Cloned Xylanolytic and Cellulolytic Enzymes from an Extreme Thermophile

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Abstract

Heat treatment was a successful purification step for five xylanolytic and cellulolytic enzymes cloned from the extreme thermophile '*Caldocellum saccharolyticum*' strain Tp8T6.3.3.1. Temperature and length of heat treatment can be altered to give high recovery, or a high degree of purification. Higher purification could be gained by heat treatment at an acidic pH. Various studies showed that further factors, such as protein concentration, were also important.

The substrate specificities of the five enzymes were investigated. The β-xylosidase had a very narrow substrate specificity, with high activity only on *o*- or *p*-nitrophenyl β-D-xylopyranoside and no activity on xylobiose. It showed some transferase activity. The xylanase had high activity on xylan, releasing xylobiose and some xylotriose. It also hydrolysed carboxymethylcellulose, 4-methylumbelliferyl β-D-cellobioside (MUC) and *p*-nitrophenyl β-D-cellobioside but could not hydrolyse xylobiose. It showed transferase activity on *p*-nitrophenyl β-D-xylopyranoside. The β-glucosidase also had broad substrate specificity, with activity on cellobiose and aryl glycosides, but not xylobiose. The carboxymethylcellulase acted as an endoglucanase, with high activity only on carboxymethylcellulose. The MUCase appeared to have two catalytic sites. The N-terminal domain acted as an exoglycobiohydrolase, with high activity on xylan, MUC and *p*-nitrophenyl β-D-cellobioside, while the C-terminal domain acted as an endoglucanase, with high activity on carboxymethylcellulose.

The xylanase was purified and found to have a molecular weight of 42 000 and an isoelectric point of approximately 5.0. It showed optimum activity at pH 5.0 - 7.7 and had an activation energy of 44kJmol^{-1}. The half life at 75°C was 20 minutes in the presence of 0.5mgml^{-1} bovine serum albumin. The xylanase was stable at room temperature at pH 4.5 - 11.5 in the presence of 0.5mgml^{-1} bovine serum albumin. Xylose did not appear to inhibit the xylanase, but xylobiose may be inhibitory. The K_m for oat spelts xylan was 0.02% (w/v) at pH 6.0. The xylanase had higher activity on oat spelts and sugar cane bagasse xylan than larchwood xylan. The two catalytic residues were predicted to be positioned at the N-terminus of the xylanase.

A truncated MUCase containing only one catalytic domain (the exoglycobiohydrolase) was studied. It retained activity on the substrates that the entire protein (containing all domains) hydrolysed. The truncated MUCase had a molecular weight of 84 000. Activities on MUC and Avicel were stable for at least 24 hours at 75°C. The activation energy of the truncated MUCase on MUC was lower ($39 kJmol^{-1}$) than that of the entire protein ($55 kJmol^{-1}$). The two catalytic residues of the exoglycobiohydrolase domain were predicted to be positioned at the N-terminus of the MUCase. The two catalytic residues of the endoglucanase domain, present in the entire protein, were predicted to be in the middle of the C-terminal endoglucanase domain.

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Preface

In order to gain a more complete picture of the process and /or system being studied, results from various co-workers are presented in this thesis. The results are clearly denoted in the text and are listed below:

Hudson *et al.* (in press), parts of Table 3.4, Table 3.6 and Section 4.1;
Neal (1987), parts of Table 3.5, Table 3.6 and Section 4.4;
Neal (unpublished results), part of Table 3.5;
Patchett (unpublished results), part of Table 3.5;
Plant *et al.* (1988), parts of Table 3.5, Table 3.6 and Section 4.3, and
Schofield *et al.* (1988), part of Table 3.4.

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Abbreviations

Bicine	*N,N*-bis[2-hydroxyethyl]glycine
Bis-Tris	bis[2-hydroxyethyl]imino-tris[hydroxymethyl]methane
Bis-Tris propane	1,3-bis[tris(hydroxymethyl)methylamino]propane
BSA	bovine serum albumin
CAPS	3-[cyclohexylamino]-1-propanesulphonic acid
CM-	carboxymethyl-
CMC	carboxymethylcellulose
CMCase	carboxymethylcellulase
DEAE-	diethylaminoethyl-
DMF	*N,N*-dimethylformamide
DNA	deoxyribonucleic acid
DNase	deoxyribonuclease
DNS	3,5-dinitrosalicylic acid
DTT	DL-dithiothreitol
EDTA	ethylenediaminetetraacetic acid
EPPS	*N*-[2-hydroxyethyl]piperazine-*N*'-[3-propanesulphonic acid]
HCA	hydrophobic cluster analysis
HEC	hydroxyethylcellulose
HEPES	*N*-[2-hydroxyethyl]piperazine-*N*'-[2-ethanesulphonic acid]
HPLC	high pressure liquid chromatography
IEF	isoelectric focusing
MES	2[*N*-morpholino]ethanesulphonic acid
MOPS	3[*N*-morpholino]propanesulphonic acid
MU	4-methylumbelliferone
MUC	4-methylumbelliferyl β-D-cellobioside
MUCase	4-methylumbelliferyl β-D-cellobiosidase
MUG	4-methylumbelliferyl β-D-glucopyranoside
MUX	4-methylumbelliferyl β-D-xylobioside
*o*NP	*o*-nitrophenol
*o*NPX	*o*-nitrophenyl β-D-xylopyranoside
*o*NPXX	*o*-nitrophenyl β-D-xylobioside
ORF	open reading frame
PAHBAH	*p*-hydroxybenzoic acid hydrazide
PMSF	phenylmethylsulphonyl fluoride

*p*NP	*p*-nitrophenol
*p*NPAf	*p*-nitrophenyl α-L-arabinofuranoside
*p*NPAp	*p*-nitrophenyl α-L-arabinopyranoside
*p*NPC	*p*-nitrophenyl β-D-cellobioside
*p*NPG	*p*-nitrophenyl β-D-glucopyranoside
*p*NPL	*p*-nitrophenyl β-D-lactopyranoside
*p*NPX	*p*-nitrophenyl β-D-xylopyranoside
*p*NPXX	*p*-nitrophenyl β-D-xylobioside
*p*NPXXX	*p*-nitrophenyl β-D-xylotrioside
PT	proline threonine
SDS	sodium dodecyl sulphate
SDS-PAGE	sodium dodecyl sulphate-polyacrylamide gel electrophoresis
TEMED	*N,N,N',N'*-tetramethylethylenediamine
Tricine	*N*-tris[hydroxymethyl]methylglycine
Tris	tris[hydroxymethyl]aminomethane
UV	ultraviolet

Chapter One

Introduction

Previous efforts to study the cellulolytic enzymes from the cellulose degrading, extreme thermophile, '*Caldocellum saccharolyticum*' using conventional methods were not successful. Gene cloning has enabled many of the difficulties to be overcome and a few of the xylanolytic and cellulolytic enzymes have been studied individually. The objective of this thesis was to carry out an overall study of five cloned xylanolytic and cellulolytic enzymes. The work was dependent on the provision of clones by a collaborating group. A heat treatment method of purification for the five enzymes was developed after preliminary experiments measuring the thermostabilities of proteins were made. The substrate specificities of the five enzymes (β-xylosidase, xylanase, β-glucosidase, CMCase and MUCase) were studied. The xylanase and the MUCase were studied in further detail. This introduction backgrounds each area investigated by discussing the most relevant literature to hand.

1.1 Thermostability and heat treatment

1.1.1 Theory

Native water soluble proteins generally have hydrophobic amino acids on the interior of the molecule and polar or more hydrophilic amino acids on the exterior. All buried polar atoms are hydrogen bonded (Chothia, 1975). The principle determinants of thermostability are the non-covalent forces that dictate the secondary and tertiary structure of proteins (Ponnuswamy *et al.*, 1982). Such forces include hydrogen bonding, hydrophobic interactions, ionic interactions and van der Waals interactions (Schulz and Schirmer, 1979). Disulphide bonds do not contribute significantly (Ponnuswamy *et al.*, 1982). The combination of these factors ultimately reflects the primary structure or amino acid sequence (Ward and Moo-Young, 1988). Stabilizing and destabilizing forces are very large (Brandts, 1969), so the difference or net free energy of stabilization is very small (Pace, 1975 and Tanford, 1970). Very small changes in the overall structure giving rise to additional hydrogen bonds, hydrophobic interactions or salt bridges can easily account for drastic changes in thermostability. Evidence for this has been provided by point mutation experiments and studies on differences in the amino acid sequences of related

proteins, for example, Langridge (1968), Grutter *et al.* (1979), Perutz and Raidt (1975) and Matthews *et al.* (1974). The change of one amino acid usually has little effect on the overall three dimensional structure, but can cause marked stabilization or destabilization (for example, Yutani *et al.*, 1977, Holmes and Matthews, 1982 and Matsumura *et al.*, 1984). External environmental factors can also influence thermostability. Such factors include cations, substrates, co-enzymes, modulators, polyols, proteins and pH (for example, Schmid, 1979 and Citri, 1973). Thermostability in dilute buffered solution is usually very different from that inside the cell (Ng and Kenealy, 1986).

Enzyme denaturation by heat occurs in two steps (Brandts, 1967 and Klibanov, 1983). In the first step reversible denaturation occurs. Increased temperatures cause the native catalytically active enzyme to become a randomly coiled catalytically inactive denatured form. Low energy bonds stabilizing the three dimensional enzyme conformation are broken due to increased vibrational energy and collisions with water molecules. Even higher temperatures in the second step cause irreversible denaturation. Covalent modification of the denatured form can be due to deamidation reactions, hydrolysis reactions, reactions involving sulphur-containing amino acids or conformational changes (for example, Ahern and Klibanov, 1985, Zale and Klibanov, 1984 and 1986 and Whitaker and Fujimaki, 1980). Non-covalent inter- and intramolecular interactions between exposed hydrophobic regions in the unfolded protein cause protein coagulation and precipitation.

1.1.2 Measurement of thermostability used in this study

(a) Protein assay

Protein thermostability can be quantified by measuring the amount of protein remaining soluble after a heat treatment. The protein concentration must be sufficient for denatured protein to aggregate and precipitate. Additionally the amount of insoluble or precipitated protein can be measured. The most commonly used methods of protein measurement include Biuret, Lowry, dye-binding and UV spectrophotometry.

The Biuret reaction involves a strongly alkaline copper reagent which produces a purple colouration with protein. There are two different ways of applying the Biuret reaction. The first involves a copper complex (often formed with $CuSO_4$ and tartrate (Gornall *et al.*, 1949)) soluble in NaOH. When protein

is added copper is displaced from the tartrate forming a copper-protein complex of different colour and greater absorbance intensity. However at higher protein concentration values, the colour intensity is not proportional to protein concentration due to competition between protein and complexing agent for the available copper. In the second method the protein is dissolved in NaOH and then $CuSO_4$ added to it (Robinson and Hogden, 1940). Excess copper is removed by centrifugation as the precipitated hydroxide, leaving the coloured copper-protein complex in solution. The Biuret colour is strictly proportional to protein added. All proteins give very similar Biuret values because the copper reagent reacts with the peptide chain rather than side groups. The method is simple to carry out and gives extremely reproducible results. The main disadvantage is a relative lack of sensitivity. A method for determining the total protein in whole microbial cells has been described by Herbert *et al.* (1971). It involves boiling a washed cell suspension with strong NaOH for five minutes before the addition of the copper reagent.

Lowry *et al.* (1951) developed a method of protein determination. A combination of the copper reagent used in the Biuret method and the reaction of the Folin-Ciocalteu reagent (Folin and Ciocalteu, 1927) with phenols, such as tyrosine in proteins, gives a strong dark blue colour with proteins. However the amount of colour produced varies greatly with different proteins as it is due to their content of tyrosine and tryptophan. The method is less reproducible than the Biuret method and much more subject to interference (see for example: Peterson, 1983). The great advantage of the Lowry method is its sensitivity. Herbert *et al.* (1971) applied the method to suspensions of whole microorganisms using a step of heat treating in strong NaOH. Peterson (1983) also described a method for measuring insoluble protein.

The dye-binding method was first developed by Bradford (1976). Coomassie Blue G-250 turns a red-brown colour when dissolved in perchloric acid. When it binds to protein in this solvent, the blue colour of the dye is restored. The colour developed depends on the composition of the protein to a limited extent. The method is fast and very sensitive. The main problem is that the dye adsorbs to glassware and cuvettes. However, it can be removed by quickly rinsing with dilute SDS (Scopes, 1982).

There are various ways of measuring protein using UV absorption. Proteins absorb at 250 - 310nm almost entirely because of tyrosine and

tryptophan residues. Since the content of these two amino acids varies enormously, a simple 280nm reading gives only a rough idea of actual protein content (Scopes, 1982). However, measurement of the absorbance at two different wavelengths allows the tyrosine and tryptophan content of a protein to be determined (Beaven and Holiday, 1952). A correction can be made for nucleic acid and nucleotide content using measurements at both 280nm and 260nm (Warburg and Christian, 1941). Absorbance in the far UV, around the peptide bond, is far more sensitive and much less affected by amino acid composition. The most consistent results are obtained around 205nm. The values vary from protein to protein because aromatic and some other residues also add variable contributions in this range and the secondary structure has some influence on the shape and exact position of the peptide absorbance peak. Difficulties with measurements in this range include absorbance by oxygen, most salts and buffers. Nevertheless, UV absorbance methods are fast and non-destructive (Scopes, 1982).

(b) UV difference spectrophotometry

The UV absorbance spectrum of a protein can be divided into three regions (Wetlaufer, 1962). In the region 250 - 310nm absorbance is due to the aromatic amino acids tyrosine, tryptophan and phenylalanine. Below 210nm down to the limit of detection protein absorbance is complex and due to a number of chromophores, the most important being the peptide bond. Protein absorbance between 210nm and 250nm is also due to a number of contributions, not all of which have been identified. However the aromatic amino acids also show a weak band of absorbance at approximately 210 - 220nm.

UV difference spectophotometry is useful as an indicator of changes in protein conformation (Wetlaufer, 1962) as minor features of an absorbance curve can be emphasized. Difference spectra of proteins often show a peak in the region 210 - 250nm that is more prominant than that in the 250 - 310nm region. The former peak is due to the conformation dependence of peptide band absorbance. The absorbance of the peptide band changes significantly with change in the conformation of peptide chains whereas the absorbance of aromatic side chains is much less sensitive to environmental change (Wetlaufer, 1962). Several factors are capable of inducing small spectral perturbations in proteins by causing changes in the environment of protein chromophores. Denaturation causes changes in the environments of protein

chomophores, usually due to changes in the three dimensional protein structure on denaturation. Such changes can be

(i) the orientation of the solvent around the solute chromophore,
(ii) solvent anions or cations near the chromophore,
(iii) the solvent hydrogen bonding to the chromophore,
(iv) ionization of groups nearby the chromophore (Leach and Scheraga, 1960),
(v) changing location of ionic groups nearby the chromophore and
(vi) generating new ionizing groups near the protein chromophore (Wetlaufer, 1962).

Thermostabilities of pure proteins have been examined using UV difference spectophotometry (Wetlaufer, 1962). However the overall thermostabilities of protein mixtures appear not to have been studied using this method.

(c) Enzyme activity

The measurement of the total activity of an enzyme before and after heat treatment is a simple functional method that has conventionally been used to study the thermostability of enzymes. The usual way of reporting enzyme thermostabilities is as half lives. The half life ($t_{1/2}$) of an enzyme is the length of time for half the activity to be lost at a specified temperature. Such measurements can be made whether the enzyme is pure or in a mixture of proteins as long as it is the only activity of its type present. However, the results in each situation will be different as thermostability is also dependent on environment.

1.1.3 Use of the property of thermostability in enzyme purification by heat treatment

The use of heat treatment to purify enzymes by selective denaturation and subsequent precipitation of denatured protein is a simple, rapid and well established procedure. Successful applications are limited to those few enzymes that possess a thermostability considerably higher than the majority of cell proteins. The introduction of thermostable enzymes into the protein population of a mesophile by cloning offers a clear opportunity to use a heat treatment method of purification to its full advantage. Subsequent purification of cloned thermostable enzymes by heat treatment will also inactivate contaminating activities of the host organism. Work done to date suggests that qualitative retention of product thermostability generally results when this method is used.

There are many reports of the use of heat treatment in the purification of cloned thermostable hemicellulases and cellulases in the literature; some examples are Beguin *et al.* (1983), Joliff *et al.* (1986), Petre *et al.* (1986), Schwarz *et al.* (1986) and Patchett *et al.* (1989).

1.2 Enzymatic degradation of xylan

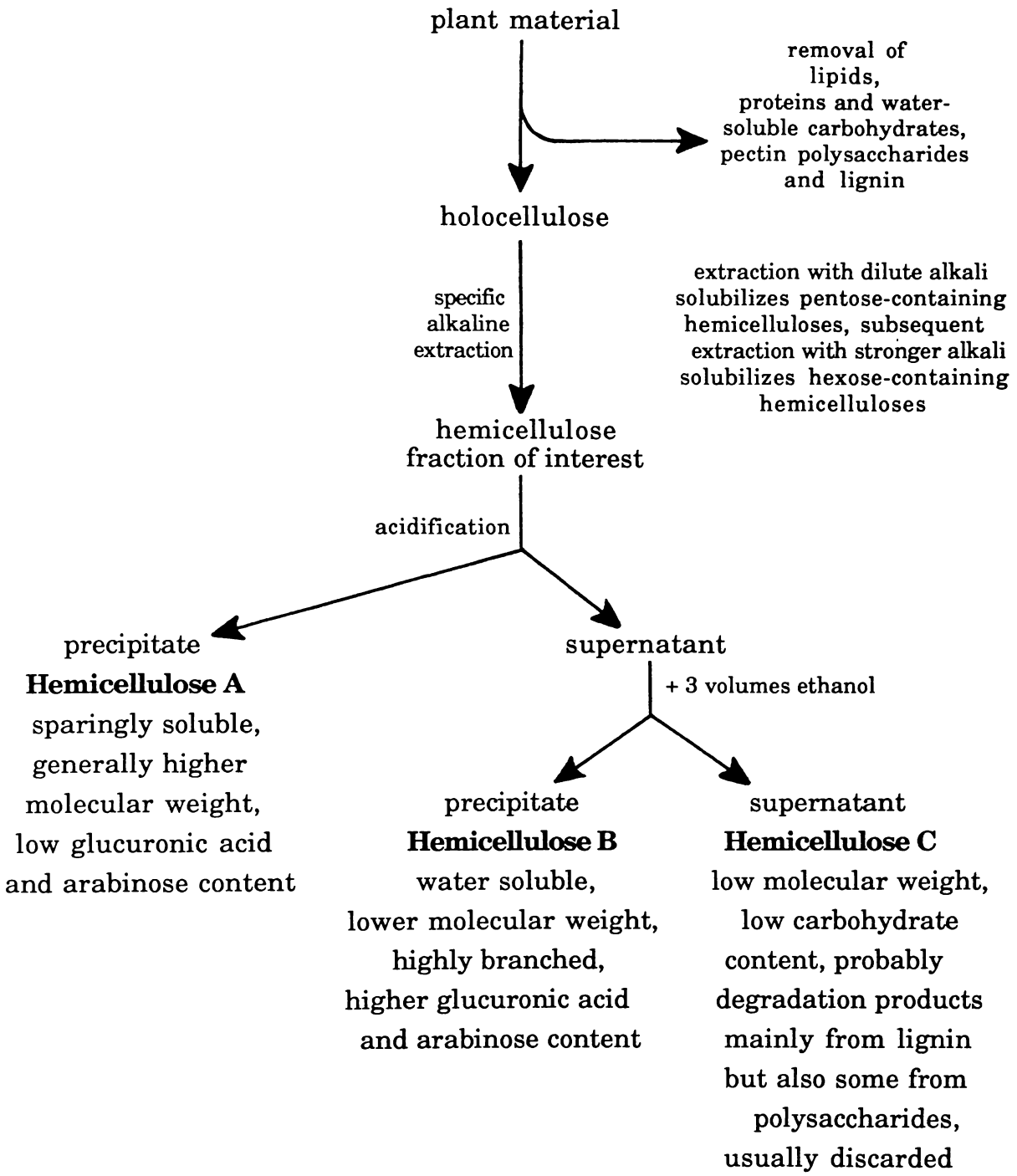
1.2.1 Hemicellulose

Hemicelluloses have historically been defined as the polysaccharide constituents of plant cell walls that are soluble in alkali. They are the second most abundant polysaccharides after cellulose. They are present in all layers of the plant cell wall, concentrated mainly in the secondary layer and occur in close association with cellulose and lignin. They are usually heteropolysaccharides made up of at least 2 - 4 different types of sugar residues. The naming convention is based on the dominant sugar residue. The three predominant types of hemicelluloses are 1,3- and 1,4-β-D-galactans, 1,4-β-D-mannans and 1,4-β-D-xylans, only the latter will be dealt with in detail here. Different types of sugar residues, often as short mono- or oligosaccharide appendages, are usually linked to the main glycan backbone. A less reliable method of hemicellulose classification is based on the method of extraction (Figure 1.1).

1.2.2 Hemicellulose hydrolysing enzymes

Hemicellulases are enzymes that attack the backbone chain of hemicellulose. The three main types are therefore β-D-galactanases, β-D-mannanases and β-D-xylanases. There are endo- and exo-acting hemicellulases. Endo-acting hemicellulases are the most common. They progressively degrade the hemicellulose into shorter fragments by cleaving bonds throughout the main backbone chain of the molecule, giving a product with retention of configuration. Exo-acting hemicellulases successively remove terminal sugars or oligosaccharide units from the main backbone chain of hemicellulose in a stepwise manner. They give products of inverted configuration. Other enzymes required for the complete hydrolysis of hemicellulose are β-glycosidases which hydrolyse low molecular weight glycosides and enzymes that cleave short chain or monosaccharide appendages from the main backbone chain. Examples are α- and β-galactosidases, β-D-mannosidases, β-D-xylosidases and α-L-arabinosidases. Hemicellulases can be

Figure 1.1 The extraction and classification of hemicelluloses.
(The classes shown can be subfractioned further.)
Based on Blake *et al.* (1971), Dekker (1979)
and Halliwell and Halliwell (1984b).



found in a wide range of organisms including bacteria (marine and terrestrial), fungi (terrestrial), yeast, rumen microorganisms (bacteria and protozoa), caecal bacteria (non-ruminant and ruminant), xylophagous insects, certain molluscs (for example, snails), crustaceans, marine algae and germinating seeds (land plants) (Dekker and Richards, 1976). Only xylan degrading enzymes will be discussed in further detail.

1.2.3 Xylan

Xylan is a hemicellulose with an essentially linear backbone of 1,4-β-D-linked xylose. Side chains can include one or more of α-L-arabinose, D-glucuronic acid, 4-O-methyl-D-glucuronic acid and acetyl groups. Xylans are mainly secondary wall components but are also found in the primary walls of monocotyledonous plants. They are more abundant in angiosperms than gymnosperms. The distribution of xylan across the cell wall is dependent on the species of plant, cell growth and differentiation. Xylan plays a major role in cell wall cohesion. It is the major interface between lignin and other carbohydrates in the secondary plant cell walls. It is probably covalently linked to phenolic residues via arabinosyl and glucuronosyl residues (Wong *et al.*, 1988). The degree of branching and frequency and composition of side chains vary depending on the source of xylan (Table 1.1) and its method of isolation. The modes of attachment of single residue side chains are well established for many xylans but the lengths and exact structures of more extended side chains remain to be established with precision (Aspinall, 1980). The solubility of xylan is directly proportional to its degree of substitution. However, the extent to which a xylan substrate can be enzymatically degraded is inversely proportional to the degree of substitution (Wong *et al.*, 1988).

1.2.4 Xylan hydrolysing enzymes

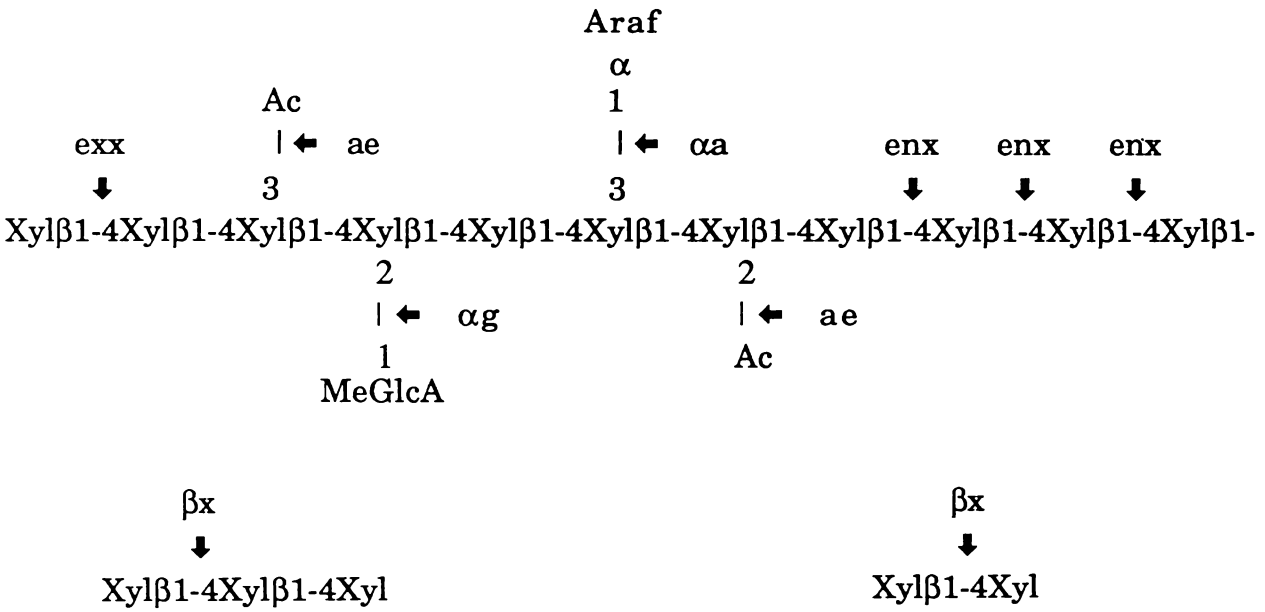
(a) Individual components of the system

Due to the heterogeneity of xylans several types of enzymes are required for its complete hydrolysis, those that degrade the main backbone chain and those that cleave side chains (Figure 1.2).

Table 1.1 Types of xylan, by source.
Based on Aspinall (1980), Halliwell and Halliwell (1984b) and Wong *et al.* (1988).

Angiosperms		Gymnosperms
Monocots	Dicots	
A. Cereal endosperms eg. wheat, rye **L-arabinoxylans** 35-40% of xylose units have α-L-arabino-furanose linked to C3. Devoid of D-glucuronic acid. Highly branched. B. Grasses eg. maize, barley, oat, sugarcane **L-arabino-(4-O-methyl-)D-glucurono-xylans** α-L-arabinofuranose linked to C3. (4-O-methyl-)D-glucuronic acid linked to C2. Some galactose residues. Highly branched.	Hardwoods eg. larchwood, birch, aspen, beech, elm, also flax, lentils **O-acetyl-4-O-methyl-D-glucuronoxylans** 70% of xylose units have O-acetyl groups, C3 linkage occurs more often than C2. 10% of xylose units have 4-O-methyl-D-glucuronic acid linked to C2. Only infrequently contain α-L-arabino-furanose. Degree of polymerization 150 - 200. Slightly branched.	Softwoods eg. conifers, pines **L-arabino-4-O-methyl-D-glucuronoxylans** 18% of xylose units have 4-O-methyl-D-glucuronic acid linked to C2. 12% of xylose units have α-L-arabino-furanose linked to C3. Not acetylated. Higher proportions of 4-O-methyl-D-glucuronic acid than hardwoods. Shorter chain length, degree of polymerization 70 - 130. Less branched than hardwoods.

Figure 1.2 A hypothetical plant xylan and the sites of its attack by microbial xylanolytic enzymes.
Based on Biely (1985).



Ac	acetyl group
Araf	L-arabinofuranose
MeGlcA	4-O-methyl-D-glucuronic acid
Xyl	D-xylose

αa	α-arabinosidase
αg	α-glucuronidase
βx	β-xylosidase
ae	acetylesterase
enx	endoxylanase
exx	exoxylanase

Endoxylanases (1,4-β-D-xylan xylanohydrolases, EC 3.2.1.8) cleave β-1,4-xylosidic bonds throughout the main backbone chains of xylan molecules to produce xylooligosaccharides with retention of configuration. They cause a rapid decrease in the degree of polymerization. The linkages that are attacked are determined by the length of the substrate, the degree of branching, the presence of side chains and the subsite pattern of the enzyme (Dekker, 1979). In general endoxylanases do not cleave linkages adjacent to the residues carrying a side chain (Biely, 1985). Fungal endoxylanases are often divided into two groups, those capable of releasing L-arabinose and those incapable. To date, no bacterial endoxylanases capable of releasing L-arabinose have been reported. Fungal non-debranching endoxylanases are found to have limited action against arabinoxylans. The action of endoxylanases is negatively influenced by the presence of acetyl side groups.

Exoxylanases (1,4-β-D-xylan xylohydrolases, EC 3.2.1.37) cleave xylose from the non-reducing ends of xylan chains in a stepwise manner, with inversion of configuration. Very few bacterial exoxylanases have been described to date.

β-xylosidases (β-D-xyloside xylohydrolase, EC 3.2.1.?) cleave short xylooligosaccharides to produce xylose with retention of configuration. This group includes xylobiases that cleave xylobiose to produce xylose. There is much confusion in the literature concerning exoxylanases and β-xylosidases. Unfortunately, the Enzyme Commission lists β-xylosidase and xylobiase as further names for EC 3.2.1.37, the systematic name of which indicates that the substrate of the enzyme is xylan. A further contradictory note indicates that the enzyme can hydrolyse xylobiose. This class either needs to be split into two (as listed here) in a similar way to the β-glucosidases (β-D-glucoside glucohydrolase, EC 3.2.1.21) and exoglucosidases (1,4-β-D-glucan glucohydrolase, EC 3.2.1.74), or redefined so that the systematic name correctly describes the enzymes discovered to date.

α-arabinosidases (α-L-arabinofuranoside arabinofuranohydrolase, EC 3.2.1.55) liberate arabinose side groups.

α-glucuronidases (α-D-glucuronoside glucuronosohydrolase, EC 3.2.1.?) liberate (4-O-methyl-) D-glucuronic acid side chains. However these enzymes are not found in many xylanolytic organisms (Biely, 1985).

Acetylesterases (acetylesterase, EC 3.1.1.6) remove O-acetyl groups.

Only those enzymes having activity on the main backbone chain or hydrolysis products of the main backbone chain of xylan will be discussed further.

(b) Multiplicity

There are many examples of endoxylanase multiplicity, however the production of more than two endoxylanases is rare (Gilbert *et al.*, 1988). The heterogeneity of the substrate is considered to be the main cause of multiplicity, leading to a system of enzymes each with specialized functions which enhance the utilization of xylan. Xylosidic linkages are not all equivalent and accessible and accessibility changes throughout hydrolysis (Wong *et al.*, 1988). Xylan is an important factor in fibre cohesion and multiple xylanases may be required for destruction of fibre integrity. Varying debranching activities may be required for the removal of substituents on xylan. Enzymes able to hydrolyse other xylose containing polysaccharides or secondary substrates or to act as transferases may be required. Multiple enzymes may have varying abilities to interact with other enzymes. Xylanases with different properties may be required for hydrolysis under various conditions.

Some organisms may appear to produce multiple xylanases but the activities on xylan may be due to a number of different enzymes (for example, cellulases and xylanases) all having broad substrate specificities. Multiplicity may also be mistaken in cases where degeneration of microbial culture filtrates or attack by other enzymes (for example, proteases) on the xylanase during purification has occurred (Wong *et al.*, 1988).

On a molecular level multiplicity is due to either genetically determined differences in primary structure (separate genes coding for different endoxylanases) or by modification of the same primary sequence (one gene coding for different endoxylanases). Modification (for example: signal processing, glycosylation, limited proteolysis and disulphide bond formation) is usually due to attack by other enzymes during production. These molecular differences lead to individual endoxylanases with distinct, but still similar, physicochemical properties.

(c) Mode of action

The mode of attack of xylan has been reported (Dekker, 1985) to be either through attack by glycosidases exposing the backbone chain and reducing steric hindrance or by endoxylanase attack on unbranched or moderately branched chains giving an array of oligosaccharides for further endo- and exo- attack. However in the fungal xylanolytic system these models fail to account for the action of those endoxylanases that are also able to release some side chain substituents. It may be that all the enzymes involved act simultaneously rather than sequentially.

Many bacterial hemicellulase (and cellulase) activities have been found to be associated with large extracellular complexes (Hespell, 1988). *Ruminococcus* spp. have highly structured complexes of high molecular weight. They are composed of protein and polysaccharide and contain cellulase and other enzymes. Xylanase activity in *Bacteroides* spp. is also associated with large complexes. Such complexes may aid in the association of the cells with insoluble plant materials and hinder diffusion of the enzymatically generated products away from the cell. The mechanism of hydrolysis by endoxylanases is assumed to be the same as that for endoglucanases and will therefore be discussed in Section 1.3.2 (e).

1.3 Enzymatic degradation of cellulose

1.3.1 Cellulose

Cellulose is a homopolymer of β-1,4-linked glucose residues. Each residue is rotated 180° about the main axis with respect to its neighbouring residues. The basic recurring unit is therefore cellobiose. The most likely model of cellulose is the folding chain model as described by Coughlan (1985) and Marsden and Gray (1986). The basic unit is a platellite which has a degree of polymerization of up to 10 000. Several platellites associate together, oriented in parallel and staggered with respect to partners. Many hydrogen bonds strongly bind them together to form a crystallite (or elementary fibril). The hydrogen bonding network consists of inter- and intramolecular bonds between successive and adjacent glycosyl residues. Intramolecular bonds help to maintain the rigidity of the cellulose chain. Chain length and the degree of interaction between chains varies widely in celluloses from different origins

(Mandels *et al.*, 1976). Sixteen crystallites pack together to form a fibril and four fibrils combine to form a microfibril. The microfibrils form insoluble fibres of great strength that are embedded in a matrix of other polysaccharides and lignin, to produce the rigid and inert wall structure characteristic of the primary and secondary walls of higher plants. Within cellulose fibres regions of complete order alternate with less well ordered regions. Over three quarters of the cellulose structure is considered to be in a crystalline form, which is enzymatically resistant compared to amorphous areas (Lamed and Bayer, 1988). There are no sharp boundaries between two regions. The degree of crystallinity within cellulose fibres varies with the source of the cellulose.

1.3.2 Cellulose hydrolysing enzymes

(a) Individual components of the system

Due to the complexity of native cellulose, a number of enzymes that act in both synergy and a complex manner are required for its complete hydrolysis. Such a system of enzymes is called a cellulase. It is generally accepted that there are three main types of enzymes involved in the hydrolysis of cellulose (for example, Reese *et al.*, 1968, Reese, 1977, Lee and Fan, 1980, Ryu and Mandels, 1980, Mandels, 1982 and Gilbert and Tsao, 1983). Much of the evidence for this has come from studies on fungal cellulases, in particular *Trichoderma* spp.. Cellulases can also be found in a wide variety of organisms, similarly to hemicellulases (see Section 1.2.2).

Endoglucanases (1,4-β-D-glucan glucanohydrolase, EC 3.2.1.4) cleave bonds throughout cellulose chains to produce water soluble oligosaccharides with retention of configuration. They have a preference for larger molecules, show no activity on cellobiose and when purified generally show no activity on crystalline cellulose. There can be some transglycosylation activity. They cause a rapid decrease in the degree of polymerization, or chain length, as shown by a rapid decrease in the viscosity of a solution of CMC. However, only a slow increase in reducing sugars is observed.

Exoglucanases remove cellobiose or glucose from the non-reducing ends of cellulose molecules. The product released is inverted to α–configuration. There is no activity on cellobiose and transglycosylation activity has not been observed to date. End product inhibition occurs and is most marked on crystalline and resistant substrates. The main type of exoglucanases are

exocellobiohydrolases (1,4-β-D-glucan cellobiohydrolase, EC 3.2.1.91) which cleave cellobiose from cellulose. Less well studied are exoglucosidases (1,4-β-D-glucan glucohydrolase, EC 3.2.1.74) which cleave glucose from cellulose.

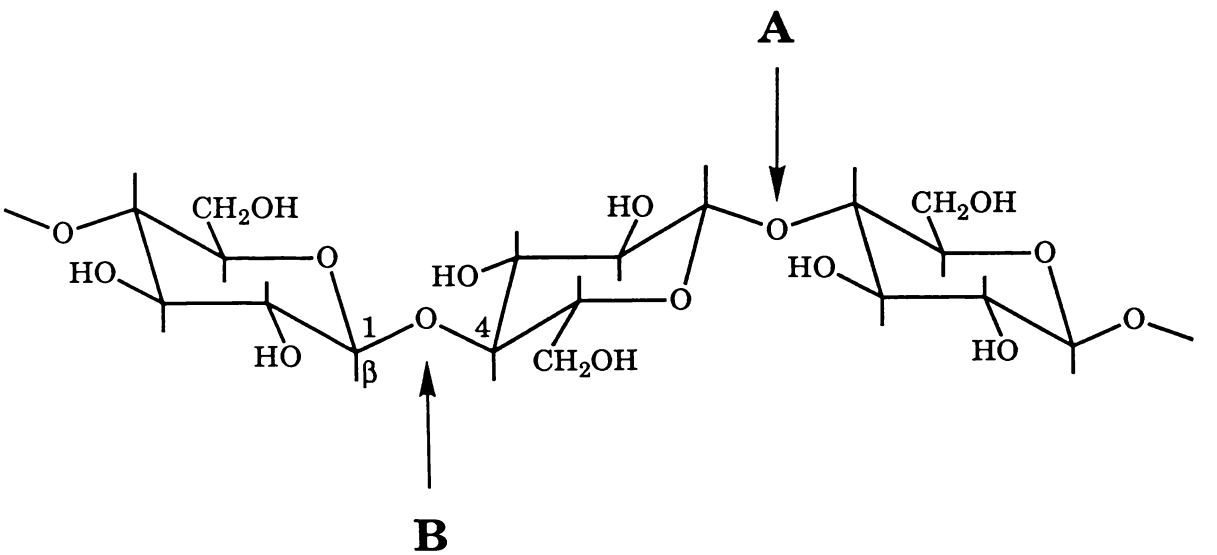
β-glucosidases (β-D-glucoside glucohydrolase, EC 3.2.1.21) act on cellobiose and small oligosaccharides to produce glucose with retention of configuration. They are subject to end product inhibition. This group includes cellobiases that cleave cellobiose to produce glucose. Dimers containing β-1,2, β-1,3, β-1,4 and β-1,6 bonds are all generally hydrolysed. There is no activity on cellulose.

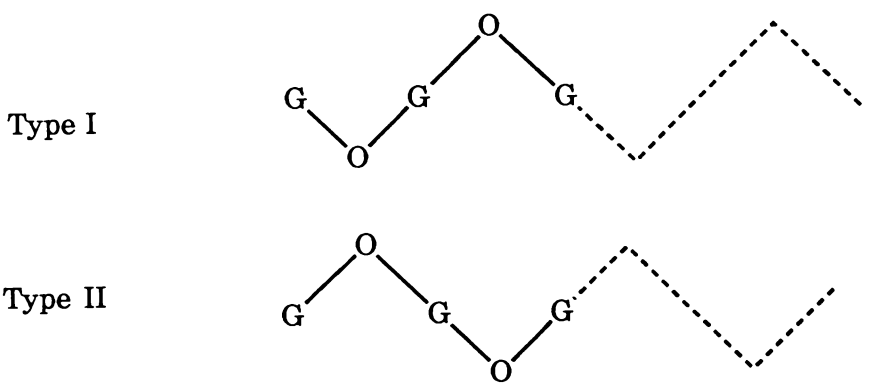
(b) Multiplicity

Although the magnitude of glucanase multiplicity is just becoming apparent, the reasons for such multiplicity are far from understood. A common observation is that glucanase multiplicity gives glucanases a diversity of substrate specificities (Knowles *et al.*, 1987). Whereas the heterogeneity of xylan explains xylanase multiplicity, cellulose is a homopolymer. However, some similarities with xylan can be seen. Not all glucosidic linkages are equivalent and accessible and accessibility changes during hydrolysis (Knowles *et al.*, 1987). Wood (1981) has argued that for stereochemical reasons, at least two types of endoglucanases and two exocellobiohydrolases must be required for the hydrolysis of cellulose. Glucosidic linkages between successive residues are locked in position by hydrogen bonds, in different planes (Figure 1.3). The array of protons and hydroxyls on successive residues differ and therefore at least two endoglucanases differing in stereospecificity must be required for the cleavage of glucosidic linkages in native cellulose. Depending on the linkage cleaved, at least two different non-reducing end groups could be generated so at least two different stereospecific exocellobiohydrolases are also required.

Multiple glucanases may be required for the destruction of fibre integrity or crystallinity in cellulose. Different glucanases may be required depending on the nature of the cellulose: amorphous or crystalline. Cellulose does not occur in pure form in any natural source. It is always associated with a variety of other polysaccharides, particularly lignin and hemicellulose, so enzymes with broad specificities may be required (Knowles *et al.*, 1987). Enzymes possessing transferase activity may also be required. Glucanases with different properties may be required for hydrolysis under various conditions.

Figure 1.3 Diagrammatic representation of the two types of glucosidic linkages in cellulose and the two types of non-reducing end groups produced by their hydrolysis.
Cleavage at A gives end group type I, cleavage at B gives end group type II. Based on Coughlan (1985).





On a molecular level glucanase multiplicity is due to the same factors as xylanase multiplicity. These are genetically determined differences and modifications of the same primary sequence, for example by signal processing, glycosylation, limited proteolysis or disulphide bond formation. Although there are only a few examples of glycosylated bacterial proteins, most bacterial glucanases are glycosylated. All glucanases share similar physicochemical properties (Enari and Niku-Paavola, 1987).

(c) Mode of action

The most widely accepted hypothesis of the mode of attack of native cellulose has been descibed clearly by Coughlan (1985) and Sharrock (1988). The ordered structure of cellulose is maintained by inter-chain hydrogen bonds. Intra-chain glucosidic bonds cleaved by endoglucanases would therefore rapidly reform unless exocellobiohydrolases were immediately on hand to remove cellobiose units. Exocellobiohydrolases are also required to solubilize fragmented outermost chains on the microfibril surface to allow access of endoglucanases to internal cellulose chains. Attack by endoglucanases on crystalline cellulose is only successful in the presence of exocellobiohydrolases. Exocellobiohydrolases act synergistically with endoglucanases to break down crystalline cellulose against which neither group has significant activity (Marsden and Gray, 1986). Such synergism is most marked with highly crystalline substrates, low with amorphous celluloses and absent with soluble derivatives of cellulose.

The bacterial hydrolysis of cellulose shows some differences from the fungal cellulolytic system. Many bacterial cellulolytic systems studied to date consist of high molecular weight cellulolytic complexes that are often associated with exocellular structures whereas fungi usually excrete large amounts of cellulase. *Cl. thermocellum* has a discrete cell surface organelle called a cellulosome. It is a high molecular weight, multisubunit protein aggregate possessing both multiple cellulase activities and cellulose binding properties. *Ruminococcus* spp., *Bacteroides* spp. and *Acetivibrio* spp. also form high molecular weight complexes. The complex is often responsible for the adherance of the bacterium to the cellulose substrate so may be designed for the effective delivery of the enzyme to the substrate. The various complementary enzymes are brought into close proximity and possibly into the correct juxtaposition with respect to the substrate to allow synergy among the

components. A high concentration of cellulase can be brought into close contact with the substrate to enable highly efficient disruption of the crystalline structure of the substrate. The complex may be structured in such a way as to enable the protection of various product intermediates and to facilitate their transfer to other cellulase components for further hydrolysis. For example, cellobiose and short chain oligosaccharides produced by the *Cl. thermocellum* cellulosome are metabolized by β-glucosidases or cellobiose oxidases in the periplasmic space. The organism may also conserve cellulase by preventing its random secretion into the medium. Cellulolytic bacteria and fungi differ in their metabolism of glucose. Bacteria take up cellobiose and hydrolyse it intracellularly either by hydrolysis or phosphorylysis. Fungi secrete extracellular β-glucosidases (Beguin *et al.*, 1987).

(d) Adsorption and accessibility

Adsorption and accessibility of a cellulase (or the individual enzymes of the system) to the cellulose substrate depend on many factors. Native cellulose can vary in the extent of crystalline or amorphous areas, the extraneous materials present (for example, lignin or hemicellulose) and the degree of swelling. The topography and surface area also change as degradation proceeds. Additionally, pretreated cellulose substrates vary according to the method of pretreatment (physical, chemical or enzymatic). Substituted celluloses contain groups such as methyl, ethyl, carboxymethyl or hydroxyethyl. The enzyme/substrate ratio, the affinity of the enzyme for the substrate and the physical properties of the enzyme must be considered. The diffusion of the enzyme into or on the substrate is a function of both the nature of the substrate and the physical properties of the enzyme. Other factors important for adsorption and accessibility include the diffusion of products from the cellulose, the effects of such products on the enzyme, the environment (salt concentration, pH and temperature) and other substances that might promote or inhibit adsorption or accessibility (Mullings, 1985, Coughlan, 1985, Marsden and Gray, 1986 and Lamed and Bayer, 1988).

(e) Mechanism of hydrolysis

Endoglucanase and endoxylanase (and β-glucosidase and β-xylosidase) hydrolysis is generally accepted to occur by an acid catalysis mechanism similar to that of lysozyme. Lysozyme hydrolyses a polysaccharide present in bacterial cell walls which is a β-1,4-linked polymer of alternating *N*-acetyl

muramic acid and *N*-acetyl glucosamine. The glycosidic bond is specifically cleaved with retention of configuration (Paice and Jurasek, 1979). Hydrolytic reactions catalysed by endoglucanases and endoxylanases provide overall retention of configuration, similar to lysozyme.

Binding of the substrate positions the glycosidic bond in close proximity to the active residues Glu and Asp (in lysozyme) (Figure 1.4). A proton is transferred from the carboxyl group of the conserved active site Glu residue to the glycosidic bond, which is thereby cleaved. A conformational flattening of the pyranosyl ring accompanies the formation of a carbonium intermediate which is stabilized by a conserved Asp residue. Some of the products diffuse away at this stage. The hydrolysis reaction is completed by the addition of a hydroxyl to the carbonium intermediate and a proton to the side chain of Glu and the release of the remaining product. The active residue in endoglucanases and endoxylanases that donates a proton for the cleavage of the glycosidic bond is thought to be Glu or Asp, while the residue that stabilizes the carbonium intermediate is thought to be Asp or Glu or His (Paice and Jurasek, 1979, Coughlan, 1985, Knowles *et al.*, 1987 and Henrissat *et al.*, 1989).

