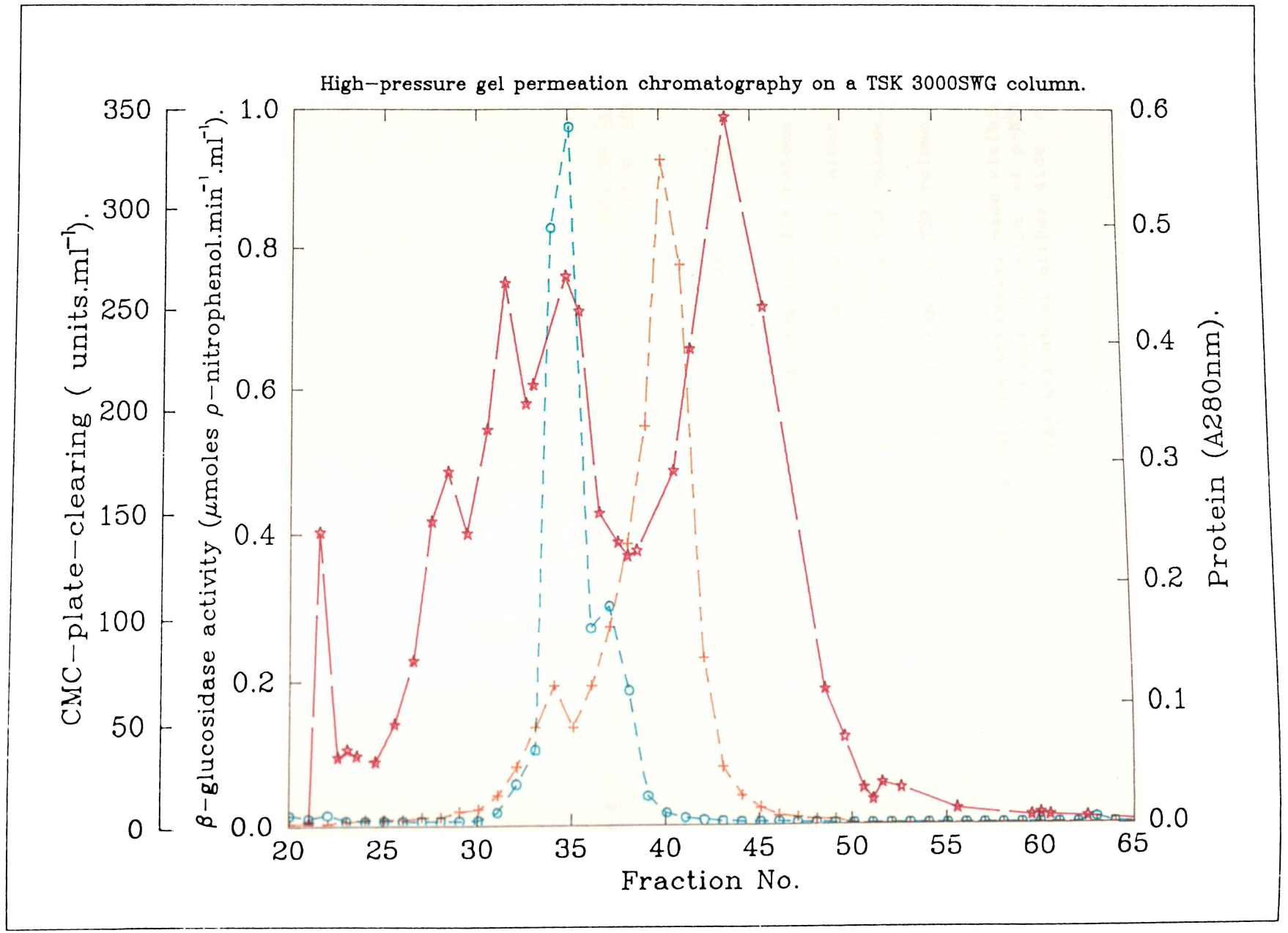
Figure 6.36 High pressure gel permeation chromatography on a TSK-3000SWG column of ß-glucosidase-containing fractions from the DEAE-cellulose column of Fig.6.35.

+ = CMC-plate-clearing activity

○ = ß-glucosidase activity

✡ = total protein (A_{280nm})

Sample applied:	1.4ml concentrate of fractions 25-34 of the DEAE-cellulose column of Fig.6.35.
Eluent:	50mM sodium phosphate buffer pH7.6.
Flow rate:	4ml.min^{-1}.
Fraction volume:	4ml
Column dimensions:	2.15cm i.d. x 60cm.


High-pressure gel permeation chromatography on a TSK 3000SWG column.
CMC-plate-clearing (units.ml-1).
β-glucosidase activity (μmoles ρ-nitrophenol.min-1.ml-1).
Protein (A280nm).
Fraction No.
0
50
100
150
200
250
300
350
0.0
0.2
0.4
0.6
0.8
1.0
0.0
0.1
0.2
0.3
0.4
0.5
0.6
20
25
30
35
40
45
50
55
60
65

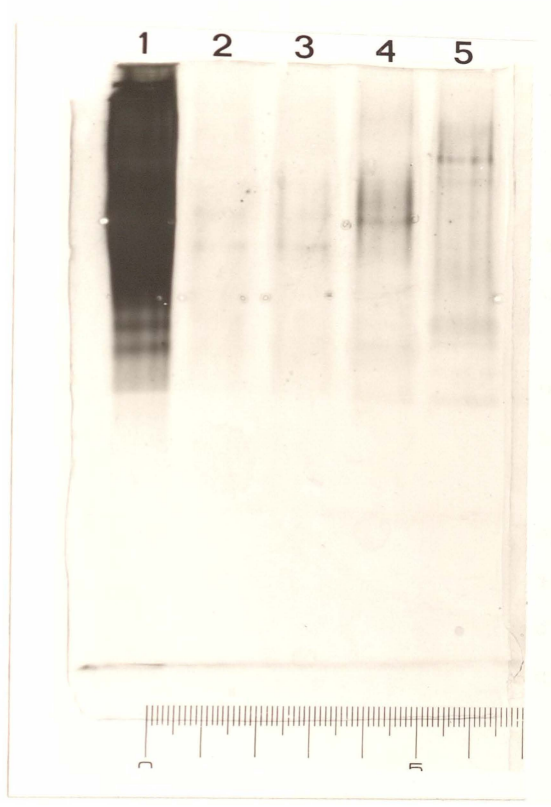


Figure 6.37 Non-denaturing electrophoresis of the proteins from the two peaks of ß-glucosidase activity from the HPLC separation of Fig. 6.36.

Lane 1 5µl of the concentrate applied to the TSK column.

Lane 2 40µl of Fctn. 38 from the TSK column.

Lane 3 40µl of Fctn. 37 from the TSK column.

Lane 4 40µl of Fctn. 35 from the TSK column.

Lane 5 40µl of Fctn. 33 from the TSK column.

Proteins with ß-glucosidase activity, made visible prior to silver staining by their release of p-NPG, were marked by puncturing the gel at at either side of each band.

6.5.6.1 Analytical IEF of the ß-glucosidase from the preparative electrophoresis IEF of the washings of the ß-glucosidase band (Fig. 6.38 Lanes 2 and 3) showed a large number of bands of protein. The dominant band was very much less obvious in the IEF patterns produced by washings of the slices cut from immediately above and below the active slice on the preparative gel (Lanes 4 and 5, Fig. 6.38) suggesting that this particular band might have been associated with the ß-glucosidase activity.

The Serva Protein Test Mixture 9 pI markers (Lanes 1 and 8) indicated a pI of 4.4 - 4.6 for the dominant band in the ß-glucosidase-containing material (Lanes 2 and 3).

The aim of obtaining a ß-glucosidase which was free of cellulolytic activity had been achieved, so no further purification of it was attempted.

6.5.7 Unsuccessful approaches to ß-glucosidase purification

6.5.7.1 SDS-PAGE A small-scale trial showed the SDS-PAGE system of Laemmli (1970) to be inappropriate for preparative use in ß-glucosidase purification since only a 3% yield of activity resulted, even without any pretreatment of the sample. We subsequently found that the ß-glucosidase activity was irreversibly inhibited by the electrode buffer solution used in this system.

6.5.7.2 Attempted adsorption of contaminating cellulases onto cellulose or CMC A CMC/agarose activity zymogram produced from the SDS-PAGE gel revealed that at least 10 enzymes with CMC-plate-clearing activity were present in the ß-glucosidase-containing fractions following the DEAE-cellulose step. Attempts to adsorb these extraneous activities onto crystalline cellulose or coarse insoluble CMC were unsuccessful. Note however that these particular cellulases had already been grouped together on the basis of their failure to bind to crystalline cellulose during the initial harvesting step (Section 6.4.2). This original cellulose-binding step was certainly of great assistance in separating the majority of the cellulases from the ß-glucosidase.

Figure 6.38 I.E.F. of proteins recovered from the preparative electrophoresis of fractions 34 and 35 from the TSK column of Fig. 6.36.

Lane 1 25µg Serva Protein Test Mixture 9.

Lane 2 25µl of the washings of the slice cut from the centre of the zone of ß-glucosidase activity.

Lane 3 5µl of the washings of the slice cut from the centre of the zone of ß-glucosidase activity.

Lane 4 25µl of the washings of the slice cut from immediately above the zone of ß-glucosidase activity.

Lane 5 25µl of the washings of the slice cut from immediately below the zone of ß-glucosidase activity.

Lane 6 5µl of the material applied to the preparative PAGE (i.e. combined concentrate of fractions 34 and 35 from the TSK gel.)

Lane 7 1µl of the material applied to the preparative PAGE (i.e. combined concentrate of fractions 34 and 35 from the TSK gel.)

Lane 8 25µl of Serva Protein Test Mixture 9.

6.5.8 Km for cellobiose

All assays of the activity of this ß-glucosidase up to this point had been performed using p-NPG. Cellobiose was confirmed as a substrate however, and a Km for cellobiose of 8.5mM was obtained from a Lineweaver-Burk plot (Fig. 6.39).

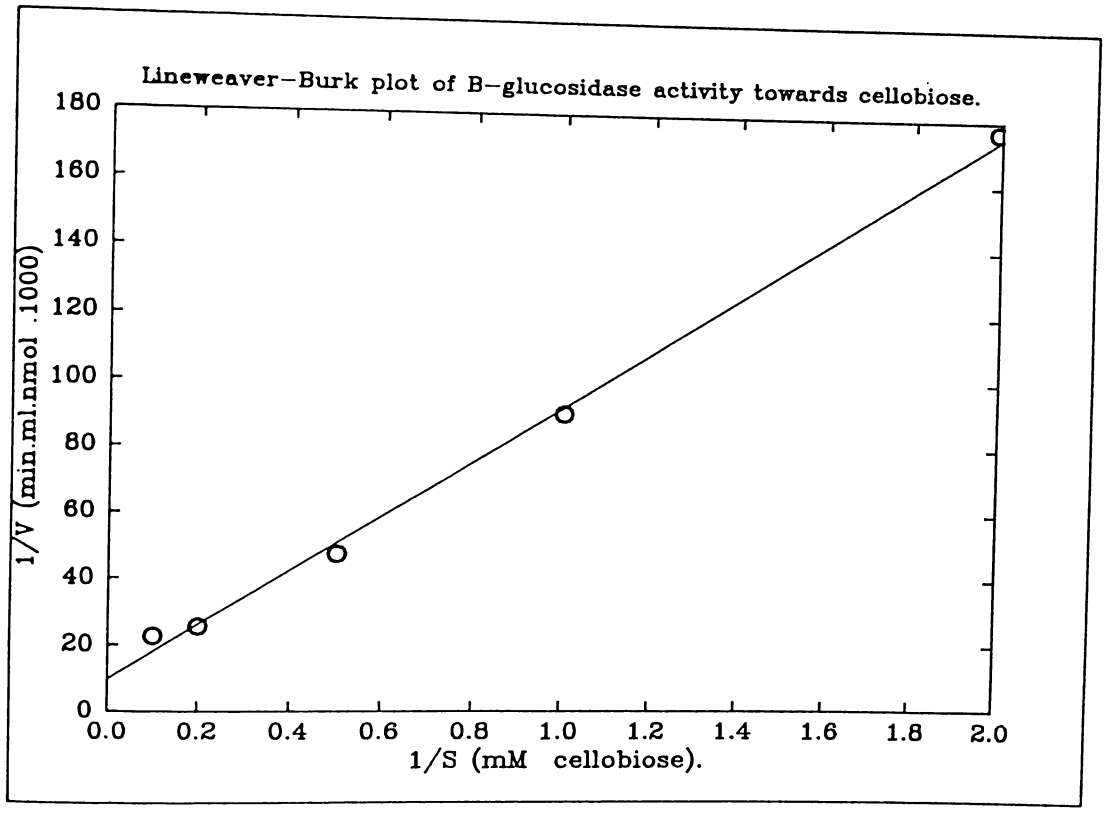


Figure 6.39 Lineweaver-Burk plot of B-glucosidase activity towards cellobiose.

Incubation was for 41 min at 75°C in 0.25M sodium acetate buffer pH5.7. Glucose was measured by the GOD-Perid. method.

6.6 SYNERGISMS BETWEEN COMPONENTS OF THE TP8.T6.3.3.1 CELLULASE COMPLEX

Yields of avicelase activity from most purification steps were lower than those of other types of cellulolytic activity. This suggested that avicelase activity by the complete cellulase complex might have involved synergistic action by two or more components. In order to test this theory we looked for evidence of any synergisms between the three peaks resulting from fractionation of crude concentrated culture supernatant on a column of crystalline cellulose (Fig. 6.2). Peak I shall be referred to as "the ß-glucosidase peak", Peak II as "the endoglucanase peak" and Peak III as "the exoglucanase peak" on the basis of the data of Table 6.3, as discussed in Section 6. 3, and despite the fact that activity from none of the peaks was

confined strictly to any one of these classes.