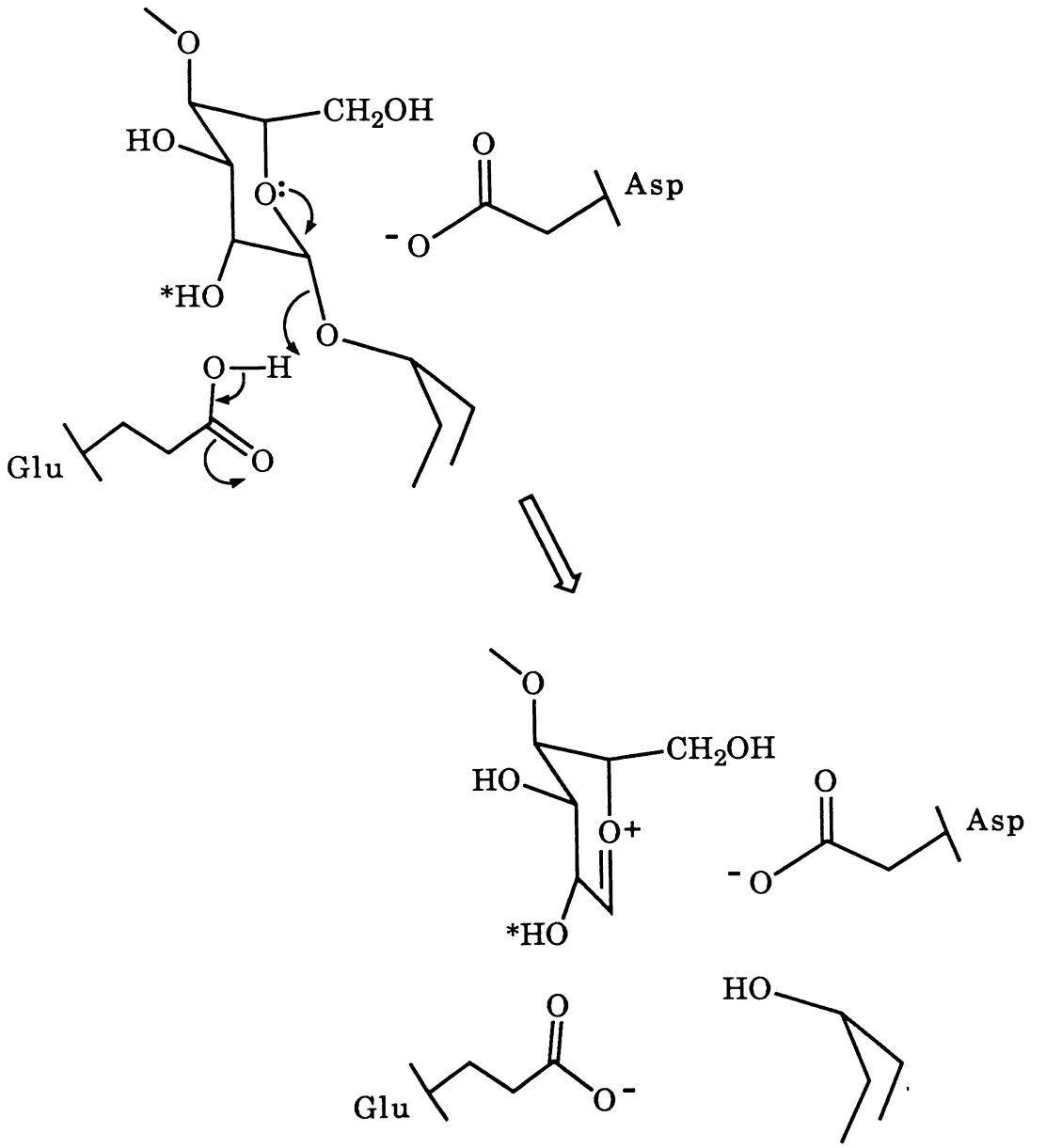
Exoglucanases and exoxylanases produce a product of inverted configuration. No hypothesis has yet been proposed for the hydrolysis mechanism of these enzymes. It is possible that they use a very similar hydrolysis mechanism as the endoglucanases and endoxylanases as the substrates are the same. However, the substrate may be bound in a different orientation with respect to the active residues (West *et al.*, 1989), or the active residues may be oriented differently with respect to the substrate in order to produce a product of inverted configuration. Recently Kanda *et al.* (1986) proposed that glucanases have the potential to catalyse reactions with different substrates by different mechanisms. They found that two fungal glucanases were able to create product configuration de novo, that is, always produce one particular anomeric form without reference to the donor substrate configuration. Claeyssens *et al.* (1990) have found that two fungal cellobiohydrolases also create the anomeric configuration of their reaction products by means that are independent of the substrate configuration. However, these findings may have little relevance in vivo where endoglucanases (and endoxylanases) and exocellobiohydrolases appear to hydrolyse only β-1,4 linkages. Under these circumstances, it can be assumed that only one mechanism is used by each enzyme.

Figure 1.4 Proposed mechanism for endo-catalytic cleavage of β-1,4-linkages in cellulose by analogy with the mechanism of action of lysozyme.

The polysaccharide degraded by lysozyme contains an AcHN- group in place of the *HO- group in cellulose.

The amino acids Glu and Asp appear at the active site in lysozyme, while Glu or Asp, and Asp, Glu or His (respectively) could appear in cellulases.

Based on Paice and Jurasek (1979) and Coughlan (1985).



Although the primary structures of the active sites of lysozymes from different sources are variable, the tertiary structures are conserved. Comparison of the primary structures of glycanases with those of lysozymes has generally been unsuccessful. Indeed, West *et al.* (1989) state that the identification of the active site on the basis of comparison with lysozyme is probably incorrect. A comparison of all carbohydrate binding proteins with known three dimensional structures shows that there is much diversity in structure (Knowles *et al.*, 1987). However, more recent studies in this area have shown some similarities between different glycanase structures. A common structural design is often found in different cellulases, from both the same organism and different organisms. There appear to be separate functional domains. A non-conserved catalytic core protein is linked by a flexible hinge usually rich in proline and hydroxyl amino acids to a highly conserved tail region at either the N- or C- terminal end which is involved in substrate binding or solubilization (McGavin and Forsberg, 1989). Shuffling of gene segments coding for functional domains has been proposed to be the mechanism of protein evolution (Knowles *et al.*, 1987). The clustering of cellulase genes conforms with a pattern well established for gram negative bacteria in which functionally related genes are clustered on the main chromosome (Wynne and Pemberton, 1986).

Henrissat *et al.* (1989) compared the amino acid sequences of 21 β-glycanases by hydrophobic cluster analysis (HCA) and classified them into six families of homologous proteins. HCA is a powerful method for comparing amino acid sequences. It can clearly detect similarities in the three dimensional folding of proteins of very low sequence identity and is very effective in finding homologous domains which are separated by variable segments of widely differing sizes. The comparisons are based on the two dimensional topology and distribution of the hydrophobic clusters along the sequence. Nearly all of these clusters correspond to regular secondary structure elements which constitute the main three dimensional folding cores of globular proteins. The comparisons then, are not based on the maximization of sequence identities but on the successive correspondence of two dimensional structuring elements (hydrophobic clusters). Henrissat *et al.* (1989) listed the possible catalytic residues (Glu, Asp, His) which were conserved in homologous regions for three of the families. In the largest family (of solely endoglucanases) only two such residues were conserved, a Glu and a His. Two other families contained at least one endoglucanase and one exocellobiohydrolase. Another family

contained one exocellobiohydrolase and two endoxylanases. This may indicate that the hydrolysis mechanism of exoglycanases is very similar to that of endoglycanases.

Despite the differences between fungal and bacterial glycanases, a number of homologies still exist. The modes of action show some similarity.

1.4 Some bacterial cellulolytic and xylanolytic systems

1.4.1 *Clostridium* species

The most well studied cellulolytic bacterium is *Cl. thermocellum*. It is an anaerobic thermophile, capable of degrading cellulose and xylan. It produces a particulate, multisubunit protein complex that contains multiple cellulase activities and cellulose binding ability and that is termed a cellulosome (Ait *et al.*, 1979, Ng and Zeikus, 1981, Petre *et al.*, 1981 and Lamed *et al.*, 1983). Xylanase activity has also been found to be associated with the cellulosome. The cellulosome has been described as an integrated multipurpose machine, performing all steps of natural holocellulose degradation (Grepinet *et al.*, 1988a). It is made up of 10 - 50 polypeptides, depending on the strain of *Cl. thermocellum* and the method of analysis. The cellulosome is responsible for the adherence of cells to cellulose (Lamed *et al.*, 1983 and Bayer *et al.*, 1985).

Cellulosomes are either tightly bound or loosely bound, and both forms can be found free in the medium or bound to cellulose. Loosely bound cellulosomes can disaggregate to form free polypeptides (Mayer *et al.*, 1987). The partial disaggregation of free cellulosomes leads to a loss of binding ability (Hon-nami *et al.*, 1985 and Hon-nami *et al.*, 1986). In the cellulosome similarly sized polypeptide subunits are spaced equidistantly and in apparently identical orientation, in rows parallel to the major axis of the cellulosome (Mayer *et al.*, 1987). In some strains the cellulosomes are tightly packed together to form polycellulosomes, which are only found bound to cellulose (Coughlan *et al.*, 1985 and Mayer *et al.*, 1987). Polycellulosomes do not disaggregate easily (Hon-nami *et al.*, 1986). *Cl. thermocellum* has also been found to produce a yellow affinity substance (YAS) which binds to cellulose and facilitates the adherence of the polycellulosome to cellulose (Coughlan *et al.*, 1985). Cellulosomes and polycellulosomes are found to be cell bound early in culture growth but are released later on (Coughlan *et al.*, 1985).

Cellulosomes are not distributed in a random fashion, but appear centralized on protuberant structures which decorate the cell surface at roughly periodic intervals. Such polycellulosomal protuberances are the major cellular loci of bacterial attachment to cellulose (Bayer *et al.*, 1985 and Lamed and Bayer, 1988). Fibrous structures have been found connecting the cells to cellulosomes which are bound to cellulose. They are not directly responsible for binding but may act as contact corridors to direct products to the cell surface (Lamed and Bayer, 1988).

Cellulose-bound polycellulosomes, cellulose-bound cellulosomes and free bindable cellulosomes require Ca^{2+} and DTT for activity on microcrystalline cellulose, but not for activity on CMC (Johnson *et al.*, 1982 and Johnson and Demain, 1984). The largest component of the cellulosome is non-catalytic and may be responsible for anchoring the cellulosome to the cell surface (Hon-nami *et al.*, 1986). Alternatively it may be responsible for the organization of component parts into a complex or may have a cellulose binding role (Lamed and Bayer, 1988). Wu *et al.* (1988) found two components from the cellulosome that could act synergistically to produce activity on Avicel. The smaller component had activity on CMC while the larger component had no activity. It was suggested that the larger component may act as an anchoring component.

When bound to cellulose the cellulosome may provide for simultaneous multicutting events by endoglucanases, thereby alleviating the necessity for exocellobiohydrolases. The existence of exoglucanases in the *Cl. thermocellum* cellulolytic system has yet to be shown. Cellulosomes are resistant to dissociation with the retention of activity of individual components making study of this system difficult, a problem to which gene cloning is a useful solution.

A large number of genes coding for cellulolytic and hemicellulolytic enzymes have been identified in *Cl. thermocellum* (Hazlewood *et al.*, 1988). They include at least fifteen endoglucanases, a β-glucosidase and two xylanases. Some of the gene products have been purified and/or characterized and compared to the native enzymes. Five endoglucanases, named endoglucanase A, B, C, D and E with corresponding genes labelled *celA*, *B*, *C*, *D* and *E* respectively, are all active on CMC but with differing specific activities. Endoglucanases A and B are not active on MUC or *p*NPC whereas endoglucanases C, D and E are active. Endoglucanases A and C have high

activity on lichenan (a β-1,3-1,4-glucan) and very high activity on barley β-glucan (also a β-1,3-1,4-glucan). These endoglucanases may non-specifically hydrolyse 1,3 and 1,4 bonds (Schwarz *et al.*, 1986 and Schwarz *et al.*, 1988). Additionally endoglucanase C has very high activity on *p*NPC releasing only cellobiose, which is also the product released from cellotriose, cellotetraose and cellopentaose (Petre *et al.*, 1986). Endoglucanase E also has activity on xylan (Hall *et al.*, 1988). Endoglucanase D binds Ca^{2+} and this increases its thermostability (Chauvaux *et al.*, 1990).

Endoglucanases A, B, D and E all contain a homologous sequence approximately 23 amino acids long that is reiterated and preceded by a PT box or PT-like region. It appears at the C-terminal end of endoglucanases A and B and in the middle of endoglucanase E (Beguin *et al.*, 1985, Grepinet and Beguin, 1986 and Hall *et al.*, 1988). Deletion of the reiterated sequence in endoglucanase E has shown that it is not important for catalytic activity. It is possibly required for the binding of crystalline substrates or to anchor the enzyme in the cellulosome structure. Although it shows similarities with the Ca^{2+}-binding sites of several Ca^{2+}-binding proteins, the reiterated sequence seems not to be required for Ca^{2+} binding, by endoglucanase D at least (Chauvaux *et al.*, 1990). Xylanase Z (with a gene labelled *xynZ*) also contains the reiterated sequence in the middle of the enzyme but it is not preceded by a PT box. However, there are two small PT boxes elsewhere in the sequence. The C-terminal third of the enzyme has been found to be responsible for catalytic activity (Grepinet *et al.*, 1988b). Xylanase Z releases xylobiose and xylotriose from xylan. It has very high activity on *p*NPXX, low activity on *p*NPC, *p*NPX and *p*NPG and no activity on CMC, cellobiose, cellotriose, cellotetraose and cellopentaose. MacKenzie *et al.* (1989) have described a further three xylanases produced by *Cl. thermocellum*. One produces low molecular weight products and extensive reducing sugars from xylan substrates. The other two are atypical xylanases, producing high molecular weight products from aspen and larchwood xylans and having no activity on oat spelts xylan. At least two β-glucosidases are produced, one of which has activities in the following order: MUC > MUG > cellobiose > cellotriose > cellotetraose > cellopentaose, with no activity on CMC (Kadam and Demain, 1989).

The cellulase complex of *Cl. cellulovorans* has been investigated by Shoseyov and Doi (1990). They found a large component that is non-catalytic, but has a very high affinity for cellulose. Although CMCase and

cellobiohydrolase components could bind to microcrystalline cellulose, the large non-catalytic component was required for activity. They suggested that the large component could serve as a core protein to which other subunits bind. The complex formed may then facilitate cooperative action between CMCases and cellobiohydrolases. Alternatively, the large component may convert cellulose into a structure amenable to attack by subunits.

Two xylanases have been purified from the culture supernatant of *Cl. acetobutylicum*. Xylanase A has a higher molecular weight and high activities on xylan and CMC while xylanase B has a lower molecular weight and high activities on xylan and lichenan. Both produce xylobiose and xylotriose on prolonged hydrolysis of xylan. Xylanase A also produces higher xylooligosaccharides. The smallest xylooligosaccharides degraded by xylanases A and B are xylohexaose and xylotetraose, respectively (Lee *et al.*, 1987). Zappe *et al.* (1987) have cloned a xylanase gene, the product of which appears similar to xylanase B.

Cl. stercorarium has been shown to produce an endoglucanase, exocellobiohydrolase, xylanase and a cell-bound β-glucosidase (Creuzet *et al.*, 1983). No yellow pigment is produced (Berenger *et al.*, 1985). One endoglucanase, three exoglucanases and a β-glucosidase were identified by Bronnenmeier and Staudenbauer (1988). Three cellulase genes have been cloned by Schwarz *et al.* (1989). *CelZ* codes for an endoglucanase having activity on CMC, low activity on Avicel and no activity on *p*NPC, *p*NPG or cellobiose. An exocellobiohydrolase is encoded by *celX*. It has activity on MUC, low activity on CMC and no activity on *p*NPG, MUG and cellobiose. Finally a β-glucosidase is coded for by *bglZ*. It has activity on MUC, MUG, *p*NPC, *p*NPG and cellobiose with low activity on CMC. Three xylanases (A, B and C) have been purified. All are of high molecular weight, have similar properties and are not active on CMC, *p*NPX and *p*NPG. Xylobiose and xylotriose are released on prolonged hydrolysis of xylan. The three xylanases may be formed by the partial proteolysis of a common enzyme precursor (Berenger *et al.*, 1985).

1.4.2 *Cellulomonas* species

Ce. fimi is a facultatively anaerobic, mesophilic coryneform bacterium. Its cellulolytic system appears to consist of three components with great affinity for insoluble cellulosic substrates. Enzymes free in the supernatant are subject to proteolysis and deglycosylation, with up to ten products being formed. All

have reduced ability to bind to insoluble cellulosic substrates, though some retain enzyme activity (Langsford *et al.*, 1984). The three components are an exocellobiohydrolase and two endoglucanases (A and B). The former two components have been purified from culture supernatants. The genes for all three components have been separately cloned and genes for the former two components have been sequenced (Gilkes *et al.*, 1984 a and b and O'Neill *et al.*, 1986). The overall structures show three distinct domains. The exocellobiohydrolase has an N-terminal catalytic domain and a C-terminal binding domain separated by a PT box. Endoglucanase A has an N-terminal binding domain and a C-terminal catalytic domain separated by a PT box. The binding domains show homology and the PT boxes are highly conserved. The binding regions are irregular, rich in hydroxy amino acids and have a low charge density. The catalytic domains are ordered and have a higher charge density. The PT boxes may be a hinge region and are a site of glycosylation. It is proposed that the genes arose through duplication and shuffling (Warren *et al.*, 1986 and Langsford *et al.*, 1987). The catalytic domain of the exocellobiohydrolase shows homology with *Bacillus* sp. strain C125 xylanase A and the N-terminal fragment of *Cryptococcus albidus* xylanase. Endoglucanase A shows homology with *Trichoderma reesei* exocellobiohydrolase II (West *et al.*, 1989). A bifunctional fusion protein of the exocellobiohydrolase and endoglucanase catalytic regions has been made and expressed in *E. coli*. It hydrolysed CMC and *p*NPC but did not bind to microcrystalline cellulose (Warren *et al.*, 1987). The binding domains of exocellobiohydrolase and endoglucanase A have been used to make hybrid proteins for the purification and/or immobilisation of a β-glucosidase and an alkaline phosphatase (Ong *et al.*, 1989 and Greenwood *et al.*, 1989). The exocellobiohydrolase acts on MUC, *p*NPC, CMC, lichenan (releasing cellobiose with inversion of configuration) and xylan. Endoglucanases A and B have activity on CMC and lichenan. Endoglucanase A has been shown to release products with retention of configuration. None of the enzymes have activity on *p*NPG or *p*NPX (Gilkes *et al.*, 1984b, O'Neill *et al.*, 1986 and Gilkes *et al.*, 1988). The xylanolytic activities (if any) of *Ce. fimi* have not been studied.

Some enzymes from *Cellulomonas* sp. ATCC21399 have been studied. Three endoglucanases (A, B and C) have been purified and one xylanase partially purified from the culture supernatant. All of the endoglucanases have activity on CMC, β-1,3-1,4-glucans and xylan. However, the specific activities of endoglucanases A, B and C on xylan are 2.7-, 4.0- and 3.5-fold higher

(respectively) than those on CMC. These enzymes may be better classified as endoxylanases. Endoglucanase A has a slightly higher specific activity on β-1,3-1,4-glucans than xylan and also has low activity on Avicel. The xylanase only has activity on xylan (Poulsen and Petersen, 1989 a and b).

Ce. flavigena has bound and free cellulase activities (Sami *et al.*, 1988). Two cellulase genes from *Ce. flavigena* have been cloned in to *E. coli*. One is homologous to *Cl. thermocellum celB* and the other to both *Ce. fimi* exocellobiohydrolase and endoglucanase A (Akhtar *et al.*, 1988).

Two cellulase components (A and B) from *Ce. fermentans* have been purified. Both have activity on CMC and low activity on *p*NPC and xylan (Bagnara *et al.*, 1986).

1.4.3 *Bacillus* species

Two xylanases (A and B) and a β-xylosidase secreted by *B. circulans* have been purified. Xylanase A has a high molecular weight and produces xylose, xylobiose and higher xylooligosaccharides from xylan. Xylanase B has a low molecular weight and produces xylobiose and higher xylooligosaccharides from xylan (Esteban *et al.*, 1982). Genes for two xylanases (P and R) have been cloned from a different strain of *B. circulans* into *E. coli*. Xylanase P has a high molecular weight but is not similar to Xylanase A. Xylanase P is unable to extensively hydrolyse xylan and has limited reducing sugar production. Xylanase R shows some similarity to xylanase B and also shows homology with *B. subtilis* xylanase, *B. polymyxa* xylanase and *B. pumilus* xylanase (Yang *et al.*, 1988 and Yang *et al.*, 1989 a and b).

The *B. subtilis* xylanase has a low molecular weight and has activity on xylan but not CMC or *p*NPX. It shows homology with *B. pumilus* xylanase (Bernier *et al.*, 1983 and Paice *et al.*, 1986). An endoglucanase from *B. subtilis* has also been purified. It has activity on CMC, low activity on *p*NPC and none on *p*NPG (Nakamura *et al.*, 1987).

Three enzymes from *B. pumilus* IPO have been studied. *XynA* codes for a xylanase having greatest affinity for the second to sixth linkages from the terminus of larchwood xylan. It produces xylobiose and higher xylooligosaccharides from xylan. This low molecular weight enzyme has no activity on xylobiose, a very low amount of activity on CMC and shows

transxylosylation activity on xylooligosaccharides. A β-xylosidase is encoded by *xynB* (Panbangred *et al.*, 1983). *XynA* and *xynB* are found to be closely linked on the chromosome (Moriyama *et al.*, 1987). Finally a β-xylosidase is coded for by *xynC*. It prefers *p*NPX rather than xylobiose (Panbangred *et al.*, 1985).

The alkalophilic *Bacillus* sp. strain C125 produces two xylanases (A and N) which have high and low molecular weights (respectively). Both produce xylobiose and higher xylooligsaccharides from xylan, show transxylosylation activity and do not hydrolyse xylobiose or xylotriose (Honda *et al.*, 1985 a and b).

The alkalophilic *Bacillus* sp. strain N-4 resembles *B. pasteurii*. Three cellulase genes show partial homology with each other and have possibly arisen by gene duplication. *CelA* and *celB* contain a sequence approximately 60 amino acids long that is reiterated in *celB*. It appears at the C-terminal end of both sequences. These two genes are in tandem location on the chromosome (Fukumori *et al.*, 1986, 1987 and 1989).

The thermophilic, acidophilic *Bacillus* sp. strain 11-1S produces an extracellular xylanase and a cytoplasmic β-xylosidase. The xylanase produces mainly xylobiose and xylotriose from xylan, and cellobiose and higher oligosaccharides from CMC (Uchino and Nakane, 1981). The xylanase has a higher specific activity on CMC than on xylan and may be better classified as a broad specificity endoglucanase.

Bacillus sp. strain W1 (JCM2888), *Bacillus* sp. strain W2 (JCM2889) and *Bacillus* sp. strain K17 each produce two xylanases. In each case xylanase I has a low molecular weight and produces xylobiose and higher xylooligosaccharides from xylan, and xylanase II has a high molecular weight and produces xylose, xylobiose and higher xylooligosaccharides from xylan. Both xylanases from *Bacillus* sp. strain W1 (JCM2888) and *Bacillus* sp. strain W2 (JCM2889) show transferase activity (Okazaki *et al.*, 1985 and Kang *et al.*, 1986).

Cellulase K produced by alkalophilic *Bacillus* sp. strain KSM-635 has activity on CMC, very low activity on *p*NPC and Avicel and no activity on xylan and *p*NPG. The molecular weight of cellulase K is high and varies depending on culture conditions (Ito *et al.*, 1989).

Bacillus sp. strain KSM-522 is taxonomically similar to *B. pumilus*. It produces two alkaline endoglucanases, the properties of which are indistinguishable from each other. They have activity on CMC, low activity on xylan and no activity on *p*NPC, *p*NPG, cellobiose, cellotriose, cellotetraose and Avicel. They produce glucose, cellobiose and cellotetraose from cellopentaose and cellohexaose. A further endoglucanase is produced that has activity on CMC and lichenan and shows no activity on *p*NPC, *p*NPG, cellobiose and Avicel. It degrades cellotriose and cellotetraose to glucose and cellobiose, and cellopentaose and cellohexaose to cellobiose and cellotriose (Kawai *et al.*, 1988 and Okoshi *et al.*, 1990).

1.4.4 *Ruminococcus* species

R. albus is an anaerobic rumen bacterium. In some strains adherence to cellulose and plant fibres is a prerequisite for effective cellulose degradation (Morris, 1988). *R. albus* produces both high and low molecular weight cellulase complexes, composed of numerous polypeptides. These large surface bound complexes contain cellulase enzymes and are similar to the cellulosomes of *Cl. thermocellum*. Ten distinct cellulase genes including three β-glucosidase genes were reported to have been found in a large gene library. Two had activity on CMC but not *p*NPC or *p*NPG. Four had activity on CMC and MUC, low activity on *p*NPC and none on *p*NPG. One had activity on CMC, MUC and *p*NPC, with lower activity on *p*NPG. Three had activity on *p*NPG, with lower activity on MUC and *p*NPC and low activity on CMC (Howard and White, 1988). Ware *et al.* (1989) isolated four cellulase genes and studied two in detail. Both had high activities on CMC, low activities on crystalline cellulose and none on MUC and MUG. One also had high activity on xylan.

R. flavefaciens is also an anaerobic rumen bacterium that produces a high molecular weight cellulase complex. Exoglucanase A was purified from the culture supernatant and has activity releasing cellobiose from *p*NPC. It also has some activity on *p*NPL, low activity on *p*NPX and none on *p*NPG and CMC. It is subject to cellobiose inhibition and requires Ca^{2+} for maximum activity (Gardner *et al.*, 1987). One large gene library produced 26 clones with activity on CMC that fell into four groups. Two groups had activity only on CMC, another group had activity on CMC, MUC, *p*NPC and lichenan with variable activities on Avicel and the final group had activity on CMC with variable activities on MUC, MUG, *p*NPC, *p*NPG, cellobiose, xylan, lichenan and

Avicel (Huang *et al.*, 1989). A gene library from a different strain produced five clones having activity on xylan, one also had activity on MUX while another also had activity on CMC. All those without activity on CMC had no activity on *p*NPC, *p*NPL, *p*NPG and Avicel (Flint *et al.*, 1989).

1.4.5 Other species

A gene cluster from *Cellvibrio mixtus* has been cloned into *E. coli*. Cellulase activity was found to be encoded, as were a yellow water insoluble pigment and the characteristic of an increased adherence to solid surfaces (Wynne and Pemberton, 1986).

Acetivibrio cellulolyticus cells do not aggregate around cellulose particles in the culture. *A. cellulolyticus* has a soluble cellulase system which adsorbs to cellulose in the culture medium. The complex consists of an array of proteins and requires Ca^{2+} and DTT for maximum hydrolysis of Avicel. The culture supernatant includes a β-glucosidase which has a strong preference for *p*NPG over cellobiose. The exoglucanase studied produces cellobiose from Avicel (Saddler and Khan, 1981, MacKenzie and Bilous, 1982 and MacKenzie *et al.*, 1987).

One of the most active cellulolytic rumen microorganisms is the anaerobic bacterium *Bacteroides succinogenes*. It produces an extracellular endoglucanase complex containing a multiplicity of cellulases. Some of the cellulolytic activity is membrane bound. A gene has been found that produces an enzyme with high activity on lichenan, CMC and *p*NPC and low activity on xylan. *p*NPC is hydrolysed to cellobiose and *p*NP (Taylor *et al.*, 1987). Endoglucanase 2 has a high affinity for amorphous cellulose. Proteolysis decreases binding ability but not endoglucanase activity. Endoglucanase 2 has a bifunctional organisation, consisting of a substrate binding domain and a catalytic domain (McGavin and Forsberg, 1989). A xylanase has been cloned in to *E. coli* that has high activity on xylan and very low activity on CMC (Sipat *et al.*, 1987).

An endoglucanase from *Butyrivibrio fibrisolvens* has been cloned and sequenced. It has high activity on CMC and lichenan, low activity on xylan and *p*NPC and has no activity on *p*NPG or *p*NPX. It shows homology with *Cl. thermocellum* endoglucanase E (Berger *et al.*, 1989).

Three xylanases (L, M and S) that possess no homology with each other have been found in the alkalophilic *Aeromonas* sp. no.212 (ATCC31085). Xylanase L appears to have the highest molecular weight of any bacterial xylanase reported to date (Kudo *et al.*, 1985 and Kato *et al.*, 1986).

Pseudomonas fluorescens subsp. *cellulosa* produces multiple cellulase activities. One membrane bound endoglucanase and two extracellular endoglucanases, one a post translational modification of the other, have been described (Lejeune *et al.*, 1988). Hong *et al.* (1990) have cloned and purified two endoglucanases, one of which appears to be a post translational modification of the other. These endoglucanases are very similar to those described by Lejeune *et al.* (1988). A different endoglucanase gene has been sequenced, the product of which undergoes proteolysis in bacteria to produce multiple endoglucanases of lower molecular weights. This enzyme has an N-terminal catalytic region (Hall and Gilbert, 1988). A xylanase gene is adjacent to the endoglucanase gene. Three xylanase clones all had activity on xylan, MUC and *p*NPC. Two showed no activity on CMC, xylobiose and *p*NPG (Gilbert *et al.*, 1988).

Streptomyces lividans produces two xylanases. Xylanase A progressively fragments in to several lower molecular weight species that are still active in the culture supernatant. It is active on xylan, producing mainly xylobiose, some xylooligsaccharides and a small amount of xylose. It does not have activity on CMC, *p*NPX or xylobiose (Morosoli *et al.*, 1986). Xylanase B produces mainly xylobiose and xylotriose, and some larger xylooligosaccharides from xylan. It has no activity on CMC or MUX. Xylanase B preferentially degrades longer water-insoluble xylan molecules, while xylanase A hydrolyses short chain xylooligosaccharides such as those produced by xylanase B (Kluepfel *et al.*, 1990).

The extremely thermophilic anaerobe strain NA10 (IFO14436) produces a multicomplex of cellulases. Three genes have been cloned into *E. coli*. Two genes code for endoglucanases that have activity on CMC, lichenan and one of MUC or *p*NPC and low activity on xylan. The third gene may code for a laminarinase as there is high activity on laminarin and *p*NPG and none on CMC, *p*NPC, MUC or cellobiose (Honda *et al.*, 1988 a and b).

Strain BW is different from *Cl. thermocellum*. It produces cellulase and xylanase but no yellow pigment. An endoglucanase has been purified that has activity on CMC and low activity on xylan (Creuzet and Frixon, 1983).

1.4.6 *'Caldocellum saccharolyticum'*

'C. saccharolyticum' strain Tp8T6.3.3.1 is the source of the enzymes under study in this thesis. It is an extremely thermophilic bacterium isolated from decomposing wood in a thermal spring near Lake Taupo, New Zealand (Sissons *et al.*, 1987). This organism has an optimum growth temperature of 68°C and produces a very thermostable cellulase complex. It degrades cellulose, xylan, starch and a wide variety of simple sugars. The end products of fermentation are CO_2, H_2, acetate and propionate. *'C. saccharolyticum'* is an obligate anaerobe and has a pH optimum of 7. The cells are rod-shaped with tapered ends, they are non-motile and do not form spores. The results of DNA-DNA hybridisation and other experiments have confirmed that this isolate is not closely related to the thermophilic cellulose degraders *Cl. thermocellum* and *Cl. stercorarium* (Donnison *et al.*, 1986 and 1989). Cellulolytic activity is bound to cellulose in the culture medium, however, there is not a high degree of bacterial binding to cellulose (Reynolds *et al.*, 1986).

Sharrock (1985) attempted to purify cellulolytic enzymes from the native organism but had difficulty due to the multiplicity of the enzymes. Genes coding for *'C. saccharolyticum'* cellulolytic and hemicellulolytic enzymes were cloned in to *E. coli* (Bergquist *et al.*, 1987) leading to more successful purification. The enzymes derived from clones have been given trivial names based on the substrate they were able to hydrolyse, until the mode of action could be studied in detail and the enzyme classified.

Luthi *et al.* (1990) found five open reading frames (ORF1, 2, 3, 4 and 5) in tandem location on the chromosome (Figure 1.5). ORF1 is named *xynA* and codes for a xylanase. Luthi *et al.* (in press a) have made further studies of the xylanase. This xylanase was investigated in detail in this study. Results of heat treatment purification appear in Section 3.2, substrate specificity experiments in Section 4.2 and purification and characterization in Chapter 5.

ORF2 is named *xynC*, possibly codes for an acetyl esterase and is further described by Luthi *et al.* (in press b). No activity could be detected in clones containing ORF3 and ORF4.

ORF5 is named *xynB* and codes for a β-xylosidase (Luthi and Bergquist, 1990). The β-xylosidase was purified and studied by Hudson *et al.* (in press) and described as an aryl β-xylosidase. In this thesis, results of heat treatment

purification of the β-xylosidase appear in Section 3.2 and substrate specificity experiments in Section 4.1.

A β-glucosidase encoded by *bglA* (Love *et al.*, 1986, Love and Streiff, 1987 and Love *et al.*, 1988) was purified and studied in detail by Plant *et al.* (1988). They found it had a broad substrate specificity. Section 3.2 of this study contains results of heat treatment purification and Section 4.3, results of substrate specificity experiments.

A CMCase is encoded by *celA* (Streiff *et al.*, 1986) (Figure 1.6). It was studied in outline by Neal (1987) who found it had activity on CMC only, with insignificant activity on xylan, Avicel and MUC. The CMCase was also investigated in this thesis. Heat treatment purification results are in Section 3.2 and substrate specificity results in Section 4.4.

CelB codes for an enzyme with activity on both MUC and CMC (Figure 1.6). The enzyme has three domains separated by PT boxes. MUCase activity is coded for by the N-terminal domain and CMCase activity by the C-terminal domain (Saul *et al.*, 1989). In this study results of heat treatment purification appear in Section 3.2, substrate specificity results in Section 4.5, and results of further studies in Chapter 6.

The clones used in this study are listed in Table 1.2. Each clone is known by a PB number. The enzyme activity of interest expressed by each clone and the DNA fragment (known by a pNZ number) incorporated into each clone are listed in Table 1.2. Those DNA fragments that are smaller fragments of each other, or that are found close to each other on the chromosome are shown in Figures 1.5 and 1.6.

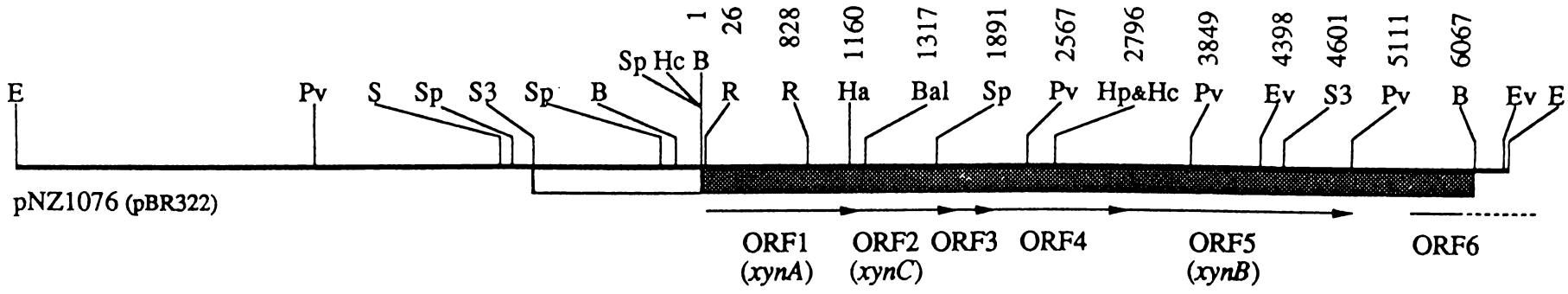
1.5 Methods of assay

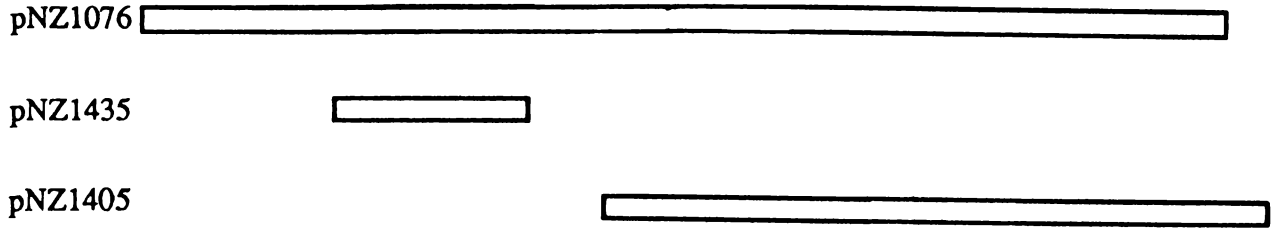
1.5.1 Nature of the substrate

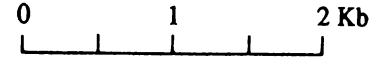
Natural substrates are often insoluble and structurally variable and rarely totally pure. They are undefined with respect to concentration and chemical form (Ghose, 1987). Pretreatment of substrates by the manufacturer or laboratory worker also leads to variable purity and form. Pretreatments may be physical or chemical and can include ballmilling, pulping, cutting, grinding, steam explosion, chemical treatments with NaOH, acid, NH_3 or SO_2, heat,

Figure 1.5 Gene maps of xylanolytic enzymes from '*C. saccharolyticum*'.

Based on Luthi *et al.* (1990).







ORF1 (*xynA*) :	xylanase	40.5kD
ORF2 (*xynC*) :	acetyl esterase	30.6kD
ORF3 :	not known	10.7kD
ORF4 :	not known	36.5kD
ORF5 (*xynB*) :	ß-xylosidase	56.4kD or 55.5kD

Restriction enzyme abbreviations

B	*Bam*HI	Hc	*Hinc*II
Bal	*Bal*I	Pv	*Pvu*II
E	*Eco*RI	R	*Rsa*I
Ev	*Eco*RV	S	*Sal*I
Ha	*Hae*III	S3	*Sau*3AI
Hp	*Hpa*I	Sp	*Sph*I

Figure 1.6 Gene maps of cellulolytic enzymes from '*C. saccharolyticum*'.

Based on Streiff *et al.*, (1986) and Love and Saul (unpublished results).

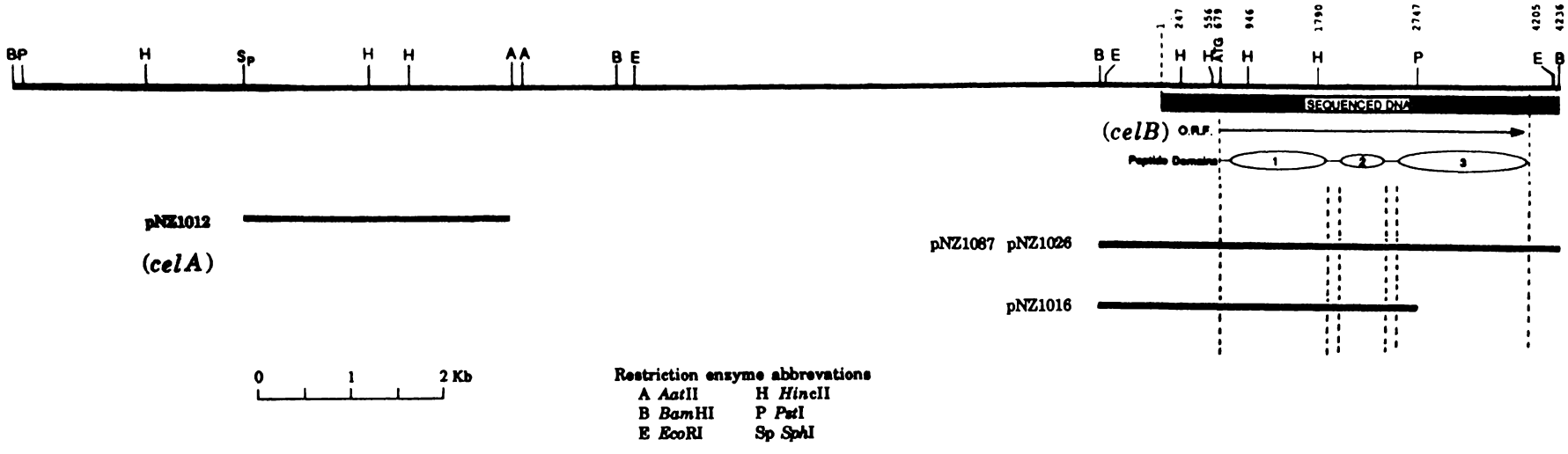


Table 1.2 List of clones used in this study, showing their PB numbers and the DNA fragments incorporated.

Enzyme activity of interest expressed by clone	Clone number	DNA fragment
β-xylosidase	PB4855[a]	pNZ1405
xylanase	PB4716[b]	pNZ1076
xylanase	PB4887[c]	pNZ1435
β-glucosidase	PB4551[d]	pNZ1001
CMCase	PB4563[b]	pNZ1012
MUCase	PB4800[a]	pNZ1087
MUCase	PB4567[b]	pNZ1016
MUCase	PB4574[b]	pNZ1026
MUCase	X[a]*	pNZ1016

* no PB number exists for this clone

E. coli host strains: [a] PB2946
[b] PB2477
[c] TG1
[d] PB2481

solubilization by sonication, removal of low molecular weight substances, removal of insoluble particles, swelling by water and the covalent linking of substituent groups (such as carboxymethyl or hydroxyethyl) or dyes to the substrate. Substituent groups make cellulose less crystalline, more soluble in water and more susceptible to degradation. Most pretreatments serve to decrease crystallinity and particle size and to increase surface area and solubility (Mullings, 1985 and Marsden and Gray, 1986).

The hydrolysis rate of a substrate is affected by its source and pretreatment and furthermore, the structure of the substrate probably changes throughout hydrolysis. Often a fast rate of release of reducing sugars from

crystalline substrates is observed initially followed by a reduced rate, a phenomenon due to amorphous regions being attacked first. Initial rates of hydrolysis may therefore be meaningless (Coughlan, 1985). Such variables make the measurement of activity on any substrate not only difficult, but incomparable theoretically to any other measurements. The best way of reducing these variables is to use widely available substrates with little or no laboratory pretreatment. If pretreatment must be used it should be totally reproducible.

1.5.2 Methods measuring enzymatic hydrolysis of substrates

Most enzyme theory is based on reactions in solution. The cellulolytic system involves an interface between enzymes (either free in solution or bound to the cell surface) and an insoluble substrate surface (Sharrock, 1988). The hydrolysis of insoluble cellulose is inconvenient to assay and there are no simple enzyme assays to accurately measure activity on crystalline cellulose (Knowles *et al.*, 1987). For this reason and those reasons in the preceding section (Section 1.5.1), there are almost as many cellulase (and hemicellulase) assays as there are workers in the field. Only the more common methods will therefore be described.

The three main types of assay measure physical changes, the total loss of substrate and products accumulating during hydrolysis (Mullings, 1985). There are disadvantages with each type. The first two are relatively insensitive and the latter is often complicated by transferase activity (Paice and Jurasek, 1979). Only the product actually measured can be stated to be produced. For example, activity on *p*NPG produces *p*NP but not necessarily glucose in stoichiometric amounts. Further complications arise when impure enzyme preparations or culture supernatants are assayed. Not only may there be activity by several components but the components may act synergistically. It is most important to include appropriate controls, such as enzyme-only and substrate-only, in assays (Ghose, 1987). Attempts to standardize xylanolytic and cellulolytic assays have been made (Ghose and Bisaria, 1987 and Ghose, 1987).

(a) Total sugars

Total soluble sugars can be assayed by the cysteine/phenol/sulphuric acid method. All traces of substrate must be removed before adding the

reagent. The method is much more sensitive to glucose than galactose and pentoses such as xylose and arabinose (Halliwell and Halliwell, 1984 a and b).

(b) Reducing sugars

There are four main methods of reducing sugar measurement. All involve the reduction of a substance to give a product that can be measured colourimetrically. In the DNS method, 3,5-dinitrosalicylic acid is reduced to 3-amino-5-nitrosalicylic acid which is a red colour. This method was first developed by Sumner (1921) (and Sumner and Howell, 1935) and modified by Miller (1959). In the Nelson and Somogyi method, Cu(II) salts are reduced to Cu(I)oxide which reduces arsenomolybdate to molybdenum blue (Nelson, 1944 and Somogyi, 1952). A further method involves the reduction of ferricyanide to ferrocyanide which reacts with ferric ions to produce Prussian blue (Halliwell, 1965 and Halliwell and Riaz, 1970). The final method is the PAHBAH method developed by Lever (1973). *p*-hydroxybenzoic acid hydrazide is reduced to *p*-hydroxybenzoic acid hydrazone which then forms a yellow coloured calcium chelate.

There are several disadvantages to measuring reducing sugars. The same sample will give differing results depending on the reducing sugar assay system used. The response also varies from sugar to sugar as the aldehyde group reacts differently depending on the residue to which it is attached. The final ratio of various products affects the results and this ratio often changes during hydrolysis (Sharrock, 1988). Furthermore, various substances can interfere with the assay. Koziol (1981) has described the effect of some compounds on the PAHBAH assay and lists interferences to other reducing sugar assays.

(c) Specific sugars and oligosaccharides

The total amount of glucose present can be measured using the glucose oxidase/peroxidase colorimetric assay (Raabo and Terkildsen, 1960). Alternatively, total sugars present as small oligosaccharides can be measured by converting all sugars to glucose using a β-glucosidase. These assays require that no inhibitory substances are present in the assay.

Cellobiose produced during the cellulase reaction can be assayed continuously using a cellobiose dehydrogenase assay (Canevascini, 1985).

Cellobiose is oxidised with ferricyanide and a cellobiose dehydrogenase using a modified method of Ameyama (1982). This enzyme is specific so glucose does not contribute to the result. β-glucosidase interference can be eliminated by D-glucono-1,5-lactone inhibition. One advantage of this method is the lack of product inhibition during the cellulase assay, due to the continuous removal of cellobiose. The disadvantage is that the enzyme cellobiose dehydrogenase is not readily available.

Sugars and oligosaccharides present at any stage of cellulose hydrolysis can be measured using HPLC, with refractive index detection. This method has the advantage of qualitative product identification but the disadvantage is that it is time consuming and not suited for large numbers of assays (Sharrock, 1988).

(d) Viscometry

When acting on soluble cellulose derivatives, endoglucanases cause a rapid decrease in chain length and hence viscosity. Plots of increase in relative fluidity versus reducing equivalents released may be used to compare individual endoglucanases and serve as an indicator of the 'randomness' of attack on the substrate. However, ionic substituted celluloses (such as CMC) are not the ideal substrates for viscometric assays as the viscosity of an ionic substrate depends on pH, ionic strength and polyvalent cations. Non-ionic substituted celluloses (for example, HEC) are preferred for the determination of low endoglucanase activities. Temperature also has a significant effect on CMC viscosity. Chain length and degree of substitution can vary depending on the source of CMC. Viscometry is also not suited for large numbers of assays (Sharrock, 1988 and Enari and Markkanen, 1977).

The rapid decrease in chain length of substrates incorporated into solid media (agar plates for bacterial growth, electrophoresis gels or activity overlays) can be detected by staining with Congo Red (Wood, 1980). Congo Red is an anionic dye that complexes with polysaccharides containing five or more contiguous β-1,4-linked glucose units, so unstained areas indicate activity.

(e) Chromogenic substrates

Many substrates are now available based on derivatives of *p*NP (or *o*NP) and MU. These substrates are small and are generally used to assay exo-acting

enzymes or β-glycosidases. Details on many MU substrates and their use in assays are given by Leaback (1975).