Samples from each of the three peaks were diluted with water as shown on Table 6.9 to give similar concentrations of avicelase activity. These dilutions were assayed for avicelase activity separately and in various combinations, as shown in Table 6.9. Each resulting supernatant was assayed in triplicate for total soluble carbohydrates by the phenol/sulphuric acid method, for reducing sugars by the DNSA and PABAH methods, and for glucose by the glucose oxidase/peroxidase method. Corrections were made allowing for the preincubation levels of these various parameters and for slight increases in these levels which resulted from incubating buffer blanks with cellulose.

All combinations tested exhibited higher avicelase activities by all the methods of measurement than were to be predicted from the means of individual component activities. In cases where unequal volumes were mixed, predicted values were weighted means. The factor given in brackets beside the measured avicelase activity for each combination in Table 6.9 is the factor by which the expected (i.e. mean) activity must be multiplied to obtain the measured value. Thus a value of 1 would have indicated the absence of synergism.

Greatest synergism was displayed by the combinations involving both the ß-glucosidase and the exoglucanase peaks. The magnitude of the apparent synergism depended on the detection method employed, with the value of ca. 2.5-fold obtained by the phenol-sulphuric method being the most reliable (for reasons discussed below). When the exoglucanase was acting alone, glucose was only a minor product (Table 6.9). However, in combination with the ß-glucosidase, glucose compromised 77 - 83% of the products on a molar basis. Probably the synergism between these components was due to an alleviation of end-product inhibition which was occurring when the exoglucanase operated alone, rather than to an alteration of the mechanism of cellulose hydrolysis.

The most reliable indication of the degree of synergistic increase of cellulose hydrolysis was the avicelase assay based on total soluble carbohydrate measurement. All of the other assays had the potential to indicate false synergisms which could merely have amounted to cleavage of cellodextrins or cellobiose to smaller, more numerous reducing sugars, and thus to produce higher assay responses, without any increase in the rate of cellulose solubilisation having occurred. The reducing sugar and glucose oxidase assays had a value, nonetheless, in revealing something about the composition of the hydrolysis products. Normally

Table 6.9 Avicelase activity of three peaks from the cellulose column separation of Fig. 6.2, measured both separately and after recombination.

Enzymes incubated	Avicelase activity (nmoles.min^{-1}.ml^{-1}) By phenol-sulphuric	By DNSA	By PABAH	By glucose-oxidase	Glucose produced reducing sugars (PABAH)
0.6ml ß-glucosidase	4.1	10.7	8.8	7.4	0.84
0.6ml endoglucanase	9.9	8.1	4.1	0.84	0.21
0.6ml exoglucanase	9.3	9.7	6.4	1.9	0.29
0.4ml ß-glucosidase + 0.2ml endoglucanase	10.1 (x1.7)*	13.5 (x1.4)	12.5 (x1.7)	10.6 (x2.0)	0.85
0.2ml ß-glucosidase + 0.4ml exoglucanase	20.8 (x2.7)	25.0 (x2.5)	20.7 (x2.9)	15.9 (x4.3)	0.77
0.2ml endoglucanase + 0.4ml exoglucanase	14.0 (x1.5)	12.6 (x1.4)	7.9 (x1.4)	1.9 (x1.2)	0.24
0.2ml ß-glucosidase + 0.2ml endoglucanase + 0.2ml exoglucanase	18.6 (x2.4)	21.6 (x2.3)	18.0 (x2.8)	14.9 (x4.4)	0.83

* Brackets contain the factor by which the predicted level is multiplied to obtain the observed level of activity. Each value is the mean of triplicate assays.

Peak fractions 6, 16 and 28 of the separation shown in Fig. 6.2 were diluted as follows:
"ß-glucosidase" = 1 part Fraction 6 + 1 part water; "endoglucanase" = 1 part Fraction 16 + 1 part water;
"exoglucanase" = 1 part Fraction 28 + 34 parts water.
All assays involved incubation with 1% Sigmacell crystalline cellulose for 350min at 75°C in 0.05M sodium acetate buffer, pH 5.6.

the glucose oxidase assay would be expected to give the lowest value for avicelase activity, since only glucose is detected. The PABAH assay should respond to glucose plus exposed reducing end-groups of cellobiose and cellodextrins, whereas DNSA gives a somewhat greater assay by partially splitting cellobiose and probably some cellodextrins (Section 3.5.1.2). The greatest apparent avicelase activity would be expected to result from a measurement of total soluble carbohydrates which pays no regard to their form.

In fact, the above predictions did not hold for Peaks I and III in the synergism experiment. The explanation highlights another danger in the use of reducing sugar measurements to assess avicelase activities. The total soluble carbohydrate assay registered significantly higher background levels in Peaks I and III than did the other assay methods. Presumably this was due to soluble cellodextrins which contained few exposed reducing ends. These would have been introduced into the assay incubations as readily hydrolysable alternative substrates to the cellulose, and yet would have gone largely undetected by the reducing sugar assays. However, during incubation at 75°C, these cellodextrins would have been hydrolysed, releasing reducing sugars indistinguishable from those arising from hydrolysis of the cellulose supplied as substrate for the assay. Thus unless a further set of controls consisting of enzyme incubated without added cellulose substrate is included, only the total soluble carbohydrate assay allows complete compensation to be made for these pre-existing soluble cellodextrins.

Synergism evident between the endoglucanase and the exoglucanase peaks was not as great as between the ß-glucosidase and the exoglucanase but was clearly not attributable to any alleviation of inhibition by cellobiose since the ratio of glucose to total reducing sugars remained as it was in hydrolysates produced by the endo- and exoglucanase peaks individually.

The degree of synergism which occurred when all three peaks were recombined was not quite as great as that resulting from combining only the ß-glucosidase and the exoglucanase. This may have been due to the different proportions in which the latter two activities were mixed in the two combinations however, and doesn't necessarily mean that the endoglucanase peak was redundant in the presence of the other two peaks. The fact that the endoglucanase peak showed definite synergism in combination with each of the other peaks individually indicates that indeed it did make some unique contribution to the hydrolysis of

crystalline cellulose.

CHAPTER SEVEN

DISCUSSION

When we began this project we had no definite evidence for the existence of extremely thermophilic cellulolytic bacteria. Clearly a niche was available for such organisms in the many natural thermal pools, which were found to contain an abundance of vegetative debris, lolly papers, ice-cream wrappers and the occasional sock. Such material often appeared to be decaying despite water temperatures in excess of 75°C.

Three out of the four thermal areas sampled in fact proved to be rich sources of cellulolytic bacteria capable of growth at 75°C, particularly when samples of partially decayed lignocellulosics were taken as inocula for enrichment cultures. Only one site gave rise to cellulase production in an aerobically grown culture. The organism responsible failed to grow in a subculture. This left us with a group of anaerobes which were capable of growth at 75°C but not at 80°C and which released varying amounts of extracellular cellulase, plus a less thermophilic enrichment culture (3WUB) which would grow well at 65°C but not at 70°C or above. This feature of the 3WUB enrichment, combined with a relatively high CMCase to avicelase activity ratio, suggested the presence of *Clostridium stercorarium* (Madden,1983). *C. stercorarium* was not available for direct comparison, but, according to Madden (1983), it has an optimum growth temperature of 65°C and growth at 75°C was not reported. We found *C. thermocellum* (ATCC 274050) would grow at 60°C but not well at 65°C and not at all at 70°C, which is in agreement with McBee (1954). Thus our organisms, which grew almost as rapidly at 75°C as at 70°C, appeared to be more thermophilic than *C. stercorarium* or *C. thermocellum*.

C. thermocellum released higher levels of extracellular cellulase activity into the growth medium than did the best of our cultures (3 to 5 times more CMCase (Fig 4.1) and 5 to 8 times more avicelase (Table 4.6)).

The production of terminal spores, which has been reported for both *Clostridium* species, was not observed amongst any of our isolates when grown on both solid and liquid media, although we were able to detect such spores in *C. thermocellum*.

The negative Gram stain reaction and a preference for a near-neutral medium pH clearly distinguished our bacteria from the thermophilic acidophilic cellulolytic *Bacillus* sp. reported by Uchino and Nakane, (1981).

It is possible that bacteria similar to ours might have been present in cellulolytic cultures from Icelandic hot springs, briefly reported by Ljungdahl *et al.*, (in 1981, after our work had begun) as being able to ferment cellulose in environments ranging from pH 2 to 8.6 and at temperatures as high as 84°C. No report of the isolation and characterisation of these organisms has yet been published.

Our isolates differed from one another in several ways. There was considerable variation in their capacities to solubilize crystalline cellulose. The strains which visibly consumed crystalline cellulose and accumulated reducing sugars in their culture media (TP8, TP10 and RT8 strains) were also the only strains for which significant levels of avicelase activity were detected in the cell-free supernatants. Other strains released CMCase but no avicelase activity into the growth medium, and grew relatively poorly unless cellobiose was supplied.

Volatile end product analysis also served to divide the isolates into three broad groups, based on the ratios of ethanol to acetate. Only the 3WUB mixed culture resembled *C. thermocellum* in this respect, although only when grown on cellobiose, which suggested that the fermenting potential was being limited by the very slow rate of cellulose hydrolysis in 3WUB. All other isolates or mixed cultures produced lower ratios of ethanol to acetate than did *C. thermocellum*. The effects on this ratio of cellobiose as a medium supplement served to further distinguish between the isolates, as did their production of other unidentified additional end-products.

Cellobiose (0.2%w/v) repressed CMCase production by cultures of most of our isolates by about 50%. Exceptions were TOK8.1 andLC4 which exhibited increased CMCase activity, WAI21 which was unaffected and the 3WUB mixed culture which showed a 90% reduction. *C. thermocellum* CMCase levels were not significantly affected by the cellobiose supplement, which is in conflict with the repression observed by Lee and Blackburn (1975) and Ng *et al.* (1977) but is supported by Enebo (1954), Shinmyo

et al. (1979) and Lamed and Zeikus (1980).

The disagreement in the literature over the effects of cellobiose on *C. thermocellum* may reflect strain differences or could be due to variations in other medium components such as yeast extract which might be required for the synthesis of certain enzymes involved in cellobiose metabolism. The extent to which cell density is affected should also be considered, as well as the effects at the individual cell level, before any effect on enzyme production by a culture as a whole can be labelled as either induction or repression. In the case of *Trichoderma*, cellobiose has been shown to cause both induction and repression of cellulases, depending on the cellobiose concentration (Reese *et al.*, 1952, Gilligan and Reese, 1954). This concentration is altered during the course of culture growth. When these complications are coupled with the facts that a multi-enzyme complex is involved and that most of the groups used different cellulase assay techniques, it is not surprising that the effects of cellobiose on the *C. thermocellum* cellulase system are still disputed.

Thermal stabilities of the CMCases of the isolates also revealed clear differences between them. CMCases from TOK8.1 and WAI21.3 were exceptionally stable, with half-lives at 85°C in excess of 10 hours. Skinner and Tokuyama (1978) found that inclusion of filter paper during the preincubation of *Thielavia terrestris* cellulase at 100°C had a marked stabilizing effect. We didn't test this for our organisms, but even without this probable aid to stability, it was clear that several of the isolates produced CMCases which were more thermostable than an endoglucanase purified from *C. stercorarium* (reported to have a half-life of 1 hour at 85°C by Creuzet and Frixon (1983)), which appears to be the most thermostable of bacterial thermophilic cellulases reported in the literature. *C. thermocellum* CMCase was markedly less stable than any of the CMCases of our isolates. We found it to have a half-life at 85°C of less than 5 minutes. The filter-paper activity of *T. terrestris* was reported to have a half-life of two hours at 100°C (Skinner and Tokuyama, 1978), which makes it the most stable cellulase system so far reported. Note however that this was only the half-life of a residual 50% of the original activity. The other 50% of the activity was much less stable at 100°C.

Brief investigation of the effects of pH and buffer composition on CMCase thermostability showed their influence to be substantial and variable amongst the different isolates tested. Clearly these factors

merit inclusion in any description of the thermostabilities of the CMCases of these organisms.

The pH optima for CMCase, avicelase and ß-glucosidase activities of the unfractionated TP8T culture supernatant were all in the range 4.5-5.7. pH optima for individual component enzymes contributing to each of these activities were not investigated, but some could conceivably lie outside the above range.

Morphologically, most of our isolates showed little variation, with the exception of WAI21 which grew as longer, thinner cells, with one end characteristically hooked. The other isolates were of similar dimensions to *C. thermocellum* (ca. 0.6x4µm), all were Gram negative and grew singly or in short chains of up to 4 cells long. TP8.T6.3.3.1 was pleomorphic, becoming thinner and much more elongated when temperatures rose slightly above 75°C. A similar response has been noted for *Acetivibrio cellulolyticus* (Patel *et al.*, 1980).

All of our isolates were anaerobes but at least one strain (TP8.T6.3.3.1) was oxygen tolerant and capable of "mopping up" low levels of oxygen in the growth medium in the absence of any reductant. This may prove to be a very useful attribute, simplifying handling and media preparation. *C. thermocellum* is known to be a strict anaerobe and will not grow in the absence of a reductant (Enebo, 1951).

It appears that our cellulolytic isolates are sufficiently different from any of the characterised cellulolytic thermophilic anaerobes to demand that several new species or possibly a new genus be created. Further characterisation of the growth requirements and morphological features will first be necessary however.