Dyes such as Remazol Brilliant Blue have been covalently linked to a number of natural substrates, for example Avicel, HEC and xylan. Enzyme action produces soluble fragments of the substrate with dye attached. These remain in solution when the large substrate molecules are removed by precipitation and/or centrifugation and can be quantified absorptiometrically. Dyed substrates can also be incorporated into solid media. Activity is detected after washing as bands of reduced staining. However the dye may be non-uniformly distributed throughout the substrate. The separation of soluble dyed polysaccharide fragments from the large substrate molecules may be affected by many parameters such as ionic strength and temperature (Sharrock, 1988). The dye may also be inhibitory.

(f) Other methods

Residual substrate can be measured by weight or chemical means. Although this is insensitive, tedious and adsorbed proteins and bacteria must first be removed, it is very reliable (Sharrock, 1988).

The tensile strength of strips of cotton subjected to enzyme treatment can be measured; but this is not widely used.

Turbidometric measurements of both finely powdered cellulose and insoluble xylan can be made, but sometimes the optical density increases before decreasing.

Finally, isotope release, that is, the appearance of ^{14}C-labelled sugars, can be used. The method is simple and sensitive, but expensive.

Chapter Two

Methods

2.1 Enzymatic assays

2.1.1 Specific assays on substrates

(a) Xylan

0.4ml of a xylan suspension (0.25% w/v) in buffer was preheated in glass tubes in at least quadruplicate. 0.1ml of an enzyme sample was added to two of the four tubes and incubated at 70°C for 10 minutes (t=10). All assays were stopped by the addition of 1.0ml PAHBAH reagent. 0.1ml of the enzyme sample was then added to the remaining two tubes (t=0). The PAHBAH method was used to detect reducing sugars released. Oat spelts xylan was routinely used, except where otherwise stated. Standards were 0.1ml of 0 - 0.8mM D-xylose (in distilled water) added to 0.4ml buffer, with reducing sugars being assayed by the addition of 1.0ml PAHBAH reagent. Unless otherwise stated, activity on xylan is expressed as μmol(xylose equivalents) $min^{-1}mg^{-1}$. Xylan suspensions were made freshly or stored at 4°C for short periods.

Dialysed xylan suspensions were made to remove any low molecular weight reducing sugars that may have been present. 25ml of a xylan suspension (4% w/v) was extensively dialysed (3 500 molecular weight cutoff, Spectra/Por 3) against 2 x 2l distilled water and 2 x 2l 0.1M sodium citrate/citric acid buffer (pH 6.0) at 4°C. The dialysed xylan suspensions were then made up to 100ml (1% w/v equivalent) with 0.1M sodium citrate/citric acid buffer (pH 6.0) for assay.

Insoluble and soluble fractions of xylan were prepared for assay by heating 100ml of a xylan suspension (0.25% w/v in 10mM MES/NaOH buffer pH $6.0_{70°C}$) to 70°C for 60 minutes, with occasional agitation, for maximum dissolution under assay conditions and then centrifuging (47 800 xg, 30min, 20°C). The supernatant was retained as the soluble fraction. The pellet was washed with 10mM MES/NaOH buffer (pH $6.0_{70°C}$) and centrifuged, as above. The resulting pellet was resuspended in 100ml 10mM MES/NaOH buffer (pH $6.0_{70°C}$).

The proportion of insoluble xylan in a xylan suspension was measured by heating 80ml of a xylan suspension (0.25% w/v in distilled water) to 70°C for 60 minutes, with occasional agitation, for maximum dissolution under assay conditions and then centrifuging (47 800 xg, 30min, 20°C). The supernatant was discarded and the pellet dried in an oven at approximately 100°C and weighed.

(b) Dyed xylan

Dyed xylan was prepared from oat spelts xylan and Remazol Brilliant Blue following the method of Biely *et al.* (1985). 10g oat spelts xylan, 10g Remazol Brilliant Blue, 27g sodium acetate (anhydrous) and 350ml distilled water were mixed. 6g NaOH pellets were added and the volume was made up to 400ml with distilled water. The mixture was stirred for 90 minutes at room temperature. The dyed xylan was precipitated by the addition of 800ml 96% ethanol. The mixture was stirred overnight, then centrifuged (16 300 xg, 15min, 20°C). The pellet was resuspended in 1l 96% ethanol : 0.05M sodium acetate in distilled water (2:1 v/v). The mixture was centrifuged and the pellet resuspended, both as above. The mixture was then filtered (Whatman GF/A) and the precipitate was washed with 96% ethanol : 0.05M sodium acetate in distilled water (2:1 v/v) until the filtrate was colourless. The dyed xylan was then washed with 1l 96% ethanol and finally with 1l acetone before drying at room temperature. The expected yield (according to Biely *et al.* (1985)) was 11g and the actual yield was 11.42g. The dyed xylan dissolved best in water or very low concentration buffers.

Soluble dyed xylan was prepared by mixing 5g dyed xylan with 150ml distilled water for 4 hours at 75°C. The mixture was then centrifuged (47 800 xg, 2min, 20°C) and the supernatant retained as the soluble portion. All dyed xylan assays were performed with soluble dyed xylan. The proportion of insoluble dyed xylan was measured by centrifuging (47 800 xg, 5min, 20°C) 10ml of a dyed xylan suspension (1% w/v in distilled water). The pellet was dried in an oven at approximately 100°C and weighed. The amount of dye in the soluble dyed xylan was calculated using an extinction coefficient (ε_{595}) for Remazol Brilliant Blue of $9.25 \times 10^3 l mol^{-1} cm^{-1}$.

Initially dyed xylan assays were performed as follows: 0.4ml of a dyed xylan solution (approximately 0.6% w/v soluble dyed xylan) in buffer was preheated in at least duplicate. 0.1ml of an enzyme sample was added to the

substrate and incubated at 70°C for 10 minutes. The assays were stopped by the addition of 1.0ml ethanol. After standing for 10 minutes the assays were centrifuged (12 800 xg, 1min, 20°C) and the absorbance of the supernatant was read at 595nm.

It was found that the precipitation of dyed xylan could be improved by dissolving the dyed xylan in 10mM buffer rather than distilled water and adding a solution of NaCl immediately before the ethanol used to precipitate the dyed xylan. The NaCl solution and ethanol could not be mixed together before addition to the dyed xylan. Larger volumes of NaCl solution and/or ethanol improved precipitation, with 1M NaCl being better than 6M NaCl. Sodium acetate was found to be just as efficient as NaCl.

The assay method was modified and the improved method was used for most dyed xylan assays. 0.2ml of a soluble dyed xylan solution (approximately 0.6% w/v soluble dyed xylan) in 10mM MES/NaOH buffer (pH $6.0_{70°C}$) was preheated in at least duplicate. 50µl of an enzyme sample was added to the substrate and incubated at 70°C for 10 minutes. The assays were stopped by the addition of 0.1ml 1M NaCl immediately followed by 0.7ml ethanol. After standing for 5 minutes the assays were centrifuged (12 800 xg, 1min, 20°C) and the absorbance of the supernatant was read at 595nm.

(c) CMC

0.4ml of a CMC (sodium salt, low viscosity) solution (2% w/v) in buffer was preheated in glass tubes in at least quadruplicate. 0.1ml of an enzyme sample was added to two of the four tubes and incubated at 70°C for 10 or 20 minutes (t=10 or 20). All assays were stopped by the addition of 1.0ml PAHBAH reagent. 0.1ml of the enzyme sample was then added to the remaining two tubes (t=0). The PAHBAH method was used to detect reducing sugars released. Standards were 0.16ml of 0 - 0.32mM D-glucose (in distilled water) added to 0.34ml buffer, with reducing sugars being assayed by the addition of 1.0ml PAHBAH reagent. Unless otherwise stated, activity on CMC is expressed as µmol(glucose equivalents)$min^{-1}mg^{-1}$. CMC solutions were made freshly or stored at 4°C for short periods.

(d) Avicel

Activity on Avicel was measured by incubating the enzyme sample with a slurry of Avicel and determining the reducing sugars released using the PAHBAH system. Initially the conditions of Reynolds *et al.* (1986) were used, that is, a 17 hour incubation at 70°C with shaking. The method was modified by decreasing the time, increasing the proportion of enzyme and decreasing the total assay volume. Most of the assays with MUCase extracts were performed at a lower temperature (45°C) due to the uncertainty of the MUCase being stable. All assays included t=0 (non-incubated) backgrounds which were subtracted from the incubated samples to give only reducing sugars produced during incubation. Unless otherwise stated, assays with MUCase extracts were performed either (i) with the inclusion of substrate-free backgrounds (non-incubated again subtracted from incubated) which were subtracted from substrate-containing assays to get activity on Avicel only or (ii) using extracts which had any contaminating activity inactivated by heat treatment or separated by gel filtration.

The assay used was as follows: 0.25ml Avicel slurry (10% w/v) in buffer was dispensed by autopipette using tips with approximately 2mm cut off the ends. The slurry was shaken between each dispensing. Quadruplicate substrate-containing and substrate-free (containing 0.25ml buffer instead of Avicel slurry) assays were performed. 0.25ml of an enzyme sample was added to two of the substrate-containing and two of the substrate-free tubes and they were incubated at 70°C or 45°C for 3 hours (t=3hr) submerged in a shaking (100 strokes/min) waterbath. The remaining two of each (t=0) were either incubated at 0°C for 3 hours and then 0.25ml of the enzyme sample (which had also been incubated at 0°C) was added or the enzyme sample was added initially followed by incubation at 0°C for 3 hours. All assays were stopped by placing in ice cold water. The tubes were mixed and centrifuged (12 800 xg, 1min, 4°C) to remove residual Avicel. 2x 0.18ml aliquots of the supernatant were removed from each tube and reducing sugars assayed (usually immediately, sometimes after storage overnight) by the addition of 1.0ml PAHBAH reagent to each aliquot. Standards were 0.18ml of 0 - 0.2mM D-glucose (in distilled water), with reducing sugars being assayed by the addition of 1.0ml PAHBAH reagent. All absorbance readings were converted to amounts of glucose before activity calculations. Unless otherwise stated, activity on Avicel is expressed as

μmol(glucose equivalents)$min^{-1}mg^{-1}$. Assays on Avicel in the substrate specificity work (Chapter 4 and Sections 5.3.8 and 6.3.7 and the last two MUCase examples in Table 3.5) were incubated for 2 hours. Avicel slurries were made freshly or stored at 4°C for short periods.

(e) Dyed Avicel

Dyed Avicel was prepared from Avicel and Remazol Brilliant Blue following the method of Leisola *et al.* (1975). 10g Avicel was mixed with 100ml distilled water at 50°C. 100ml of a Remazol Brilliant Blue dye suspension (1% w/v) was added followed by 20g Na_2SO_4. The mixture was agitated for 45 minutes. 20ml Na_3PO_4 (10% w/v) was added and the mixture agitated for a further 60 minutes. The dyed Avicel was collected by filtration (Whatman no.1). The dyed Avicel was washed with hot water (60°C) until the filtrate was colourless. The dyed Avicel was then washed with acetone and ether and then dried in a vacuum. The yield was 9.86g.

Initial assays on dyed Avicel were as follows; 1ml dyed Avicel slurry (8 concentrations, 0.01 - 5% w/v) in buffer was dispensed by autopipette using tips with approximately 2mm cut off the ends. The slurry was shaken between each dispensing. Duplicate substrate-containing assays were performed at each substrate concentration. 0.1ml of an enzyme sample was added to one assay tube (t=60) and both tubes were incubated at 45°C for 60 minutes submerged in a shaking (100 strokes/min) waterbath. All assays were stopped by placing in ice cold water. 0.1ml of the enzyme sample was added to the assay tube containing only substrate (t=0). The tubes were mixed and centrifuged (12 800 xg, 1min, 20°C) to remove residual dyed Avicel. The absorbance of the supernatant was read at 595nm.

The final attempt to detect activity on dyed Avicel used the following modifications to the above method: duplicates of 0.6ml dyed Avicel slurry (2% w/v) in buffer and 0.6ml enzyme sample and duplicates where buffer replaced the enzyme sample were incubated at 45°C for 72 hours (t=72hr). Singles of each were also prepared at the end of the 72 hour incubation (t=0). The tubes were mixed and centrifuged (12 800 xg, 5min, 20°C).

Dyed Avicel slurries were made freshly.

(f) MUC

The model substrate MUC was used in the screening of clones by Bergquist and co-workers (see for example Streiff *et al.*, 1986) and is thought to indicate exocellobiohydrolase activity. The assay on MUC used here is based on the discontinuous absorptiometric assay of Heptinstall *et al.* (1986). The assay procedure generally used is as follows: 0.3ml of a MUC solution (2mM) in distilled water together with 0.1ml buffer were preheated in glass tubes in at least duplicate (t=20). Tubes containing 0.3ml distilled water instead of MUC solution were also preheated in at least duplicate (t=0). 0.1ml of an enzyme sample was added to all of the tubes and they were incubated at 70°C for 20 minutes. All assays were stopped by the addition of 3.5ml 0.5M glycine/NaOH buffer (pH 10.4). After mixing the absorbance of the solution was read at 365nm. However in the substrate specificity work (Chapter 4 and Sections 5.3.8 and 6.3.7 and the last two MUCase examples in Table 3.5) a smaller assay volume was used, as follows: 0.1ml of a MUC solution (10mM) in buffer together with 0.1ml buffer were preheated in glass tubes in at least duplicate (t=20). Tubes containing 0.1ml distilled water instead of MUC solution were also preheated in at least duplicate (t=0). 50µl of an enzyme sample was added to all of the tubes and they were incubated at 70°C for 20 minutes. All assays were stopped by the addition of 1.75ml 0.5M glycine/NaOH buffer (pH 10.4) and cooled in running water. After mixing the absorbance of the solution was read at 365nm.

Solutions of MUC (that is, enzyme-free assays) were found to have insignificant absorbance readings which did not change on incubation under the assay conditions. The assays containing enzyme but no MUC (t=0) allowed for any absorbance change due to denaturation of protein during incubation. This was noticeable with crude extracts or with enzyme samples of high protein concentration.

A standard curve was prepared from MU. The MU was recrystallized from hot ethanol and a stock solution of 20mM made up in methanol. Standards in the range 0 - 1.6mM were then made up from the stock solution in distilled water. The standard curve was prepared by mixing 0.1ml of each standard with 0.4ml distilled water and adding 3.5ml 0.5M glycine/NaOH buffer (pH 10.4). The absorbance of the solution was read at 365nm. An extinction coefficient (ε_{365}) of 18.4 x $10^3 l mol^{-1} cm^{-1}$ was obtained and used to calculate

activities. Heptinstall *et al.* (1986) obtained an extinction coefficient (ε_{365}) of 18.0 x $10^3 l mol^{-1} cm^{-1}$. Unless otherwise stated, activity on MUC is expressed as $\mu mol(MU) min^{-1} mg^{-1}$. MUC solutions were stored at -20°C and incubated at 50°C for approximately 15 minutes to ensure dissolution prior to assay.

(g) *p*NP-substrates

Two assay procedures were used. The first procedure used a smaller assay volume and was used in the substrate specificity work (Chapter 4 and Sections 5.3.8 and 6.3.7 and the second β-glucosidase example in Table 3.5). 0.2ml of a *p*NP-substrate solution in buffer was preheated in glass tubes in at least quadruplicate. 50µl of an enzyme sample was added to two of the four tubes and incubated at 70°C for 20 minutes (t=20). All assays were stopped by the addition of 0.5ml 1M Na_2CO_3. 50µl of the enzyme sample was then added to the remaining two tubes (t=0). All of the tubes were cooled in running water before the absorbance was read at 400nm. The substrate concentrations were: *p*NPC, *p*NPG and *p*NPX, 25mM; *p*NPL, 15mM and *p*NPAp, 11mM. The concentrations were different due to solubility.

The second procedure was used with *p*NPX as the substrate in the remaining Sections of Chapter 5 and the third β-xylosidase example in Table 3.4. 0.4ml of a *p*NPX solution (10mM) in buffer was preheated in glass tubes in at least quadruplicate. 0.1ml of an enzyme sample was added to two of the four tubes and incubated at 70°C for 10 minutes (t=10). All assays were stopped by the addition of 1.0ml 1M Na_2CO_3. 0.1ml of the enzyme sample was then added to the remaining two tubes (t=0). All of the tubes were cooled in running water before the absorbance was read at 400nm. *p*NP-substrate solutions were made freshly or stored at 4°C for short periods.

A standard curve was prepared from *p*NP. A stock solution of 0.2mM was made up in buffer. Standards in the range 0 - 0.2mM were then made up from the stock solution in buffer. The standard curve was prepared by mixing 0.25ml of each standard with 0.5ml 1M Na_2CO_3. The absorbance of each solution was read at 400nm. An extinction coefficient (ε_{400}) of 17.5 x $10^3 l mol^{-1} cm^{-1}$ was obtained and used to calculate activities. Unless otherwise stated, activity on each *p*NP-substrate is expressed as $\mu mol(pNP) min^{-1} mg^{-1}$.

2.1.2 Detection of products - other methods

(a) Sugars and oligosaccharides - HPLC

The end products of β-xylosidase activity on xylobiose, cellobiose and *o*NPX were determined as follows: 0.1ml xylobiose (18mM), cellobiose (20mM) or *o*NPX (20mM) dissolved in 50mM MES/NaOH buffer (pH $5.85_{70°C}$) was incubated with 0.1ml of an enzyme sample at 70°C for 40 minutes. All assays were stopped by cooling in ice cold water and the tubes were then centrifuged (12 800 xg, 2min, 20°C). 20µl samples were analysed by HPLC on a 7.8mm x 30cm Aminex HPX-87H column (Biorad, California, USA) operated at 50°C. Filtered, vacuum degassed 0.01N H_2SO_4 was used as the solvent at a flow rate of $0.5 ml min^{-1}$. Saccharides were detected using a refractive index detector. Standards were xylose, xylobiose, glucose and cellobiose. Control samples were *o*NPX and the enzyme sample.

The end products of xylanase activity on xylobiose, cellobiose, *p*NPX, *p*NPX in the presence of 10mM xylose and oat spelts xylan and β-glucosidase activity on xylobiose and cellobiose were determined as follows: 50µl xylobiose (18mM) or cellobiose (40mM) dissolved in 50mM MES/NaOH buffer (pH $5.85_{70°C}$) was incubated with 50µl enzyme sample at 70°C for 60 minutes. 80µl *p*NPX (25mM) dissolved in 50mM MES/NaOH buffer (pH $6.0_{70°C}$) was incubated with 20µl enzyme sample at 70°C for 60 minutes. 0.2ml oat spelts xylan (0.25% w/v) in 50mM MES/NaOH buffer (pH $6.0_{70°C}$) was incubated with 25µl enzyme sample at 70°C for 15 and 30 minutes and 2, 4 and 24 hours. All assays were stopped by cooling in ice cold water and the tubes were then centrifuged (12 800 xg, 2min, 20°C). 20µl samples were analysed by HPLC on a 7.8mm x 30cm Aminex HPX-42A column (Biorad, California, USA) operated at 85°C. Hot filtered, degassed Milli-Q water was used as the solvent at a flow rate of $0.6 ml min^{-1}$. Saccharides were detected using a refractive index detector. Standards were xylose, xylobiose, glucose, cellobiose, maltose, maltotriose, maltotetraose, maltopentaose, maltohexaose and maltoheptaose. Control samples were *p*NPX, oat spelts xylan and the enzyme samples. Retention times for xylooligosaccharides were predicted using the standards and the results of xylan and *p*NPX hydrolysis by xylanase.

The HPLC system used would not have detected hydrolysis products representing less than 0.25% of the initial amount of xylobiose or cellobiose. Xylobiose and cellobiose solutions were stored at -20°C.

(b) PAHBAH method of reducing sugar determination

The method is based on that of Lever (1973). The stock solutions are: 0.5M trisodium citrate, 1M Na_2CO_3, 0.2M $CaCl_2$ and 5M NaOH. The first two were stored in dark bottles and all were stored at 4°C. The PAHBAH reagent was made by mixing 10ml of each stock solution, in the order they are listed, with mixing between each addition. 1.52g *p*-hydroxybenzoic acid hydrazide (PAHBAH) was added and the volume made up to 100ml. The PAHBAH reagent was made freshly each day and stored in a dark bottle at room temperature.

When determining the amount of reducing sugars in a sample, the stated volume of PAHBAH reagent was added to the sample, it was mixed and immediately boiled for 5 minutes. After cooling in running water the absorbance was read at 420nm.

All of the buffers used in assays in which reducing sugars were measured were found to have insignificant effect on colour production in the PAHBAH system. Phosphate buffer was found to interfere with the PAHBAH assay.

2.1.3 Buffers used

Buffers that were generally used to dissolve or suspend substrates, to prepare enzyme extracts and for enzyme dilution prior to assay are as follows: β-xylosidase and MUCase, 0.2M sodium acetate/acetic acid buffer (pH $5.6_{20°C}$); xylanase, usually 50mM MES/NaOH (pH $6.0_{70°C}$), early work with 50mM sodium citrate/citric acid buffer (pH $6.0_{20°C}$); β-glucosidase and CMCase, 50mM sodium citrate/citric acid buffer (pH $6.4_{20°C}$) and host *E. coli* extracts, the same buffer as used for the clone in each comparison experiment.

2.2 Protein measurement

2.2.1 Use in measurement of thermostability

(a) Soluble and insoluble protein

Two methods were used to determine the protein concentration of both the supernatant and the pellet of the heat treated extracts.

The Biuret assay used was based on that of Herbert *et al.* (1971). Soluble protein in supernatants was assayed by adding 0.5ml 3M NaOH to 0.5ml of the supernatant (or standard) and standing for 10 minutes. 0.5ml 2.5% (w/v) $CuSO_4.5H_2O$ was added and the solution stood for 10 minutes before centrifuging (12 800 xg, 2min, 20°C). The absorbance was read at 555nm. Insoluble protein in protein precipitates in the form of drained pellets was measured by adding 1ml buffer followed by 1ml 3M NaOH to the pellet and mixing well. The solution was heated at 90°C for 5 - 10 minutes, cooled, 1ml 2.5% (w/v) $CuSO_4.5H_2O$ was added and the solution left to stand for 10 minutes before centrifuging (12 800 xg, 2min, 20°C). The absorbance was read at 555nm. Standards were 1 - 4mgml^{-1} BSA in buffer and they were stored at -20°C.

The Lowry method used was based on that of Peterson (1983). The reagents were:

A 0.1% (w/v) $CuSO_4.5H_2O$
0.2% (w/v) sodium potassium tartrate
10% (w/v) Na_2CO_3

B 5% (w/v) SDS

C 0.8M NaOH

D 50ml A + 100ml B + 50ml C

E 50ml 2M Folin-Ciocalteau reagent
+ 250ml distilled water.

The sample for assay was 0.5ml supernatant (or standard) or a pellet to which 0.5ml buffer had been added. 2ml reagent E was added, the solution was mixed and left to stand for 10 minutes. 1ml reagent D was then added, the solution was mixed and left to stand for 30 minutes. The absorbance was read at 750nm. Standards were 0.25 - 1.0mgml^{-1} BSA in buffer and they were stored at -20°C.

(b) UV difference spectrophotometry

A Shimadzu UV-250 UV-visible recording spectrophotometer with both a Shimadzu graphic printer PR-1 and a Shimadzu option program/interface OP1-4 attached was used. The option program/interface allowed subtraction of one scan from another to give a difference spectrum. Matched quartz cuvettes were used. They were regularly cleaned by boiling in nitric acid.

2.2.2 Protein measurement in enzyme activity measurement

Two methods of dye-binding protein measurement were used. A method based on that of Peterson (1983) was used in trials and preliminary experiments. The stock reagent was 0.9g Coomassie Blue dissolved in 200ml 85% (w/w) orthophosphoric acid. 100ml 95% (v/v) ethanol was then added and the solution stirred for one hour. The assay reagent was made by mixing 30ml of the stock reagent with 90ml 85% (w/w) orthophosphoric acid and then 40ml 95% (v/v) ethanol. The solution was made up to 600ml and filtered (Whatman no.1). The assay reagent was discarded after two weeks. Protein samples were assayed in duplicate by adding 1.5ml of the assay reagent to 1ml sample (or standard). After standing for 10 minutes the absorbance at 595nm was read. Standards were 0 - 30μgml^{-1} BSA in buffer and they were stored at -20°C.

The method used for most of the work was based on the 'Pierce Procedures and Reference Guide for Coomassie Blue G-250' leaflet. The reagent was made by dissolving 100mg Coomassie Blue in 50ml 95% (v/v) ethanol and adding 100ml 85% (w/w) orthophosphoric acid. The solution was made up to 1l with distilled water. Protein samples were assayed in triplicate by adding 5ml of the reagent to 0.1ml sample (or standard). After mixing well and standing for 5 minutes the absorbance was read at 595nm. A small volume assay was also used in which 1ml reagent was added to 20μl sample (or standard). Standards were 0 - 1$mgml^{-1}$ BSA in buffer and they were stored at -20°C.

Glass cuvettes were used and they were rinsed with 1% (w/v) SDS to remove bound dye.

2.2.3 Protein composition of extracts

The protein composition of heat treated and non-heat treated extracts was investigated by HPLC using a TSKgel column Type G3000 SWG (21.5mm x

60cm, Toyo-Soda Manufacturing Co., Tokyo, Japan). The solvent was 0.2M ammonium acetate pH $6.9_{20°C}$.

2.3 Source of bacteria - growth media

2.3.1 Source of bacteria and maintenance

The *Bacillus stearothermophilus* used was DSM22. The *Thermus aquaticus* used was ATCC25104. The *Thermus* sp. strain T-351 used was ATCC31764. *Thermotoga* sp. strain FjSS3B.1 was obtained from B. Huser, Thermophile Research Unit, University of Waikato, Hamilton, New Zealand. All *E. coli* strains (both host strains and clones) were obtained from P.L. Bergquist and co-workers, Department of Cellular and Molecular Biology, University of Auckland, Auckland, New Zealand. Clone strains were PB4551, PB4563, PB4567, PB4574, PB4716, PB4800, PB4855, PB4887 and X (further details are given in Table 1.2). Host strains were PB2477 (=C600: F^-, *thi*-1, *leu*-6, *thr*-1, *lac*Y1, *sup*E44, r_k^- m_k^+), PB2946 (=JM83: ara, Δ[lac,pro], thi, ϕ80dlacZ ΔM15 strA, I^- F^-) and TG1 (*Δ[lac,pro], supE, thi, hsdS,* F'[*traD36, proAB*$^+$, *lacI*^{q}, *lacZΔM15*]). Various clones became available at different stages throughout this study. Host *E. coli* strains were maintained on LB agar slopes and plates, stored at 4°C. *E. coli* clones were maintained on LB-Amp agar slopes and plates, stored at 4°C.

2.3.2 Growth media

LB medium was made by dissolving $5.0gl^{-1}$ trypticase peptone, $5.0gl^{-1}$ yeast extract and $5.0gl^{-1}$ NaCl in distilled water. The pH was adjusted to 7.0 and the medium autoclaved. 1.75% agar was added for solid medium.

LB-Amp medium was LB medium supplemented with not less than $50mgl^{-1}$ active ampicillin. For large volumes of medium (>4l), filter sterilized ampicillin was asceptically added to the autoclaved LB medium. For smaller volumes of medium, $100mgl^{-1}$ ampicillin was added before the LB medium was autoclaved.

Oshima's medium was made by dissolving $8.0gl^{-1}$ polypeptone peptone, $4.0gl^{-1}$ yeast extract and $3.0gl^{-1}$ NaCl in distilled water. The pH was adjusted to 7.6 and the medium autoclaved.

2.4 Preparation of enzyme samples

Two sonicators were used throughout the work, a Kontes micro-ultrasonic cell disrupter fitted with a micro-probe (K) and a Dynatech sonic dismembranator fitted with a medium sized probe (D).

2.4.1 Protein extracts used in the thermostability work

Four batches of extracts were prepared. The buffer used throughout was 0.1M sodium phosphate buffer pH $7.0_{20°C}$ (Na_2HPO_4 and NaH_2PO_4).

The first batch was prepared from 1l *Thermus aquaticus* ATCC25104 culture (grown on Castenholz medium D (Castenholz, 1969 and Ramaley and Hixson, 1970) at 70°C), 1l *B. stearothermophilus* DSM22 culture (grown on Oshima's medium at 55°C), both culture volumes were obtained from Y. Casey, Thermophile Research Unit, University of Waikato, Hamilton, New Zealand and 2l *Thermotoga* sp. strain FjSS3B.1 culture (grown on Mineral Salts medium (Huser *et al.*, 1986) at 85°C), obtained from B. Huser, Thermophile Research Unit, University of Waikato, Hamilton, New Zealand. In each case the culture volume was centrifuged (23 500 xg, 15min, 4°C), the cells were washed once with phosphate buffer and centrifuged again (as above). The cells were resuspended in phosphate buffer, sonicated (K) for 40 minutes (*T. aquaticus* and *B. stearothermophilus*) or 25 minutes (*Thermotoga* sp.) in an ice bath and centrifuged (300 000 xg, 2hr, 4°C).

Further extracts were prepared from 16.69g *Thermus* sp. strain T-351 ATCC31764 cells and 11.80g *T. aquaticus* ATCC25104 cells both supplied from storage at -196°C by Y. Casey, Thermophile Research Unit, University of Waikato, Hamilton, New Zealand. The cells were resuspended in phosphate buffer containing 0.02% (w/v) sodium azide, centrifuged (23 500 xg, 15min, 4°C) and resuspended (as above). The resuspension was frozen (-20°C), thawed and 0.6ml (*Thermus* sp.) or 0.2ml (*T. aquaticus*) of 10 $mgml^{-1}$ DNase in 0.15M NaCl was mixed in. The resuspension was then frozen (-20°C) and thawed, sonicated (D) for 20 minutes in an ice bath and centrifuged (300 000 xg, 3hr, 4°C).

Two extracts were prepared each from 4l *B. stearothermophilus* DSM22 culture (grown in 1l Oshima's medium in 2l flasks, with a 2% inoculum, at 55°C in an orbital shaker overnight). In each case the culture volume was centrifuged (23 500 xg, 15min, 4°C), the cells were washed once with phosphate

buffer containing 0.02% (w/v) sodium azide and centrifuged again (as above). The cells were resuspended in 0.45ml/g wet weight cells of phosphate buffer containing 0.02% (w/v) sodium azide. 0.4ml of 10mgml^{-1} DNase in 0.15M NaCl was mixed into one extract. Each resuspension was frozen (-20°C) and thawed twice, sonicated (D) for 25 minutes (DNase-containing extract) or 30 minutes (extract containing no DNase) in an ice bath and centrifuged (300 000 xg, 3hr, 4°C).

Finally, an extract was prepared from 1l *B. stearothermophilus* DSM22 culture (grown in 1l Oshima's medium in a 2l flask at 55°C in an orbital shaker overnight). The culture volume was centrifuged (23 500 xg, 15min, 4°C), the cells were washed once with phosphate buffer containing 0.02% (w/v) sodium azide and centrifuged again. The cells were resuspended in phosphate buffer containing 0.02% (w/v) sodium azide, sonicated (K) for 30 minutes in an ice bath and centrifuged (300 000 xg, 2hr, 4°C).

Heat treatment of extracts for the soluble and insoluble protein measurement experiments was performed at temperatures from 60°C - 95°C, with a control temperature of 20°C. Triplicate 1ml aliquots of the extract in 8ml sealed glass centrifuge tubes, were incubated in a water bath for a specific time at an appropriate temperature. The incubation was stopped by cooling the tubes in ice. The tubes were then centrifuged (4 000 xg, 15min, 4°C). The supernatant in each tube was decanted and the tubes were inverted and drained on a paper towel (whether or not they contained pellets).

The latter three *B. stearothermophilus* extracts were used for the UV difference spectrophotometry work. In these experiments heat treatment was performed (as above) on 3ml aliquots of appropriately diluted extracts.

2.4.2 Enzyme preparations from *E. coli* clones

(a) General methods

The method of preparation depended on the scale. Small (<5l) or very large (>200l) volumes of culture were harvested by centrifugation, whereas moderate volumes (10 - 50l) were harvested using a hollow fibre column. Small amounts of cells were lysed by sonication, large amounts using a lysozyme method. Unless otherwise stated, all *E. coli* extracts were made by small scale growth with the cells being harvested by centrifugation and subjected to at least

one freeze/thaw cycle. The resuspended cells were sonicated and the cell debris removed by centrifugation. Such cell-free extracts are generally referred to as crude extracts, while extracts that have also been heat treated are generally referred to as heat treated extracts. Detailed descriptions of the general methods follow, with extracts made by different methods described after that (Section 2.4.2 (b)).

The *E. coli* strain was grown in 1l LB (host strains) or LB-Amp (clones) medium contained in a 2l flask, with a 1% inoculum, at 35°C - 37°C, with vigorous agitation and aeration, overnight. Up to 22 flasks could be grown simultaneously.

When harvesting by centrifugation (23 500 xg, 20min, 4°C or 13 700 xg, 20min, 4°C) all cell pellets were transferred to one centrifuge tube, resuspended in buffer and centrifuged again (23 500 xg, 20min, 4°C). The cell pellet was transferred to a glass beaker, covered and frozen (-20°C).

Alternatively, an Amicon hollow fibre column (H1MP01-43, 0.1µm cutoff, with a DC10LA pump unit, both Amicon) was used for harvesting cells. The concentrate was kept at 4°C using a cooling probe (Julabo). The cells were washed by dilution with buffer at least once. The concentrate was then centrifuged (23 500 xg, 20min, 4°C) and the cell pellet transferred to a glass beaker, covered and frozen (-20°C).

One method of cell disruption used was sonication. Thawed cells were mixed with 1 - 2v/wet cell weight of buffer. The mixtures were sonicated (D) for 10 - 25 minutes and centrifuged (47 800 xg, 30min, 5°C followed by 69 000 xg, 60min, 4°C or 156 000 xg, 30min, 4°C). The supernatant was removed carefully using a pasteur pipette.

The lysozyme method of cell disruption was based on the lysozyme/non-ionic detergent/osmotic shock method of Schwinghamer (1980). This method was used for the lysis of large amounts of cells. Three extracts were made using this general method and all are detailed further in the following section (Section 2.4.2 (b)). Thawed cells were frozen (-20°C), thawed again and mixed with approximately one third of their net weight of glycerol. When the slurry was sufficiently liquid, 1% (v/wet cell weight) of 10% (w/v) Triton X-100 and 0.1% (v/wet cell weight) β-mercaptoethanol were added and stirred for 1 - 2 hours at 4°C. 6 (first xylanase extract in Section 2.4.2 (b)), 4 (second xylanase extract in

Section 2.4.2 (b)) or 3 (first MUCase extract in Section 2.4.2 (b)) volumes of 50mM MOPS/KOH buffer (pH $7.0_{20°C}$) containing 7.5mM β-mercaptoethanol, 7.5mM disodium EDTA, 0.02% sodium azide and 0.02% lysozyme (and 0.2mM $MgCl_2.6H_2O$ for the first MUCase extract in Section 2.4.2 (b)) were then added rapidly. The temperature was raised to 35°C and 0.02% (v/wet cell weight) of DNase (or 0.001% for the first MUCase extract in Section 2.4.2 (b)) and 0.05% (v/wet cell weight) of PMSF (as a 1% w/v solution in acetone) added. 0.03% of Pepstatin A was also added to the first MUCase extract in Section 2.4.2 (b). The slurry was stirred for 40 - 60 minutes and cooled on ice. The first MUCase extract in Section 2.4.2 (b) was centrifuged (23 500 xg, 15min, 4°C), the other two extracts were not centrifuged.

The general method of heat treatment involved incubating the extract at 70°C for 30 minutes in a sealed or capped tube. The incubation was stopped by cooling the tube in an ice bath. Usually 0.5 - 1.5ml aliquots in sealed Eppendorf tubes or 1.5 - 3.0ml aliquots in capped (with a rubber bung) glass tubes were heat treated. After mixing the extract was centrifuged (12 800 xg, 5min, 20°C and/or one of 39 200 xg, 15min, 15°C or 69 000 xg, 15min, 4°C or 156 000 xg, 60min, 4°C). Heat treatment at acidic pH was performed by changing the pH of the extract to the appropriate pH using 5M acetic acid (pH 4.5, NaOH). After heat treatment at acidic pH the extract was neutralised using 5M ammonia (pH 9.5, HCl).

(b) Extracts made by different methods

(i) A β-xylosidase extract was obtained from R.C. Hudson, Thermophile Research Unit, University of Waikato, Hamilton, New Zealand. It was used in the substrate specificity screen (Section 4.1). Briefly, the sample was purified by Hudson *et al.* (in press) as follows; *E. coli* clone PB4855 cells were lysed using a lysozyme method and centrifuged to remove cell debris. The extract was heat treated, centrifuged and subjected to DEAE-Sepharose anion exchange chromatography. After concentration and dialysis the extract was applied to a CM-Sepharose cation exchange column and after subsequent concentration to a TSK Fractogel gel filtration column. The purification factor at this stage was 32.5 (Hudson *et al.*, in press).

(ii) A xylanase extract was made from *E. coli* clone PB4716. The extract was used in Sections 3.2.1 and 3.2.2 (e). The cells were grown following the general method of growth (see Section 2.4.2 (a)) but 1.5l of medium was contained in

each of 18 x 2l flasks. The cells were harvested using the general method of harvesting using a hollow fibre column (see Section 2.4.2 (a)). The buffer used was 50mM MOPS/KOH (pH $7.0_{20°C}$). The lysozyme method described in the general method of cell breakage (see Section 2.4.2 (a)) was used. There was no centrifugation step before heat treatment. The extract was heat treated by incubation (in a glass container) in a water bath at 70°C with frequent agitation. When the extract reached 70°C it was held at this temperature for 30 minutes then cooled rapidly in an ice bath before centrifuging (23 500 xg, 2hr, 5°C).

(iii) A xylanase extract was made from *E. coli* clone PB4716. The extract was used for the purification and characterization of the xylanase (Sections 5.2 and 5.3) as well as in Sections 3.2.1 and 4.2. As the DNA fragment in this clone additionally coded for β-xylosidase, this activity was also measured before and after heat treatment of the extract and the results appear in Section 3.2.1.

The inoculum was prepared by growing the strain in 8 x 1l of medium contained in 2l flasks as for the general method of growth (see Section 2.4.2 (a)). The growth medium concentrate consisted of 3kg yeast extract and 1.5kg NaCl dissolved in 20l distilled water and 3kg trypticase peptone and 1.5kg NaCl dissolved in a further 20l distilled water.

The 800l fermenter was sterilized by steaming with 1% formalin for 25 minutes and steam rinsed twice with tap water sterilized by filtering through successive 10μm, 1μm and 0.2μm maxicapsule filters (Gelman). After approximately 500l of filter sterilized tap water had been added, 25g ampicillin dissolved in 2.5l sterile distilled water and the medium concentrates were pumped in through the same filters. Further water was added to bring the final volume to approximately 600l. When the medium was equilibrated at 37°C and pH 7.0, the 8l inoculum was added through a vent in the top of the fermenter using a sterilized funnel.

The fermenter was stirred slowly with aeration (filter sterilized air) throughout the growth period. Samples of the culture were taken asceptically by syringe, through the rubber sealed sampling vent on the side of the fermenter, at regular intervals throughout the growth period. Cell density was monitored by reading the absorbance of the sample at 650nm. The pH of each sample was also noted. Purity of the culture was checked by growing pre-inoculum and post-inoculum samples on both LB-Amp and LB agar plates.

Harvesting of cells began 17 hours after inoculation, when the culture had an optical density (A_{650}) of 1.16. A continuous flow centrifuge (Sharples, model 6) was used (12 000g) with a flow rate of approximately 2lmin^{-1}. Harvesting took approximately 4.5 hours. The cells were scraped out of the centrifuge rotor cylinder in to a plastic container and stored by freezing at -20°C. The yield was 1.5kg.

1.2kg cells were lysed using the lysozyme method described in the general method of cell breakage (see Section 2.4.2 (a)). There was no centrifugation step before heat treatment. Heat treatment was carried out by incubating the extract (in a metal container) in a water bath maintained at 70°C, with constant stirring. When the extract reached 70°C it was held at that temperature for 30 minutes, then cooled rapidly (with stirring) in an ice bath. The extract was centrifuged (23 500 xg, 2hr, 5°C) and the supernatant stored at 4°C.

A trial of ammonium sulphate precipitation was made by dissolving ammonium sulphate in 1ml samples of the heat treated extract to obtain 0, 40, 50, 60 and 70% saturation. After standing for 30 minutes the samples were centrifuged (13 000 xg, 5min, 20°C) and the supernatants assayed for remaining activity. In the large scale purification, 1.664kg ammonium sulphate was dissolved by stirring in the 5.3l heat treated supernatant to give a concentration of 50% saturation. The extract was stored at 4°C for 21 days and then centrifuged (13 500 xg, 20min, 5°C). 1.210kg ammonium sulphate was dissolved by stirring into the supernatant to give a concentration of 80% saturation and the solution was stored overnight at 4°C. After centifuging (13 500 xg, 20min, 5°C) a pellet weighing approximately 205g was obtained. 80ml 50mM MOPS/KOH (pH $7.0_{20°C}$) was added to the pellet and it was dialysed (3 500 molecular weight cutoff, Spectra/Por 3) at 4°C against 3 x 20l distilled water. The membrane split, so the 20l was concentrated by ultrafiltration through a spiral wound membrane (S10Y10, 10 000 molecular weight cutoff, with a DC10LA pump unit, both Amicon) using a cooling probe (Julabo) to keep the concentrate at 5°C. The concentrated extract was then freeze dried. 13.7g powder was obtained.

Various chromatographic trials were made. In the cation exchange MONO S 5/5 (Pharmacia) trial, a sample volume of 0.2ml was loaded containing 2mg freeze dried powder. The elution buffer was 50mM MES/NaOH (pH $6.0_{70°C}$)

and the flow rate 1mlmin^{-1}. After an elution volume of 14ml, a 40ml gradient of 0 - 0.86M NaCl (in 50mM MES/NaOH (pH $6.0_{70°C}$)) was started. The anion exchange MONO Q 5/5 (Pharmacia) trial involved loading a 0.5ml sample containing 5mg freeze dried powder. The elution buffer was 20mM Bis-Tris/HCl (pH $6.55_{20°C}$) and the flow rate 1mlmin^{-1}. After an elution volume of 10ml, a 40ml gradient of 0 - 0.86M NaCl (in 20mM Bis-Tris/HCl (pH $6.55_{20°C}$)) was started. In the hydrophobic interaction Phenyl Superose 5/5 (Pharmacia) trial, a sample volume of 0.5ml was loaded containing 1mg freeze dried powder. The elution buffer was 50mM MES/NaOH (pH $6.0_{70°C}$) containing 1.7M ammonium sulphate and the flow rate 0.3mlmin^{-1}. After an elution volume of 13.5ml, a 15ml gradient of 1.7 - 0M ammonium sulphate (in 50mM MES/NaOH (pH $6.0_{70°C}$)) was started. A trial on a TSKgel column Type G3000 SW (7.5mm x 60cm, Toyo-Soda Manufacturing Co., Tokyo, Japan) was made. The trial involved loading a 20µl sample containing 0.5mg freeze dried powder. The elution buffer was 0.1M ammonium acetate (pH $6.9_{20°C}$) and the flow rate 1mlmin^{-1}. The first Sepharose CL-6B trial involved loading a 1ml sample containing 50mg freeze dried powder. A column was made in which the gel volume was approximately 250ml (2.6cm x 47cm). The elution buffer was 0.1M ammonium acetate (pH $6.75_{20°C}$) and the flow rate 1mlmin^{-1}. A second trial loaded a 1ml sample containing 5mg freeze dried powder, with a flow rate of 1.5mlmin^{-1}. An Analytical Superose 6 (Pharmacia) trial involved loading a 0.1ml sample containing 0.5mg freeze dried powder. The elution buffer was 0.1M ammonium acetate (pH $6.75_{20°C}$) and the flow rate 0.5mlmin^{-1}.

For the final purification sequence of the xylanase a small amount of material (40 mg freeze dried powder) was further purified using three chromatography steps. A 4ml sample containing 40mg freeze dried powder was loaded on a MONO Q 10/10 (Pharmacia) column. The elution buffer was 20mM Bis-Tris/HCl (pH $6.55_{20°C}$) and the flow rate 3mlmin^{-1}. After an elution volume of 88ml, a 60ml gradient of 0 - 0.22M NaCl (in 20mM Bis-Tris/HCl (pH $6.55_{20°C}$)) was started. The final conditions of this gradient were continued for 262ml, before a further 60ml gradient of 0.22 - 0.32M NaCl (in 20mM Bis-Tris/HCl (pH $6.55_{20°C}$)) was started. The final conditions of this gradient were continued for 67ml, before a final 60ml gradient of 0.32 - 1.0M NaCl (in 20mM Bis-Tris/HCl (pH $6.55_{20°C}$)) was started. The active fractions totalled 37ml when pooled and 5.29g ammonium sulphate was added to bring the concentration to 1M. This sample was loaded on a Phenyl Superose 5/5 column. The elution buffer was 20mM Bis-Tris/HCl (pH $6.55_{20°C}$) containing 1M ammonium

sulphate and the flow rate 0.4mlmin^{-1}. After an elution volume of 42ml, a 6ml gradient of 1 - 0.5M ammonium sulphate (in 20mM Bis-Tris/HCl (pH $6.55_{20°C}$)) was started. The final conditions of this gradient were continued for 6.6ml, before a final 14ml gradient of 0.5 - 0M ammonium sulphate (in 20mM Bis-Tris/HCl (pH $6.55_{20°C}$)) was started. The active fractions totalled 20ml when pooled. The sample was concentrated by ultrafiltration, 10-fold using a PM10 membrane (10 000 molecular weight cutoff, Amicon) and a further 4-fold using a microconcentrator (molecular weight cutoff). Two runs were made on an Analytical Superose 6 column. Each involved loading 0.2ml of the concentrated sample. The elution buffer was 50mM MES/NaOH (pH $6.0_{70°C}$) and the flow rate 0.5mlmin^{-1}. The active fractions totalled 3.75ml when pooled.

(iv) A CMCase extract was obtained from the Thermophile Research Unit, University of Waikato, Hamilton, New Zealand. It was used in the substrate specificity screen (Section 4.1). Briefly, the sample had been purified by Patchett (unpublished results) as follows: *E. coli* clone PB4563 cells were lysed using both a lysozyme method and sonication. Cell debris were removed by centrifugation and the extract was heat treated at 80°C for 15 minutes. After centrifugation ammonium sulphate was added to the supernatant to a concentration of 50% saturation. Following further centrifugation the pellet was dissolved and the solid matter removed by centrifugation. The sample was dialysed to remove ammonium sulphate and freeze dried. The purification factor at this stage was 24.4 (Patchett, unpublished results).

(v) A MUCase extract was made from *E. coli* clone PB4567. The extract was used in Section 6.3.2. The cells were grown following the general method of growth (see Section 2.4.2 (a)). The cells were harvested using the general method of harvesting using a hollow fibre column (see Section 2.4.2 (a)). The buffer used was 50mM MOPS/KOH (pH $7.0_{20°C}$). The lysozyme method described in the general method of cell breakage (see Section 2.4.2 (a)) was used. Experiments with this extract that involved heat treatment used the general method of heat treatment (see Section 2.4.2 (a)).

(vi) A MUCase extract was made from *E. coli* clone PB4567. The extract was used in gel filtration and ultrafiltration experiments (Sections 6.3.1 and 6.2.2). The cells were grown following the general method of growth (see Section 2.4.2 (a)) but 1.5l of medium was contained in 22 x 2l flasks. The cells were harvested by the general method of harvesting using a hollow fibre column (see Section

2.4.2 (a)). The buffer used was 20mM sodium acetate/acetic acid (pH $5.6_{20°C}$). Cell breakage was by sonication (D), using the general method (see Section 2.4.2 (a)). Experiments with this extract that involved heat treatment used the general method of heat treatment (see Section 2.4.2 (a)).