Preliminary characterisation of the factors influencing growth and cellulase production of the TP8T isolates was restricted to a brief study of factors which could be of assistance in subsequent cellulase production and purification. Growth and cellulase production on a defined medium, completely lacking yeast extract and trypticase peptone, was impractically slow. Subsequently Dr. Paul Reynolds (per. comm.) has found that glutamate serves as an effective substitute for yeast extract and trypticase peptone in allowing growth of TP8.T6.3.3.1.

None of the soluble carbon sources tested proved to be useful alternatives to crystalline cellulose for the production of avicelase activity by TP8.T6.3.3.1. Soluble CMC also produced very poor growth, whereas growth on glucose, cellobiose and sucrose was more rapid than on crystalline cellulose. Neither *C. thermocellum* nor *C. stercorarium* are

capable of fermenting sucrose (McBee,1954, Madden,1983).

Mild agitation during growth, just sufficient to maintain the cellulose in suspension, was clearly stimulatory to growth and hastened cellulose degradation. The reverse was found for *C. thermocellum* by Ng *et al.* (1977) and Zertuche and Zall (1982), although Bayer *et al.* (1983) also found that shaking improved growth and cellulase production. It is possible that the effects of agitation are due, at least in part, to an improved distribution of the gas phase through the medium. This may be particularly important at high temperatures due to reduced solubilities as we suspect that CO_2 stimulated growth of TP8.T6.3.3.1. This has been demonstrated for *C. thermocellum* (Enebo, 1951, Zertuche and Zall, 1982). Oxygen, on the other hand, is clearly inhibitory to the growth of both organisms. Hence differences in the relative levels of CO_2 and O_2 in the head spaces could explain the conflicting effects of agitation observed. Alternatively, certain agitation methods may have produced greater denaturation by mechanical forces such as surface tension and shear forces created by flowing liquids (Halliwell, 1961, Reese and Ryu, 1980).

A concentrate of the cell-free supernatant of TP8.T6.3.3.1 was found to be capable of producing reducing sugars from finely ground *Pinus radiata, Eucalyptus* and protein-extracted lucerne. Solubilisation of the substrates was incomplete, with the levels of reducing sugars produced accounting for only 30-40% of the cellulose content in the case of the lucerne and substantially less for the more lignified materials (Table 4.11). However a similar degree of solubilisation (ca. 30%) resulted when pure crystalline cellulose was the substrate of the cellulase concentrate, so factors other than non-cellulosic components were probably limiting hydrolysis of the cellulose in the natural substrates also (e.g. inhibition by end-product accumulation). The decreased rate of hydrolysis of all the substrates, including crystalline cellulose, resulting from the second addition of the enzyme was probably due to the increased crystallinity of the residual material. The substrates were thoroughly washed between enzyme additions. No further enzyme additions were made, so the limits of the hydrolyses possible for each substrate using the cell-free cellulase were not discovered.

Whole cultures (i.e. including growing cells) of TP8.T6.3.3.1 were capable of completely solubilizing crystalline cellulose or sulphite-bleached *P. radiata* wood pulp, supplied at a 0.2%(w/v) level.

Complete solubilisation took about 2 weeks, or less if the culture was agitated. A cell-bound factor may therefore be vital for the complete solubilisation of crystalline cellulose.

Concentration of our cellulases without incurring major activity losses was a problem which had to be addressed before purification could begin. Ammonium sulphate precipitation gave excellent yields of avicelase activity, provided reasonably pure ammonium sulphate (lab. grade or better) was used. However, this was prohibitively expensive for concentrating cellulases from the large volumes of culture which we were necessitated by the low avicelase activity in the culture supernatants (about 300 times less than the activity quoted by Ng and Zeikus (1981a) for *Trichoderma* cultures).

Ultrafiltration gave disappointing yields of avicelase activity on most occasions (ca. 30%), despite the use of polysulfone rather than cellulose-based membranes. Other types of cellulolytic activity and also ß-glucosidase appeared to be much more amenable to ultrafiltration.

As an alternative method of concentration, adsorption to crystalline cellulose had many merits. The majority of the avicelase plus a smaller component of the CMCase activity became readily bound while other proteins, including the ß-glucosidase, did not bind. Subsequent elution facilitated by heating and washing with distilled water was practical on a small scale, and offered excellent yields of each type of activity plus a useful degree of separation.

However major problems were encountered in eluting the enzymes from cellulose when the system was scaled up to involve more cellulose and a higher loading of enzyme per gram of cellulose. The problems probably stemmed from the partial hydrolysis of the cellulose, resulting in a decreased particle size and consequent extremely poor flow properties. A solution to this problem may have been to perform the initial binding on a column of pharmaceutical gauze (Toyama, 1969) with much better flow properties than powdered cellulose, or possibly the use of other eluents may have improved recovery. Ng *et al.* (1977) employed 40% glycerol in 0.1M ammonium acetate buffer, pH 5.0 or 0.1M Tris-HCl pH 9.0, while Langsford *et al.* (1984) found 8M guanidine HCl in 10mM Tris/HCl pH 7.0 buffer to be appropriate for recovering bacterial cellulases from cellulose. Lamed *et al.* (1983) employed 1% triethylamine, and neutralized the eluent immediately. Reese (1982) tested a wide range of methods for eluting *Trichoderma* cellulases from cellulose and finally favoured 6M urea or 4M guanidine. Alternatively, relatively low salt

concentrations at a pH of 10 or more, brought about elution of cellulase but prolonged exposure caused denaturation of the enzymes.

Cross-linked cellulose has been used successfully for the binding of cellulases and their subsequent fractionation by sequential elution (Weber *et al.*, 1980, 1983). The cross-linking was reported to prevent hydrolysis of the cellulose by the bound cellulases. However we found that cross-linking also prevented the initial binding of the bulk of our avicelase and CMCase activities.

Bacterial cellulases are notoriously difficult to fractionate and purify. Wood *et al.* (1982) found that components of the *Ruminococcus albus* cellulase complex had an unpredictable tendency to aggregate and disaggregate. Ljungdahl *et al.*, (1983) encountered a glue-like polymer when they attempted to concentrate endoglucanases from *C. thermocellum* which had not bound to the substrate cellulose. This effectively prevented the use of column chromatography, which deterred them from attempting to purify the component enzymes. The relatively low cellulase activity in bacterial broth (about 100 times less per volume of *C. thermocellum* broth than for *Trichoderma* (Ng and Zeikus, 1981a)) has been a serious problem plaguing the study of extracellular bacterial cellulases in general. In a comprehensive review of research into thermophilic clostridial cellulases, Duong *et al.*, 1983 conclude that purification and characterization of *C. thermocellum* cellulase components has been hindered by adherence of enzymes to cellulose, by a tendency of the proteins to form aggregates, and by very low recovery of the components (especially the avicelase activity) during purification. Lee and Blackburn (1975) recovered approximately 20% of the endoglucanase activity by initial ammonium sulphate precipitation, but further attempts to purify this concentrate were unsuccessful as massive losses of activity occurred during column chromatography. Separation on an electro-focussing column was more successful, with the endoglucanase being separated into four fractions with a total recovery of activity of 128%. The fraction with a pI of 6.8 contained 40% of the crude endo-ß-glucanase activity but the protein content was too low to be measured by the Lowry procedure, so no purification factor could be calculated.

Adsorption of the enzymes to cellulose substrate and large losses of activity during purification due partly to adsorption onto Sephadex and DEAE-cellulose also hindered attempts of Ait *et al.* (1979b) to purify the cellulase components from *C. thermocellum*. They finally

resorted to preparative non-denaturing electrophoresis which allowed separation of a single mobile band accounting for 10% of the activity applied. A significant amount of the cellulase did not migrate and was found at the top of the gel after electrophoresis (a problem which we also encountered using non-denaturing gels). The mobile band contained 10% (w/w) carbohydrate, and eluted as a single peak with an apparent M.W. of about 125,000 after gel filtration on acrylamide Bio-gel P-200. ~~and with an apparent molecular weight of about 125,000.~~ However the purity of this band of activity was doubted since it showed activity towards both CMC and Whatman CC41 cellulose and could be split into at least five bands when subjected to SDS-PAGE. Only one of these 5 fragments retained detectable CMCase activity, but no mention was made of any attempts to renature the proteins after the SDS-PAGE.

The nature of the protein-carbohydrate association in the native complex was not investigated. Ait *et al.* (1979b) suggested that the attached carbohydrate could have simply been tightly bound cellodextrins resulting from the incomplete hydrolysis of the substrate. Alternatively, the enzyme(s) might be true glycoproteins. It would have be interesting to observe the effects of added cellodextrin phosphorylase or some other active degrader of cellodextrins on the molecular weight and carbohydrate content of the complex. If in fact it is the carbohydrate which binds the enzymes together, such a treatment might prove very useful in obtaining their separation without having to resort to the use of denaturing conditions. The added carbohydrase could then be selectively denatured by heating, provided the components of the complex were significantly more heat-stable. Whatever the nature of the carbohydrate-protein binding, they concluded that the *C. thermocellum* CMCase and cellulase are exoenzymes which aggregate in a soluble complex of constant molecular weight.

Lamed *et al* (1983) found a cellulose-binding protein of *C. thermocellum* that behaved as a single unit with an apparent M.W. of 2 million during gel permeation chromatography and analytical ultrafiltration, even when treated with 8M urea. It would not migrate into 5% native acrylamide electrophoresis gels, and appeared under the electron microscope to contain structures of uniform size (ca. 18nm) although irregular in shape which could be seen to be comprised of several different subunits, with a cleft or central channel being visible in some molecules. SDS-PAGE brought about fractionation of the complex into 15 or so polypeptides, of which at least 8 showed

endoglucanase activity. It has been shown that the endoglucanase fraction which does not bind to cellulose produces a markedly different pattern of bands upon SDS-PAGE to that of the water extract of the bound endoglucanase fraction (Ljungdahl *et al.*, 1983), so presumably the total number of endoglucanases produced is well in excess of the eight which Lamed *et al.* extracted from cellulose.

In addition it appears likely that an exoglucanase might be involved, since this is normally the case amongst "complete" extracellular cellulase complexes. No exoglucanase has yet been isolated from *C. thermocellum*. In fact, there is no conclusive evidence for the production of an exoglucanase by *C. thermocellum*. Johnson and Demain (1984) found that avicelase activity but not CMCase activity was inhibited by oxidation or sulphydryl reagents, and speculated that the latter were acting upon an exoglucanase which is vital for avicelase activity only. However, Ljungdahl *et al.* (1983) found that the yellow affinity substance which was involved in binding the cellulase complex to the cellulose was oxygen sensitive. Its deactivation by oxygen could thus explain the oxygen-sensitivity of the avicelase activity without necessarily involving an exoglucanase. This affinity substance, which was not water soluble, may obviate the need for an exoglucanase by improving the efficiency of endoglucanase hydrolysis of crystalline cellulose. Another cellulose binding factor was identified as a high M.W. (ca. 210,000) polypeptide component of the water-soluble cellulose-binding complex (Lamed *et al.*, 1983). This factor exhibited no endoglucanase activity on gel overlays but was not isolated from the electrophoresis gel and so was not tested for exoglucanase activity.

Purification of an endo-ß-1,4 glucanase from *C. thermocellum* by Ng and Zeikus (1981b) involved firstly 3 ion exchange steps, after which the amount of enzyme recovered was so low that further purification on conventional gel-permeation chromatography was not considered feasible. Further purification of the endoglucanase was achieved by preparative gel electrophoresis, with a 50% yield of activity. The purified endoglucanase was found to produce a single band under IEF. SDS-PAGE revealed a single band (apparent M.W. 90,000) on gels containing 0.1% SDS, but two additional minor bands (M.Ws 60,000 - 80,000) were produced if the enzyme was pretreated with 1% SDS at 90°C for 5 min. Analytical ultracentrifugation indicated that only one protein component of about M.W. 85,000 was present. The pI was 6.7, similar to that of the dominant endoglucanase of Lee and Blackburn's IEF separation (1975).

A smaller endoglucanase (M.W. 56,000 and a pI of 6.2) was isolated from *C. thermocellum* by a single ion-exchange separation in 6M urea (Petre *et al.*, 1981). It contained the vast majority of the TNP-CMCase of the crude extract. Despite the simplicity of the separation procedure, the protein produced only one band upon SDS-PAGE, and IEF in 8M urea revealed only two minor impurities.

The same French group recently engineered the transfer of two endoglucanase genes from *C. thermocellum* into *E. coli*, which expressed them intracellularly (Cornet *et al.* 1983a,b). One of these genes was shown to code for the endoglucanase which they had already purified from *C. thermocellum* culture supernatant (see above) whereas the other coded for a slightly larger endoglucanase (M.W. 66,000) which was much less active towards TNP-CMC than the first. They purified this second enyzme by a novel approach involving production of the enzyme by the *E. coli* strain carrying the appropriate gene followed by sonication to rupture the cells and heat precipitation to remove the other normal products of *E. coli* protein synthesis (Cornet *et al.*, 1983a). Further purification proved necessary however, involving ion exchange and gel permeation chromatography, but was greatly facilitated by the absence of high molecular weight aggregates like those formed by *C. thermocellum* extracellular proteins (Shinmyo *et al.*, 1979, Ait *et al.*, 1979b, Petre *et al.*, 1981, Ljungdahl *et al.*, 1983).

Three endoglucanases have thus been purified from *C. thermocellum*. This small group of endoglucanases which have proven amenable to purification comprises only a fraction of the *C. thermocellum* cellulase complex, in view of the work described earlier (Ait *et al.*, 1979b, Lamed *et al.*, 1983, Ljungdahl *et al.*, 1983).