(vii) MUCase extracts were made on a small scale for the efficiency of extraction experiments (Section 3.2.2 (f)). These extracts were also used in Section 6.2.3 and for parts of Sections 6.3.5 and 6.3.6. *E. coli* clone PB4567 (unless otherwise stated) was grown following the general method of growth (see Section 2.4.2 (a)). The cells were harvested using the general method of harvesting by centrifugation (see Section 2.4.2 (a)). The cell pellet was transferred to Eppendorf tubes (instead of a glass beaker) in 0.5g quantities and frozen at -20°C. 0.8ml of an appropriate buffer was mixed with the thawed cells. The mixture was sonicated (K) for 2.5 minutes in an ice bath and then centrifuged (12 800 xg, 20min, 4°C followed by 39 200 xg, 90min, 15°C or 69 000 xg, 60min, 4°C). Experiments with these extracts that involved heat treatment used the general method of heat treatment (see Section 2.4.2 (a)).

2.5 Xylanase characterization experiments

2.5.1 Molecular weight

(a) SDS-PAGE

SDS-PAGE was performed using a PhastSystem (Pharmacia). A PhastGel Gradient 10 - 15 and PhastGel SDS Buffer Strips were used (both Pharmacia). The PhastGel was polyacrylamide on a polyester backing. The overall dimensions were 43 x 50 x 0.45mm and the backing was 0.175mm thick. The gel had a 13mm stacking gel zone (4.5% T, 3% C) and a 32mm continuous 10 - 15% gradient gel zone with 2% crosslinking. The buffer system in the gel was 0.112M acetate (leading ion) and 0.112M Tris, pH 6.4. The buffer system in the PhastGel Buffer Strips was 0.20M Tris and 0.55% SDS, pH 7.5. The Buffer Strips were made of 2% Agarose IEF and had dimensions 41 x 10 x 6mm, with a volume of approximately 2.5ml. Low Molecular Weight Calibration Kit standards (Pharmacia) were dissolved in 10mM Tris/HCl (pH $8.0_{20°C}$) containing 1mM EDTA, 2.5% SDS and 5% β-mercaptoethanol. The molecular weight standards and a sample of the purified xylanase were heated at 100°C for 5 minutes. 0.01% Bromophenol Blue was added to the molecular weight standards. 1µl of the xylanase sample (containing approximately 50ng protein)

and of the molecular weight standards were loaded on to the gel. The gel was run and silver stained under the conditions specified in the PhastSystem Separation Technique File No.110 and PhastSystem Development Technique File No.210 (Pharmacia). The running conditions were 250V, 10.0mA and 3.0W at 15°C for 60Vh. The sample loading was from 1 - 10Vh. Silver staining was performed with the following solutions for the time, and at the temperature, specified: 50% ethanol and 10% acetic acid, 2min, 50°C; 10% ethanol and 5% acetic acid, 2min, 50°C; 10% ethanol and 5% acetic acid, 4min, 50°C; 8.3% glutardialdehyde, 6min, 50°C; 10% ethanol and 5% acetic acid, 3min, 50°C; 10% ethanol and 5% acetic acid, 5min, 50°C; Milli Q water, 2min, 50°C; Milli Q water, 2min, 50°C; 0.25% silver nitrate, 13min, 40°C; Milli Q water, 0.5min, 30°C; Milli Q water, 0.5min, 30°C; 0.04% formaldehyde in 2.5% sodium carbonate, 0.5min, 30°C; 0.04% formaldehyde in 2.5% sodium carbonate, 4min, 30°C; 5% acetic acid, 2min, 50°C and 10% acetic acid and 5% glycerol, 3min 50°C.

(b) Gel filtration

Immediately after the two Analytical Superose runs in the final step of the purification sequence, 100µl of Gel Filtration Molecular Weight Standards (Biorad) were loaded. Identical conditions were used, that is, the elution buffer was 50mM MES/NaOH (pH $6.0_{70°C}$) and the flow rate $0.5 ml min^{-1}$.

2.5.2 Isoelectric point

Isoelectric focusing was performed in agarose gels, as there was a possibility that this could be used as a purification step. Elution from agarose gels is generally easier than from polyacrylamide gels. The methods were based on those in the Pharmacia IEF Handbook. Initially 20ml gels were made containing sorbitol and Pharmalytes. The gels were modified to 30ml gels containing glycerol and Ampholines, they were also made wider. Electrolytes were modified from 0.05M H_2SO_4 and 1M NaOH or 0.05M H_2SO_4 and 0.2M L-histidine to 1M H_3PO_4 and 1M NaOH (anolyte and catholyte respectively). The length of prefocusing and focusing were shortened and the voltage increased. The final conditions and methods follow.

An agarose gel was made by dissolving 0.3g Agarose IEF in 3.78g glycerol and 27ml distilled water by bringing the mixture to the boil. The solution was cooled in a 75°C water bath and 0.38ml 4 - 6, 0.76ml 3.5 - 5 and

0.76ml 5 - 8 LKB Ampholines were added. A 25cm length of Gel Bond was rinsed with distilled water, dried and adhered to a glass plate using a few drops of water. The glass plate rested on and between two supports and the surface was level. The Gel Bond and glass plate arrangement was warmed using either a hairdrier or a plastic bag containing warm water. The gel was poured on to the middle of the Gel Bond and spread quickly with a prewarmed spreader. After the gel had set it was removed from the glass plate and stored over night in a humidity box at 4°C. The gel was trimmed to approximately 9.5cm x 13cm. Its thickness was 1 - 1.5mm. Electrode strips (6 x 10mm cross section, Pharmacia) were soaked in the electrolytes and blotted thoroughly before cutting to the exact size required so that the strip did not overhang the edge of the gel. The gel was adhered to the bed of a Flatbed IEF Apparatus (Pharmacia) with a few drops of water (Gel Bond-side down). A constant temperature of 10°C was maintained throughout the experiment with circulating water supplied by a cooling unit (Julabo). A Computer Controlled Electrophoresis Supply model 3000Xi (Biorad) was used. The gel was prefocused for 15 minutes (approximately 135Vh) with limits set to 5W (constant), 1 500V and 150mA. During this time power and current remained approximately constant while voltage increased from approximately 470 to 590V. 5 - 20µl samples were loaded near the cathode on filter paper applicators cut from filter paper (Whatman no.1), unless otherwise stated. The gel was focused for approximately 95 minutes (approximately 2 000Vh) with limits set to 10W (constant), 1 500V and 150mA. During this time power decreased from approximately 10 to 8W, current decreased from approximately 13 to 6mA and voltage increased from approximately 770 to 1 500V. The sample applicators were removed after approximately 50 minutes focusing. The electrodes were blotted several times during focusing.

An activity overlay method was developed based on the methods of Sharrock (1985). A very similar method has also been developed by MacKenzie and Williams (1984). Agarose gels containing 0.1% or 0.5% (w/v) dyed xylan in citrate buffer were less successful than those containing oat spelts xylan in trials. The method finally used is as follows. An overlay gel was made by dissolving 0.06g agarose in 6ml of a solution pipetted from the top of a settled 0.25% oat spelts xylan suspension in 50mM sodium citrate/citric acid buffer (pH $6.4_{20°C}$), by bringing to the boil. The solution was cooled to 75°C in a 75°C water bath and poured onto Gel Bond as for the Agarose IEF gels above. After the gel had set it was removed from the glass plate and stored over night in a humidity

box at 4°C. The gel was trimmed to approximately 9.5cm x 13cm. Its thickness was approximately 0.5mm. Immediately after focusing the overlay gel was carefully laid on the IEF gel without trapping air bubbles between them for one minute and its position marked carefully. The overlay gel was then adhered (Gel Bond-side down) to a prewarmed thick glass plate with a few drops of water. The underside of the glass plate was in contact with the water in a 70°C water bath. The overlay was incubated uncovered at 70°C for 3 minutes. It was immediately stained by submergence in a dish of $1mgml^{-1}$ Congo Red for 10 minutes with occasional agitation. The overlay was destained by submergence in a dish of 1M NaCl for an appropriate amount of time with occasional agitation. In order to dry and store the overlay it was then submerged in a solution of 5% (v/v) acetic acid. The overlay was dried in air.

Immediately after the activity overlay gel had been removed from the IEF gel, the IEF gel was fixed for 30 minutes with constant agitation in a solution of 5% (w/v) sulphosalicylic acid and 10% (w/v) trichloroacetic acid. It was destained twice, for 25 minutes with constant agitation each time, in a solution of methanol : acetic acid : distilled water (3:1:6). The gel was dried by placing it on some filter paper and covering it with three pieces of filter paper (all Whatman no.1), a glass plate and 1kg weight for 15 minutes. It was then dried off completely in air or with a cool hairdrier. It was stained with constant agitation in 0.2% (w/v) Coomassie Brilliant Blue R-250 dissolved in destaining solution and filtered before use (Whatman no.1). It was then destained twice (as above) and dried in air or with a cool hairdrier.

All gel manipulations, that is IEF and overlay gel pouring and trimming, electrode soaking and blotting, focusing, sample loading and IEF and overlay gel developing, were performed while wearing latex gloves.

2.5.3 Thermostability

0.1ml aliquots of the xylanase (in the presence or absence of BSA) were incubated in capped Eppendorfs for specific times at appropriate temperatures. Assays for residual activity were performed as in Section 2.1.1 (a), except that 0.4ml oat spelts xylan suspension, preheated to 70°C, was added to the heat treated xylanase immediately each heat treatment incubation was completed.

2.6 MUCase purification and characterization experiments

2.6.1 Gel filtration

Gel filtration was performed on columns of TSK Fractogel. The gel was mixed with 5l of distilled water or buffer and allowed to settle for more than two hours. The liquid was decanted, more distilled water or buffer was added and the liquid was removed by vacuum filtration. Approximately 50% (v/v gel) buffer was added to the gel and the slurry was vacuum degassed. The gel was poured carefully down the side of the column and equilibrated with buffer at a constant flowrate.

A column with final dimensions of 5cm x 39cm was used in the molecular weight work (Sections 6.2.2 and 6.3.1). The elution buffer was 20mM sodium acetate/acetic acid (pH $5.6_{20°C}$) and the flow rate 4mlmin^{-1}. 6.5ml samples of MUCase and Gel Filtration Molecular Weight Standards (Sigma) (the molecular weight standards sample also contained 0.3ml glycerol) were loaded separately.

A slightly larger column with final gel dimensions of 5cm x 50cm was used in the gel filtration purification trials (Section 6.2.2). The elution buffer was 200mM sodium acetate/acetic acid (pH $5.6_{20°C}$) and the flow rate 4mlmin^{-1}. 2ml, 20ml and 200ml samples of MUCase were loaded in three separate runs. In each case the active fractions were pooled and concentrated by ultrafiltration using a PM10 membrane (10 000 molecular weight cutoff, Amicon). The protein concentration of each extract before and after gel filtration was adjusted to approximately 2.3 mgml^{-1} before heat treatment.

2.6.2 Cellulose column

The column was made and run based on the methods of Sharrock (1985). A small column was made by loading 0.2g Sigmacell 50 into a plastic dropper. The final dimensions of the Sigmacell 50 column were 4mm x 4.3cm. The column was submerged in an ice bath and equilibrated with ice cold 0.2M sodium acetate/acetic acid (pH $5.6_{20°C}$) at a flow rate of 0.33mlmin^{-1}. A 1ml MUCase sample was loaded. After an elution volume of 18ml, the elution buffer was changed to ice cold 20mM sodium acetate/acetic acid (pH $5.6_{20°C}$). After a further 13ml, the elution buffer was changed to ice cold 2mM sodium acetate/acetic acid (pH $5.6_{20°C}$) and after 11ml of this buffer, ice cold distilled

water was used. The column was moved to a 20°C water bath after an elution volume of 21ml distilled water. The temperature was slowly raised so that after 47ml distilled water the temperature had reached 50°C. 16ml 6M urea at 70°C was then passed through the column.

2.6.3 Affinity chromatography

The formation of an affinity gel was attempted by linking cellobiose to cyanogen bromide activated Sepharose 6B following the procedure in the Pharmacia Affinity Chromatography Handbook. The exact procedure used is as follows. 1.125g freeze dried cyanogen bromide activated Sepharose 6B was swollen in distilled water and washed with 100ml distilled water on a filter. It was then washed with coupling buffer (0.1M sodium borate/NaOH, pH 9.5). 2g cellobiose was dissolved in 20ml coupling buffer and then mixed with the swollen gel. The slurry was incubated for 16 hours at 45°C in a shaking water bath (100 strokes/min). The gel was washed on a filter with 100ml each of coupling buffer, distilled water, 0.1M sodium borate/NaOH (pH 8.0) and 0.1M sodium acetate/acetic acid buffer (pH 4.0). The gel was mixed with 10ml 1M ethanolamine/HCl (pH 9.0) and stored at 4°C for 23 hours. It was then washed with 100ml each of 0.1M sodium acetate/acetic acid buffer (pH 4.0), 0.5M NaCl, 0.1M sodium borate/NaOH (pH 8.0) and 0.5M NaCl and stored at 4°C.

The gel was washed with 200mM sodium acetate/acetic acid (pH $5.6_{20°C}$) containing 0.02% (w/v) sodium azide and resuspended in this buffer. A small column was made by pouring the slurry into the barrel of a 5ml syringe and equilibrating it with 200mM sodium acetate/acetic acid (pH $5.6_{20°C}$) at a flow rate of $0.5 ml min^{-1}$. The column contained 3.2ml gel. 0.3ml MUCase extract was loaded. After an elution volume of 22ml, an elution buffer (200mM sodium acetate/acetic acid (pH $5.6_{20°C}$)) containing 100mM cellobiose was used.

2.6.4 Dialysis and desalting

Dialysis was performed at 4°C in a Spectra/Por 3 membrane with a 3 500 molecular weight cutoff. Desalting was performed at room temperature on a column with a 6 000 molecular weight cutoff.

2.6.5 Molecular weight

Ultrafiltration was performed with PM30 (30 000 molecular weight cutoff) and YM100 (100 000 molecular weight cutoff) membranes (both Amicon).

In each case a 2ml MUCase sample was diluted to 10ml with buffer and concentrated to approximately 2ml, twice.

The gel filtration estimation of molecular weight is described in Section 2.6.2.

2.6.6 Temperature stability

MUCase samples were incubated in capped tubes for specific times at appropriate temperatures. The protein concentration of the MUCase sample before heat treatment was $2mgmin^{-1}$. Assays for residual activity were performed as in Section 2.1.1 (c) and (f).

2.6.7 Effect of compounds on stability

An extract of MUCase was extensively dialysed against a concentration of urea slowly increasing to 4M and decreasing to 0M. A 2.5ml sample was dialysed (3 500 molecular weight cutoff, Spectra/Por 3) against 500ml buffer (0.2M sodium acetate/acetic acid (pH $5.6_{20°C}$)) at 4°C. Urea was added in the amount listed (final urea concentration also listed) and the sample was dialysed against the resultant solution for the time listed: 1.201g (40mM), 16hr; 10.811g (0.4M), 10hr; 109.31g (4M), 21.5hr; dilution of solution 10-fold with distilled water (0.4M), 16hr; dilution of solution 10-fold (40mM), 9.5hr; dilution of solution 10-fold (4mM), 14.5hr; change solution to 0.2M sodium acetate/acetic acid (pH $5.6_{20°C}$) (0mM), 10.5hr; change solution to 0.2M sodium acetate/acetic acid (pH $5.6_{20°C}$) (0mM), 13hr.

2.7 Hydrophobic cluster analysis

The HCA used was based on that of Henrissat *et al.* (1989). The HCA plot is derived from a two dimensional pattern used by Lim (1974) for two dimensional structure predictions. The amino acid sequence, in one letter code, is drawn on a classical α-helical net (3.6 residues per turn) and duplicated (Gaboriaud *et al.*, 1987). Proline is symbolized by ★, glycine by ♦, cysteine by ©, serine by ⊡ and threonine by ◻. The following amino acids are considered as hydrophobic: V, I, L, F, W, M and Y. In a hydrophobic environment, alanine and cysteine can sometimes be considered as hydrophobic. Clusters composed of adjacent hydrophobic residues not separated by prolines are circled. The comparisons are based on the two dimensional topology and distribution of the hydrophobic clusters along the sequences.

2.8 Chemicals and their sources

The following is not an exhaustive list of every chemical used in this study, but a list of the more important or unusual chemicals used.

The following were obtained from Sigma: oat spelts xylan, larchwood xylan, pentosan polysulphate, CMC (sodium salt, low viscosity), PAHBAH, all *p*NP-substrates, *p*NP, *o*NPX, MUC, xylobiose, cellobiose, D-xylose, D-glucose, maltose, maltotriose, maltotetraose, maltopentaose, maltohexaose, maltoheptaose, ampicillin, lysozyme, DNase, BSA, PMSF, SDS, agarose, Sigmacell 50, Sepharose CL-6B, cyanogen bromide activated Sepharose 6B, Gel Filtration Molecular Weight Standards, MES, MOPS, Bis-Tris, Bis-Tris propane, Tris and Remazol Brilliant Blue.

Pharmacia supplied the following: PhastGel Gradient 10 - 15, PhastGel SDS Buffer Strips, Low Molecular Weight SDS Calibration Kit, IEF Standards, Agarose IEF and Pharmalytes. Other sources included: Asahi Chemical Industry Co. Ltd. (Japan) for Avicel PH-102; Merck for TSK Fractogel HW55 (F) and bulk ammonium sulphate; LKB for Ampholines; R.F.H. Dekker (Division of Biotechnology, CSIRO, Australia) for hemicellulose A and B from sugar cane bagasse; Eastman Kodak for MU and Biorad for Gel Filtration Molecular Weight Standards and Gel Bond.

Where possible analytical grade reagents were used. Although the term distilled water is used throughout this work, it was the minimum grade of water used (unless otherwise stated). Milli-Q grade water was generally used, especially in chromatography experiments.

Chapter Three

Thermostability and heat treatment

A heat treatment method of purification can be used for thermostable proteins. Thermostability and heat denaturation of proteins were studied initially. A systematic study of heat treatment as a method of purification of five cloned xylanolytic and cellulolytic enzymes was then made. The aim was to develop a simple method of purification.

3.1 Measurement of thermostability

Four methods were investigated to measure the overall thermostability of crude protein extracts: assaying soluble and insoluble protein, UV difference spectrophotometry, fluorescence spectrometry and enzyme activity meaurements. The latter will be discussed in Section 3.2. Fluorescence was attempted as it has been reported to be a more sensitive method than absorbance (Wetlaufer, 1962). Preliminary fluorescence experiments gave results that were difficult to interpret, possibly due to the complex composition of the protein extracts. No further fluorescence experiments were attempted. For these reasons the preliminary fluorescence results have not been included.

3.1.1 Soluble and insoluble protein

Cell free protein extracts of organisms with different growth temperatures were subjected to various heat treatments. Table 3.1 shows that protein extracts of organisms with higher growth temperatures (*T. aquaticus* and *Thermotoga* sp.) had more protein remain in solution after heat treatment. This is not unexpected, as proteins from thermophiles are generally more stable than those from mesophiles. Daniel *et al.* (1982) showed that heat induced insolubility of protein extracts correlates with the growth temperature of the organism.

There is a negligible difference in the proportion of soluble and insoluble protein when protein extracts of various protein concentrations are heat treated (Table 3.2). Thermostability appears to be independent of protein concentration at higher protein concentrations ($>0.87 mgml^{-1}$). Intracellular protein concentrations are very high, so any variation in the thermostabilities of intracellular proteins is likely to be independent of the intracellular protein

concentration. As the heat treatment temperature is increased, the amount of protein remaining soluble decreases and more protein is detected as insoluble (Table 3.3). The proportions of soluble and insoluble protein do not usually total 100% due to the inaccuracy in measuring insoluble protein.

Typical standard curves for the protein assays used are shown in Plots A and B of Figure 3.1.

3.1.2 UV difference spectrophotometry

A UV scan (from 190nm to 320nm) of a protein extract was made and stored in the memory of the spectrophotometer. A difference spectrum was then obtained by subtracting the stored scan from another scan being made. The difference spectra were broad with no distinctive peaks, due to the protein extracts containing many proteins. In contrast, difference spectra of pure proteins typically have distinctive peaks that show clear changes on chemical or physical treatment.

Protein extracts heated at high temperatures were generally found to have UV scans more different to those of the controls (non-heat treated protein extracts), than protein extracts treated at lower temperatures (Figure 3.2). This can be seen more clearly in the difference spectra (Figure 3.3).

In order to compare different treatments more easily, one wavelength was chosen at which to take all further UV measurements. Heat treatment temperature versus difference in absorbance (as a percentage of the control) at six wavelengths was plotted (Figure 3.4). 240nm was chosen as the most reliable wavelength to show large differences. At this wavelength the problems associated with lower (far UV) wavelengths are avoided, while peptide band absorbance still contributes and amino acid composition has less affect. Difference spectra are reported to have a more prominant peak in the range 220 - 240nm than 280 - 290nm (Wetlaufer, 1962). Figure 3.4 also shows that the *B. stearothermophilus* protein extract shows little difference from the control when heat treated at 60°C. After heat treatment at 70°C a significant difference is observed. Heat treatment at 80°C gives only a slight increase to that at 70°C, but a 90°C heat treatment gives a very large difference compared to the control. The absorbance change is not directly proportional to the temperature of heat treatment, therefore the amount of denaturation is likely to be not proportional to the temperature of heat treatment. Proteins can be expected to be

Table 3.1 Effect of organism's growth temperature on the proportion of protein remaining soluble and insoluble after heat treatment of a protein extract at 85°C for one hour.

Organism	Growth temp (°C)	Initial protein conc ($mgml^{-1}$)	Protein remaining soluble (%)	insoluble (%)
B. stearothermophilus	55	0.78	40	nd
		0.78	48	67
T. aquaticus	70	0.80	79	nd
		0.83	84	0
Thermotoga sp. strain FjSS3B.1	85	0.85	96	nd
		0.77	77	6

nd = not determined

Table 3.2 Effect of initial protein concentration on the proportion of protein remaining soluble and insoluble after heat treatment of a protein extract at 75°C for one hour.

Organism	Growth temp (°C)	Initial protein conc ($mgml^{-1}$)	Protein remaining soluble (%)	insoluble (%)
B. stearothermophilus	55	18.5	52	52
		4.06	53	48
		1.38	64	57
Thermus sp. strain T-351	70	19.2	94	9
		3.87	94	7
		0.87	94	0

Table 3.3 Effect of heat treatment temperature on the proportion of protein remaining soluble and insoluble after heat treatment of a protein extract for one hour.

Organism	Growth temp (°C)	Initial protein conc ($mgml^{-1}$)	Protein remaining after 1 hour heat treatment at 60°C sol (%)	60°C ins (%)	75°C sol (%)	75°C ins (%)	85°C sol (%)	85°C ins (%)	90°C sol (%)	90°C ins (%)	95°C sol (%)	95°C ins (%)
B. stearothermophilus	55	1.38	90	36	64	57	nd	nd	52	74	nd	nd
Thermus sp. strain T-351	70	0.87	106	0	94	0	nd	nd	79	29	nd	nd
Thermotoga sp. strain FjSS3B.1	85	0.77	nd	nd	nd	nd	77	6	72	23	62	28

nd = not determined

Figure 3.1 Protein standard curves.

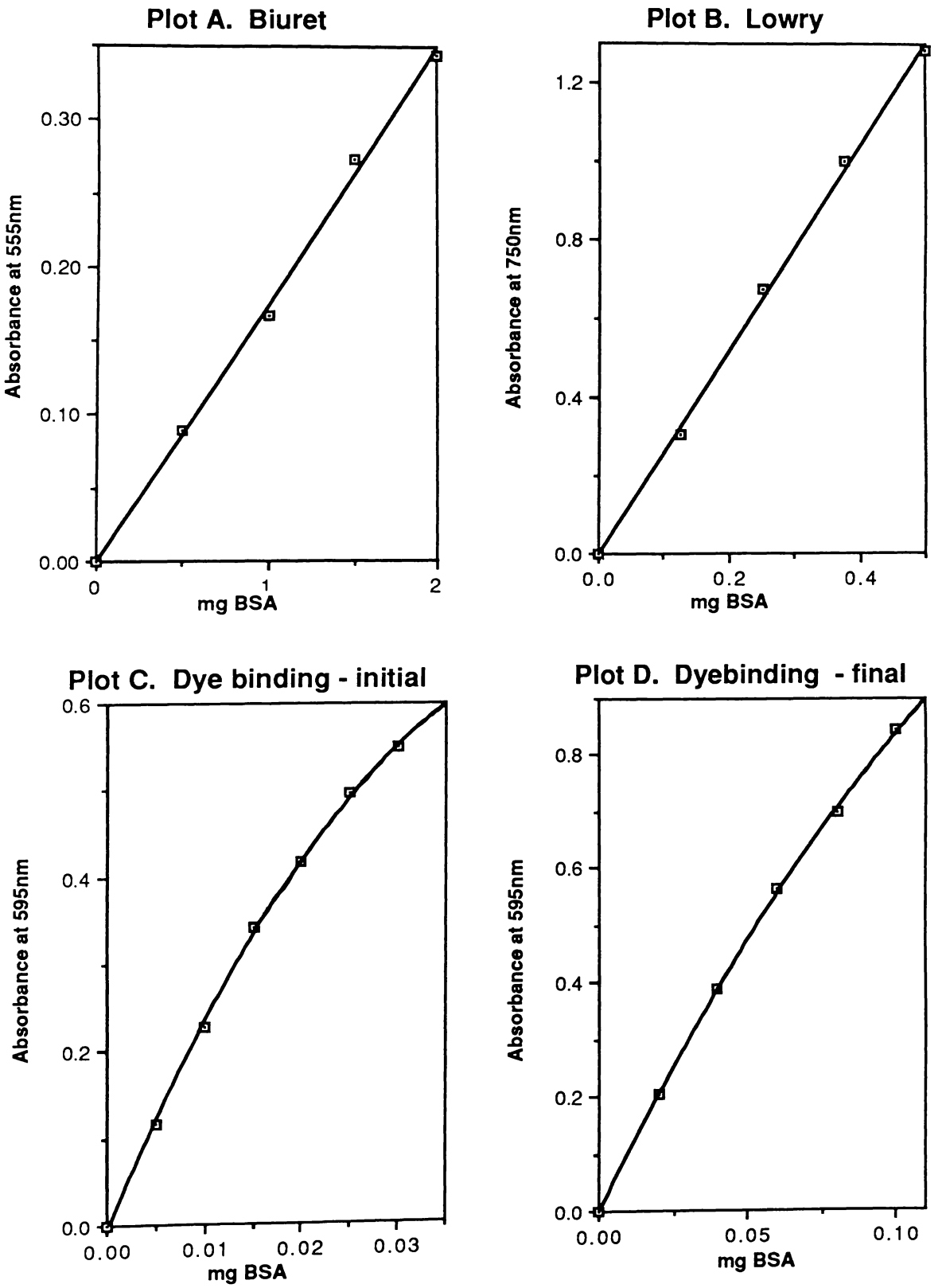


Figure 3.2 UV scans of a *B. stearothermophilus* protein extract ($43\mu gml^{-1}$), before and after heat treatment at 60°C, 70°C, 80°C and 90°C for one hour.

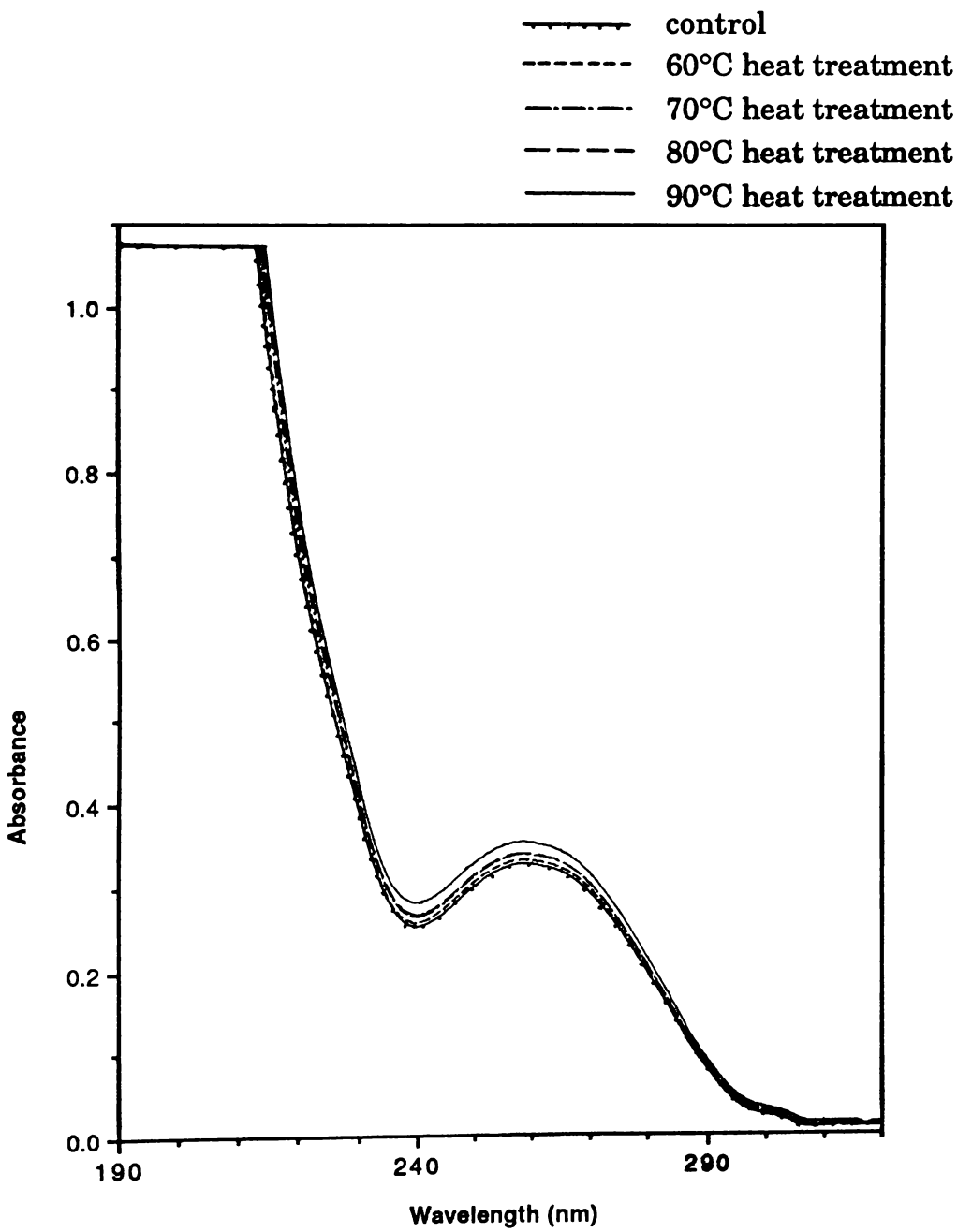


Figure 3.3 Difference spectra of a *B. stearothermophilus* protein extract (43μgml^{-1}), before and after heat treatment at 60°C, 70°C, 80°C and 90°C for one hour.

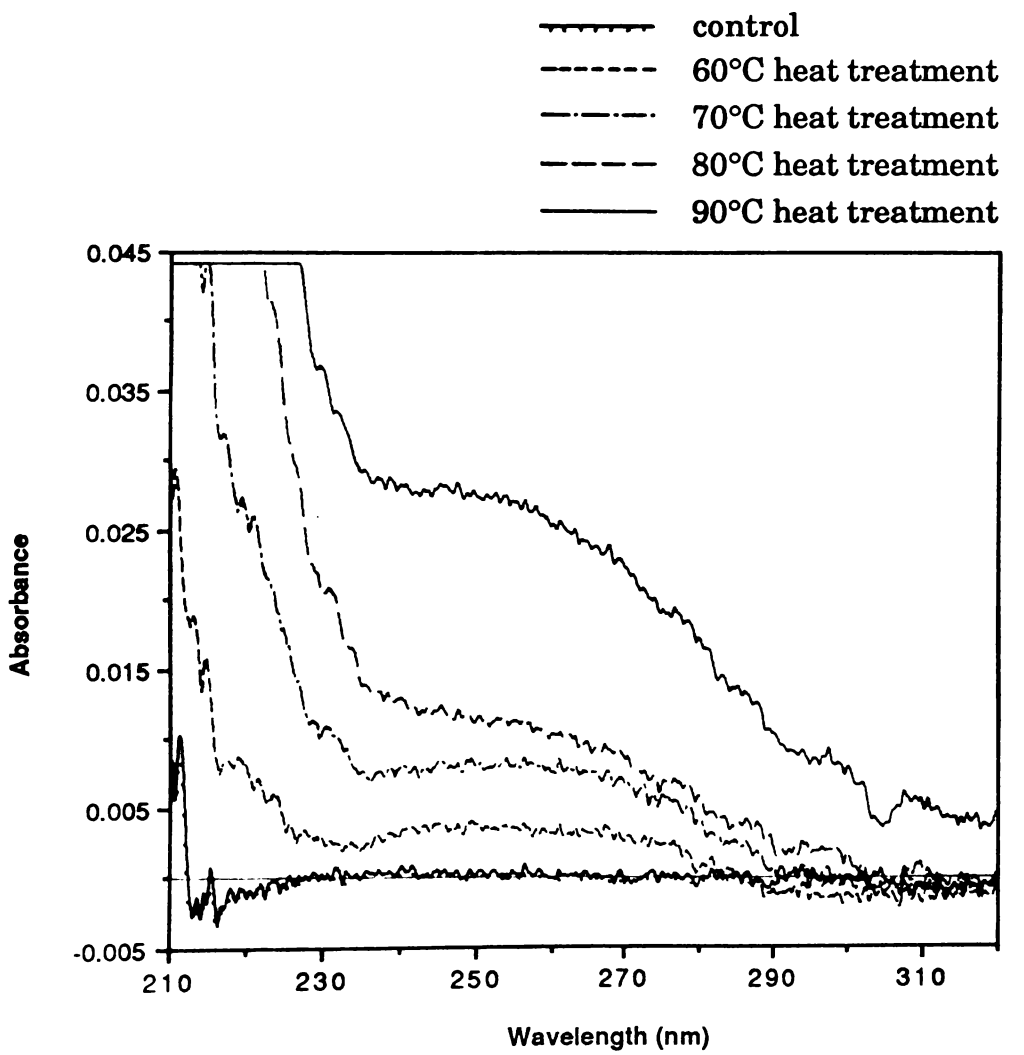
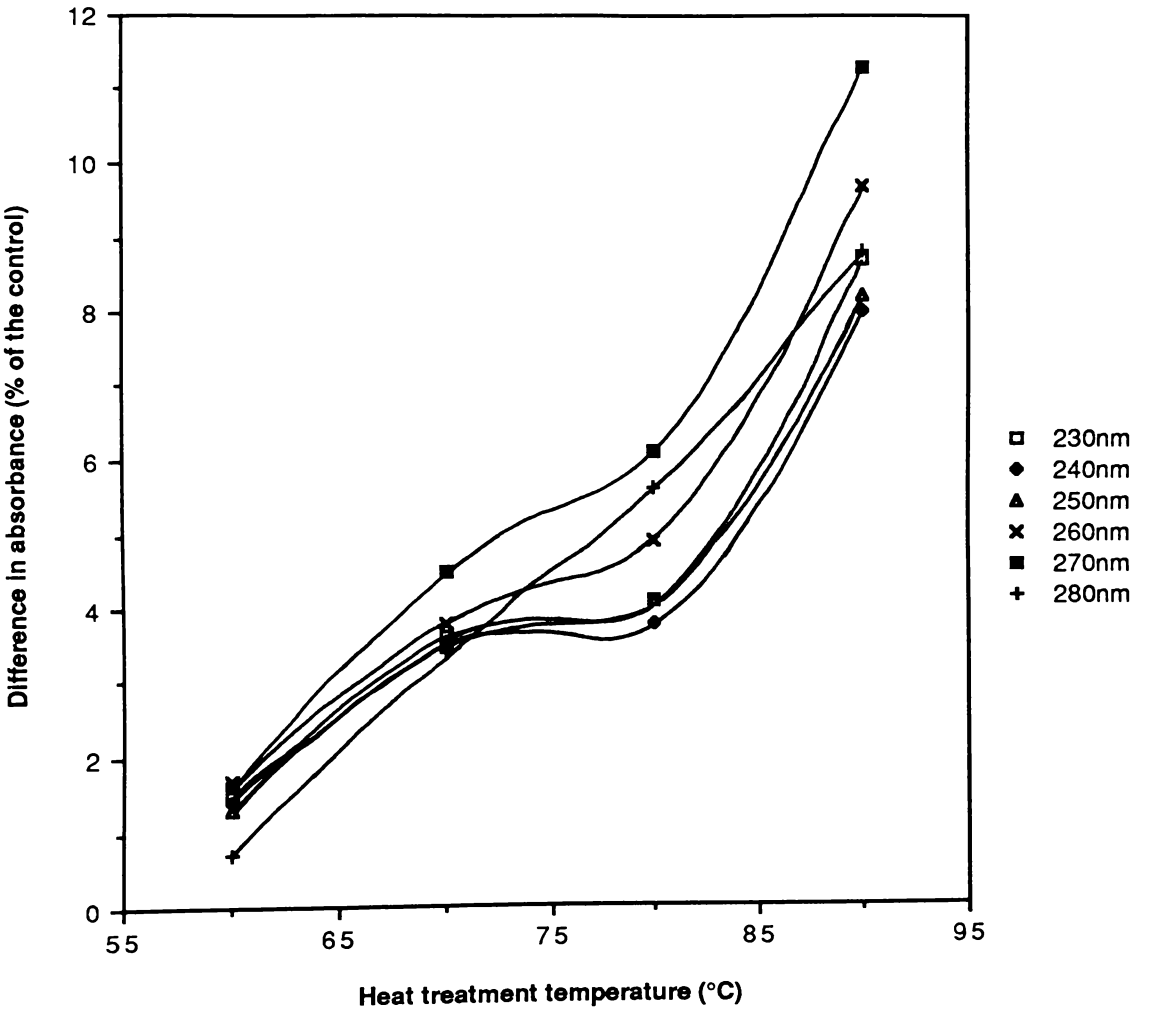


Figure 3.4 Heat treatment temperature versus difference in absorbance at six wavelengths.
B. stearothermophilus protein extract
($43\mu gml^{-1}$) heat treated for one hour.



thermostable at temperatures close to the growth temperature of the organism. At higher temperatures proteins will be less thermostable.

The effect of protein concentration on the thermostability of protein extracts was examined. Only a small protein concentration range could be used as the dilution of a heat treated extract gave a UV scan inconsistent with that of an extract that had been diluted then heat treated. Dilution after heat treatment possibly altered the denatured or undenatured state of some of the proteins in the extract. The absorbance (A_{240}) of the control and heat treated protein solutions was directly proportional to the protein concentration (Figure 3.5) in each case. The percent difference was approximately related to protein concentration at any heat treatment temperature (Figure 3.6). At protein concentrations above approximately 120μgml^{-1} differences were approximately constant for heat treatment at any temperature. At lower protein concentrations, increasingly greater differences were seen as the temperature of heat treatment increased. The data in Figure 3.6 (initial protein concentration versus percent difference for four heat treatment temperatures) were replotted as heat treatment temperature versus percent difference for five initial protein concentrations (Figure 3.7). Lower concentration protein extracts were shown to be affected more significantly (showed a larger difference) by heat treatment than protein extracts of high initial protein concentration. Also, large differences were caused by heat treatment at temperatures significantly higher than the growth temperature of the organism. These results are consistent with those seen using the soluble and insoluble protein method.

Overall, the measurement of soluble and insoluble protein was simple, and useful for protein mixtures. Results from UV difference spectrophotometry showed that this method was promising, but would probably be better for pure proteins rather than protein mixtures. In those cases it may be possible to predict what is happening on a molecular level. Although the inaccuracy in measuring insoluble protein in the soluble and insoluble protein method, and the very small differences in absorbance in the UV difference spectrophotometry method, lead to some variability in the results, they are still useful. Thermostability was found to approximately correlate to the growth temperature of the organism from which the protein extract was made. The amount of denaturation was not directly proportional to heat treatment

Figure 3.5 Protein concentration versus absorbance for a *B. stearothermophilus* protein extract heat treated at 60°C, 70°C, 80°C and 90°C for one hour.

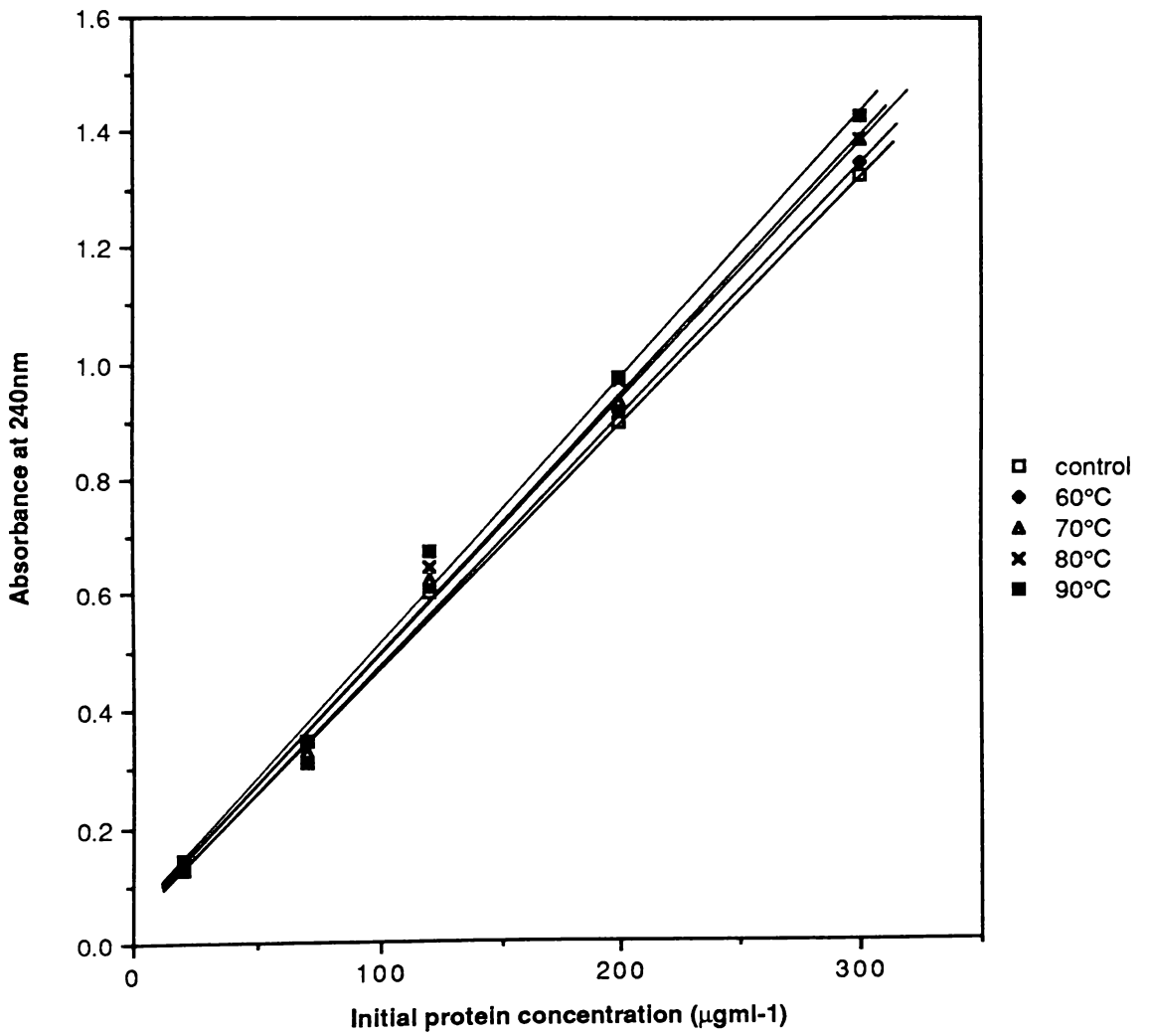


Figure 3.6 Protein concentration versus difference in absorbance for a *B. stearothermophilus* protein extract heat treated at 60°C, 70°C, 80°C and 90°C for one hour.