Two components of the *C. stercorarium* cellulase complex have been isolated by ion-exchange chromatography, an endoglucanase of M.W. about 95,000 and pI 3.85 (Creuzet and Frixon, 1983) and an exoglucanase (Creuzet *et al.*, 1983), the characteristics of which have not yet been published. Both produced single bands upon polyacrylamide gel electrophoresis, despite being the products of a purification sequence involving only two steps. No indication of the total number of components in the cellulase complex of *C. stercorarium* has been published. The exoglucanase was claimed to be a cellobiohydrolase, based on the following observations: (i) very limited ability to produce reducing sugars from soluble CMC, (ii) inability to attack crystalline cellulose unless acting in conjunction with the

endoglucanase, (iii) production of cellobiose as the major product when acting alone on highly hydrated (Walseth) cellulose.

The only other published report of bacterial production of an exo-ß-(1,4)-glucanase appears to be that of Storvick and King (1960), relating to *Celvibrio (Cellulomonas) gilvus*.

Our bacterium produced a cellulase complex which could be readily subdivided into three groups of enzymes on the basis of their cellulose-binding characteristics: (i) those which would not bind to crystalline cellulose, (ii) those which would bind to cellulose but which were released merely by warming the cellulose, and (iii) those which were bound most strongly to cellulose and required very low ionic strengths to release them from it. Each of these groups was shown to produce multiple bands when run on SDS-PAGE, and each group acted synergistically with the others in hydrolysing crystalline cellulose (Section 6.6). The extent of the synergisms was not great (up to 2.7-fold), but it should be remembered that the components had not been thoroughly purified before being recombined. The only published evidence of synergism between components of a bacterial cellulase complex was that of Creuzet *et al.* (1983), relating to *C. stercorarium*.

The group (i) proteins (which did not bind to cellulose) contained all of the extracellular ß-glucosidase activity. Preparative SDS-PAGE of this material followed by assay of the gel slice washings for ß-glucosidase (aryl-glucosidase) activity revealed at least three different aryl-ß-glucosidases with molecular weights of around 25,000, 60,000 and 120,000 (Fig. 6.15). In addition at least 10 endoglucanases were detected in this unbound group of enzymes by a CMC zymogram of an SDS-PAGE separation (See Section 6.5.7.2). One of the ß-glucosidases from group (i) was purified to the extent that it no longer contained any cellulolytic activity. The steps involved are summarised in Fig. 6.34. The ß-glucosidase was found to be active towards both cellobiose and p-nitrophenolglucopyranoside.

The cellulose-bound proteins (groups (ii) and (iii)) became the subjects of our most intensive fractionation programme. Fig. 6.16 shows a step-wise outline of this programme. Conventional purification tables were inappropriate since the separation steps would involve loss of synergistic interactions, with an unknown effect on the apparent activities of the components.

The group (ii) and (iii) proteins which had been previously separated using small cellulose columns (e.g. Fig. 6.1) were not

separated during the large scale cellulose batch-wash procedure (Section 6.4.3.). Subsequent gel permeation chromatography on Biogel P-10 divided them into two groups (Fig. 6.18). Only the group of enzymes which eluted in the P-10 column void volume (i.e. M.Ws > 20,000) was further fractionated.

The next step, ion exchange chromatography on CM-Sepharose CL-6B did not produce a worthwhile fractionation of the cellulolytic activity, so all the active fractions were bulked.

This bulked activity from the ion exchange step was then split into 5 protein peaks, each with different relative activities towards various cellulosic substrates by gel permeation chromatography on Ultrogel AcA44 (Fig. 6.19). The elution volumes of these peaks were repeatable if they were reapplied separately, indicating compositional stability, despite the fact that analytical SDS-PAGE (Section 6.4.6.1) and analytical IEF (Section 6.4.6.2) showed that each of the Ultrogel peaks contained several proteins. Inclusion of 0.1% SDS in the buffer used to elute the column did not produce further fractionation of the protein profile.

Preparative IEF (Section 6.4.7) and preparative SDS-PAGE served to split the individual peaks collected from the Ultrogel column into many components. Almost all were found to carry a share of the cellulolytic activity. Ten to fifteen distinct bands of CMC-plate-clearing activity were produced on zymograms after preparative IEF or SDS-PAGE separation of Ultrogel Peaks 3, 4, and 5 (Figs 6.21, 6.24, 6.25). The enzymes eluted from these bands were found to exhibit definite differences in their relative reaction rates towards different cellulosic substrates (see Sections 6.4.7.1 and 6.4.8.1 and Tables 6.6, 6.7 and 6.8).

A standard test for artefacts of IEF and PAGE systems is to rerun individual bands on the system which produced their separation originally. If the individual bands in turn give rise to a fresh set of bands covering the same range of mobilities as the first set, the separation may be deemed an artefact of the system. This was not found to be the case for the SDS-PAGE-separated bands. When rerun, most produced single sharp protein bands (Section 6.4.8.2, Figs 6.26, 6.27). However, the same could not be said for the I.E.F.-produced bands from Peak 4. When resubjected individually to IEF they did not refocus as single bands (Fig. 6.23). This may have been due, at least in part, to the use of a different brand of ampholyte in the second gel (Otavsky and Drysdale, 1975, Bredenkamp and Joubert, 1982). SDS-PAGE of selected bands from the preparative IEF separation showed that the latter had

achieved a substantial fractionation of the Peak 4 components, and different bands had major components with clearly different mobilities on SDS-PAGE (Fig. 6.22). However, all of the actively cellulolytic bands tested from the IEF separation gave rise to more than one band on SDS-PAGE. Conversely, all of the active bands from the preparative SDS-PAGE separations of Peaks 3 and 5 despite rerunning as single bands on SDS-PAGE, gave rise to multiple bands upon IEF. All were focussed within the range 4.5-5.0.

Since it appeared that we still had not completely purified any of the component enzymes, the two most actively cellulolytic "bands" from the preparative SDS-PAGE fractionation of Peak 5 were next pretreated with 4M urea and subjected to preparative IEF over the pH range 4.5-5.0. One split into about 10 bands with detectable CMC-plate-clearing activity on the zymogram (Fig. 6.32) while the other gave rise to about 7 less well defined active bands (Fig. 6.33). The very low levels of cellulolytic activity in each of these bands following IEF precluded meaningful comparison of their relative reaction rates on the various substrates. An attempt to rerun two of the most active bands on analytical I.E.F. as a check for artefacts gave an inconclusive silver stain due to insufficient protein. In fact we had, to all intents and purposes, fractionated our most active cellulolytic activities into virtual oblivion.

The question as to whether or not these multiple bands produced from single SDS-PAGE bands were really different proteins thus remained unanswered. Production of a single sharp band of protein on SDS-PAGE does not seem to be a satisfactory criterion for purity when cellulolytic complexes are concerned. Sprey and Lambert (1983) clearly demonstrated that protein which ran as a single band under conventional SDS-PAGE and IEF could be further fractionated into six component proteins, including a CMCase, a xylanase and two ß-glucosidases. This was achieved using IEF following pretreatment of the complex with urea and octylglucoside (a detergent).