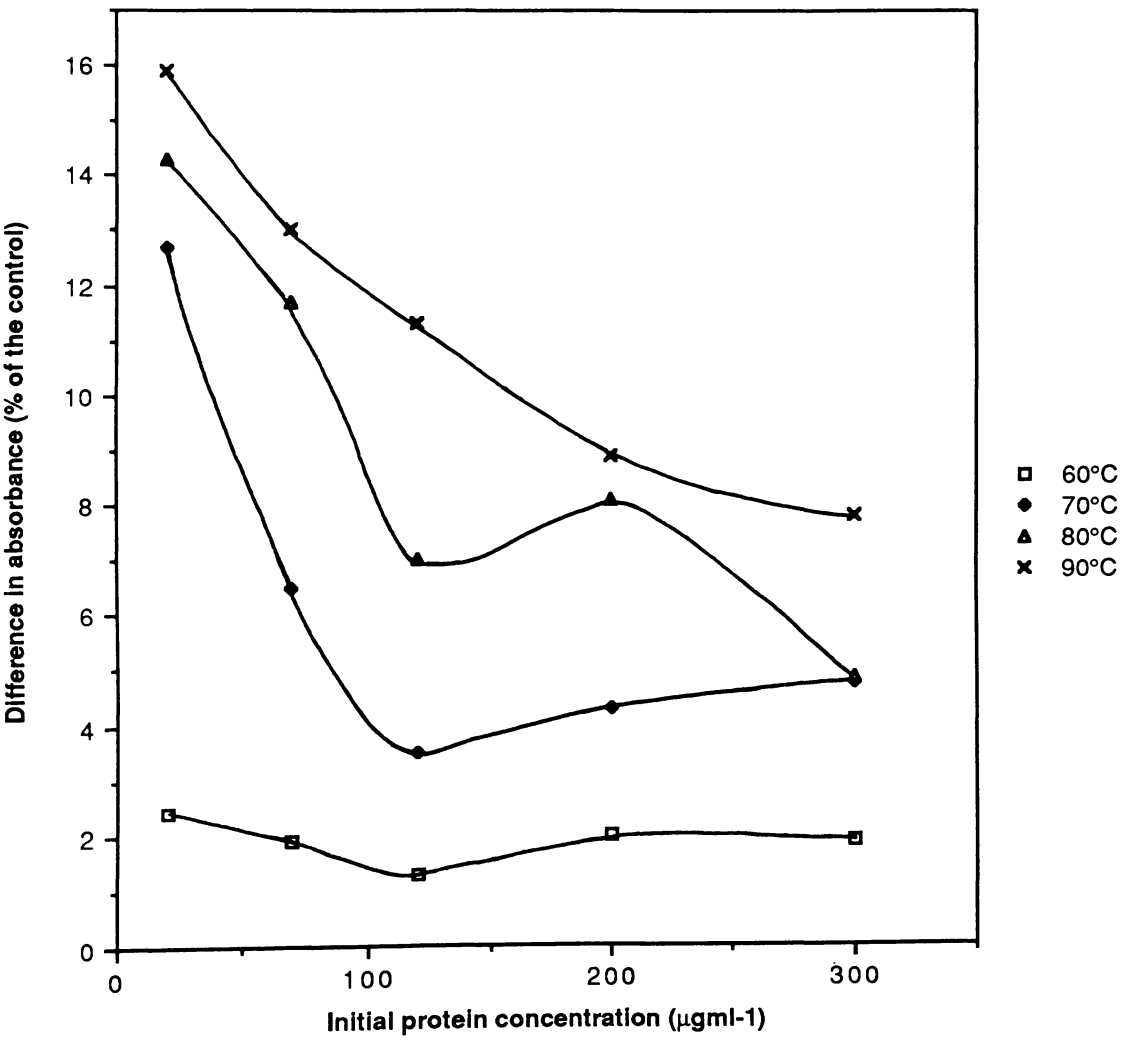
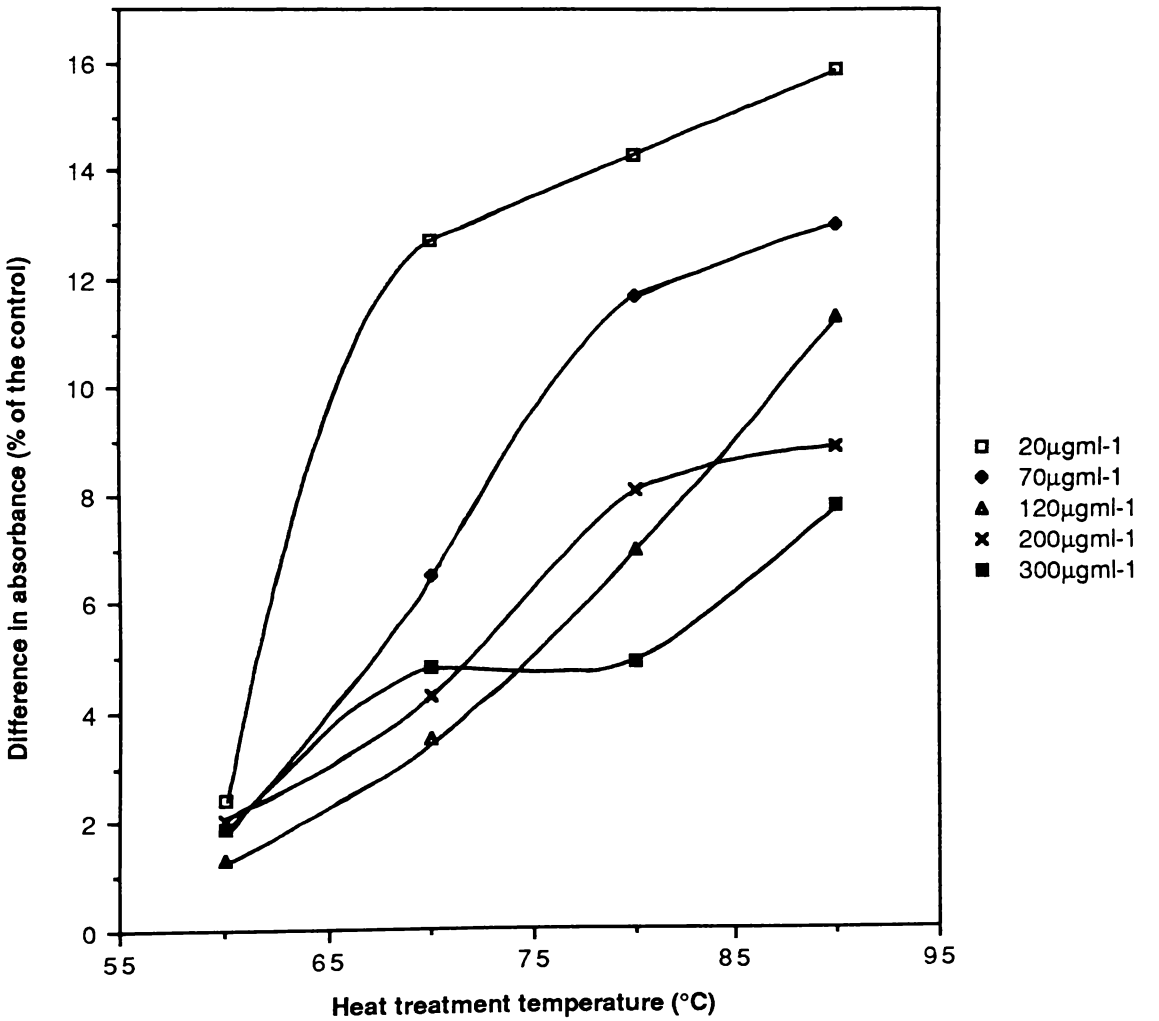


Figure 3.7 Heat treatment temperature versus difference in absorbance for a *B. stearothermophilus* protein extract heat treated at five protein concentrations.



temperature. The amount of denaturation, was found to be independent of the protein concentration as long as the protein concentration was above approximately $0.4 mgml^{-1}$. On heat treatment, very low protein concentrations showed the largest differences compared to the controls, in UV difference experiments. These protein concentrations are impractically low for use in a heat treatment method of purification.

3.2 Use of the property of thermostability in the purification of enzymes from *E. coli* clones by heat treatment

When thermostable enzymes are introduced into the protein population of a mesophile by cloning, a heat treatment method of purification can be used. Additionally, an idea of the thermostability of an enzyme can be gained by the measurement of total activity before and after heat treatment. Many of the findings reported in this section have been published (Patchett *et al.* (1989) (see Appendix).

3.2.1 Heat treatment of protein extracts of *E. coli* clones

Protein extracts made from a number of clones containing genes coding for cellulases and hemicellulases were subjected to heat treatment. Enzyme activities and soluble protein were measured before and after heat treatment (Tables 3.4 and 3.5). In the examples given, heat treatment was used either to prepare extracts for further study or as the first step in a purification sequence. The choice of temperature and time of heat treatment therefore reflect the opposition to gain maximum purification and maximum recovery.

Typical standard curves for the protein assays are shown in Plots C and D of Figure 3.1. Typical standard curves used to calculate activities of the enzymes are shown in Figures 3.8 and 3.9.

Heat treatment resulted in a 5 - 19 fold purification for the five enzymes. Heat treatment of β-xylosidase protein extracts showed high recoveries and good purification factors (Table 3.4). Heat treatment at 80°C for 30 minutes gave the highest purification factor but the lowest recovery of the four β-xylosidase examples. Xylanase extracts gave high recoveries and good purification factors (Table 3.4).

Table 3.4 Heat treatment purification of thermostable xylanolytic enzymes expressed in *E. coli*.

Enzyme	Clone	Heat treatment conditions	Total activity ($\mu molmin^{-1}$)		Soluble protein ($mgml^{-1}$)		Specific activity ($\mu molmin^{-1}mg^{-1}$)		Purification	Recovery (%)	Source
			before	after	before	after	before	after			
β-xylosidase	PB4716	80°C 30min	2.32	1.51	31.6	2.1	0.07	0.72	9.8	65	Schofield *et al.*, 1988
	PB4716	70°C 30min	2.59	2.30	31.0	3.5	0.08	0.66	8.0	89	Schofield *et al.*, 1988
	PB4716	70°C 35min	18265	14098	18.4	2.97	0.15	0.90	5.9	77	this study
	PB4855	70°C 60min	2981	2549	16.7	2.10	0.089	0.717	8.1	86	Hudson *et al.*, in press
xylanase	PB4716	70°C 35min	245700	179140	18.4	2.97	2.05	11.4	5.6	73	this study
	PB4716	70°C 30min	15.0	15.5	6.63	0.84	2.26	20.5	9.1	103	this study
	PB4716	70°C 30min	6715	6643	5.24	0.80	3.0	21.6	7.2	99	this study
	PB4716	70°C 30min	14.2	11.3	4.30	0.63	3.31	20.1	6.1	80	this study
	PB4887	70°C 30min	126.6	105.6	1.97	0.276	21.4	131.7	6.2	83	this study

Table 3.5 Heat treatment purification of thermostable cellulolytic enzymes expressed in *E. coli*.

Enzyme	Clone	Heat treatment conditions	Activity on substrate	Total activity (μmolmin^{-1}) before	Total activity (μmolmin^{-1}) after	Soluble protein (mgml^{-1}) before	Soluble protein (mgml^{-1}) after	Specific activity (μmolmin^{-1}mg^{-1}) before	Specific activity (μmolmin^{-1}mg^{-1}) after	Purification	Recovery (%)	Source
β-glucosidase	PB4551	70°C 65min		520	400	14.4	0.68	2.3	42	18.5	77	Plant *et al.*, 1988
	PB4551	70°C 30min		12.6	9.02	2.00	0.246	2.10	12.6	6.0	72	this study
CMCase	PB4563	70°C 60min		5500	2800	47.8	2.1	0.05	0.53	11.5	51	Neal, unpub. res.
	PB4563	70°C 60min		5989	2795	40.0	3.1	0.05	0.43	8.6	47	Neal, 1987
	PB4563	80°C 15min		4224	1865	20.9	2.0	0.061	0.315	5.2	44	Patchett, unpub. res.
MUCase	PB4567	70°C 30min	MUC	0.91	0.69	2.29	0.235	0.043	0.32	7.4	76	this study
			avicel	0.043	0.037	2.29	0.235	0.0021	0.0170	8.1	86	
	PB4567	70°C 30min	MUC	4.75	4.74	2.29	0.250	0.033	0.30	9.1	100	this study
			avicel	0.088	0.088	2.29	0.250	0.0006	0.0056	9.3	100	
	PB4567	70°C 30min	MUC	5.38	5.11	2.29	0.260	0.036	0.30	8.3	95	this study
			avicel	0.092	0.072	2.29	0.260	0.0005	0.0042	8.4	78	
	PB4567	70°C 30min	MUC	1.12	1.06	1.99	0.192	0.038	0.376	10.0	95	this study
			avicel	0.072	0.017	1.99	0.192	0.0024	0.0061	2.5	24	
			xylan	2.97	2.62	1.99	0.192	0.099	0.926	9.4	88	
	PB4800	70°C 30min	MUC	0.613	0.303	2.04	0.126	0.100	0.829	8.3	49	this study
			avicel	0.048	0.036	2.04	0.126	0.0078	0.099	12.6	75	
			xylan	0.612	0.464	2.04	0.126	0.100	1.27	12.7	76	

Figure 3.8 PAHBAH standard curves.

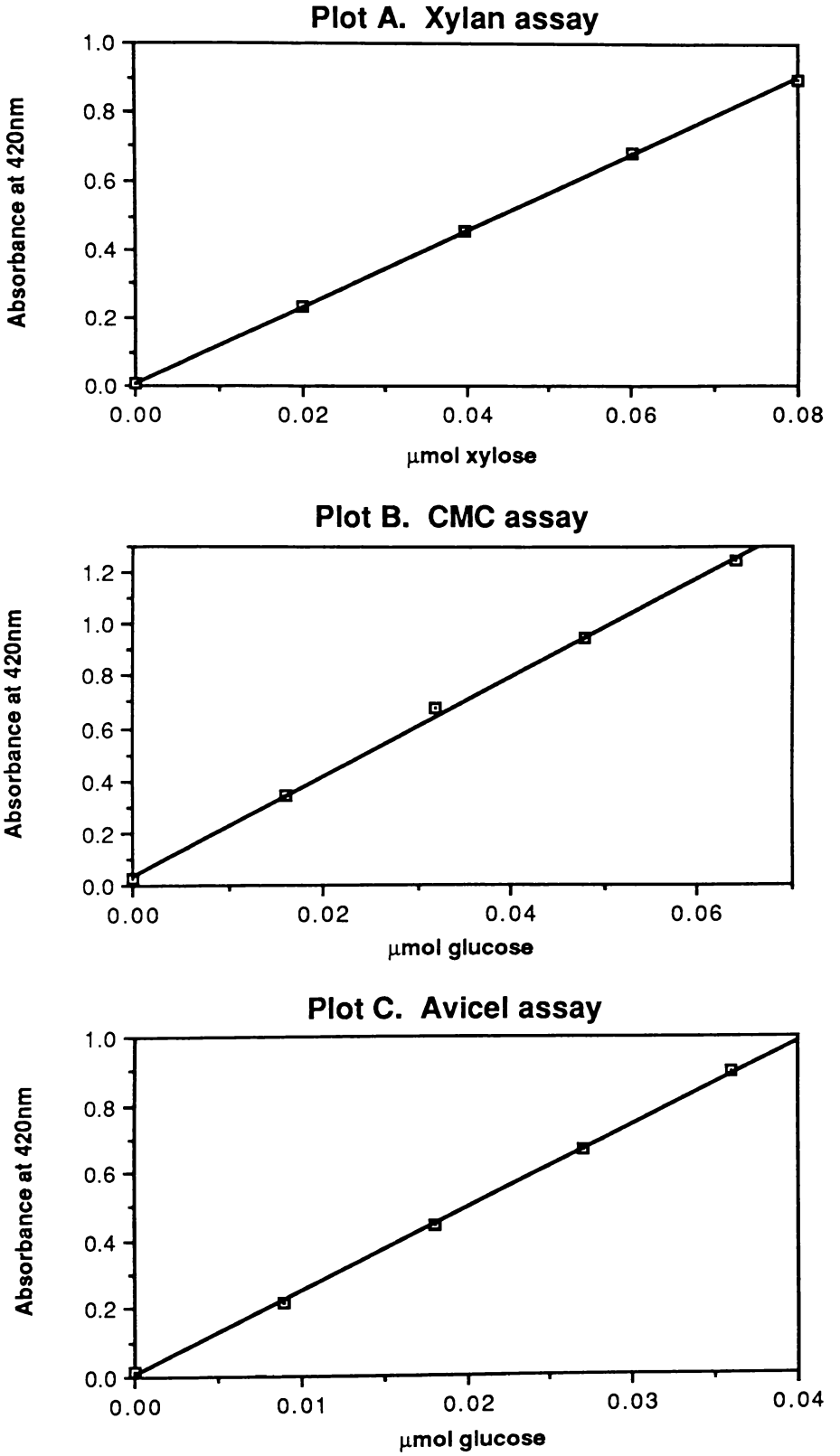
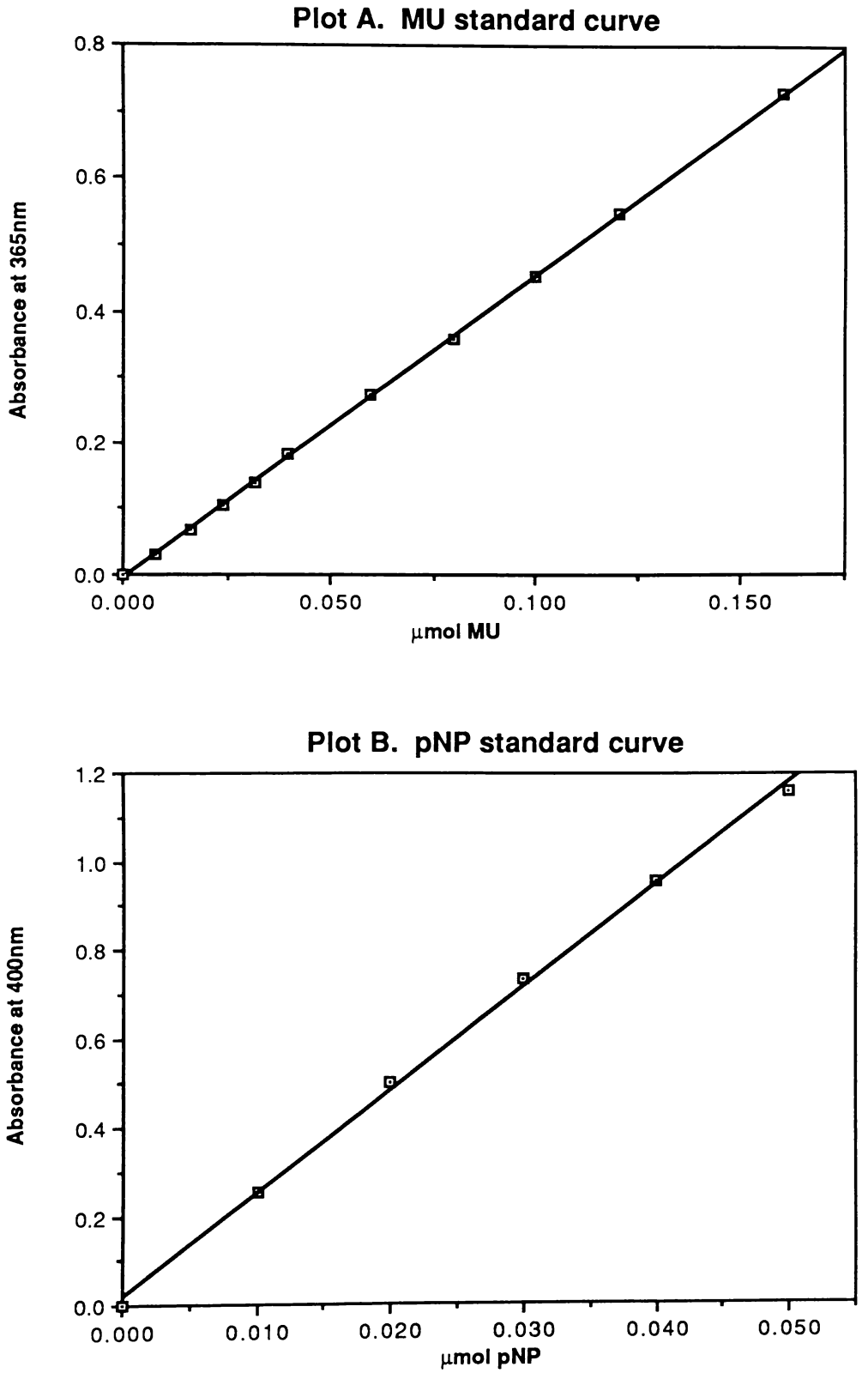


Figure 3.9 MU and *p*NP standard curves.



Heat treatment of the β-glucosidase containing extracts resulted in good recovery, and good to very high purification factors (Table 3.5). CMCase protein extracts also gave good purification factors, but only moderate recovery (Table 3.5). Losses of the CMCase are not only due to the volume of the pellet but are thought to also be due to either coprecipitation or binding of the CMCase to the precipitate (Neal, 1987).

In heat treating the MUCase protein extract, up to three activities (on MUC, Avicel and xylan) were assayed (Table 3.5). The three activities were all thought to be due to one gene product since only one gene was cloned. However, as more than one domain was present in the enzyme, several activities were assayed to see if they all behaved in a similar way. The activities on MUC and Avicel showed very high recoveries and purification factors in each of the first three MUCase examples.

In the fourth MUCase example (PB4567), recoveries of the activities on MUC and xylan were high, with good purification factors. However, there was low recovery and purification of the activity on Avicel. A possible reason for this is overestimation of the activity before heat treatment. Activity on Avicel was very low in this clone. Synergism between the enzyme and a host *E. coli* β-glucosidase present in the non-heat treated protein extracts, when assayed on Avicel, would therefore result in a much higher level of reducing sugars being produced. This would lead to an overestimation of the activity on Avicel. Such synergism is difficult to quantify and, therefore, to allow for, during calculations of activity on Avicel. If β-glucosidase activity could be completely inhibited without inhibiting the activity on Avicel (for example, by D-glucono-1,5-lactone (Heptinstall *et al.*, 1986)) an allowance for synergism could be made.

Activities on Avicel and xylan in the fifth example (PB4800) showed high purification and good recoveries. Recovery and purification of the activity on MUC was only moderate, possibly because of a high activity on MUC before heat treatment.

A heat treatment temperature of 70°C was a good compromise between protein precipitation and loss of activity (for example, see the β-xylosidase examples in Table 3.4). Loss of soluble enzyme activity during heat treatment and subsequent centrifugation could be caused by thermal denaturation or

coprecipitation. Some of the activity losses (for example, see the CMCase examples in Table 3.5) seem too high to be compatible with thermal denaturation in the light of thermostability data (Table 3.6). When heat treated at 70°C for 30 - 60 minutes only small activity losses due to thermal denaturation should be incurred under the conditions used in this study. On the other hand, some of the activity losses were unexpectedly low (for example, see the β-xylosidase examples in Table 3.4) in the light of the data in Table 3.6. It is possible that the β-xylosidase was stabilized by high protein concentrations.

Table 3.6 Thermostability data for thermostable xylanolytic and cellulolytic enzymes expressed in *E. coli*.

Enzyme	Clone	Half life at temperature indicated	Source
β-xylosidase	PB4855	40min at 70°C 3min at 80°C	Hudson *et al.*, in press
xylanase	PB4716	20min at 75°C	this study, Section 5.3.5
β-glucosidase	PB4551	>4-9 days at 70°C 2-8 days at 75°C 15 hours at 80°C	Plant *et al.*, 1988
CMCase	PB4563	7.5 hours at 70°C 6.3 hours at 80°C	Neal, 1987
MUCase	PB4567	>1 day at 75°C	this study, Section 6.3.4

The method of cell breakage (sonication versus a lysozyme method, see Section 2.4.2 (a)) appeared to have no significant effect on recovery or purification. Sonication results in very small fragments of debris compared to a gentler method such as the lysozyme method. Liquid/solid separation is thought to be easier following a gentler treatment (Watson *et al.*, 1987). The centrifugation steps used in this study seem to have resulted in efficient liquid/solid separation regardless of the cell breakage method used.

As the protein composition of each *E. coli* host is essentially the same for all enzymes, the variation in degree of purification suggests that further optimization of the heat treatment step is possible for some of the enzymes.

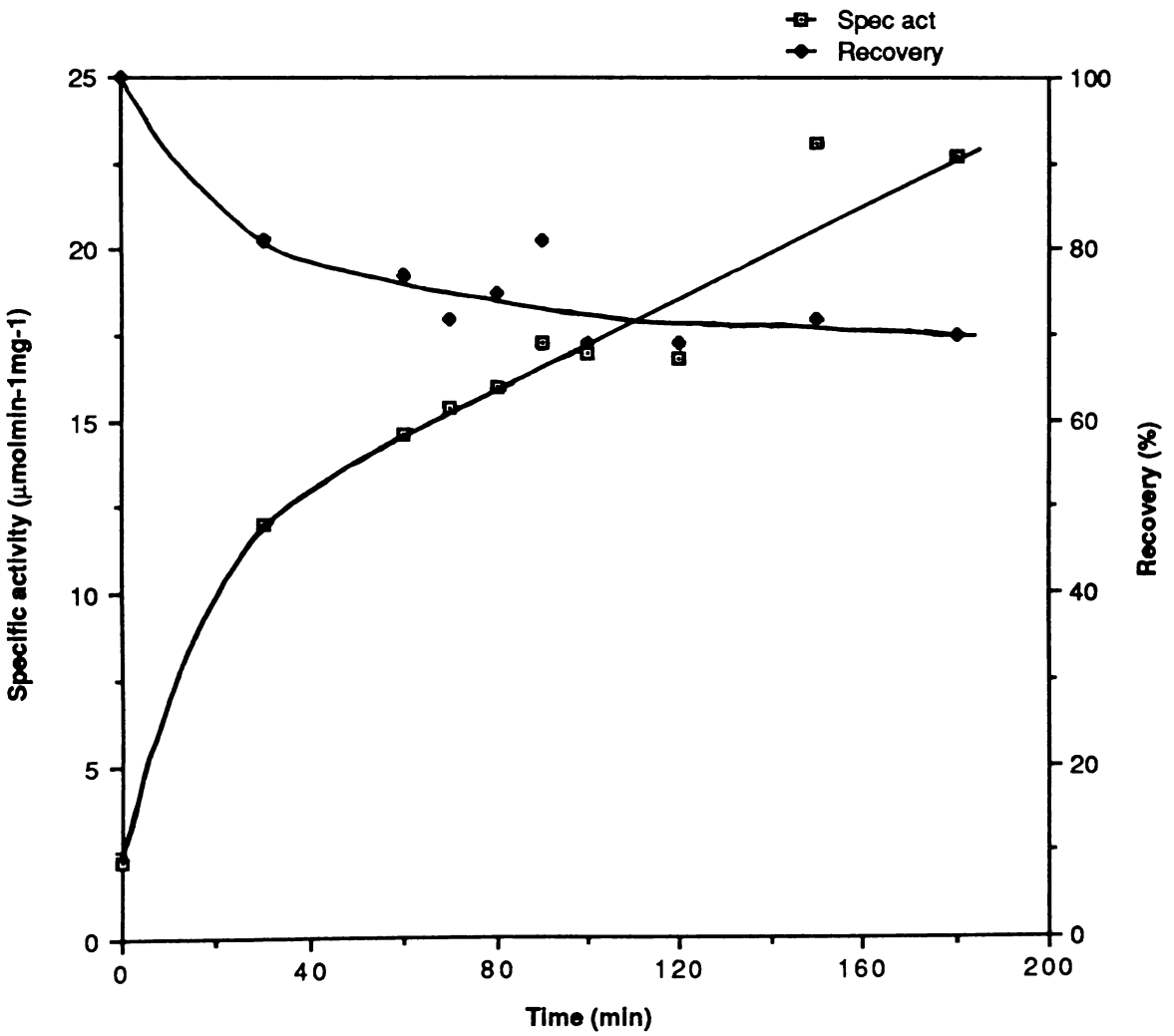
3.2.2 Preliminary optimization experiments

Preliminary experiments to increase recovery and/or the degree of purification were undertaken. The experiments were performed with enzymes that were later studied in more detail, that is the xylanase and MUCase. The CMCase, which showed the lowest recoveries of the five enzymes studied, was not used.

(a) Time of heat treatment

In the example of heat treatment of a xylanase extract at 70°C (Figure 3.10) specific activity rose quickly in the first 30 minutes, then showed a much smaller increase as protein ceased to be precipitated. Recovery decreased quickly in the first 30 minutes and then remained approximately constant after 60 minutes. Heat treatment for less than 30 minutes was insufficient for an adequate degree of purification. However, prolonged heat treatment usually results in insignificant gain in the degree of purification at the expense of recovery. A balance between degree of purification and recovery must be made depending on the aim of each experiment. The optimum time of heat treatment at 70°C for the xylanase under the conditions used in the study was therefore 150 - 180 minutes, or more. As a general technique, a heat treatment of 30 (- 60) minutes at 70°C proved to be a reasonable compromise, considering the thermostabilities of the enzymes.

Figure 3.10 Change in specific activity and recovery of xylanase (PB4716) in a protein extract heat treated at 70°C over time.



(b) Temperature of heat treatment

As seen in Section 3.1.1, increased temperature of heat treatment leads to increased protein precipitation and increased purification. However, increased temperature also leads to losses of activity, as can be seen in Table 3.7. High amounts of MUCase remain after heat treatment at 55°C or 70°C, but half the activity is lost after a 30 minute heat treatment at 85°C. The best temperature of heat treatment depends on the thermostability of the enzyme, but is likely to be close to the growth temperature of the organism from which the enzyme was cloned. As above, a balance between degree of purification and recovery must be made.

Table 3.7 Effect of heat treatment temperature on MUCase recovery after heat treatment of a MUCase (PB4567) protein extract for 30 minutes.

Heat treatment temperature (°C)	MUCase activity remaining (%)
55	107
70	89
85	48

(c) Location of activity on heat treatment

After heat treatment and centrifugation, 87% of the MUCase (PB4567) recovered after heat treatment is in the supernatant and the remainder is associated with the pellet. The activity associated with the pellet is due to the amount of soluble activity trapped within the pellet volume, and not to coprecipitation. Recoveries of activity after heat treatment can therefore be expected to always be less than 100% unless the pellet is washed.

(d) Initial protein concentration

Extracts of various clones of differing protein concentrations were heat treated for 30 minutes at 70°C. The proportion of protein remaining soluble after heat treatment was approximately constant (values between 6 and 15%, with an average value of approximately 10%) for extracts of initial protein concentrations greater than approximately 0.4mgml^{-1} (Figure 3.11 and Table 3.8). Below this concentration, the proportion of protein remaining soluble after heat treatment rapidly increased with decreasing initial protein concentration. The visual observation of heat treated extracts (Table 3.8) supported these results. Very low protein concentrations are too low for efficient aggregation and subsequent precipitation of denatured protein.

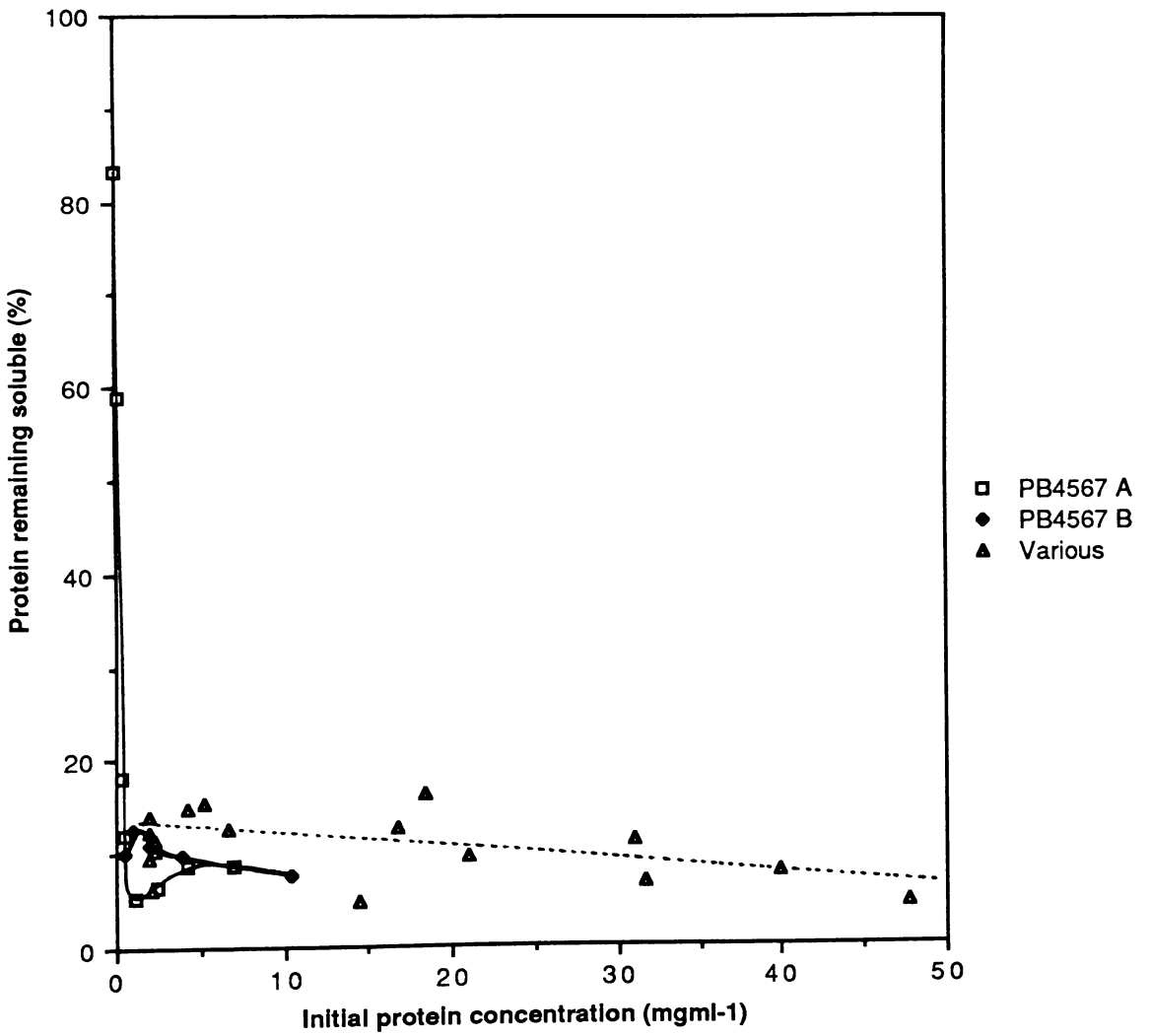
Table 3.8 Visual description of varying protein concentrations of a MUCase (PB4567) protein extract after heat treatment at 70°C for 30 minutes.

Protein concentration (mgml^{-1})		Protein remaining soluble (%)	Visual description after heat treatment
before	after		
7.03	0.61	9	creamy-yellow very thick precipitate
4.30	0.37	9	creamy-yellow thick precipitate
2.40	0.15	6	very cloudy
1.11	0.06	5	cloudy
0.42	0.05	12	slightly cloudy
0.33	0.06	18	very slightly cloudy
0.17	0.10	59	not quite clear
0.06	0.05	83	clear

Figure 3.11 Initial protein concentration versus protein remaining soluble for various *E. coli* clones heat treated for 30 minutes at 70°C.

Clone PB4567, two experiments, A and B.

Various clones, individual experiments.



(e) pH of heat treatment

Heat treatment at pH 5.4 or lower resulted in an approximately 50-fold decrease in soluble protein concentration, whereas heat treatment at pH values around neutrality decreased the soluble protein concentration by only 10-fold. Acidification to pH 5.4 or lower before heat treatment, followed by neutralisation after heat treatment, resulted in very poor recoveries of xylanase (PB4716). The xylanase may have been inactivated by the low pH. Low pH values could cause very small changes in overall protein structure (*E. coli* proteins or xylanase) giving rise to drastic changes in thermostability and therefore increased denaturation. There is potential for increasing the degree of purification of thermostable enzymes by heat treating at low pH values. However, this will only be successful for enzymes that are stable at the pH of heat treatment.

(f) Ionic strength and buffer components

Varying the ionic strength or buffer components gave insignificant increase in the recovery of MUCase (Table 3.9).

Table 3.9 Effect of ionic strength and buffer components on MUCase recovery after heat treatment of a MUCase (PB4567) protein extract at 70°C for 30 minutes.

Buffer components present during heat treatment (mM)			MUCase activity remaining (%)
acetate	NaCl	MES	
0	780	20	76
20	780	0	87
125	675	0	82
800	0	0	79
20	0	0	74
20	105	0	82

(g) Presence of various compounds

Heat treatment in the presence of various compounds showed no significant increase in recovery of MUCase (Table 3.10). The presence of ammonium persulphate markedly decreased the recovery of MUCase.

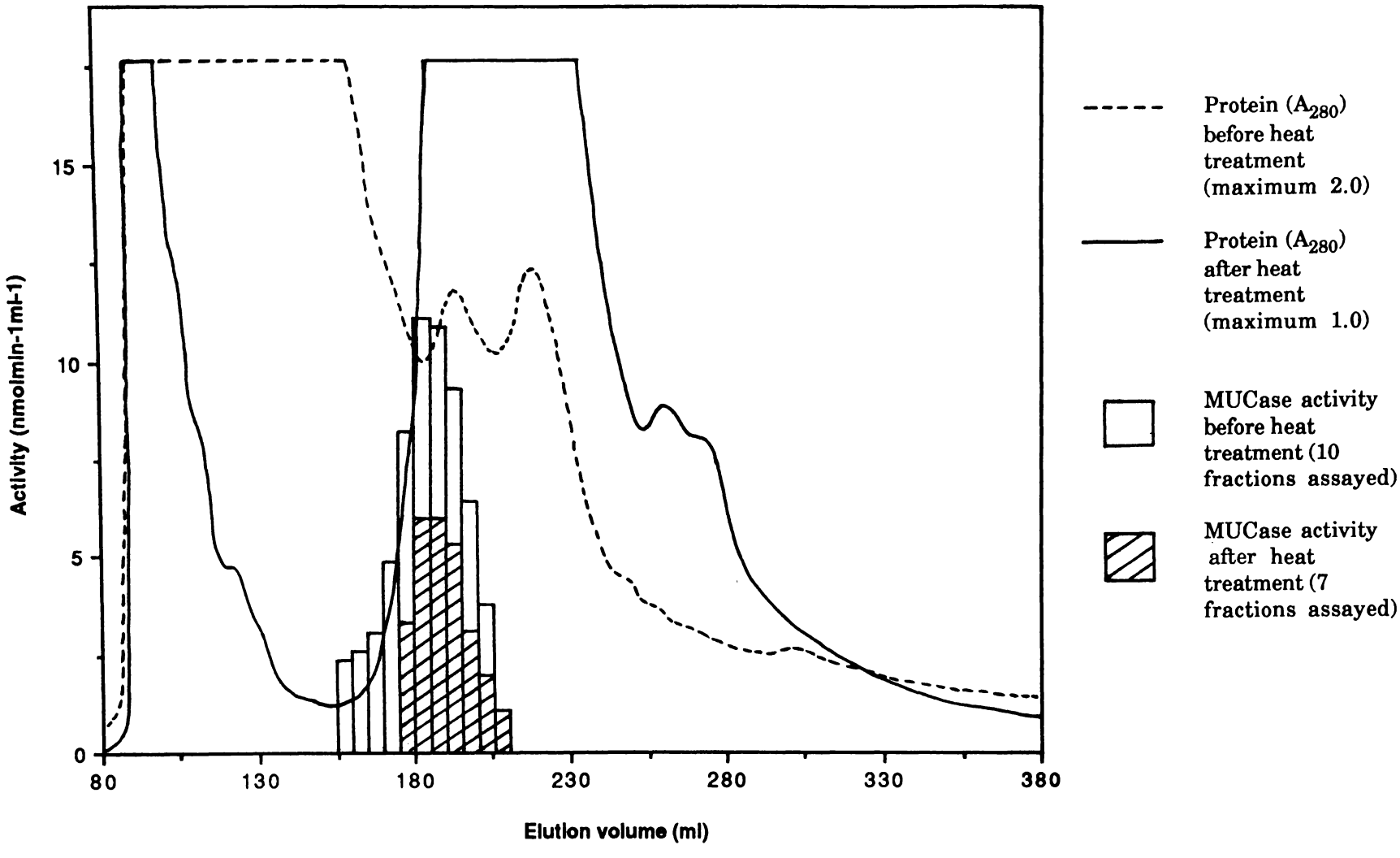
Table 3.10 Effect of various compounds present during heat treatment on recovery of MUCase after heat treatment of a MUCase (PB4567) protein extract at 70°C for 30 minutes.

Compound	MUCase activity remaining (%)
10mM β-mercaptoethanol	88
10% glycerol	87
10mM triethanolamine	83
10mM DTT	82
10mM Tris	82
10mM MOPS	81
0.1% TEMED	81
none	**80**
10mM glycine	80
0.25% DMF	79
0.05% sodium azide	76
10mM EDTA	74
10mM cellobiose	74
10mM Ca^{2+}	73
0.02 $mgml^{-1}$ Pepstatin A	72
10mM L-histidine	72
0.1% SDS	72
10mM ammonium persulphate	24

(h) Protein composition

The gel filtration elution profile of a MUCase protein extract was compared to that of the same protein extract after heat treatment (Figure 3.12). The MUCase in each protein extract eluted in the same elution volume. However, the protein traces are quite different. The crude protein extract contains mostly high molecular weight proteins, with a small amount of protein of molecular weight range slightly less than that of the MUCase. In contrast, after heat treatment a high proportion of lower molecular weight proteins is present, while most of the high molecular weight proteins appear to have been removed (by denaturation and precipitation) or degraded to smaller proteins (for example, by proteases or by the dissociation of multisubunit proteins). Purification might be faster and easier for a cloned thermostable enzyme of a molecular weight slightly higher than the MUCase (clone X), as it appears that the removal of nearly all the contaminating protein remaining after heat treatment could be accomplished by gel filtration.

Figure 3.12 Gel filtration elution profiles of a MUCase protein extract (clone X) before and after heat treatment for 30 minutes at 70°C.



Chapter Four

Substrate specificity screen

A systematic substrate specificity screen of the five cloned xylanolytic and cellulolytic enzymes was made in order to gain an idea of the mode of action of both the individual enzymes and the cellulolytic and xylanolytic systems of the original organism '*C. saccharolyticum*'. Previous studies have focussed on individual enzymes and used different sets of substrates. Some results from previous studies are discussed. All the results presented in Table 4.1 are original to this thesis. All extracts had been subjected to a heat treatment and therefore were assumed to be free of any *E. coli* contaminating activities.

4.1 β-xylosidase

This enzyme was highly specific, with significant activity only on *p*NPX and *o*NPX. There is some activity on *p*NPAp and xylan, but none on xylobiose. There was no activity on substrates consisting of hexoses, that is cellobiose, *p*NPC, *p*NPL, *p*NPG, MUC, CMC or Avicel (Table 4.1).

Hudson *et al.* (in press) have already carried out some characterization of this enzyme. They refer to it as an aryl β-xylosidase as it does not hydrolyse xylobiose. The small amount of activity on *p*NPAp and the lack of activity on *p*NPL and *p*NPG found in this study are confirmed by Hudson *et al.* (in press). They showed that other substrates that are not hydrolysed include *p*NPAf, *p*-nitrophenyl β-D-fucopyranoside, *p*-nitrophenyl β-D-galactopyranoside, *p*-nitrophenyl β-D-galactonuride, *p*-nitrophenyl β-D-maltoside and *p*-nitrophenyl β-D-mannopyranoside.

In this study, HPLC analysis of the products formed on *o*NPX showed that 2μmol *o*NPX was fully hydrolysed within 40 minutes to give 1.6μmol xylose and 0.2μmol xylobiose. The production of xylobiose indicates transferase activity. A possible mode of action on *o*NPX is outlined in Figure 4.1. The aryl β-xylosidase either hydrolyses *o*NPX to produce xylose and *o*NP, or transfers a xylosyl unit to *o*NPX from another *o*NPX molecule to produce *o*NPXX (and *o*NP). *o*NPXX can then be hydrolysed to xylobiose and *o*NP or xylose and *o*NPX. It is possible that this enzyme may not have activity on any substrates with a degree of polymerization of two as no activity was detected on xylobiose. If this is the

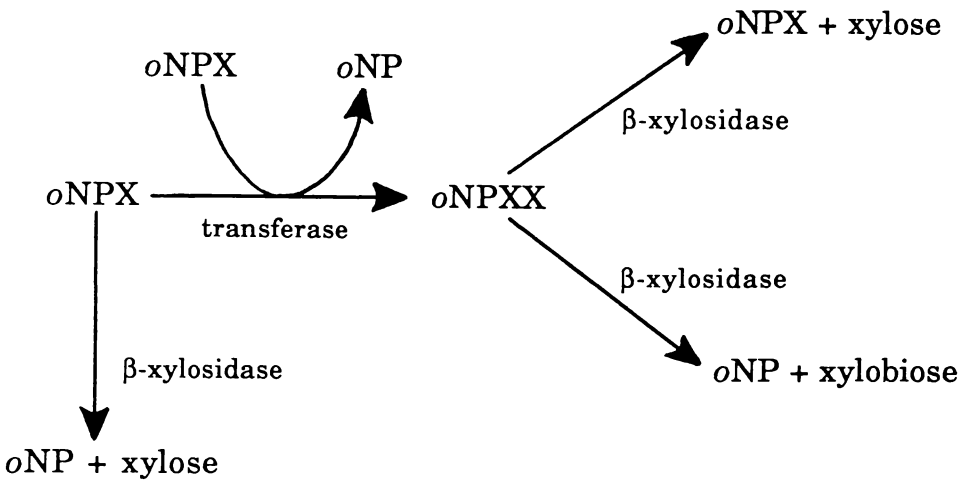
Table 4.1 Substrate specificity screen of thermostable xylanolytic and cellulolytic enzymes expressed in *E. coli*.

Upper left figure, specific activity ($\mu molmin^{-1}mg^{-1}$); lower right figure, %.

Substrate	Enzyme and clone						
	β-xylosidase (PB4855)	xylanase (PB4716)	xylanase (PB4887)	β-glucosidase (PB4551)	CMCase (PB4563)	MUCase (PB4800)	MUCase (PB4567)
oat spelts xylan	0.016 <1	175 100	157 100	0 0	0.047 4	1.33 100	0.920 100
CMC	0.003 <1	34.8 20	18.4 12	0 0	1.14 100	0.833 63	0.120 13
Avicel	0.001 <1	0.327 <1	0.023 <1	0.012 <1	0.014 1	0.106 8	0.006 <1
MUC	0.003 <1	3.85 2	1.64 1	4.41 35	0.017 1	0.677 51	0.370 40
*p*NPC	0 0	8.73 5	3.65 2	2.75 22	0 0	0.321 24	0.386 42
*p*NPL	0 0	0 0	0.122 <1	2.85 23	0 0	0.092 7	0.099 11
*p*NPG	0.008 <1	0 0	0 0	12.6 100	0 0	0 0	0 0
*p*NPX	1.82 100	0.600 <1	0 0	1.19 9	0 0	0 0	0.278 30
*p*NPAp	0.231 13	0.600 <1	0.204 <1	1.14 9	0 0	0 0	0.030 3
xylobiose	✘	✘	nd	✘	nd	nd	nd
cellobiose	✘	✘	nd	✓	nd	nd	nd

nd = not determined
✓ = activity detected by HPLC
✘ = activity not detected by HPLC

Figure 4.1 Possible mode of action of aryl β-xylosidase on *o*NPX.



case, the enzyme must act as a transferase in the first step of *o*NPX hydrolysis and not cleave *o*NPX to xylose and *o*NP.

There are two possible reasons for the apparently increased preference for aryl glycosides. Increased electron withdrawing power of the aglycone moiety may promote stability of the developing phenoxide ion during catalysis (for example, Patchett *et al.*, 1987) or there may be hydrophobic interactions between the aryl group and an aglycone subsite of the enzyme active site (De Bruyne *et al.*, 1979). Both reasons may also explain the presence of transferase activity. However, the inability to hydrolyse xylobiose and the low activity against xylan are not inconsistent with exoxylanase activity. A clear distinction between β-xylosidase activity and exoxylanase activity can be made by determining whether the products show retention or inversion of configuration. Retention indicates β-xylosidase activity and inversion indicates exoxylanase activity. β-xylosidases may be able to be divided into three groups, on the basis of substrate specificity, in a similar way to the β-glucosidases (Patchett *et al.*, 1987). The β-xylosidase described here belongs to the group of aryl β-xylosidases.

4.2 Xylanase

The substrate specificities of two extracts of xylanase were studied (Table 4.1). The extracts were made from two clones, both containing the same xylanase gene. Clone PB4716 contains a DNA fragment coding for the xylanase and four other proteins (see Table 1.2 and Figure 1.5). Xylanase purified from this clone (see Section 5.2) was used for the substrate specificity assays. Clone PB4887 contains a fragment of DNA coding for only the xylanase (see Table 1.2 and Figure 1.5). A heat treated extract of this clone was used in the substrate specificity studies.

The xylanase in each of the extracts showed significant activity on CMC, with low activity on MUC, *p*NPC and *p*NPAp and no activity on *p*NPG. The substrate specificities of the two extracts of xylanase were not identical on *p*NPL, *p*NPX and Avicel, but these activities were very low (<0.4%), and therefore difficult to measure accurately. The xylanase did not hydrolyse xylobiose (Table 4.1). The xylanase was able to cleave disaccharides from substrates, but could not hydrolyse disaccharides. This is consistent with the xylanase acting in an endo fashion. The xylanase was not completely specific for xylose containing substrates as there was some activity on substrates containing glucose or arabinose. The substrate specificity of the xylanase is discussed in more detail in Section 5.3.8.

4.3 β-glucosidase

The substrate specificity results found in this study (Table 4.1) are typical of a β-glucosidase. There is no activity on polymers such as Avicel, CMC or xylan. Activity appears to be confined to small substrates, cellobiose being the natural substrate. *p*NPG gives the highest specific activity of the glucose containing artificial substrates, with significant activities also on MUC, *p*NPC and *p*NPL. There is some activity on model substrates containing the pentoses xylose and arabinose. However, such activity may be induced by the presence of the *p*NP group, as there was no activity detected on xylobiose. Broad specificity is a common property of microbial β-glucosidases. From these results the mode of action of the enzyme appears to be the hydrolysis of β-1,4 bonds in disaccharides, with a marked preference for hexoses over pentoses.

The enzyme has been well characterized by Plant *et al.* (1988), who found activity on aryl β-glycosides, lactose and β-linked glucose dimers in the order 1,3

> 1,2 > 1,4 > 1,6. They detected no activity on aryl α-glycosides or α-linked glucose dimers.

4.4 CMCase

The CMCase acts as a typical endoglucanase with significant activity only on CMC. Thus it is specific for large soluble glucose polymers. There is a very small amount of activity on xylan, Avicel and MUC, but no activity on *p*NPC, *p*NPL, *p*NPG, *p*NPX or *p*NPAp (Table 4.1).

Neal (1987) partially characterized this enzyme, finding activity only on CMC, with insignificant activity on xylan, Avicel and MUC.

4.5 MUCase

The substrate specificities of two extracts of MUCase are shown in Table 4.1. The extracts were made from two different clones. Clone PB4800 contains a DNA fragment coding for the gene *celB* (see Table 1.2 and Figure 1.6). *CelB* codes for a protein with three domains (numbered from the N-terminal end), each separated by a PT box (a region rich in proline and threonine) (Saul *et al.*, 1989). Clone PB4567 contains a DNA fragment corresponding to the N-terminal 65% of *celB* (see Table 1.2 and Figure 1.6), which codes for a protein having only two complete domains (1 and 2) and both PT boxes.

The two MUCase extracts have similar activities on xylan, MUC, *p*NPC and *p*NPL. The catalytic site responsible for these activities must therefore be located in domain 1 or 2, but not domain 3. Activity on CMC is much less for the domain 1+2 protein compared to the entire protein and is the only activity that domain 3 shows. Domain 3 therefore appears to act as an endoglucanase. Domain 1 has significant homology with the xylanase (Luthi *et al.*, 1990), so their modes of action may be expected to be similar. Activity on MUC and *p*NPC by the domain 1+2 protein indicates release of disaccharides from these substrates and indeed the xylanase has activity cleaving disaccharides from substrates. A comparison of the activities on MUC, xylan and *p*NPC of the domain 1+2 protein and the xylanase (Table 4.1), shows the former has a much lower proportion of activity on xylan. The decreased activity on xylan may be explained by a preference by the domain 1+2 protein for smaller substrates. An alternative explanation is that a binding site that is unable to bind pentose-containing substrates may be present. Activity by the domain 1+2 protein

releasing disaccharides from substrates containing pentoses or hexoses seems to indicate that it is acting as an exoglycobiohydrolase.

The entire protein may be inactive on *p*NPX (or *p*NPAp) if domain 3 hinders the approach of more than one *p*NPX (or *p*NPAp) molecule to the active site. In the domain 1+2 protein, the absence of domain 3 may allow two *p*NPX (or *p*NPAp) molecules to approach the active site and transferase activity to be present in a similar way to the xylanase (see Section 5.3.8). The decreased activity of the entire protein on *p*NPC and *p*NPL compared to the domain 1+2 protein could also be explained in this way. The enzyme may have transferase activity on *p*NP-glycosides for similar reasons to the β-xylosidase (Section 4.1). Those reasons are the increased electron withdrawing power of the aglycone moiety (Patchett *et al.*, 1987) and hydrophobic interactions between the aryl group and the enzyme active site (De Bruyne *et al.*, 1979). MU-substrates could be expected to have a similar aryl activation effect as the pK_as of *p*NP and MU are very similar, 7.15 and 7.80 respectively (CRC Handbook of Chemistry and Physics, and Mattoo, 1958, respectively). Creme and Leaback (1980) proposed that MU gave an aryl activation effect in their study of a β-galactosidase. However, unlike activity on *p*NPC and *p*NPL, activity on MUC is not greater for the domain 1+2 protein compared to the entire protein and may be due to a lack of transferase activity on MUC, possibly for steric reasons, as MU is much larger than *p*NP.

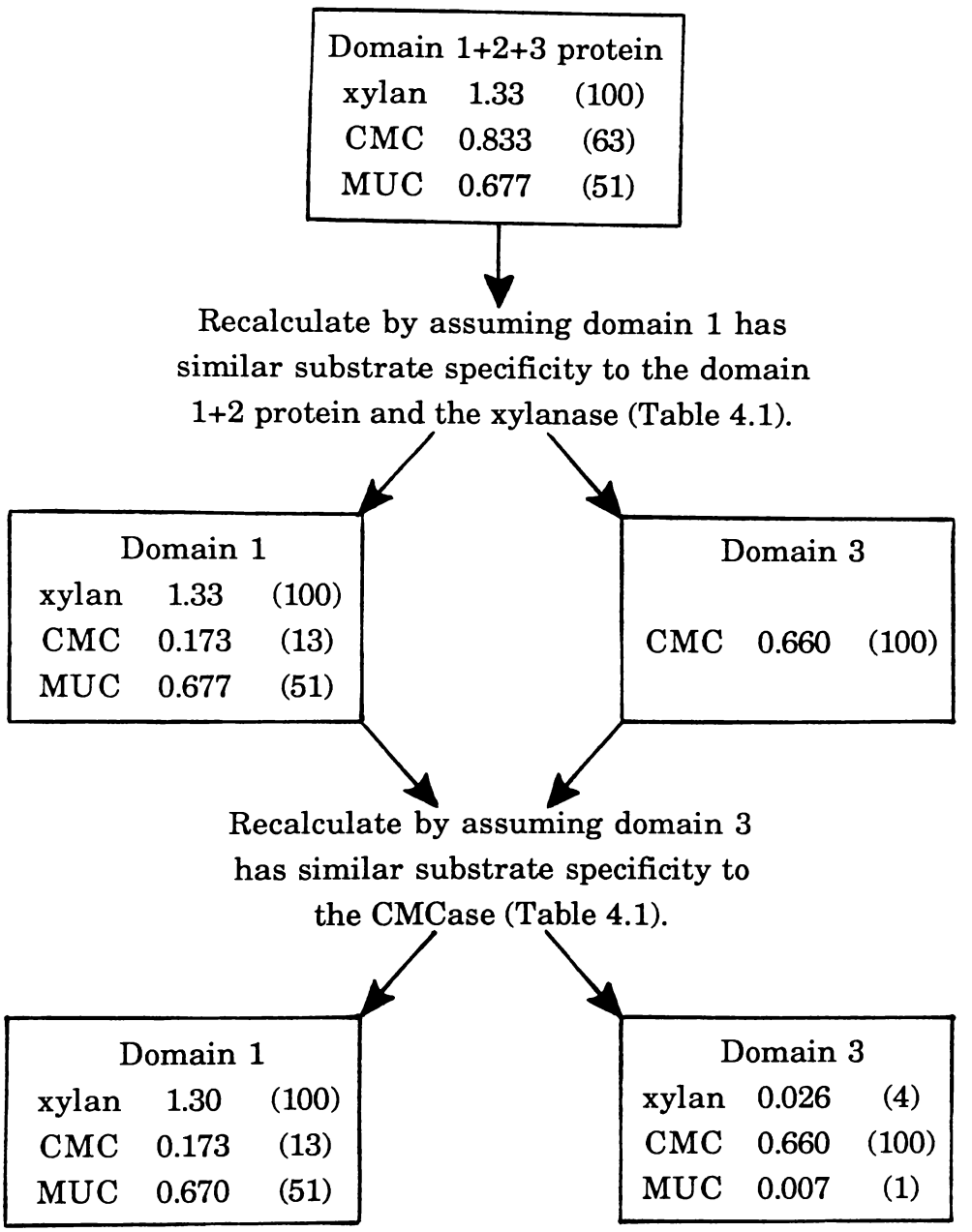
Domain 1 appears to act as an exoglycobiohydrolase and domain 3 as an endoglucanase. Domain 2 has homology with the non-catalytic region of *B. subtilis* endoglucanase and is therefore unlikely to have an active site.

The substrate specificity of each domain can be seen more clearly if the specific activities of the entire protein on xylan, CMC and MUC (Table 4.1) are calculated as those due to domain 1 and those due to domain 3. Firstly assume that 100% of the activity on xylan ($1.33\mu molmin^{-1}mg^{-1}$) is due to domain 1. Define the activity on CMC as 13% for domain 1 and the remainder as due to domain 3. 13% was chosen by comparison with the domain 1+2 protein and the xylanase (PB4887). Specific activities for each of domains 1 and 3 calculated in this way are shown in the middle of Figure 4.2. Secondly, by comparison with the CMCase (PB4563) from the same organism, assume that 100% of the activity on CMC (now $0.66\mu molmin^{-1}mg^{-1}$) is due to domain 3 and define the activity on xylan as 4% and the activity on MUC as 1% for domain 3. Define the remaining

Figure 4.2 Calculation of the specific activities on xylan, CMC and MUC of domains 1 and 3, from the specific activity of the entire MUCase (PB4800) (domain 1+2+3 protein).

Domain 2 is assumed to have no activity.

Figures are: specific activity as $\mu molmin^{-1}mg^{-1}$ and % (in brackets).



activity on xylan and MUC as due to domain 1 (lower part of Figure 4.2). The xylan, CMC and MUC specific activities for domain 1 predicted by calculation from the specific activities of the entire protein, are very similar to those actually found for the domain 1+2 protein.

We are unsure of the effect of domain 2 on the activities of domains 1 and 3, but domain 2 may have a binding function. One could expect there to be no activity on Avicel by domain 3, which acts as an endoglucanase. However the activity on Avicel of the entire protein is much greater than that of the domain 1+2 protein. It is possible that all three domains, present in the entire protein, act synergistically to produce activity on Avicel. The low activity of the entire protein on Avicel may be due to partial proteolytic cleavage of the entire protein in *E. coli* (Saul, unpublished results). *E. coli* has at least eight proteases that may selectively degrade polypeptides with abnormal conformations (Sharma, 1986). The PT boxes are certainly abnormal conformations and have previously been suggested to be sites of proteolysis. In native organisms PT boxes are often glycosylated and this is thought to act as protection against proteolysis (Langsford *et al.*, 1987). Decreased activity on insoluble substrates such as Avicel has been observed following proteolytic cleavage of a cellulase (Gilkes *et al.*, 1988). Cleavage at these sites (PT boxes) in the entire protein, would not lead to decreased activity on xylan, CMC, etc., but could lead to decreased activity on Avicel. Alternatively other factors such as Ca^{2+} and DTT or glycosylation may be required for higher activity of the entire protein on Avicel.

Domain 1 has already been shown to have a high degree of homology with '*C. saccharolyticum*' xylanase (ORF1), which in turn has been shown to have some homology to '*C. saccharolyticum*' ORF4, *Bacillus* sp. strain C125 xylanase A and *Ce. fimi* exoglucanase (Luthi *et al.*, 1990). Henrissat *et al.* (1989) found that the HCA plots of *Ce. fimi* exoglucanase, *Cl. thermocellum* xylanase Z and *Cryptococcus albidus* xylanase displayed homology and constituted cellulase family F. The HCA plots of domain 1 and the six sequences just mentioned all show homology (Figure 4.3). The encoded proteins are therefore all members of family F described by Henrissat *et al.* (1989). Family F is described as containing non-specific glycanases that hydrolyze 1,4-β-D-glucan and 1,4-β-D-xylan. The substrate specificities of this group are very similar. All except '*C. saccharolyticum*' ORF4 appear to cleave disaccharides

from substrates. The substrate specificity of domain 1 confirms its homology with this group.

Domain 3 has already been shown to have homology with *Cl. thermocellum* endoglucanase B (Saul *et al.*, 1989), which is a member of family A described by Henrissat *et al.* (1989). Family A has 10 members, all of which are endoglucanases. The HCA plot and the narrow substrate specificity of domain 3 also indicate that it is a member of this group of endoglucanases.

The HCA plot of domain 2 has homology with that of the C-terminal half of *B. subtilis* endoglucanase. Neither of these protein segments fit into any of the cellulase families of Henrissat *et al.* (1989). Indeed the N-terminal half of the *B. subtilis* endoglucanase is a member of family A, the potentially catalytic residues of which have been predicted. Unlike the entire '*C. saccharolyticum*' protein the *B. subtilis* endoglucanase does not contain PT boxes and does not appear to be divided into domains. It is unlikely that this enzyme contains a second active site. It is plausible that the C-terminal half of this enzyme is a binding region and that domain 2 is similar.

There is obvious difficulty in the classification of whole proteins into cellulase families, when some proteins consist of several domains, each domain having homology to a different family. Henrissat *et al.* (1989) have classified protein 'cores' into families but it may be better to classify all the domains of a protein into families. Members of such families will probably be found to have very similar actions or functions.

Figure 4.3 HCA plots of glycanases from family F.
Potentially catalytic residues denoted by colour.

F1 *Ce. fimi* exoglucanase (HCA from Henrissat *et al.*, 1989)

F2 *Cl. thermocellum* xylanase Z (HCA from Henrissat *et al.*, 1989)

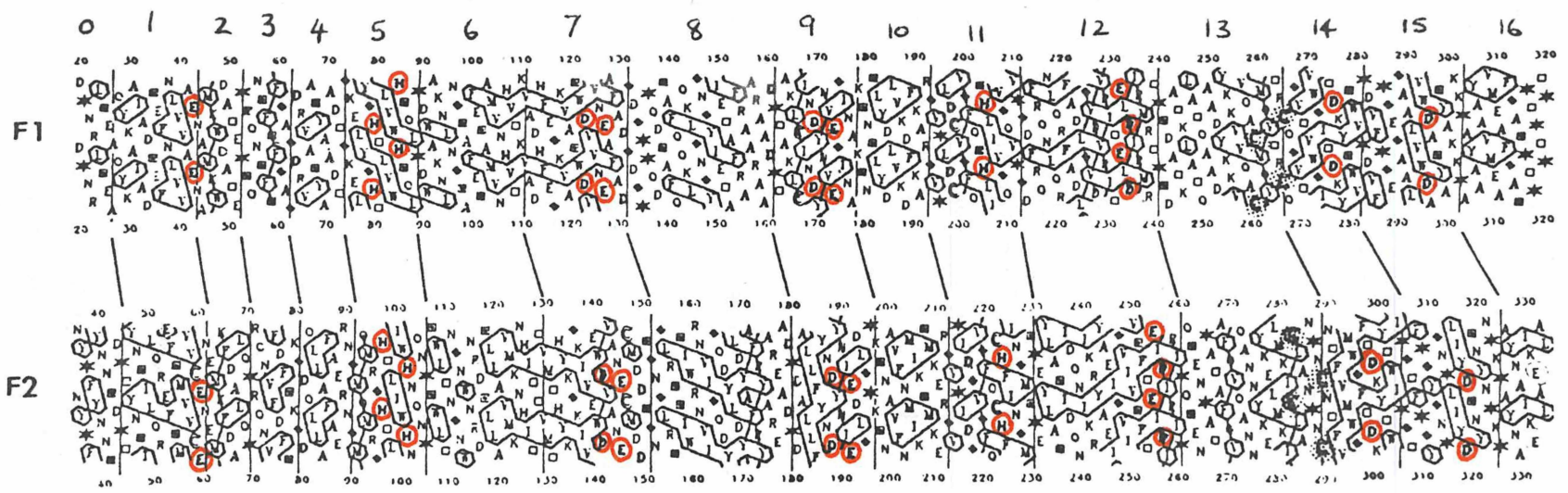
F4 *Bacillus* sp. strain C-125 xylanase A (sequence from Hamamoto *et al.*, 1987)

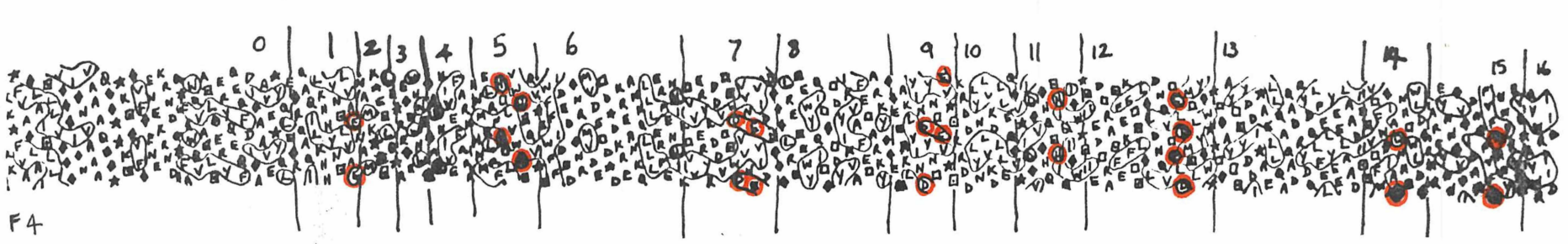
F5 '*C. saccharolyticum*' MUCase - domain 1 (sequence from Saul *et al.*, 1989)

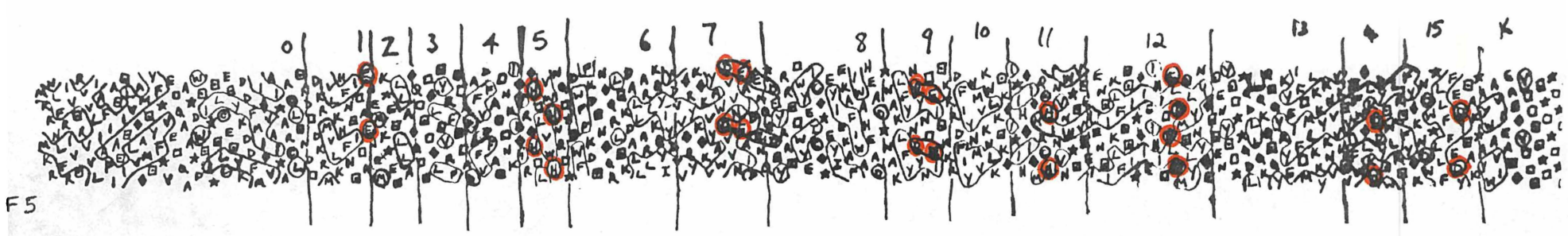
F6 '*C. saccharolyticum*' xylanase (ORF1) (sequence from Luthi *et al.*, 1990)

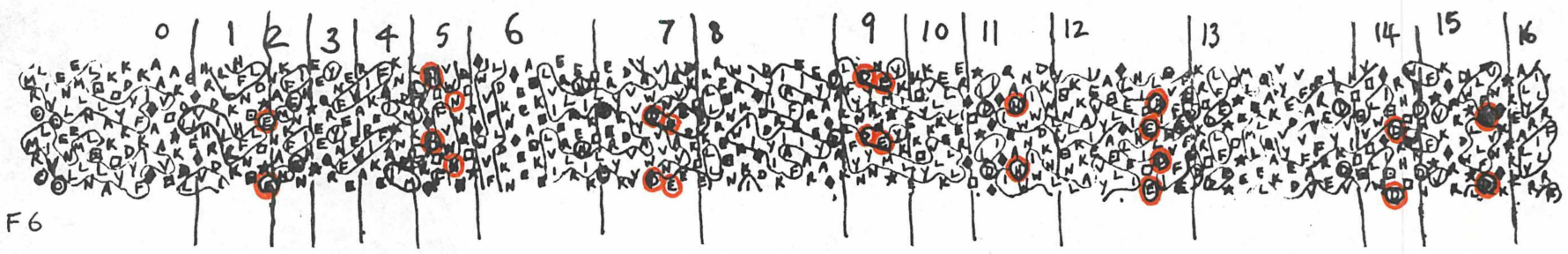
F7 '*C. saccharolyticum*' ORF4 (sequence from Luthi *et al.*, 1990)

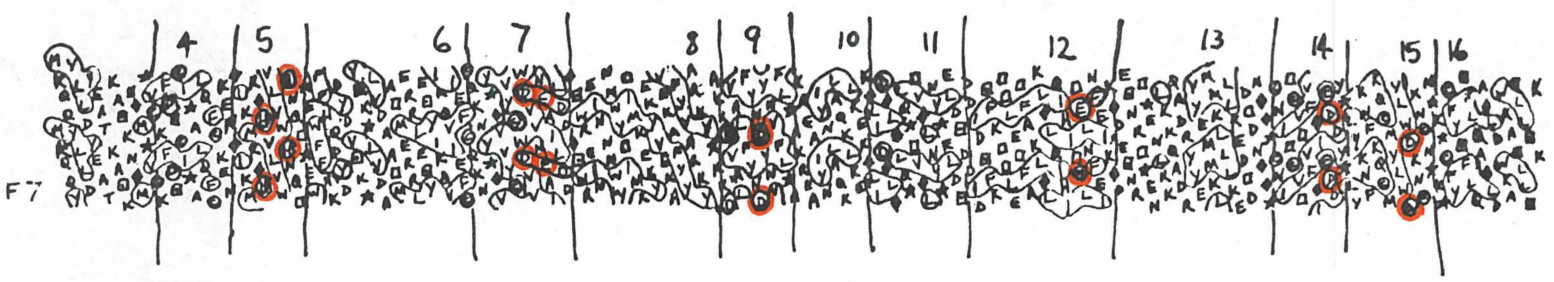
(F3 *Cryptococcus albidus* xylanase not shown)











Chapter Five

Xylanase

Results of the purification and characterization of the xylanase are presented in this chapter.

5.1 Substrates

5.1.1 Xylan substrates

Oat spelts and larchwood xylans were tested as substrates in the PAHBAH assay system. Larchwood xylan was found to have a much higher reducing sugar background than oat spelts xylan (Table 5.1). Both substrates were dialysed to remove any low molecular weight reducing sugars that may have been present. However there was no significant change in the reducing sugar background after dialysing (Table 5.1).

Table 5.1 Reducing sugar backgrounds of undialysed and dialysed oat spelts and larchwood xylans.

Xylan		nmol reducing sugars/mg xylan	µg reducing sugars/mg xylan
oat spelts	undialysed	10	1.5
	dialysed	11	1.7
larchwood	undialysed	115	17.3
	dialysed	112	16.8

The reducing sugar background of larchwood xylan was approximately ten times that of oat spelts xylan. For ease of use, oat spelts xylan was therefore chosen as the substrate for all xylanase assays. In order to use larchwood xylan at a similar concentration either the PAHBAH assay system could be

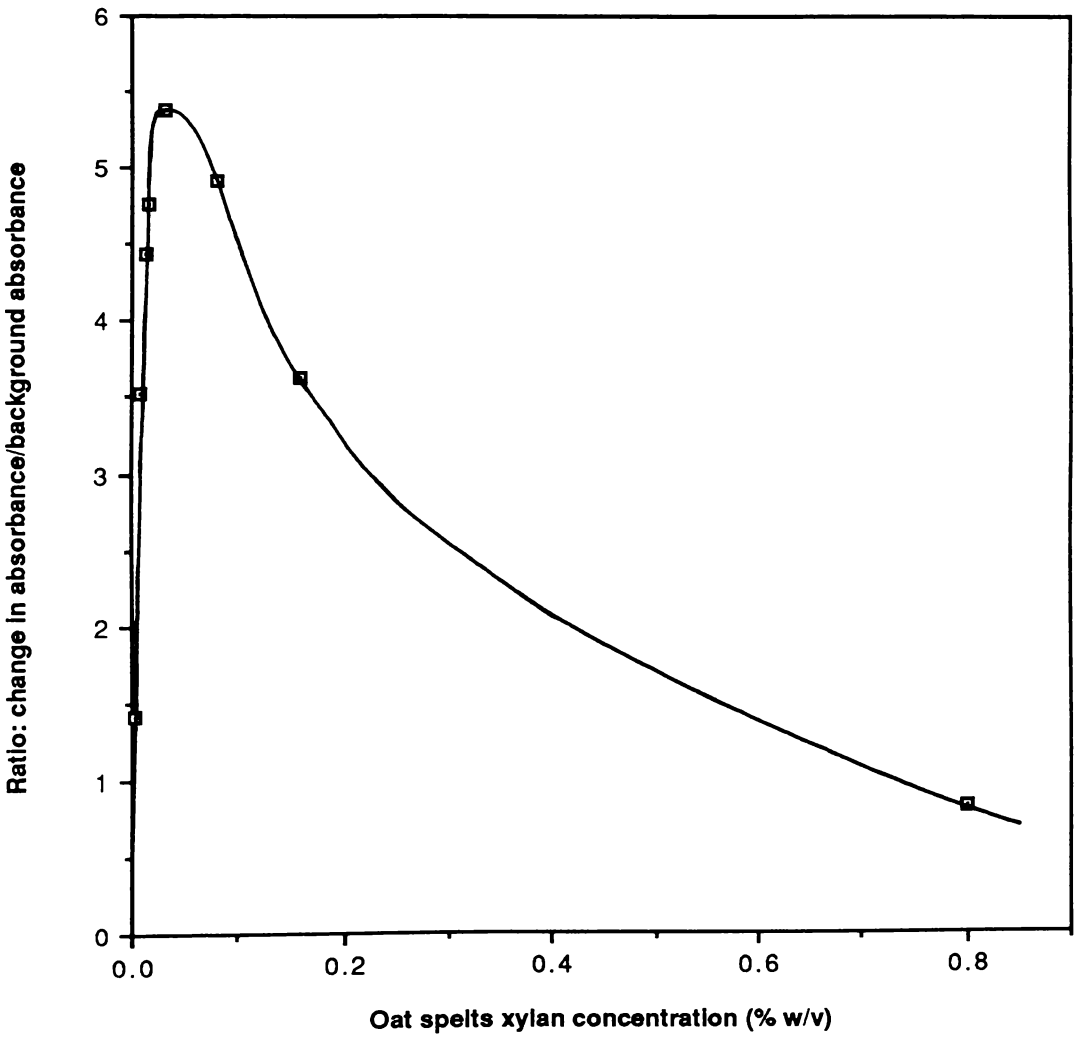
used and the final assay solutions diluted in order to read the absorbance, or a less sensitive reducing sugar assay system could be used (for example, the DNS system).

The average value of 17µg reducing sugars (xylose equivalents)/mg larchwood xylan is consistent with the 16µg reducing sugars (xylose equivalents)/mg larchwood xylan obtained by Gruninger and Fiechter (1986). They noted that this indicates the 'average' xylan molecule has 63 xylose units, if no impurities of small xylose multimers are present. The dialysis experiment above shows that no such impurities are present. Assuming larchwood xylan molecules are not branched, and thus only have one reducing end per molecule, the 'average' larchwood xylan molecule has 63 xylose units and therefore a molecular weight of 8 340. However, such figures should be treated as approximate, as xylan is a heteropolymer containing non-xylose side chains (see Section 1.2.3), and are of little value unless the size distribution of the molecules is known. Gruninger and Fiechter (1986) did not detect a reducing sugar background of oat spelts xylan using the less sensitive DNS system. Using an average value of 1.6µg reducing sugars (xylose equivalents)/mg oat spelts xylan, and assuming oat spelts xylan molecules are not branched, an 'average' oat spelts xylan molecule can be calculated to have 625 xylose units and therefore an approximate molecular weight of 83 600.

Two factors were considered in the choice of the oat spelts xylan concentration for use in the xylanase assays. It was desirable to have the reducing sugar background low and the enzyme activity near maximum. The former could only be attained at low substrate concentrations, the latter only at high substrate concentrations. A plot of substrate concentration versus the ratio enzyme activity/reducing sugar background (Figure 5.1), shows the 'optimum' oat spelts concentration to be approximately 0.032% (w/v). However when plotted in the Lineweaver-Burk form, the data (substrate concentration and enzyme activity) gave a K_m of 0.029% (w/v). As the optimum substrate concentration gave only about half maximal activity, a compromise concentration of 0.20% (w/v) was chosen. The enzyme activity was much higher at this substrate concentration, while the reducing sugar background was still manageable.

Figure 5.1 Oat spelts xylan concentration versus the ratio xylanase activity/reducing sugar background, expressed as $\Delta A_{420}/A_{420}(t=0)$.

Xylanase: crude protein extract.



A suspension of oat spelts xylan (final concentration in the assay: 0.20% w/v) was used throughout the study. No attempt was made to either solubilize or remove the insoluble fraction. However, the relative activities on the soluble and insoluble fractions were determined. A suspension of oat spelts xylan was centrifuged and the supernatant retained as the soluble fraction. The insoluble pellet was washed and resuspended to form the insoluble fraction. The insoluble fraction constituted 46% by weight of the amount of xylan originally suspended. Taking activity on the original oat spelts xylan suspension by purified xylanase to be 100%, there was 98% activity on the soluble fraction and 25% activity on the insoluble fraction. A reason for the total activity being high (123%), may be that further xylan in the insoluble fraction became soluble on resuspension. The xylanase therefore appeared to be active mainly on soluble xylan. The insoluble xylan in the suspension was not solubilized or removed as it had little effect on either xylanase activity or the performance of the assay.

5.1.2 Dyed xylan

Dyed xylan was prepared from oat spelts xylan and remazol brilliant blue, for use in an alternative assay. Enzymatic hydrolysis of dyed xylan leads to the production of xylooligosaccharides with dye attached. These remain in solution after the dyed xylan is precipitated out and can be quantified using the absorbance at 595nm. The amount of dye in the dyed xylan preparation was calculated to be 3.7% by weight. A 1% (w/v) suspension of dyed xylan was found to consist of approximately 41% insoluble dyed xylan by weight.

The assay system using dyed xylan was found to be at least 100 times less sensitive than the PAHBAH assay system. This was primarily because of the low amount of dye attached to the xylan. An approximate ratio of one dye molecule to 120 xylose molecules was calculated for the dyed xylan. With xylan (undyed) as a substrate, each β-1,4-xylosidic bond broken results in the production of a reducing sugar. However with dyed xylan, an average of 60 β-1,4-xylosidic bonds would need to be broken for the release of each dye molecule, assuming xylobiose is released from xylan and the dye is randomly distributed throughout the xylan. Further reasons for the decreased sensitivity include possible inhibition of the enzyme by the dye, or the incomplete recovery of solubilized xylooligosaccharides with dye attached in the supernatant of the assay. The enzymatically released dyed xylooligosaccharides may adsorb to the insoluble dyed xylan. The dyed xylan assay system is discussed further in

Section 5.3.6. Two buffers, citrate and phosphate, were found to interfere with the assay. Buffers compatible with the assay system included succinate, acetate, MES, HEPES, EPPS, MOPS, Bis-Tris, Bis-Tris propane, Tris, Tricine and Bicine.

The use of dyed xylan as an alternative activity overlay for detecting activity in agarose IEF gels was also attempted. However, it was less successful than overlays containing undyed xylan.

5.2 Purification

A summary of the purification of the xylanase is shown in Table 5.2. 1.2kg of cells (clone PB4716) were lysed to produce 6l lysed cell extract. Heat treatment resulted in the loss of 87% of the protein present with a 5.6-fold purification. The small loss in activity was due to a proportion of the activity remaining in the pellet after centrifugation. A small scale trial of ammonium sulphate precipitation showed that most of the xylanase was precipitated at an ammonium sulphate concentration of 70% saturation (Table 5.3). For the large scale purification, ammonium sulphate was added to a concentration of 50% saturation and precipitated protein removed and discarded. The precipitate formed by increasing the ammonium sulphate concentration from 50% to 80% saturation contained the xylanase. Ammonium sulphate precipitation removed approximately half of the remaining protein resulting in a relative purification of 11.2. After dialysing, the sample was freeze dried and the powder obtained (13.7g) was stored desiccated at room temperature. Approximately 50% of the activity was lost during freeze drying and subsequent storage over an extended period of time.

A small amount of material (40 mg freeze dried powder) was further purified (shown in the lower half of Table 5.2). A trial of a cation exchange MONO S 5/5 column proved unsuccessful. The xylanase along with the majority of the protein did not bind to the matrix. Trials of an anion exchange MONO Q 5/5 column were successful, and this was used as a step in the purification sequence. The xylanase was eluted from the MONO Q 10/10 column using a 0 - 0.22M NaCl gradient. A further gradient of 0.22 - 1.0M NaCl eluted significant protein, but no xylanase activity. The loss in activity during this step was due to the accidental loss of some active fractions, though the relative purification was good. A hydrophobic interaction Phenyl Superose 5/5

Table 5.2 Purification table of the xylanase.

Only a small portion of the activity purified by the first three steps was purified in the subsequent three steps. Recoveries were calculated as though the total material was taken through all steps.

Purification step	Total activity (μmolmin^{-1})	Total protein (mg)	Specific activity (μmol min^{-1}mg^{-1})	Recovery (%)	Relative purification
Lysed cell extract	245 700	119 600	2.05	100	1
Heat treatment and centrifugation	179 140	15 740	11.38	73	5.6
Ammonium sulphate precipitation and dialysis	166 238	7 220	23.0	68	11.2
MONO Q	176.1	1.35	130.1	24	63.5
Phenyl Superose	37.5	0.46	81.5	5	39.8
Ultrafiltration and Analytical Superose	31.4	0.19	167.6	4	81.3

column was moderately successful when tried as a purification step. A sample containing 1 mg freeze dried powder was loaded. The xylanase was eluted during the 0.4 - 0M portion of the 1.7 - 0M ammonium sulphate gradient. The recovery in this preliminary trial was 63% and the relative purification 3.6. When used as a step in the purification sequence, Phenyl Superose 5/5 was less successful. The protein loading was too high for the Phenyl Superose 5/5 column, so some of the xylanase was not retained. The remaining xylanase

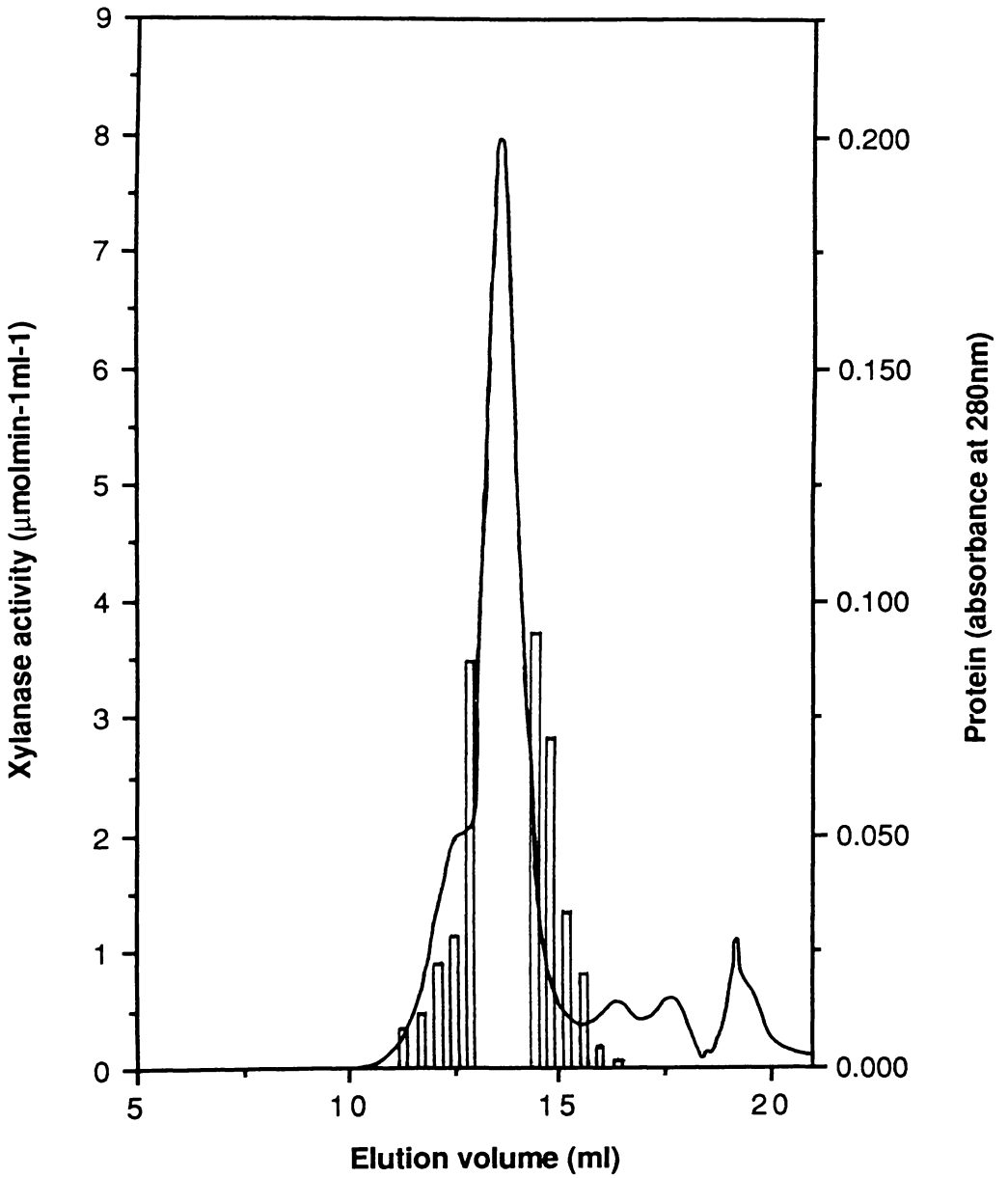
Table 5.3 Ammonium sulphate precipitation trial of the xylanase.

Ammonium sulphate concentration (%)	Xylanase activity remaining (%)
0	100
40	100
50	95
60	76
70	5

was eluted using a 1.0 - 0.5M ammonium sulphate gradient. A subsequent gradient (0.5 - 0M ammonium sulphate) eluted protein but no xylanase activity. The high loss in activity was due to selection of only those active fractions containing a low amount of protein for further purification. A trial on a TSKgel column showed it to be unsuitable due to interaction of the xylanase with the matrix, causing a large tailing effect. Sepharose CL-6B trials were more successful, resulting in the separation of the xylanase and β-xylosidase, but still gave broad peaks. Analytical Superose 6 trials showed this matrix to be the best for gel filtration of the xylanase. After concentration by ultrafiltration, the final step in the purification sequence was an Analytical Superose 6 column. 80% of the pooled material from the Phenyl Superose 5/5 step was loaded in two runs. One of the elution profiles is shown in Figure 5.2.

Overall, purification was good, but recovery low. Possible synergism between the xylanase and the β-xylosidase may have caused high activity figures early in the purification sequence. This could result in apparently lower recovery. Recovery could be increased, while still retaining good purification, by the omission of the Phenyl Superose 5/5 step. Freeze drying and storage of the resulting sample over extended periods of time should be avoided.

Figure 5.2 Elution profile of xylanase on an Analytical Superose 6 column.
(Only select fractions assayed.)



The final specific activity of the xylanase was 167.6μmolmin^{-1}mg^{-1} and the final purification factor 81.3. These values are comparable to those reported for other bacterial xylanases (Table 5.4). Purification factors are higher for xylanases purified from *E. coli* clones than from the native organisms. Xylanases are usually produced extracellularly by native organisms, whereas they must be purified from intracellular protein mixtures in *E. coli*.

The final preparation was not homogeneous with respect to protein as four bands were detectable by silver staining after SDS-PAGE. The major band, representing the xylanase, had a molecular weight of 42 000. The three minor bands had molecular weights of 60 000, 64 000 and less than 14 000. However, only one active enzyme was thought to be present and the evidence for this follows. The *E. coli* clone (PB4716) contained a fragment of DNA coding for five proteins (see Figure 1.1). The xylanase was coded for by ORF1 and had a predicted molecular weight of 40 500. ORF5 coded for a β-xylosidase of predicted molecular weight 56 000. All fractions from the Analytical Superose 6 step that were assayed for xylanase activity were also assayed for activity on *p*NPX. The only peak of activity on *p*NPX was that associated with the xylanase peak and that activity was consistent with the activity of the xylanase itself on *p*NPX (see Section 5.3.8). Previous trials of a sample containing 0.5mg freeze dried powder loaded on an Analytical Superose 6 column showed that the two enzymes were clearly distinguishable, as seen by their activities on *p*NPX (Figure 5.3). Therefore, it was concluded that the β-xylosidase was not present in the purified xylanase sample.

ORF2 coded for an acetyl esterase of predicted molecular weight 30 600. This enzyme had no activity on xylan (Luthi *et al.*, 1990). ORF3 and ORF4 coded for two unknown proteins of molecular weights 10 700 and 36 500 respectively. The protein coded for by ORF3 could not have been present in the purified xylanase sample, as the active fractions pooled from the Analytical Superose step did not include proteins having a molecular weight less than 17 000. Furthermore, no enzymatic activities could be found for ORF3 or ORF4 and poor or no translation of ORF3 and ORF4 could be expected in *E. coli* (Luthi *et al.*, 1990). There were no protein bands detected by SDS-PAGE corresponding to the proteins encoded by ORF2 and ORF4.

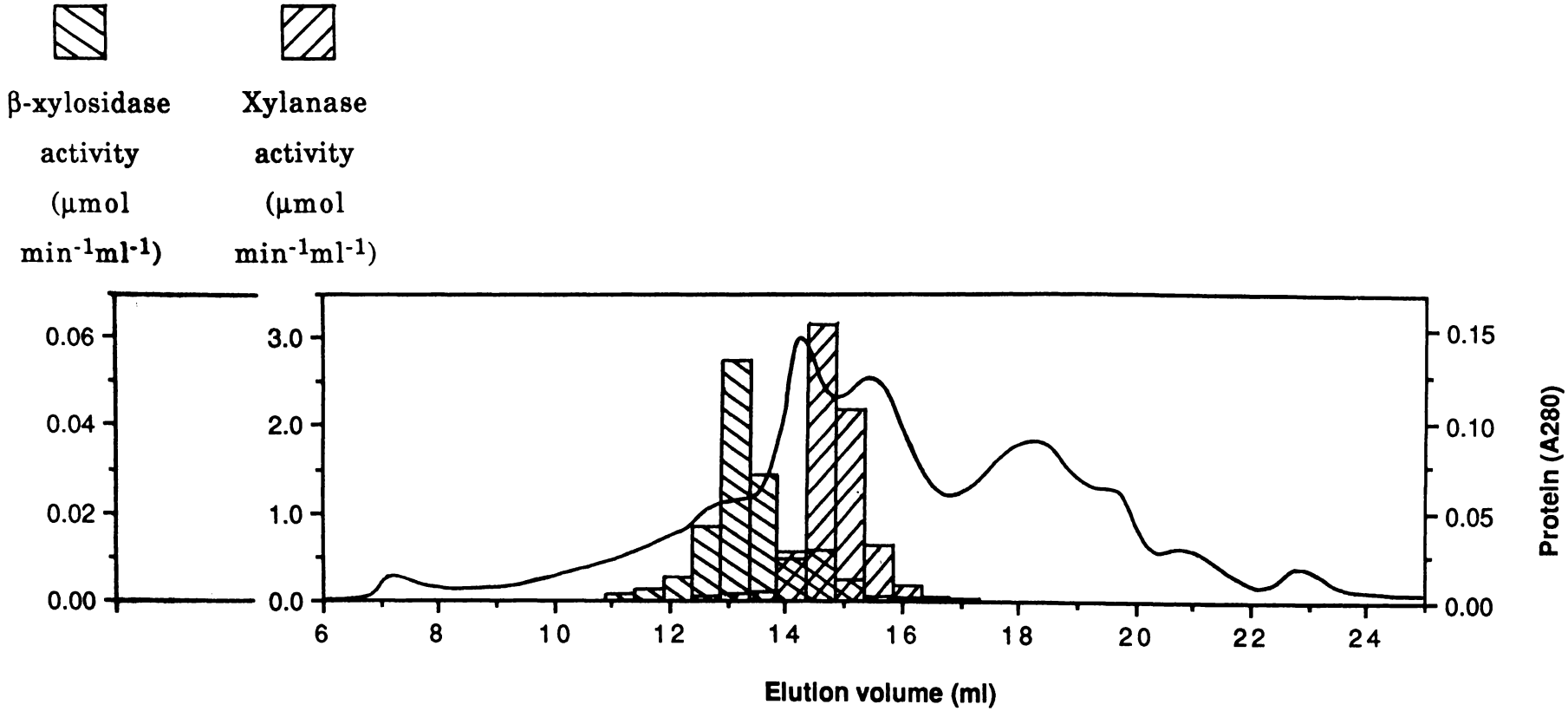
Table 5.4 Specific activities and purification factors of some purified bacterial xylanases.

Bacterium	Xylan-ase	Specific activity (μmol $min^{-1}mg^{-1}$)	Purific-ation factor	Xylan substrate	Source
Bacillus sp. strain W1	I	500	100	larchwood	Okazaki *et al.*, 1985
	II	49	96		
Bacillus sp. strain W2	I	900	333	larchwood	Okazaki *et al.*, 1985
	II	365	135		
Bacillus sp. strain K17	I	227	39.1	larchwood	Kang *et al.*, 1986
	II	113	19.5		
Bacillus sp. strain 11-1S		5.33	6.83	larchwood	Uchino and Nakane, 1981
Bacillus subtilis	*	20.8	not stated	larchwood	Paice *et al.*, 1986
Bacillus pumilus		298	18.6	larchwood	Panbangred *et al.*, 1983
Bacillus sp. strain C-125	N	159	31.0	not stated	Honda *et al.*, 1985b
	A	109	21.2		
Clostridium acetobutylicum	A	5.4	19	larchwood	Lee *et al.*, 1987
	B	6.59	23		
Clostridium stercorarium	A	3585	12.4	larchwood	Berenger *et al.*, 1985
	B	3064	13.5		
	C	3626	12.5		
Clostridium thermocellum	*	166	111	larchwood	Grepinet *et al.*, 1988a
Streptomyces lividans	A	364	31.9	oat spelts	Morosoli *et al.*, 1986
	B$^{\Delta}$	1034	8.1	oat spelts	Kluepfel *et al.*, 1990
Streptomyces sp. strain T_7		412.8	41.3	larchwood	Keskar *et al.*, 1989
'*Caldocellum saccharolyticum*'	*	167.6	81.3	oat spelts	this study
		36.9		larchwood	

* expressed in *E. coli*

$^{\Delta}$ expressed in a mutant *S. lividans* strain

Figure 5.3 Elution profile of a trial of xylanase on an Analytical Superose 6 column.
(Semi-pure xylanase: freeze dried powder sample,
purified as far as ammonium sulphate step, see Table 5.2.)



To further verify that the purified xylanase sample contained only one active enzyme, its substrate specificity was compared to that of an extract of a clone (PB4887) containing a DNA fragment coding for the xylanase only (see Table 1.2, Figure 1.5 and Table 5.5) which became available later on in the study. The two samples had identical activities except for the levels of some trace activities which were very low, and difficult to measure accurately. Heat treated extracts of the *E. coli* host strains from both xylanase-producing clones showed no activity on any of the substrates used in the above substrate specificity comparison. Any host *E. coli* enzymes were denatured by the heat treatment step during purification. The purified xylanase sample was therefore presumed to comprise a single active enzyme and this preparation was used for characterization experiments.

Table 5.5 Substrate specificity screen of two extracts of xylanase.
Specific activities expressed as %.

Substrate	Xylanase PB4716	Xylanase PB4887
oat spelts xylan	100	100
CMC	20	12
Avicel	<1	<1
MUC	2	1
*p*NPC	5	2
*p*NPL	0	<1
*p*NPG	0	0
*p*NPX	<1	0
*p*NPAp	<1	<1

5.3 Characterization

5.3.1 Molecular weight

SDS-PAGE of the purified xylanase sample showed a major band of molecular weight 42 000 (Figures 5.4 and 5.5). This is not incompatible with the molecular weight of 40 500 predicted by the DNA sequence (Luthi *et al.*, 1990) and is identical to the molecular weight observed by Luthi *et al.* (1990) using SDS-PAGE. The three minor bands could have been contaminating protein. Alternatively the enzyme may appear heterogeneous on SDS-PAGE, similarly to the purified endoglucanase A from *Cellulomonas* sp. ATCC21399 (Poulsen and Petersen, 1987).