On the other hand, there is an ever-expanding body of literature which suggests that multiple bands on IEF gels may be artefacts attributable to interaction between carrier ampholytes and macromolecules (Gianazza and Righetti, 1978, Shinjo and Harrison, 1979, Bredenkamp and Joubert, 1982, Cuono *et al.*, 1983, Basset *et al.*, 1983).

Alternatively, the multiple bands which we observed after very narrow range IEF may be simply attributable to "microheterogeneity"

which can be expected in any "population" of large protein molecules, with no two molecules being exactly alike. Aging of the proteins during storage and repeated freeze-thawing may accentuate this variability. This explanation was supported by the fact that the multiple bands revealed by IEF were generally confined to a very narrow pI range (ca.4.6-4.7) (Fig. 6.23).

A conservative estimate of the number of enzymes with endoglucanase activity which were separated from Peaks 3 and 5 combined would be 20. The bands of activity shown in Figs 6.24 and 6.25 total about 25, but some duplication is possible amongst those enzymes in the regions of overlapping mobilities with apparent M.Ws of 80,000 - 100,000. Peak 4 was subdivided into about 15 distinct bands of endoglucanase activity by IEF (Fig. 6.21). These probably included at least some enzymes that weren't present in either of Peaks 3 or 5 (Fig. 6.20). It can thus be concluded that at least 25 to 30 endoglucanases were separated from the cellulose-bound fraction of the cellulase complex of TP8.T6.3.3.1. To then suggest that individual "enzymes" of this group can be still further split into 7-10 active bands by IEF, as appeared to be the case for the two SDS-PAGE bands which were tested, invokes a complexity which is hard to accept. On top of this we have the active bands of higher molecular weights in Peaks 1 and 2 from the Ultrogel column, which were not subdivided. Further still, there are the group (i) enzymes which don't bind to cellulose and so weren't loaded onto the Ultrogel. This group was found to produce 10 or so bands of clearance on a CMC-zymogram following SDS-PAGE.

Speculation as to the adaptive significance of multiple forms of each component of a cellulase complex has been entered into in Chapter 1 (Section 1.4.5.2). Had we purified larger quantities of the multiple forms of endoglucanase from our organism, it would have been interesting to compare their sensitivities to a range of natural inhibitors from plants. A wide variety of plants produce cellulase inhibitors (Bell *et al.*, 1962, Mandels and Reese, 1963, 1965). Several of the cellulase complexes displayed inhibition curves which flattened at various degrees of inhibition, suggesting the presence of resistant and susceptible components within each complex (Mandels and Reese, 1963). It is conceivable that the multiple forms of cellulase confer on the bacterium the capacity to hydrolyse almost any natural cellulosic since no natural inhibitor is likely to be effective against all the enzyme forms.

Activity towards crystalline cellulose was always accompanied by

the CMC-plate-clearing (endoglucanase) activity. Peaks 1 and 2 from the Ultrogel separation appeared to have the highest proportion of exoglucanase activity. The predominance of cellobiose as an end-product from crystalline cellulose hydrolysis by Peak 1 was in keeping with cellobiohydrolase action, but the unidentified larger oligosaccharide which was also present (20% of the total products) suggested the activity of an endoglucanase component. The major protein components of Peaks 1 and 2 had apparent M.Ws (by SDS-PAGE) of 150,000 and 145,000 respectively.

The later-eluting peaks from the Ultrogel column encompassed a range of apparent molecular weights (based on their SDS-PAGE mobilities) from around 130,000 down to 20,000. It is intriguing that, despite their obvious complexities, the Ultrogel peaks exhibited very reproducible mobilities on Ultrogel and showed no tendency to fragment until subjected to SDS-PAGE or IEF. Perhaps these aggregates of enzymes are the true functional units *in vivo*, since there may be merit in a close spatial association of the various types of cellulase activity which they were found to contain. Protection against proteolytic degradation has also been suggested as a reason for the aggregation of cellulase component enzymes (Ait *et al.*, 1979b).

Preparative SDS-PAGE or IEF proved to be far more powerful in fractionating the cellulase complex than the more conventional column chromatography techniques. However, the former techniques are not appropriate for large scale purification unless they are used simply as preliminaries for the preparation of antibodies which could then be linked to agarose and used in immuno-affinity chromatography.

The practical application of immunoaffinity chromatography to cellulase purification has been recently demonstrated by Nummi *et al.*, (1983) using polyclonal antibodies. The preparation of enzyme-specific monoclonal antibodies and their subsequent cross-linking to an agarose matrix should obviate the need to firstly purify the cellulase component enzymes, and has been used in small-scale cellulase fractionation by Langsford *et al.*, (1984). Success of these methods depends upon the different components of the cellulase complex having different antigenic determinants. Langsford *et al.*, (1984) in fact found that several polypeptides with CMCase activity appeared to share a single determinant. They imply that this method can be used to distinguish between different gene products, with all those enzymes which bind to a single monoclonal antibody being modifications of a single gene product.

Gene cloning affords not only a fresh approach to increasing cellulase production but may also prove to be the simplest means of purifying individual components of a bacterial cellulase complex. Bacterial cellulase genes appear to be more readily cloned and expressed than fungal equivalents, with successful transfer and expression in *E. coli* being reported for two endoglucanases from *C. thermocellum* (Cornet,*et al.*,1983a,b), for an endoglucanase from *Thermomonospora* (Collmer and Wilson, 1983), *Cellulomonas fimi* (Whittle *et al.*, 1982), and for two endoglucanases from an alkalophilic *Bacillus* species (Sashihara *et al.*, 1984). Similar success has also been reported very recently (Daniel *et al.*, 1984) in the transfer to and expression by *E. coli* of genes coding for CMC-plate-clearing activity from our organism TP8.T6.3.3.1.

Purification of the products of the cloned genes appears to be relatively simple, particularly in the case of thermostable cellulases. Although the cellulase expression is almost entirely intracellular in *E. coli*, high temperatures can be used to selectively precipitate the unwanted heat-labile proteins produced by *E. coli*, as demonstrated by Cornet *et al.* (1983a). The absence of the high molecular weight aggregates of cellulases is also a major simplifying factor in purifying the individual enzymes. However, glycosylation of the cellulases was not reproduced by the *E. coli* system, which may limit the usefulness of gene cloning in cellulase production if the carbohydrate content is found to have a vital function.

The nucleotide sequence has recently been determined for the *celA* gene and other neighbouring shorter segments on a 3.2-kilobase fragment of *C. thermocellum* DNA from an *E. coli* plasmid (Beguin *et al.*, 1985). It appears that one neighbouring segment may be a transcription initiator while another may belong to a polycistronic operon including *celA*. Independent subcloning is hoped to determine the extent of their interaction and individual function.

It should be possible through the use of gene-cloning techniques to resolve the current controversy over the genetic basis for the cellulase multiplicity. An understanding of the production and purpose of these multiple enzyme forms presents an intriguing challenge for the future.

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