The molecular weight of the major peak eluted from the Analytical Superose 6 column (the last purification step) was 32 000 (Figures 5.2 and 5.6). A 3% error, either in the measurement of the elution volume of the xylanase and/or the standards, could account for the difference between this result and the molecular weight predicted by the DNA sequence. It is possible that interaction with the matrix retarded the movement of the xylanase, decreasing the apparent molecular weight. Bagnara *et al.* (1986) found that interaction between two endoglucanases from *Ce. fermentans* and the gel permeation matrix decreased the apparent molecular weights. Shoseyov and Doi (1990) observed apparently low molecular weights of *Cl. cellulovorans* cellulases on gel filtration. Warren *et al.* (1987) also observed the retardation of a cellulase from an *E. coli* clone on gel filtration.

The results indicate that the xylanase is monomeric.

5.3.2 Isoelectric point

Isoelectric focussing of the purified xylanase sample gave three major protein bands (Figures 5.7 and 5.8) which exactly matched the three major active bands in the oat spelts xylan activity overlay. The isoelectric points of these bands were 4.8, 5.0 and 5.2. Since only one xylanase gene had been cloned, these three bands must all arise from this gene. There are three possible cleavage sites of the signal sequence of the xylanase gene (Luthi *et al.*, 1990). The sites are within three amino acids of each other, so the three proteins produced would be indistinguishable from each other on a molecular weight basis. Single amino acids may change the net charge of the protein

sufficiently to produce proteins with slightly different isoelectric points, in this case 4.8, 5.0 and 5.2. Luthi (personal communication) considers it unlikely that the signal sequence of the xylanase is cleaved in *E. coli*, but Lejeune *et al.* (1988) state that in *E. coli* the precursor form of an enzyme is very rapidly processed into the mature form. There are examples of several cleavage sites of the signal sequence for one protein. Endoglucanase A from *Ce. fimi* has two cleavage sites, three amino acids apart, in *E. coli*. The molecular weight difference between the two forms was only 330 and they could not be distinguished by SDS-PAGE (Guo *et al.*, 1988). Guo *et al.* (1988) stated that signal sequence cleavage at two adjacent sites in *E. coli* is not unusual. Unfortunately the isoelectric point of endoglucanase A was not reported. Multiple active bands may be caused by other post translational modifications such as limited proteolysis and glycosylation. Proteolytic cleavage could have occurred in *E. coli* before purification.

Multiple active bands could also be an artifact of the isoelectric focussing system. However, it is unlikely in this case for two reasons. Firstly, a sample of xylanase was loaded near each of the anode and the cathode. After isoelectric focussing, identical protein and active bands were seen in each lane. Secondly, immediately after isoelectric focussing of the xylanase sample, the lane was chopped out of the gel, laid across a fresh gel and subjected to isoelectric focussing again. The protein and activity bands did not split any further and the banding pattern remained the same as the original. The inactive protein bands could have been contaminating protein.

The isoelectric points of bacterial xylanases are either acidic (3.6 - 5.2) or basic (7.8 - 9.1) and with only one exception, these correlate with high (39 000 - 85 000) and low (15 000 - 31 000) molecular weights respectively (Table 5.6). The xylanase reported here fits this pattern and falls into the former group.

Figure 5.4 SDS-PAGE of xylanase using silver staining.

Lane 1: molecular weight standards (Pharmacia): phosphorylase B, 94 000; BSA, 67 000; ovalbumin, 43 000; carbonic anhydrase, 30 000; soybean trypsin inhibitor, 20 100 and α-lactalbumin, 14 400.

Lane 2: purified xylanase.

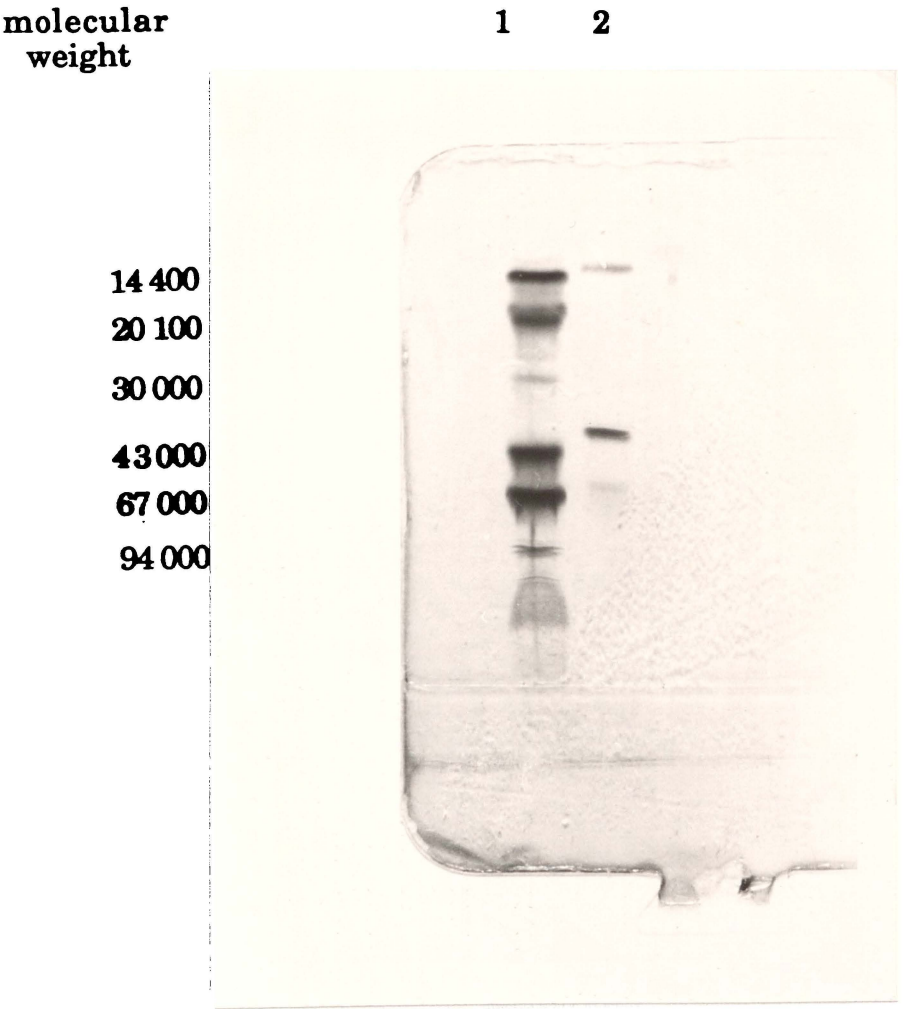


Figure 5.5 Molecular weight standard curve for SDS-PAGE of purified xylanase.

Molecular weight standards (Pharmacia): phosphorylase B, 94 000; BSA, 67 000; ovalbumin, 43 000; carbonic anhydrase, 30 000; soybean trypsin inhibitor, 20 100 and α-lactalbumin, 14 400.

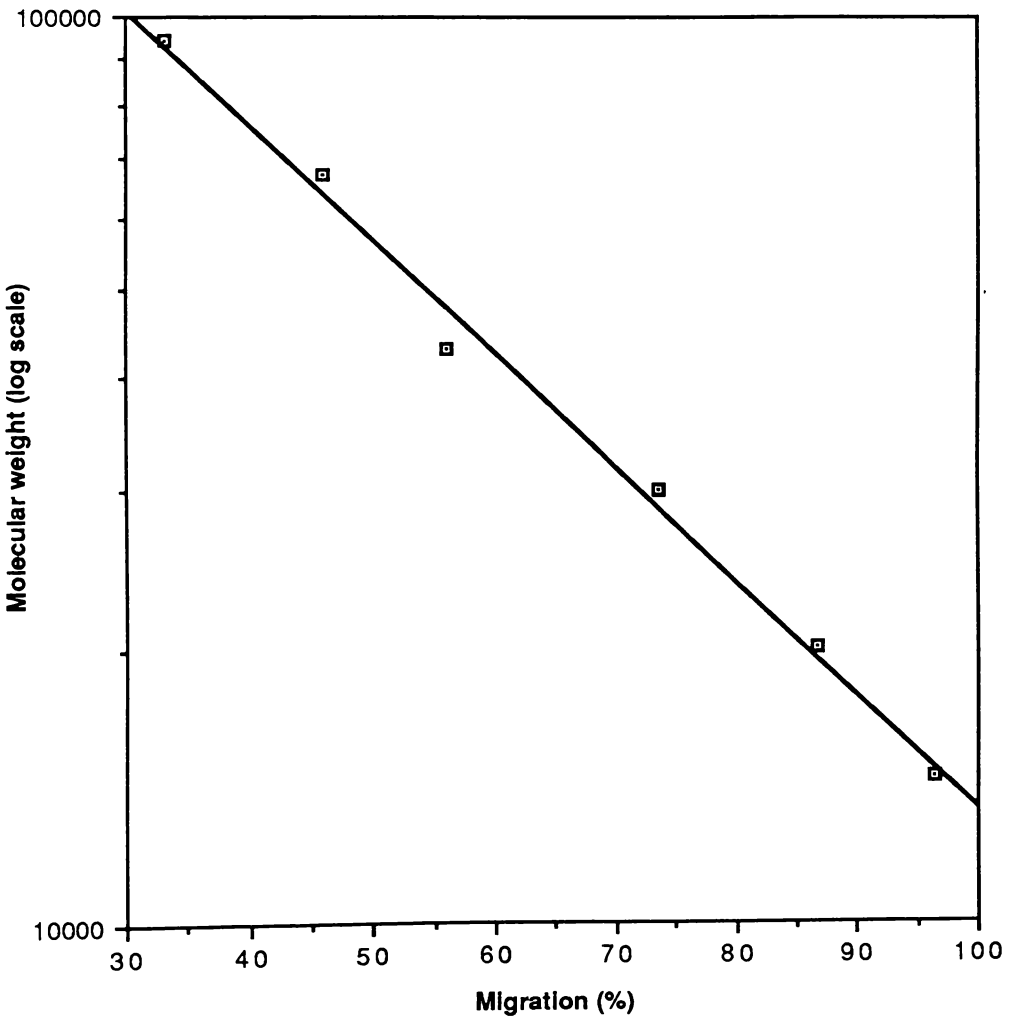


Figure 5.6 Molecular weight standard curve for Analytical Superose 6 gel filtration of purified xylanase.
Molecular weight standards (Biorad): thyroglobin, 670 000; γ-globulin, 158 000; ovalbumin, 44 000; myoglobin, 17 000 and vitamin B_{12}, 1 350.

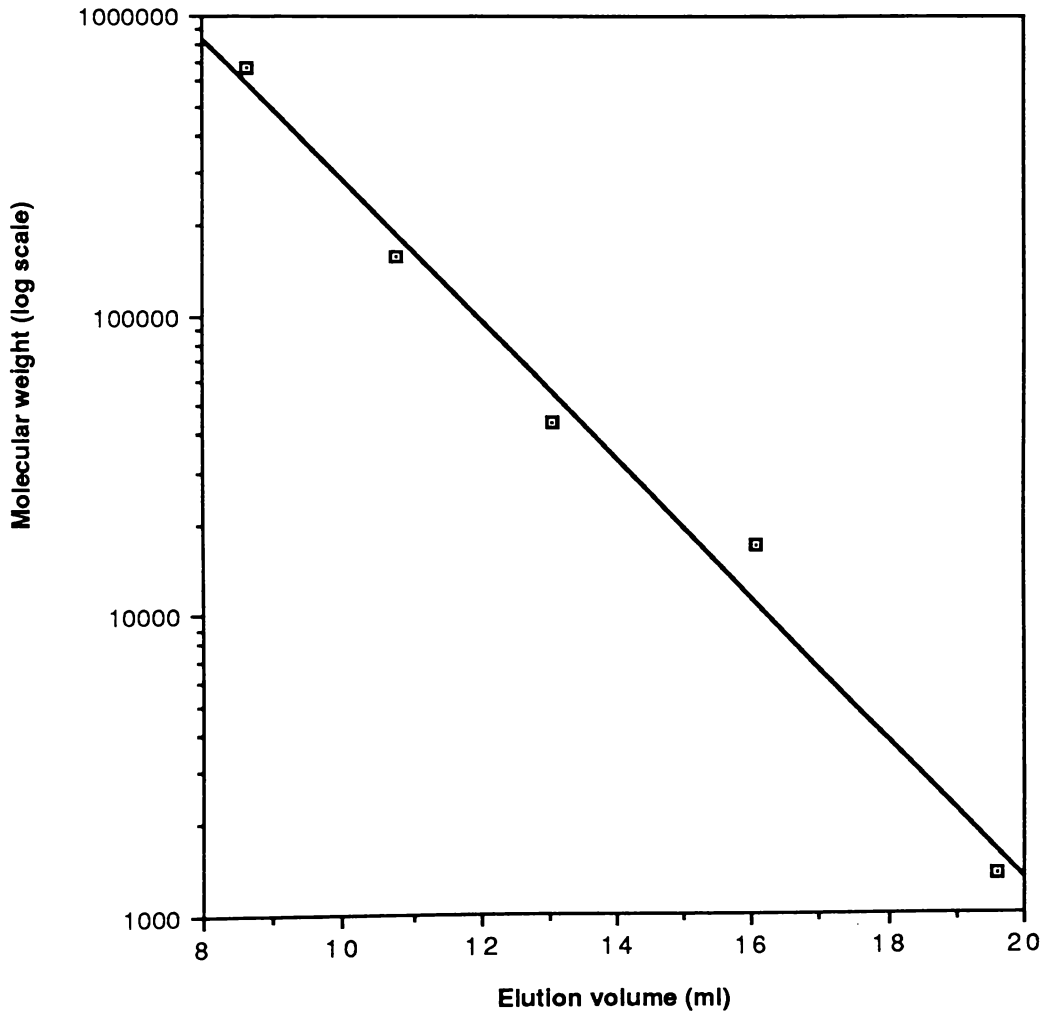


Figure 5.7 Isoelectric focusing of purified xylanase on an agarose gel (Coomassie Blue staining).

Lanes 1 and 2: isoelectric focusing standards (Pharmacia): amyloglucosidase, 3.50; methyl red, 3.75; glucose oxidase, 4.15; soybean trypsin inhibitor, 4.55; β-lactoglobulin A, 5.20; bovine carbonic anhydrase B, 5.85 and human carbonic anhydrase B, 6.55.

Lane 3: purified xylanase.

Arrows mark the three major active bands.

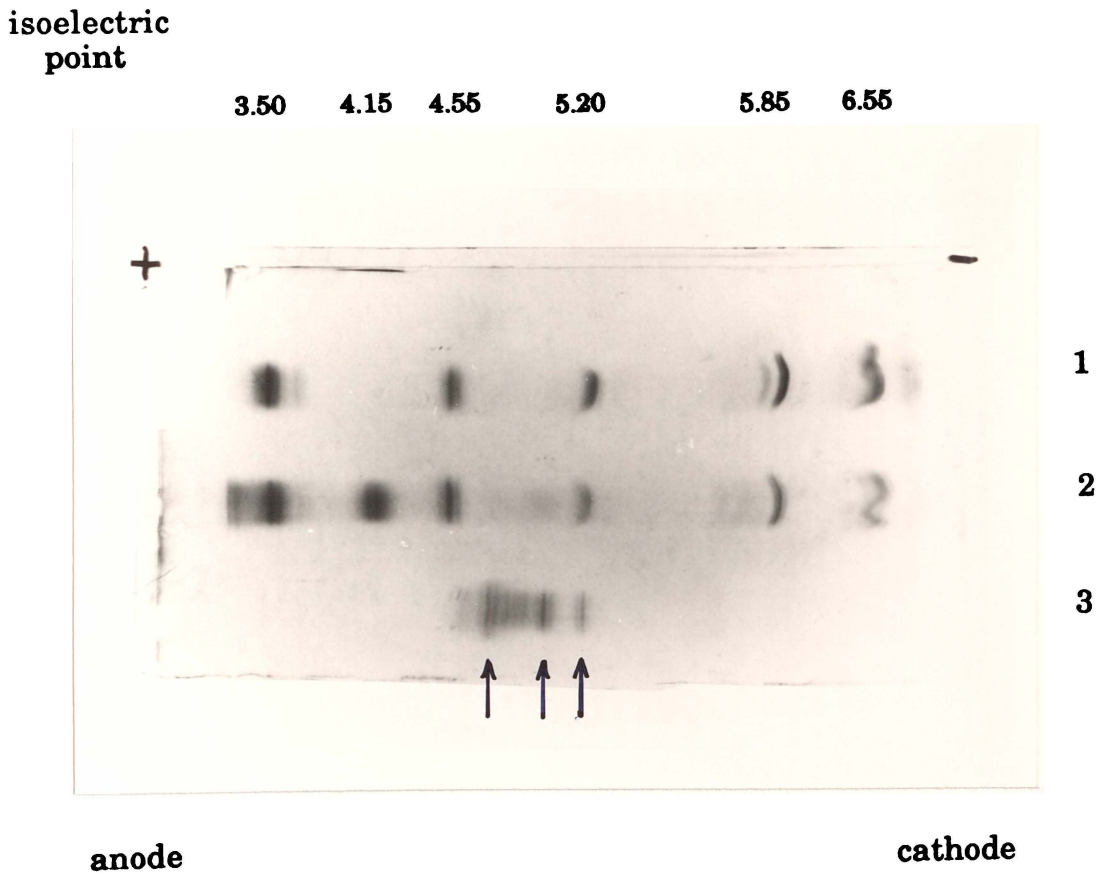


Figure 5.8 Isoelectric point standard curve of purified xylanase in an agarose IEF gel.

Isoelectric focusing standards (Pharmacia): amyloglucosidase, 3.50; methyl red, 3.75; glucose oxidase, 4.15; soybean trypsin inhibitor, 4.55; β-lactoglobulin A, 5.20; bovine carbonic anhydrase B, 5.85 and human carbonic anhydrase B, 6.55.

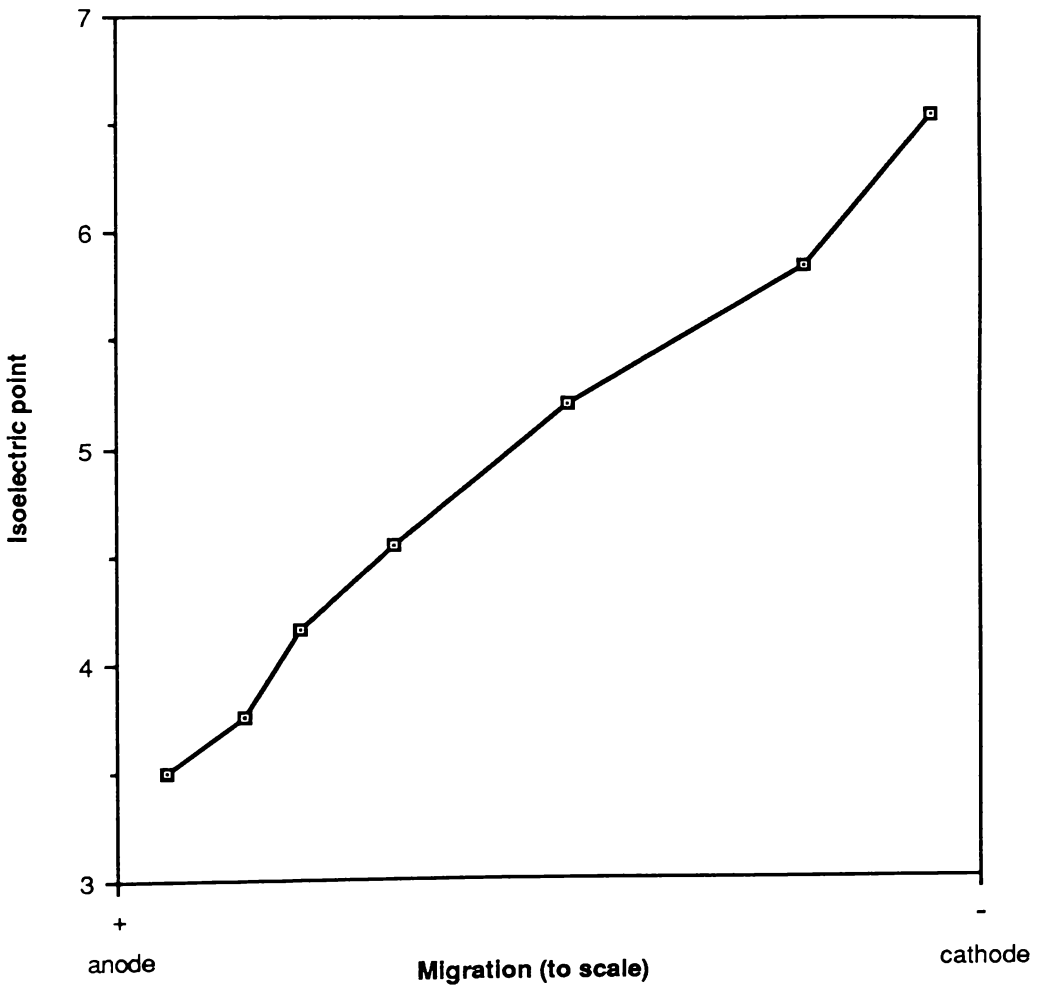


Table 5.6 Molecular weights and isoelectric points of some purified bacterial xylanases.

Bacterium	Xylanase	Molecular weight	Isoelectric point	Source
Bacillus sp. strain W1	I	21500	8.5	Okazaki
	II	49500	3.6	*et al.*, 1985
Bacillus sp. strain W2	I	22500	8.3	Okazaki
	II	50000	3.7	*et al.*, 1985
Bacillus subtilis	*	22000	8.9	Paice *et al.*, 1986
Bacillus circulans	P*†	59000	~9.0	Yang *et al.*,
	R*	22000	9.0	1989 a and b
Bacillus circulans	A†	~85000	4.45	Esteban
	B†	15000	9.1	*et al.*, 1982
Bacillus polymyxa	*	48000	4.9	Yang *et al.*, 1988
Clostridium acetobutylicum	A	65000	4.45	Lee
	B	29000	8.50	*et al.*, 1987
Clostridium stercorarium	A	44000	4.53	Berenger
	B	72000	4.43	*et al.*, 1985
	C	62000	4.39	
Clostridium thermolacticum	I	39000	4.9	Debeire *et al.*, 1990
Streptomyces lividans	A	43000	5.2	Morosoli *et al.*, 1986
	B$^{\Delta}$	31000	8.4	Kluepfel *et al.*, 1990
Streptomyces sp. strain T_7		20463	7.8	Keskar *et al.*, 1989
'*Caldocellum saccharolyticum*'	*	42000	~5.0	this study

* expressed in *E. coli*
$^{\Delta}$ expressed in a mutant *S. lividans* strain
† partially purified

5.3.3 Linearity of assay

A time course of xylanase activity was linear for 15 minutes (Figure 5.9). A plot of the results of varying enzyme concentration versus activity gave a straight line over the range 0 - 162.5ng xylanase (Figure 5.10). Throughout characterization (not purification) all xylanase assays were run over 10 minutes and performed with 32.5ng of xylanase, except where specifically stated.

5.3.4 Influence of temperature and pH on activity

Activity in a 10 minute assay increased with increasing temperature up to 70°C (Figure 5.11). A 1/T versus the log of the activity plot (apparent Arrhenius plot) of the data from 0 - 65°C gave a straight line (Figure 5.12). An activation energy (E_a) of 44kJmol^{-1} and a temperature coefficient (Q_{10}) of 1.76 were calculated from these data. The activation energy is very similar to those calculated for the three xylanases produced by *Cl. stercorarium* and the xylanase produced by *Cl. thermolacticum*. *Cl. stercorarium* xylanases A, B and C had activation energies of 43.5, 44.0 and 36.3kJmol^{-1} respectively (Berenger *et al.*, 1985). *Cl. thermolacticum* xylanase I had an activation energy of 38.8kJmol^{-1} (Debeire *et al.*, 1990).

The xylanase had a broad pH optimum, with greater than 50% activity between pH 5.0 and pH 7.7 (Figure 5.13). This pH optimum is typical of bacterial xylanases. pH optima often fall in the range 5 - 9 and are usually approximately 6 - 7.

5.3.5 Temperature and pH stability

The half life of the xylanase at 70°C in the absence of added protein was approximately 4 minutes at pH $6.0_{70°C}$ and less than one minute at pH $10.5_{20°C}$ (Figure 5.14). The plot of time versus the log of residual activity is shown in Figure 5.15. These half lives were unexpectedly low, when considered that the assay was linear up to 15 minutes. In the assay, the xylanase was probably stabilized by the presence of substrate. During heat treatment (in the absence of substrate), the low thermostability of the xylanase was probably due to the very low protein concentration (0.325μgml^{-1}). At 70°C (pH $6.0_{70°C}$) in the presence of added protein (0.5mgml^{-1} BSA) the half life was much greater than 30 minutes

Figure 5.9 Time course of xylanase assay.

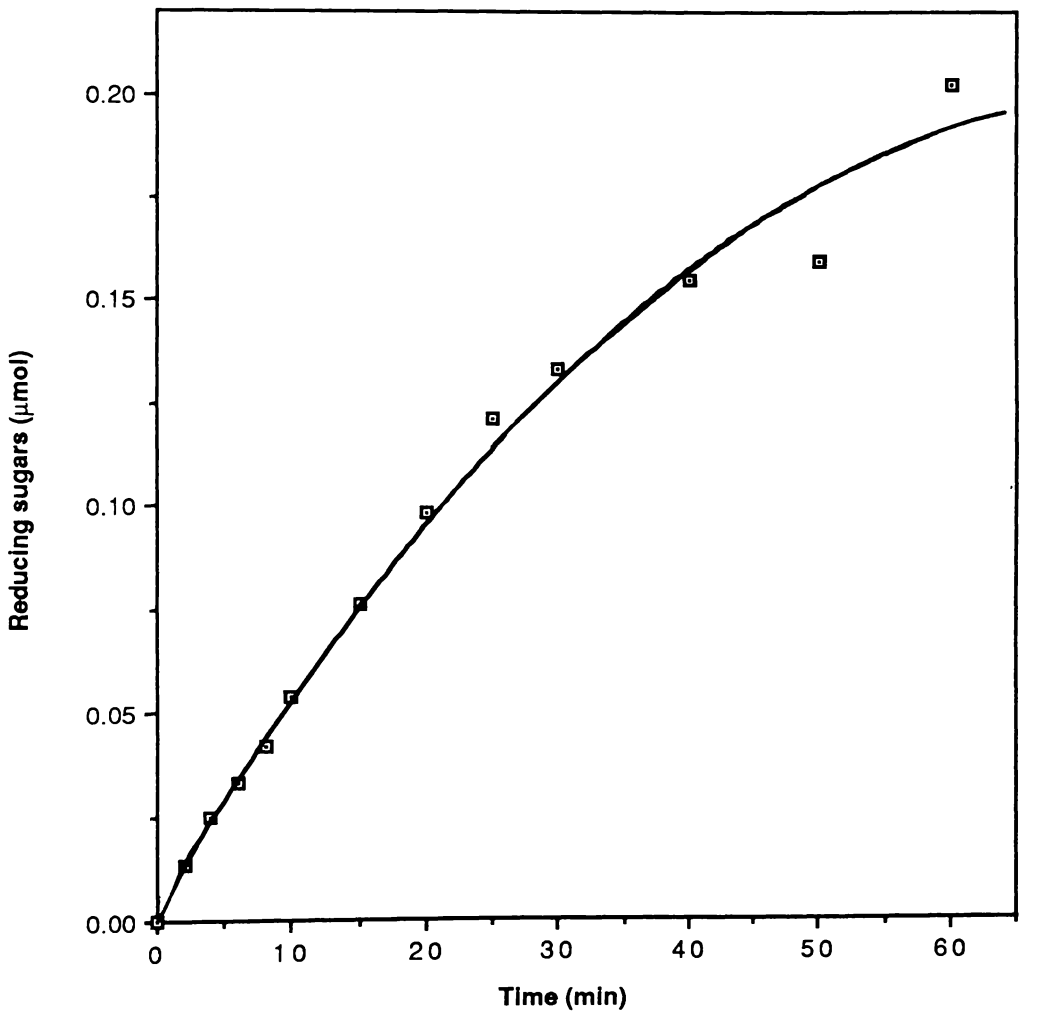


Figure 5.10 Amount of enzyme versus activity of the xylanase.

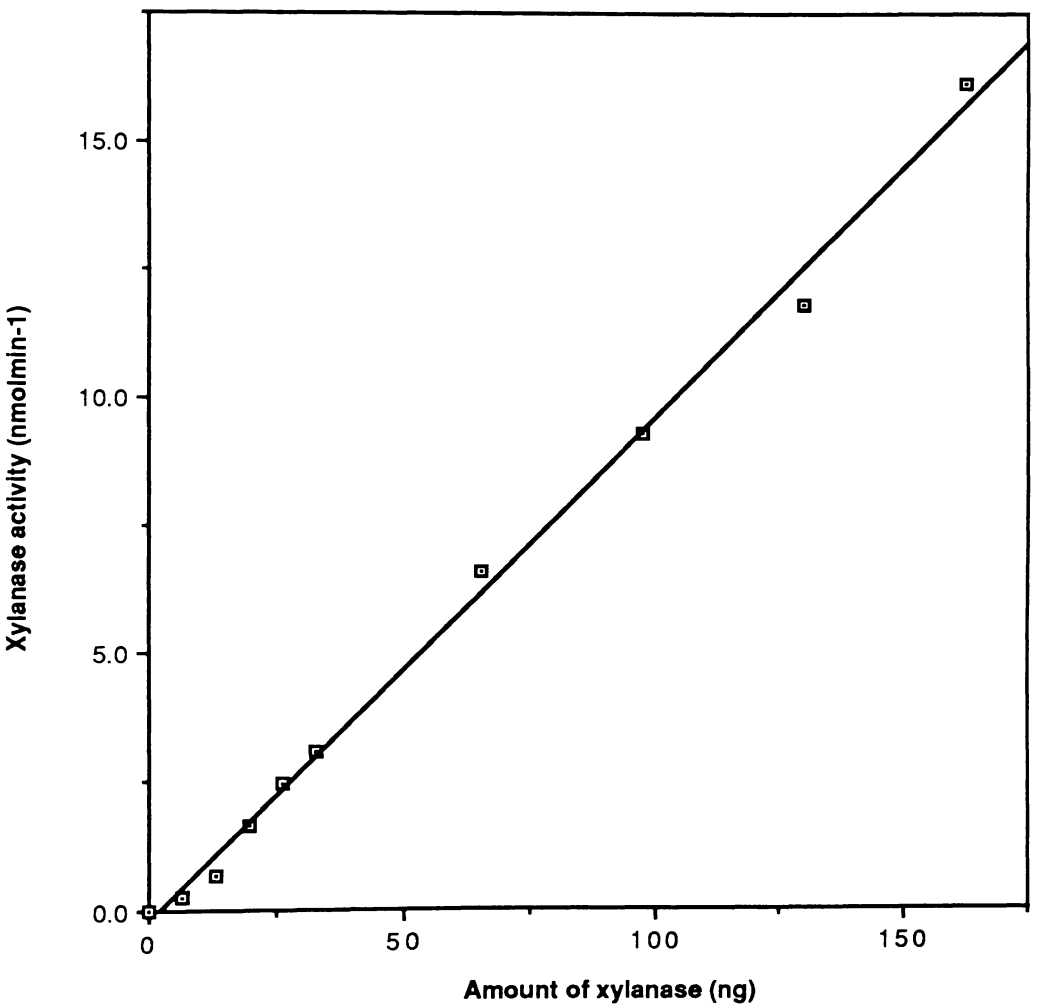


Figure 5.11 Effect of temperature on activity of the xylanase (10 minute assay at pH $6.0_{70°C}$).

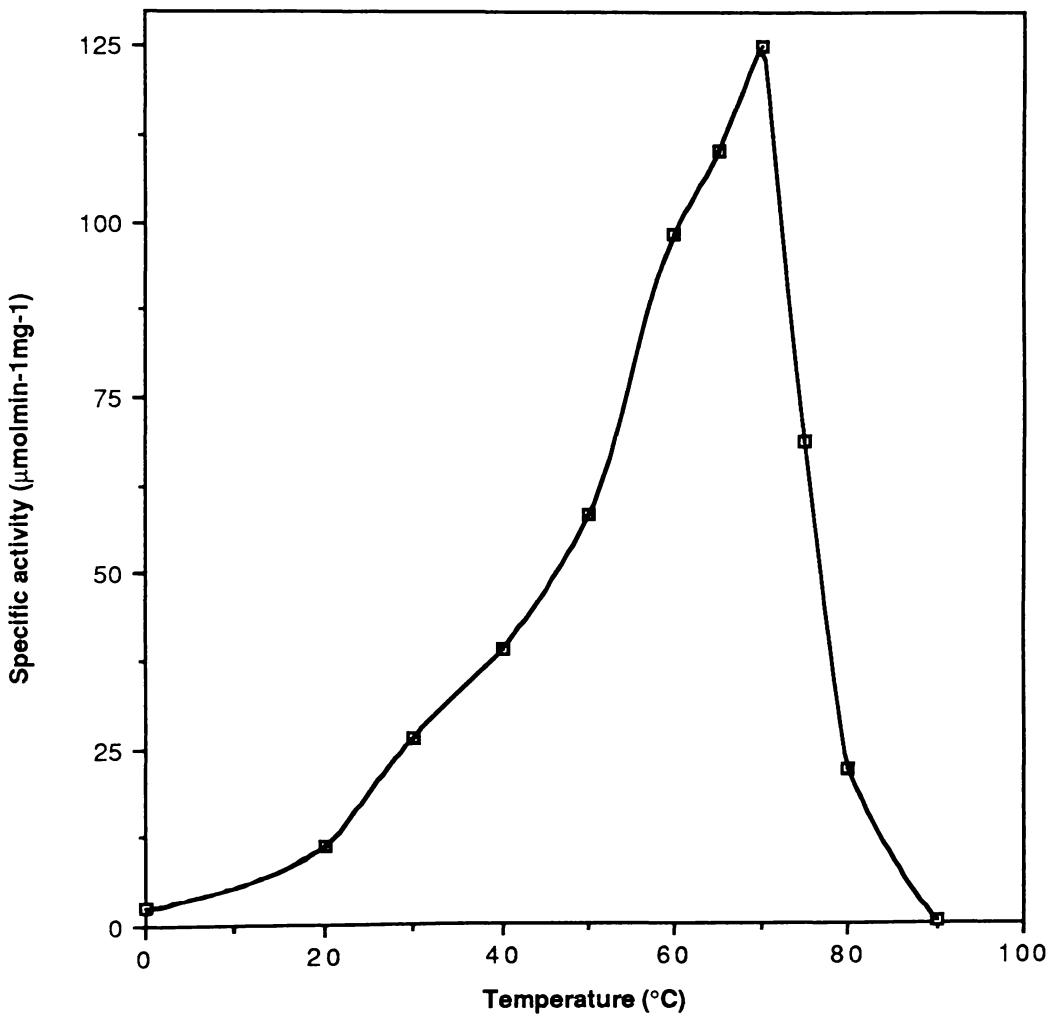


Figure 5.12 Apparent Arrhenius plot for the xylanase.

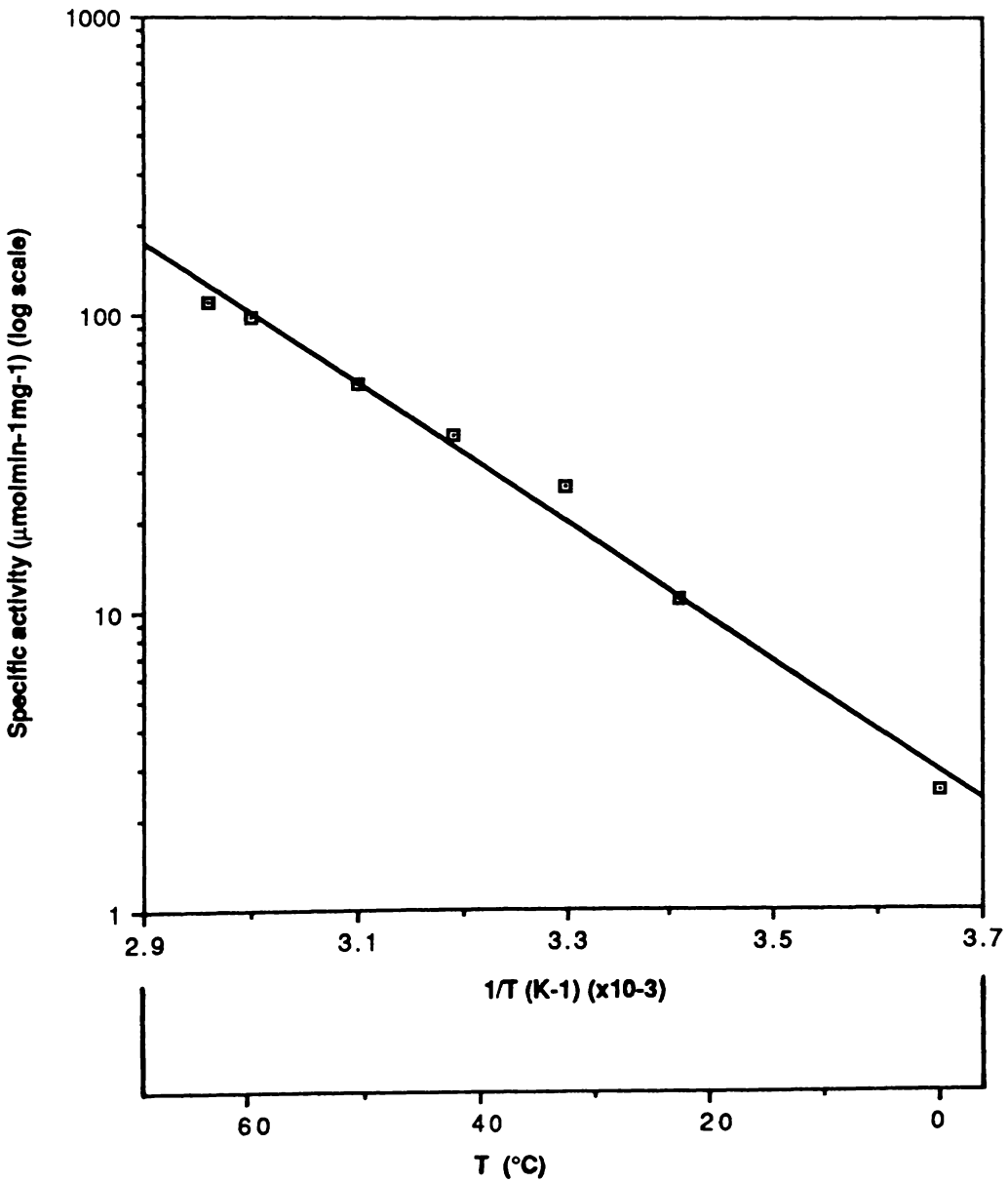


Figure 5.13 Effect of pH on activity of the xylanase.
Bis-Tris propane buffers ($pH_{70°C}$) and citrate buffers ($pH_{20°C}$) used.

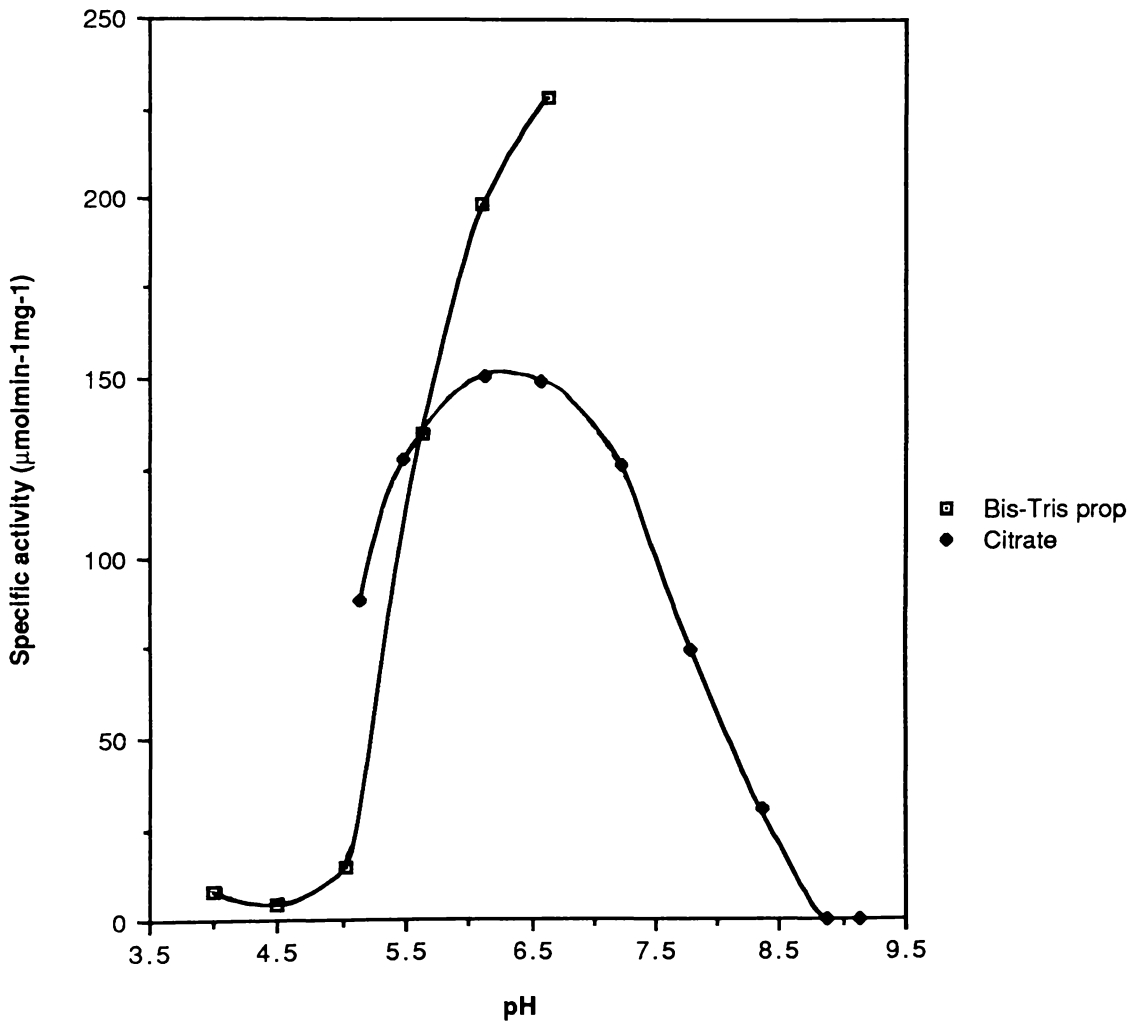


Figure 5.14 Thermostability curves for the xylanase.
Thermostabilities at: 70°C, pH $6.0_{70°C}$, no protein added (-); 70°C, pH $10.5_{20°C}$, no protein added (-); 70°C, pH $6.0_{70°C}$, BSA added (+); 75°C, pH $6.0_{70°C}$, BSA added (+).

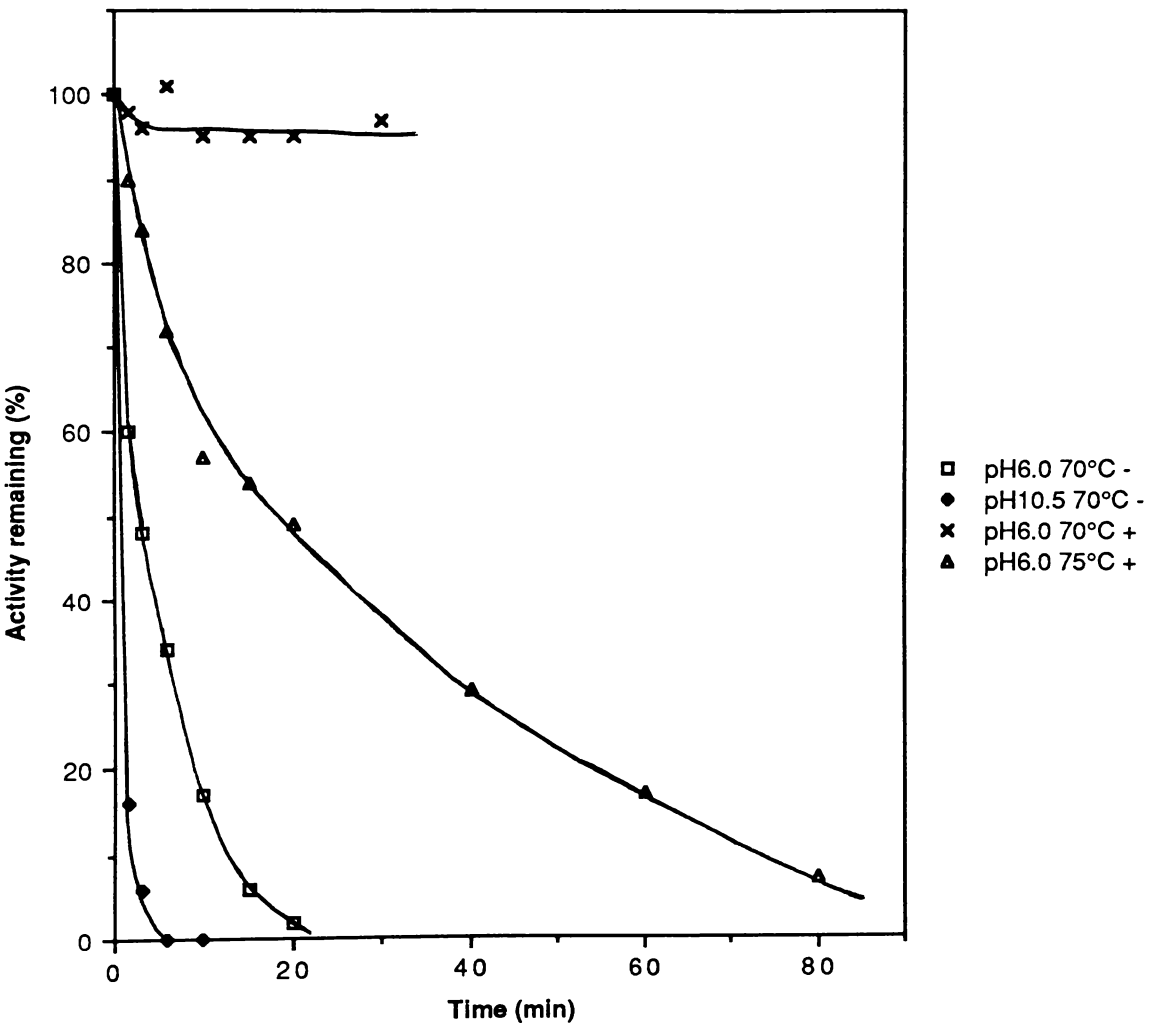
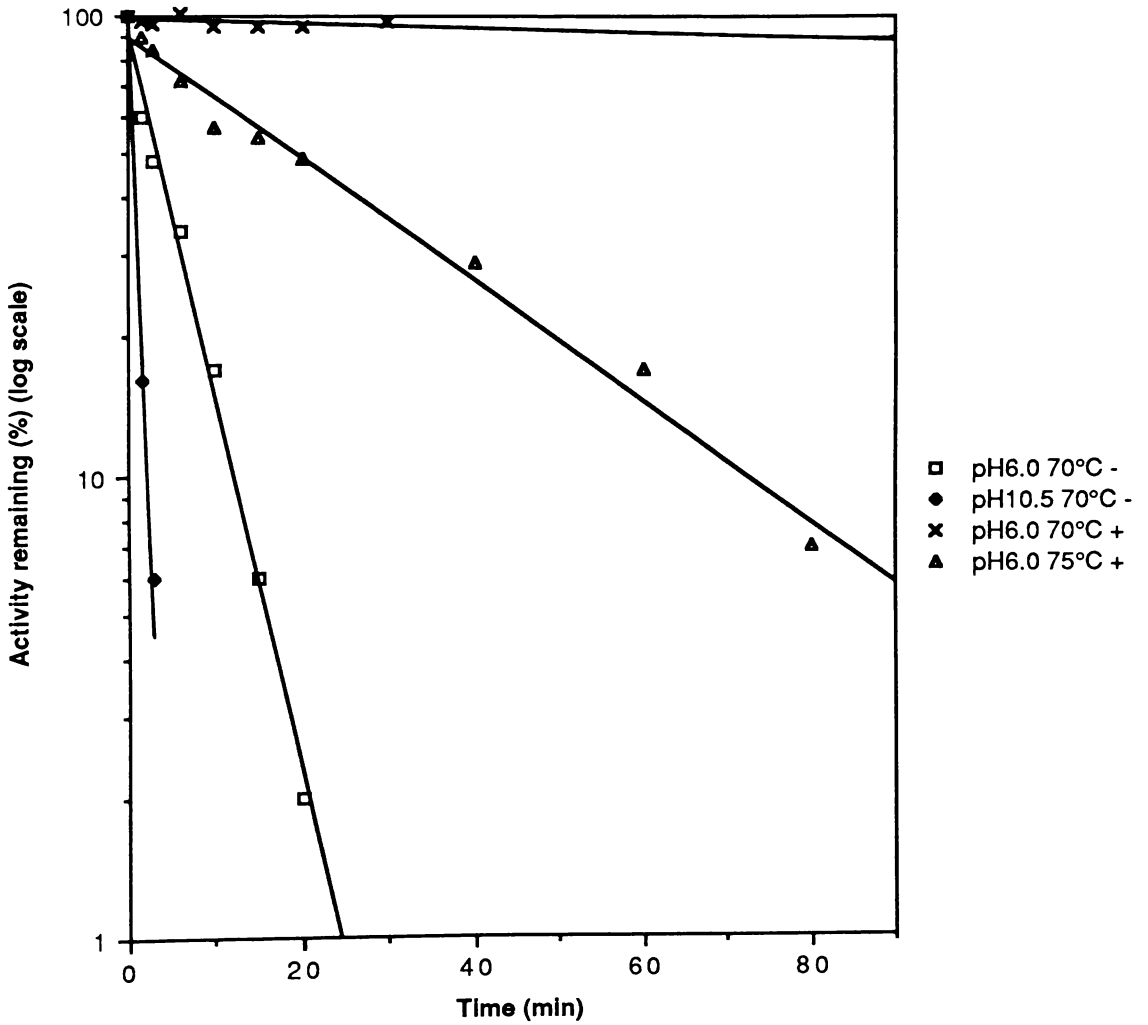


Figure 5.15 Time versus the log of residual activity for the xylanase.
Thermostabilities at: 70°C, pH $6.0_{70°C}$, no protein added (-);
70°C, pH $10.5_{20°C}$, no protein added (-); 70°C, pH $6.0_{70°C}$,
BSA added (+); 75°C, pH $6.0_{70°C}$, BSA added (+).



(Figure 5.14). At 75°C (pH $6.0_{70°C}$) in the presence of added protein, the half life was 20 minutes (Figure 5.14). The time versus log residual activity plot is shown in Figure 5.15. The xylanase is very thermostable compared to other bacterial xylanases (Table 5.7). Only *Cl. stercorarium* xylanases appear to be more thermostable.

The xylanase was treated at various pH values at room temperature for 3 hours with no added protein. It was found to be more stable at higher pH values (Figure 5.16). However, the xylanase was very stable at all pH values

Table 5.7 Thermostability data: half lives of some purified bacterial xylanases.

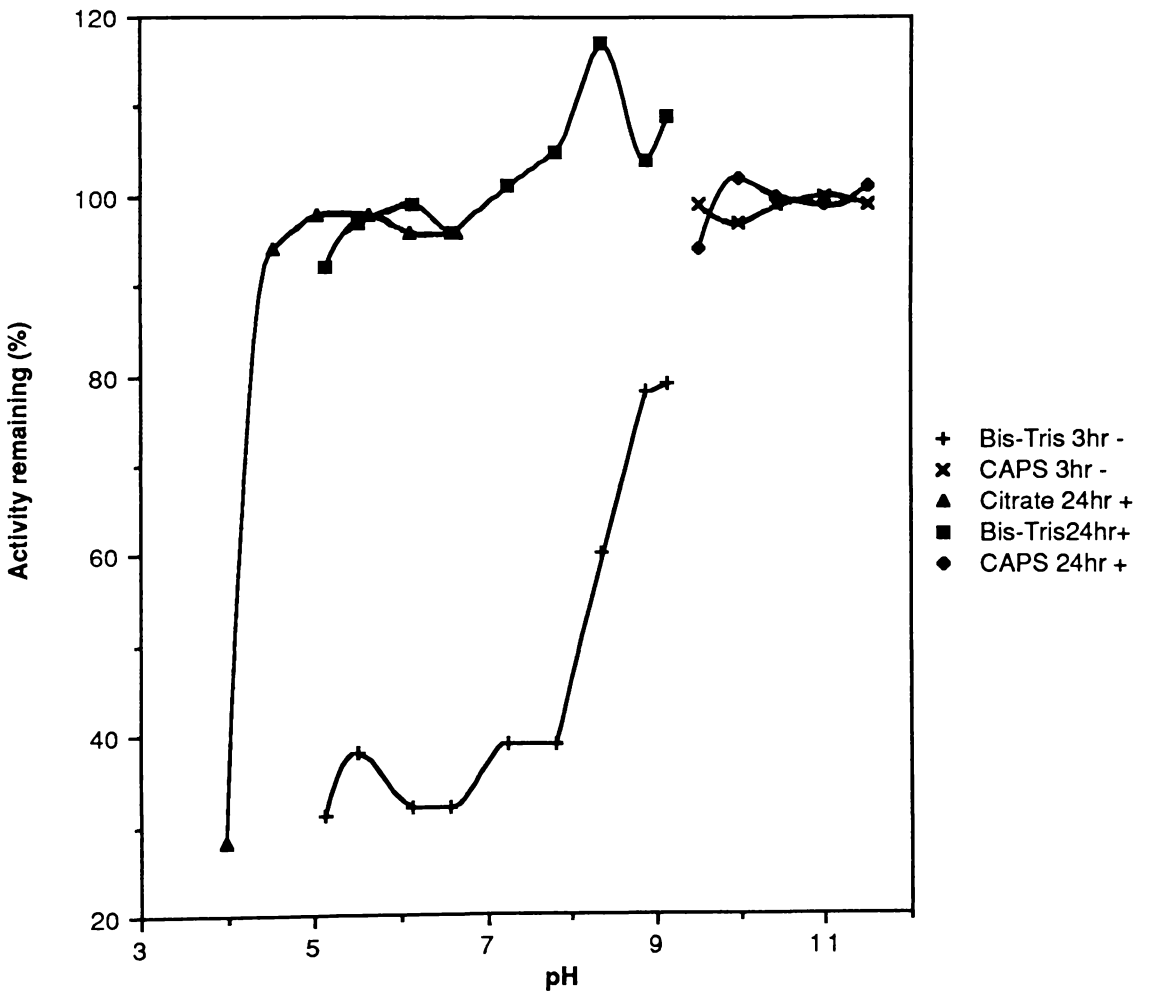
Bacterium	Xylanase	Half life (min)	Temperature (°C)	Source
Bacillus sp. strain K17	I	25	70	Kang *et al.*, 1986
	II	40	70	
Bacillus pumilus		~30	50	Panbangred *et al.*, 1983
Clostridium stercorarium	A	90	81	Berenger *et al.*, 1985
	B	2.5	81	
	C	8	81	
Streptomyces lividans	A	~180	50	Morosoli *et al.*, 1986
	B$^{\Delta}$	~270	60	Kluepfel *et al.*, 1990
Streptomyces sp. strain T_7		30	60	Keskar *et al.*, 1989
'*Caldocellum saccharolyticum*'	*	20	75	this study

* expressed in *E. coli*

$^{\Delta}$ expressed in a mutant *S. lividans* strain

Figure 5.16 Effect of pH on stability of the xylanase.

Treatments:
Bis-Tris propane buffer ($pH_{70°C}$), 3 hours, room temperature, no protein added (-);
CAPS buffer ($pH_{20°C}$), 3 hours, room temperature, no protein added (-);
citrate buffer ($pH_{20°C}$), 24 hours, room temperature, BSA added (+);
Bis-Tris propane buffer ($pH_{70°C}$), 24 hours, room temperature, BSA added (+);
CAPS buffer ($pH_{20°C}$), 24 hours, room temperature, BSA added (+).



down to pH 4.5 at room temperature for 24 hours in the presence of added protein. The low stability of the xylanase when treated for three hours at room temperature (in the absence of added protein) was probably due to the very low protein concentration (0.325μgml^{-1}) during treatment. The xylanase has a very broad pH stability (in the presence of added protein) similarly to most other bacterial xylanases (Table 5.8).

Table 5.8 pH stability ranges for some purified bacterial xylanases.

Bacterium	Xylan-ase	pH stability range	Source
Bacillus sp. strain W1	I II	4.5 - 10.0 4.5 - 10.0	Okazaki *et al.*, 1985
Bacillus sp. strain W2	I II	4.5 - 10.0 4.5 - 10.0	Okazaki *et al.*, 1985
Bacillus sp. strain K17	I II	5.0 - 9.0 6.0 - 10.0	Kang *et al.*, 1986
Bacillus sp. strain 11-1S		2.0 - 6.0	Uchino and Nakane, 1981
Bacillus sp. strain C-125	N A	4 - 12 5 - 12	Honda *et al.*, 1985b
Clostridium acetobutylicum	A B	5.5 - 7.0 3.5 - 7.0	Lee *et al.*, 1987
Clostridium thermolacticum	I	3 - 11	Debeire *et al.*, 1990
'*Caldocellum saccharolyticum*'	*	4.5 - 11.5	this study

* expressed in *E. coli*

5.3.6 Effect of various compounds on activity

Table 5.9 lists the relative activities of the xylanase in the presence of various compounds. Several cations slightly increased activity, while Rb^{+}, Ni^{2+} and Zn^{2+} inhibited activity. Iodoacetic acid decreased activity, BSA increased activity and SDS markedly decreased activity. Agents that were unable to be used due to either solubility problems or incompatibility with the PAHBAH assay system were: $BaCl_2$, AgCl, AgOAc, $CdCl_2$, $HgCl_2$, $CrCl_3$, $MnCl_2$, $FeCl_3$, $CoCl_2$, $CuCl_2$, *N*-bromosuccinamide, *p*-chloromercuribenzoate, *p*-hydroxymercuribenzoic acid, glycerol, xylose and glucose.

The effect of xylose on xylanase activity was investigated using the dyed xylan assay system. Only slight activity was found on the dyed xylan unless extra protein (BSA) was added, when the activity became very much higher. If xylose and BSA were present, the activity was only marginally higher than in the presence of BSA alone. The results in the presence and absence of added protein were not consistent with those obtained using the well defined PAHBAH assay system. It may be that having a dye linked to the substrate inhibits or destabilizes the enzyme. Added protein (BSA) may then protect or stabilize the enzyme. Alternatively, the enzyme may bind to the dye and BSA may block this binding but still allow cleavage of dyed xylooligosaccharides by the enzyme. In this case different enzyme preparations (of differing purity) could be expected to act in different ways. The dyed xylan assay system has not been well investigated. Various factors, such as ionic strength and temperature (Sharrock, 1988) appear to affect the solubilization of dyed xylooligosaccharides and precipitation of dyed xylan. It is possible that another of these factors is added protein.

The effect of xylose on *p*NPX hydrolysis by the xylanase was studied by HPLC. There was no difference in the amounts and types of products produced from *p*NPX in the presence and absence of xylose. Xylose, therefore, has no inhibitory effect on endoxylanase or transferase activity of the xylanase on *p*NPX (for the mode of action of the xylanase on *p*NPX see Section 5.3.8).

Table 5.9 Effect of various compounds on activity of the xylanase.

Compound	Relative xylanase activity (%)
none	100
0.2M LiCl	120
0.2M NaCl	118
0.2M KCl	120
0.2M RbCl	45
5mM $MgCl_2$	112
5mM $CaCl_2$	107
5mM $SrCl_2$	112
5mM $NiCl_2$	0
5mM $ZnCl_2$	0
5mM β-mercaptoethanol	96
5mM DTT	99
5mM EDTA	92
5mM iodoacetic acid	79
0.5mgml^{-1} BSA	124
1% (w/v) Triton X-100	112
1% (w/v) SDS	14

5.3.7 Kinetics

Classical Michaelis-Menten kinetics (Figure 5.17) were observed on oat spelts xylan. The data was plotted in the Lineweaver-Burk (Figure 5.18), Eadie-Hofstee and Hanes forms, and the K_m and V_{max} values calculated (Table 5.10). A figure of 5 840 β-1,4-xylosidic bonds cleaved per enzyme molecule per minute was calculated assuming a molecular weight of 42 000 and one active site. The xylanase has a very low K_m compared to other bacterial xylanases and an average V_{max} (Table 5.11), although the data for most other xylanases were measured using larchwood xylan.

Table 5.10 Kinetic data for the xylanase on oat spelts xylan.

	K_m (% w/v)	V_{max} $\mu molmin^{-1}mg^{-1}$	r
Lineweaver-Burk	0.018	132.4	0.9956
Eadie-Hofstee	0.020	137.5	-0.9878
Hanes	0.026	147.5	0.9998

Figure 5.17 Michaelis-Menten plot for the xylanase.

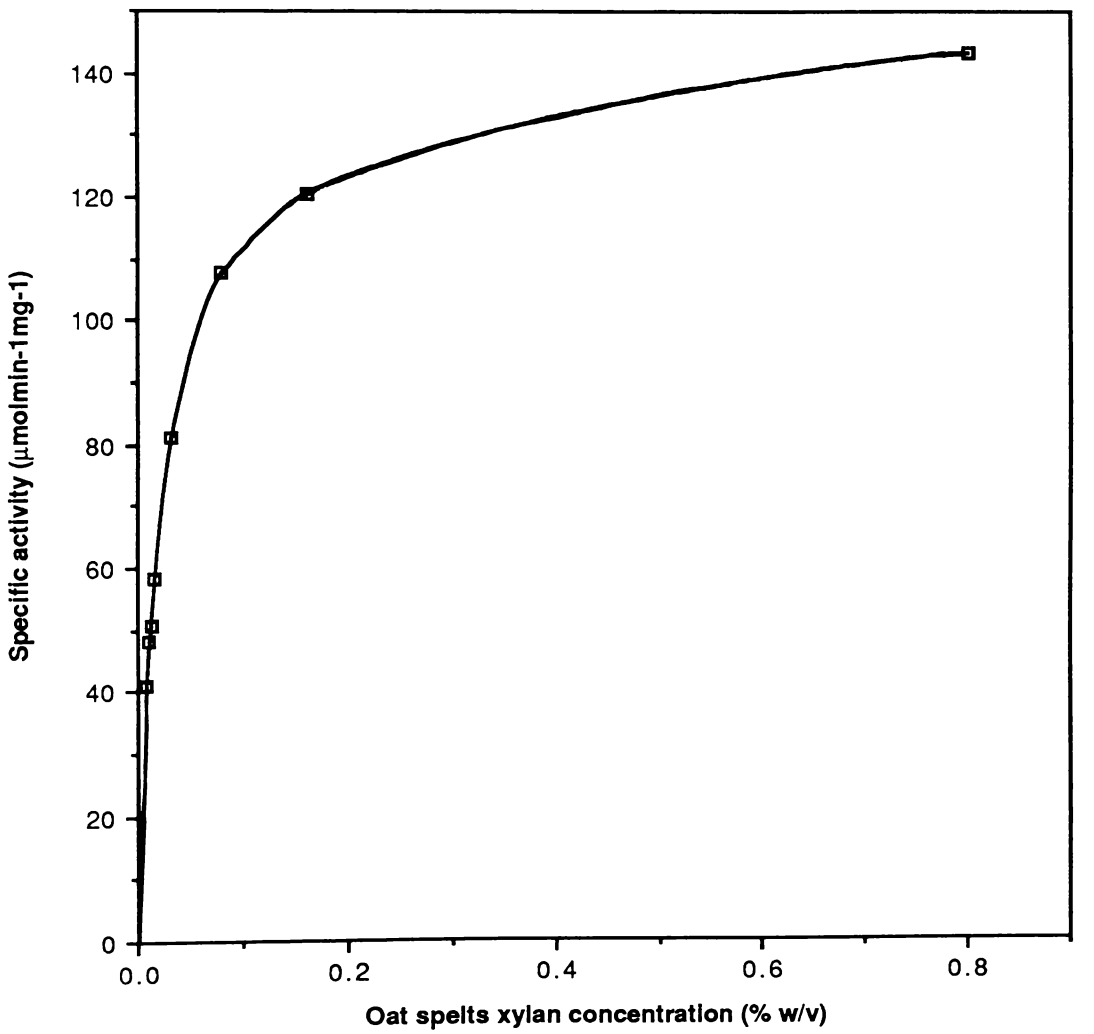


Figure 5.18 Lineweaver-Burk plot for the xylanase.

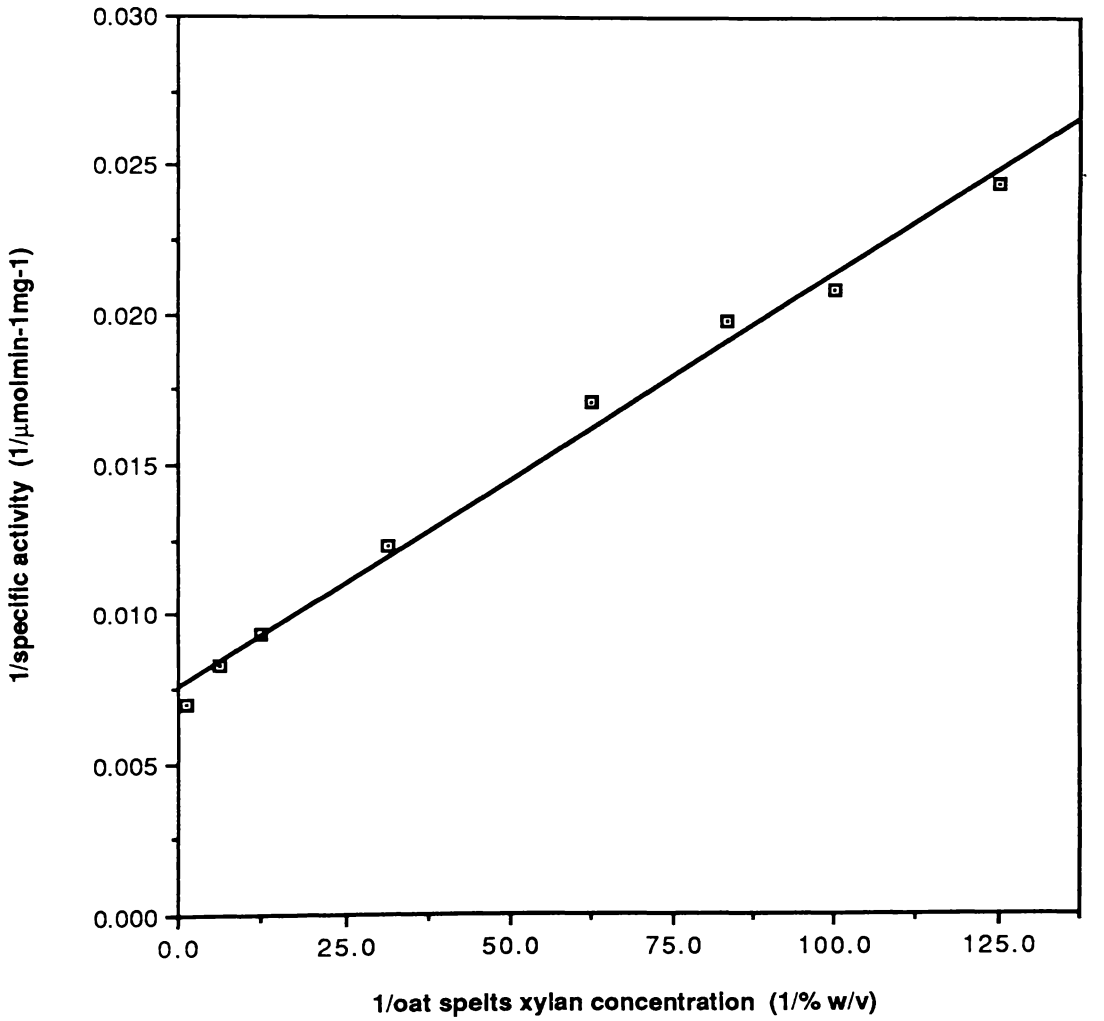


Table 5.11 Kinetic data of some purified bacterial xylanases.

Bacterium	Xylanase	K_m (% w/v)	V_{max} (μmol $min^{-1}mg^{-1}$)	Xylan substrate	Source
Bacillus sp. strain W1	I II	0.45 0.095		larchwood	Okazaki *et al.*, 1985
Bacillus sp. strain W2	I II	0.40 0.057		larchwood	Okazaki *et al.*, 1985
Bacillus sp. strain K17	I II	0.467 0.230		larchwood	Kang *et al.*, 1986
Bacillus sp. strain 11-1S			8.92	larchwood	Uchino and Nakane, 1981
Bacillus circulans	A† B†	0.8 0.4		not stated	Esteban *et al.*, 1982
Clostridium acetobutylicum	A B	0.60 0.67	22.4 22.3	larchwood	Lee *et al.*, 1987
Clostridium stercorarium	A B C	0.32 0.29 0.37	5500 3500 4000	larchwood	Berenger *et al.*, 1985
Clostridium thermolacticum	I	0.065		soluble larchwood	Debeire *et al.*, 1990
Streptomyces lividans	A	0.078	850	soluble oat spelts	Morosoli *et al.*, 1986
	B△	0.37	1960	oat spelts	Kluepfel *et al.*, 1990
Streptomyces sp. strain T_7		1.0	7600	larchwood	Keskar *et al.*, 1989
'*Caldocellum saccharolyticum*'	*	0.02	139	oat spelts	this study

* expressed in *E. coli*
△ expressed in a mutant *S. lividans* strain
† partially purified

5.3.8 Substrate specificity and mode of action

A trial experiment showed that the initial products of xylanase action on oat spelts xylan appeared to be small amounts of xylotriose and xylotetraose, and even smaller amounts of xylobiose and xylopentaose. In a subsequent experiment using higher amounts of enzyme, the hydrolysis products of oat spelts xylan were monitored for 24 hours (Figure 5.19). No xylotetraose and xylopentaose could be detected but a very small amount of xylohexaose may have been produced initially and remained constant throughout the 24 hour hydrolysis. Amounts of xylotriose and xylobiose quickly rose, with the amount of xylotriose slowly decreasing to zero over 24 hours and the amount of xylobiose continuing to increase slowly. The production of xylobiose may slow due to end product inhibition. Xylose was detected after 30 minutes and slowly increased over 24 hours. The results are consistent with the xylanase acting in an endo fashion, cleaving xylobiose and xylotriose from xylan, and having a higher affinity for the second β-xylosidic linkage over the third from the terminal of oat spelts xylan. The subsequent decrease in xylotriose is probably due to its hydrolysis to xylobiose and xylose by the xylanase. No activity could be detected when xylobiose was used as a sole substrate and this is consistent with the lack of decrease in xylobiose produced from xylan over time. The smallest xylooligosaccharide that the xylanase can hydrolyse is therefore xylotriose.

Table 5.12 shows the substrate specificity of the xylanase. The xylanase had substantial activity on sugar cane bagasse hemicelluloses (A and B), but less activity on larchwood xylan. Sugar cane bagasse xylan is more similar to oat spelts xylan than larchwood. Unlike sugar cane bagasse xylan or oat spelts xylan, larchwood xylan contains acetyl groups. Endoxylanase activity is lower on xylans containing acetyl side groups and this may be the cause of the lower activity on larchwood xylan in this study.

Figure 5.19 Hydrolysis of oat spelts xylan by xylanase. Analysis by HPLC.

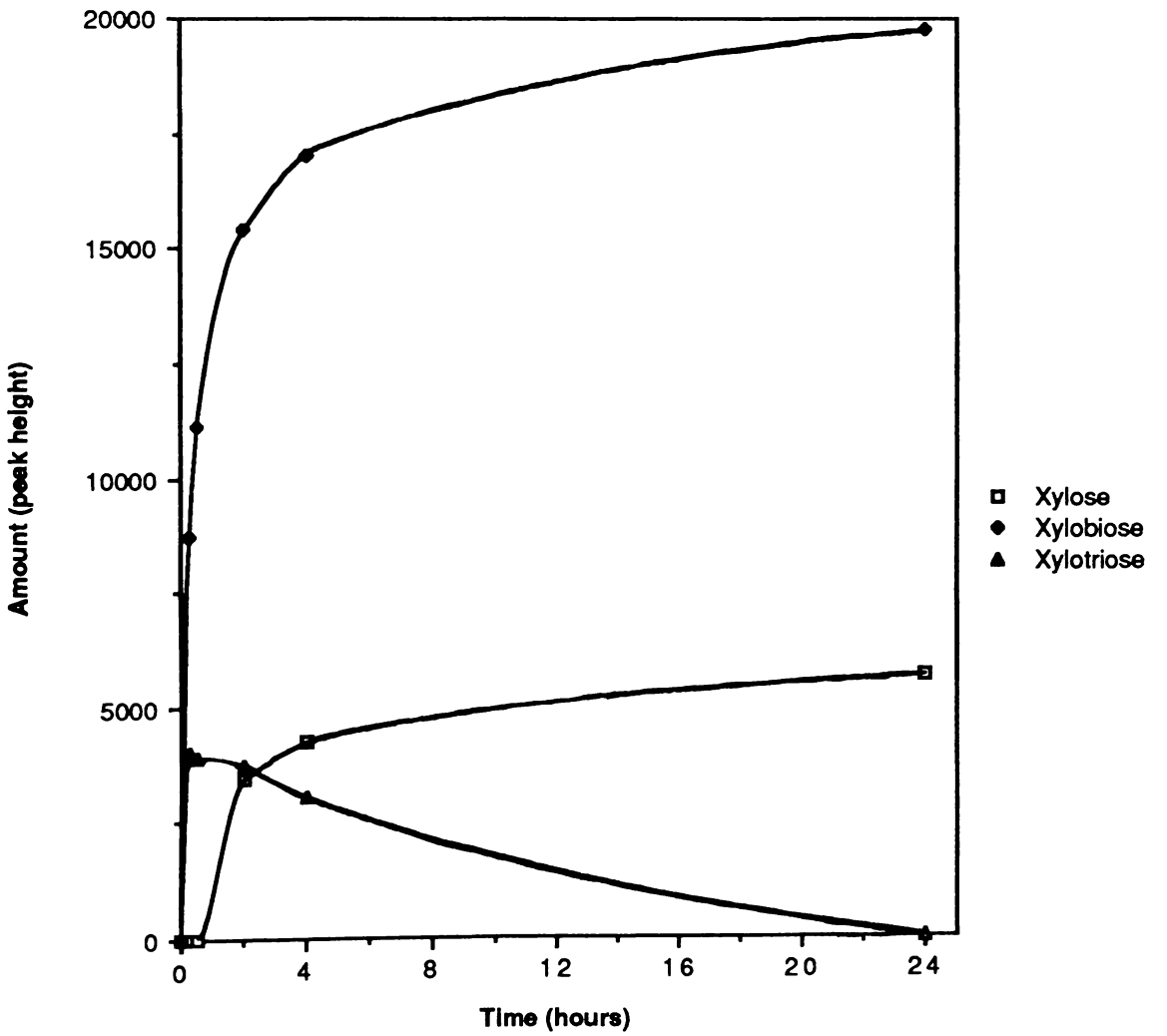


Table 5.12 Substrate specificity of the xylanase.

Specific activities expressed as %.

Substrate	Relative activity (%)	Amount of enzyme in assay ($ngml^{-1}$)
0.2% w/v oat spelts xylan	100	65
0.2% w/v larchwood xylan	22	65
0.2% w/v sugar cane bagasse hemicellulose A	74	65
0.2% w/v sugar cane bagasse hemicellulose B	69	65
0.2% w/v pentosan polysulphate	0	65
1.6% w/v CMC	20	500
5% w/v Avicel	<1	1 250
4mM MUC	2	500
20mM *p*NPC	5	500
12mM *p*NPL	0	500
20mM *p*NPG	0	500
20mM *p*NPX	<1	500
14mM *p*NPAp	<1	500
9mM xylobiose	0	25 000
20mM cellobiose	0	25 000

There was significant activity on CMC and negligible activity on Avicel. The xylanase was able to cleave MUC and *p*NPC, indicating that it was able to remove disaccharides from some substrates. However, there was no activity on *p*NPG showing it could not remove monosaccharides. These results also indicate that the xylanase acts in an endo manner. HPLC analysis of the products formed by the action of the xylanase on *p*NPX showed xylotriose and xylobiose, but no xylose (Figure 5.20). The small amount of activity on *p*NPX must have been due to initial transferase, not endoxylanase, activity of the xylanase. The proposed mode of action is shown in Figure 5.21. The transferase activity of the enzyme initially produces *p*NPXX which can then either be hydrolysed by the endoxylanase activity or serve as a substrate for the transferase. The production of xylose (and xylobiose) from xylotriose is probably not detected due to both the small proportion of xylotriose produced as well as its slow hydrolysis by the endoxylanase. Transferase activity on *p*NPXX gives *p*NPXXX which can be hydrolysed to xylobiose and *p*NPX, or xylotriose and *p*NP. The endoxylanase activity is much higher than the transferase activity, as once substrate (such as *p*NPXX) has been formed for the endoxylanase, it is hydrolysed.

The assumption by some workers that activity on *p*NPX necessarily indicates β-xylosidase activity is therefore incorrect. Only determination of the products due to such activity can indicate the type of activity involved. The small amount of xylanase activity on *p*NPAp is probably due to the same mode of action as on *p*NPX. There was no activity detected on cellobiose. The lack of activity on *p*NPG may indicate that the transferase activity of the xylanase is specific for pentose-containing substrates.

In summary, the xylanase acts in an endo fashion with preference for the second β-xylosidic linkage over the third in oat spelts xylan. It cannot release monosaccharides from substrates (except when xylobiose is released from xylotriose, then xylose is incidentally produced). The smallest xylooligosaccharide that can be hydrolysed is xylotriose. Endoxylanase activity is not completely specific for xylose containing substrates as there is some activity on substrates containing glucose. The xylanase also has some transferase activity on pentose containing substrates. Most bacterial xylanases produce xylobiose and xylotriose from xylan (see Chapter 1) and many show transferase activity on xylooligosaccharides.

Figure 5.20 HPLC profile of products of the action of purified xylanase on *p*NPX.

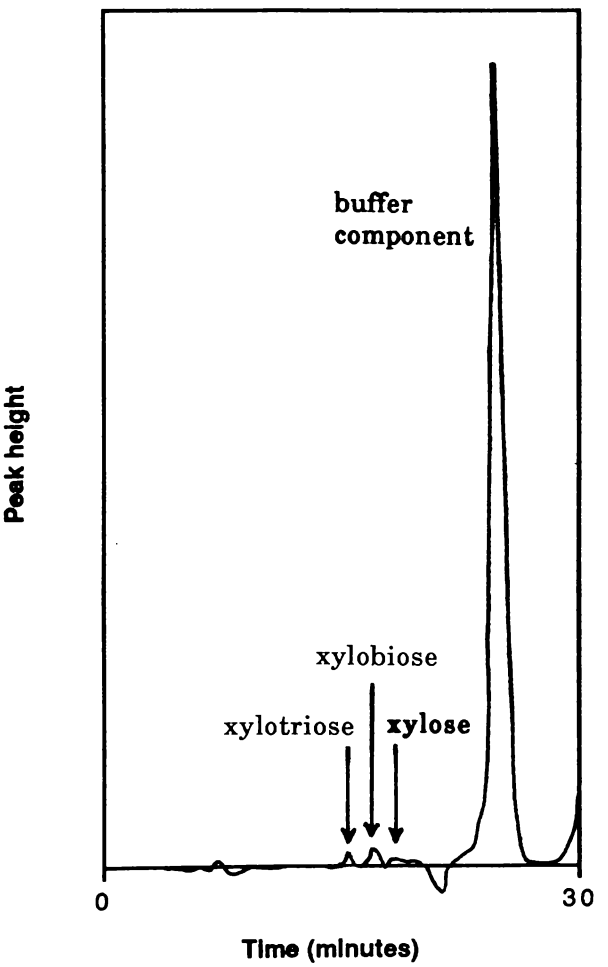
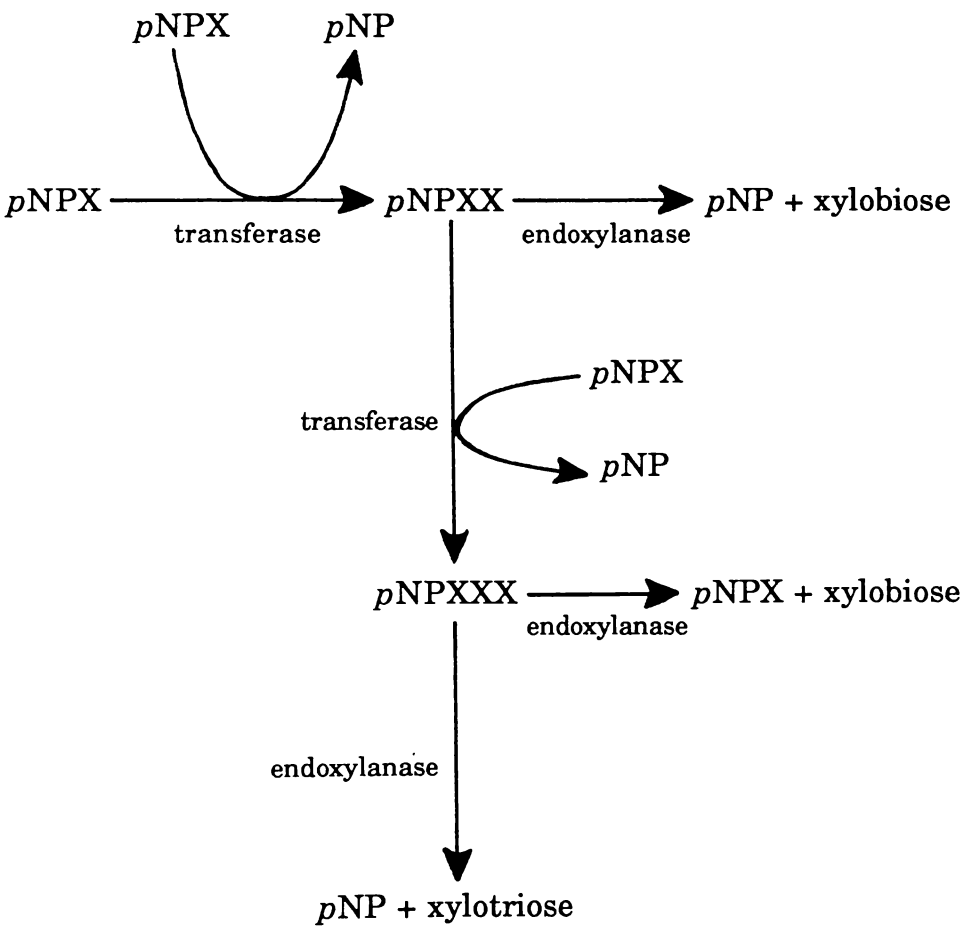


Figure 5.21 The proposed mode of action of xylanase on *p*NPX.



The xylanase belongs to cellulase family F proposed by Henrissat *et al.* (1989) (see Section 5.3.9). Family F is described as containing non-specific glycanases that hydrolyze 1,4-β-D-glucan and 1,4-β-D-xylan. *Ce. fimi* exoglucanase, also a member of cellulase family F, has been shown to release cellobiose with inversion of configuration (Gilkes *et al.*, 1988). Domain 1 of the MUCase, another member of cellulase family F, acts as an exoglycobiohydrolase (see Section 4.5). All the enzymes in cellulase family F appear to have similar modes of action, releasing disaccharides from substrates. However, under the current system of classification the xylanases are classified as endoxylanases which should produce products with retention of configuration. It is not inconceivable that xylobiose and xylotriose producing endoxylanases may produce inversion of configuration. There are no reports of the configuration of products produced by xylanases being determined. If xylanases are found to produce inversion of configuration, the name endoxylanase should be reconsidered.

5.3.9 Active site

The HCA plot of the amino acid sequence of the '*C. saccharolyticum*' xylanase is shown in Figure 4.3. Amino acid homology of the xylanase with *Ce. fimi* exoglucanase has already been shown (Luthi *et al.*, 1990) and the HCA plot confirms this. The xylanase therefore belongs to cellulase family F proposed by Henrissat *et al.* (1989). The proposed members of this family are *Ce. fimi* exoglucanase, *Cl. thermocellum* xylanase Z and *Cryptococcus albidus* xylanase. The '*C. saccharolyticum*' xylanase has amino acid homology with two other enzymes, *Bacillus* sp. strain C125 xylanase A and domain 1 of '*C. saccharolyticum*' MUCase (Luthi *et al.*, 1990). The HCA plots of these enzymes (Figure 4.3) show that they also belong to cellulase family F.

Henrissat *et al.* (1989) predict nineteen potentially catalytic residues for the three members of family F. However by comparing the HCA plots of the family F members reported by Henrissat *et al.* (1989) and those of '*C. saccharolyticum*' xylanase, *Bacillus* sp. strain C125 xylanase A and domain 1 of '*C. saccharolyticum*' MUCase, the number of potentially catalytic residues can be reduced to twelve (Table 5.13). '*C. saccharolyticum*' ORF4, which is also homologous to the '*C. saccharolyticum*' xylanase, does not appear to produce an active protein (Luthi *et al.*, 1990). The protein produced by ORF4 may be active on a substrate that has not been tested. Alternatively, activity may be prevented

by the large number of cysteine residues it contains forming disulphide bonds and holding the protein in an inactive conformation, or by the need to act in synergy with another protein (or proteins) to produce activity. A further possible reason is that *E. coli* does not possess the appropriate systems required for any post translational modifications that may be required for the production of an active form. However, if the protein is truly inactive and the lack of activity is due to the lack of a catalytic residue, the residues listed in Table 5.13 that are not conserved in '*C. saccharolyticum*' ORF4 could be considered to be the potentially catalytic residues of the '*C. saccharolyticum*' xylanase (the four **bold** residues in Table 5.13). This assumption is supported by the observation that small deletions at the N-terminus of *Ce. fimi* exoglucanase delete activity, therefore the active site residues may be close to the N-terminus (Warren *et al.*, 1986). In this case, one of the catalytic residues may be Glu 54 and the other, one of His 91 and His 96.

Table 5.13 List of potentially catalytic residues of the xylanase.

Glu 54
His 91
His 96
Asp 140
Glu 144
Asp 188
Glu 192
His 222
Glu 252
Asp 254
Asp 306
Asp 327

Chapter Six

MUCase

Results of preliminary studies of the MUCase (domain 1+2 protein, PB4567) are presented in this chapter. The enzyme was investigated as a crude or heat treated extract.

6.1 Substrates

6.1.1 MUC

Activity on the model substrate MUC is thought to indicate exocellobiohydrolase activity. MUC was used in screening clones for cellulase activity by Saul *et al.* (1989). The MUCase assay was much more convenient than the assay on Avicel. For these reasons the assay of activity on MUC was chosen for routine use.

6.1.2 Avicel

Most of the assays on Avicel were performed at a temperature lower than 70°C due to uncertainty of the enzyme being stable. Background assays corrected for the constant level of reducing sugars found in supernatants of centrifuged Avicel slurries.

6.1.3 Dyed Avicel

Dyed Avicel was prepared from Avicel and Remazol Brilliant Blue, for use in an alternative assay. Enzymatic hydrolysis of dyed Avicel leads to the production of oligosaccharides with dye attached. These remain in solution after the dyed Avicel is removed by centrifugation and can be quantified using the absorbance at 595nm. No activity, that is solubilization of dye, could be detected on dyed Avicel even after a 72 hour incubation using a crude extract. This is not surprising, as MUCase extracts showed very low reducing sugar-producing activities on Avicel and the dyed Avicel was probably even less effective than undyed Avicel. Indeed, the dyed xylan assay was found to be at least 100-fold less sensitive than the measurement of reducing sugars using PAHBAH assay system (Section 5.1.2).

There are further possible reasons for the lack of activity. The assays were stopped by cooling and centrifuging. The absorbance (at 595nm) of the supernatant was compared to that of an unincubated (t=0) sample. However, the ionic strength or temperature at which the insoluble dyed Avicel is separated from the supernatant may be important in preventing the enzymatically released dyed oligosaccharides from adsorbing to the insoluble dyed Avicel. The method of Leisola *et al.* (1975) recommends filtering the slurry at 100°C to stop the assay. Leisola and Linko (1976) report that in the presence of excess substrate some of the solubilized coloured compounds are adsorbed to the cellulose. They suggest that this adsorption can be alleviated by stopping the assays by filtering at 50°C or by heating for five minutes in a boiling waterbath. These methods were not used as it was not certain that the activity would be completely stopped. Alternatively, the dye on the Avicel may inhibit or destabilize activity of this enzyme or bind the enzyme. Leisola and Linko (1976) found that the dye group on dyed Avicel inhibited hydrolysis by 55%. However, Van Oevelen *et al.* (1975) found that the presence of dye does not alter the rate of cellulose hydrolysis.

6.2 Purification

6.2.1 Cell breakage and extraction

The effect of different buffers and ionic strengths on the efficiency of enzyme extraction were investigated. Both increasing buffer concentration (at constant ionic strength) and increasing ionic strength (at constant buffer concentration) gave no significant difference in the amount of MUCase activity extracted using sonication (Table 6.1).

6.2.2 Heat treatment and gel filtration

As described in Section 3.2.1, heat treatment of a cell free protein extract of the MUCase gave good, if somewhat variable, results. Heat treatments of protein extracts showed good MUCase recoveries after 30 minutes at 55°C and 70°C, for clones PB4567 (domain 1+2) and PB4574 (domains 1+2+3) (Table 6.2). There were lower recoveries when protein extracts were heat treated for 30 minutes at 85°C.

Gel filtration of crude extracts was not found to be adequate as an initial purification step, although any contaminating *E. coli* activity could be removed

in this way. Recoveries were moderate, but purification was low (Table 6.3). It is likely that gel filtration could be used more successfully later in the purification sequence. Heat treatment was much more efficient and required less effort as an initial purification step (Table 6.3). It removed any contaminating *E. coli* activity, gave high recovery and a high degree of purification.

Table 6.1 Effect of ionic strength and buffer components on the extraction of MUCase activity using sonication.

Buffer components present during extraction (mM)			MUCase activity (μmol $min^{-1}ml^{-1}$)
acetate	NaCl	MES	
0	780	20	0.620
20	780	0	0.579
125	675	0	0.702
800	0	0	0.549
20	0	0	0.658
20	105	0	0.615

Table 6.2 Heat treatment of two MUCase extracts, at three temperatures.

Protein extract	% MUCase activity remaining after 30min heat treatment at		
	55°C	70°C	85°C
PB4567	107	89	48
PB4574	91	90	21

(non-heat treated = 100%)

Table 6.3 Comparison of heat treatment at 70°C for 30 minutes and gel filtration, in the purification of MUCase in a crude extract.

Three trials used extracts of different volumes and protein concentrations: A, 2ml of 34.6mgml^{-1}; B, 20ml of 15.4mgml^{-1} and C, 200ml of 1.54mgml^{-1}. All samples were heat treated at a protein concentration of 2.29mgml^{-1} and results presented as if the total volume of the extract had been heat treated.

Trial	Purification step	Total activity (μmolmin^{-1})	Total protein (mg)	Specific activity (μmol min^{-1}mg^{-1})	Recovery (%)	Relative purification
A	Cell-free protein extract	1.61	69.6	0.023	100	1
	Heat treatment and centrifugation	1.15	4.22	0.273	71	11.9
	Cell-free protein extract	1.61	69.6	0.023	100 -	1
	Fractogel gel filtration and concentration	0.91	20.8	0.044	57 100	1.9
	Heat treatment and centrifugation	0.69	2.14	0.322	43 76	14.0
B	Cell-free protein extract	8.09	308	0.026	100	1
	Heat treatment and centrifugation	6.34	23.6	0.269	78	10.3
	Cell-free protein extract	8.09	308	0.026	100 -	1
	Fractogel gel filtration and concentration	4.75	144	0.033	59 100	1.3
	Heat treatment and centrifugation	4.74	15.8	0.300	59 100	11.5
C	Cell-free protein extract	8.09	308	0.026	100	1
	Heat treatment and centrifugation	6.34	23.6	0.269	78	10.3
	Cell-free protein extract	8.09	308	0.026	100 -	1
	Fractogel gel filtration and concentration	5.38	149	0.036	67 100	1.4
	Heat treatment and centrifugation	5.11	17.0	0.301	63 95	11.6

6.2.3 Cellulose and affinity chromatography

A trial column of Sigmacell 50 was run based on the methods of Sharrock (1985). MUCase in a crude extract did not bind to the column and eluted with the majority of the protein.

An affinity gel was made by linking cellobiose to cyanogen bromide activated Sepharose 6B. MUCase in a crude extract eluted from a column of this gel with the majority of the protein, but was slightly retarded. No activity eluted with the subsequent addition of cellobiose.

6.2.4 Purity - contaminating activity

Initial experiments showed the presence of a contaminating *E. coli* activity in crude extracts. It produced reducing sugars from an endogenous source, that is, in the absence of Avicel. The contaminating activity was lost on prolonged storage and it could be inactivated by heat treatment. It could be removed by gel filtration, but not by dialysis or desalting. The reducing sugars produced by the contaminating activity were significant in relation to the reducing sugars produced from Avicel by the MUCase. This was due to the low amount of activity on Avicel expressed by the clone.

The contribution by the host strain to any cloned activity must be considered. It is particularly important when a non-specific product is being measured (such as reducing sugars) and when expression of the cloned activity is very low. The thermostability of such host proteins and activities is also an important factor when using a heat treatment step in purification. The best host would have proteins and activities with low thermostabilities, in order that both were removed quickly and easily. In this regard *E. coli* could be expected to be a better host for clones with thermostable enzymes than *Bacillus* spp..

6.3 Characterization

6.3.1 Molecular weight

An ultrafiltration experiment provided an idea of the molecular weight of the MUCase (Table 6.4). MUCase in a crude extract was retained using a PM30 (30 000 molecular weight cut off) membrane, but passed through a YM100 (100 000 molecular weight cut off) membrane. Gel filtration of a crude protein extract gave an approximate weight of 84 000 for the MUCase (Figures 6.1 and

6.2). This is consistent with that of 77 000 (minimum) predicted by the DNA sequence of the cloned gene fragment (Saul, unpublished results). The molecular weight may be higher than 77 000 if part of the vector is transcribed as well as the DNA fragment cloned. This would give an indeterminate number of extra amino acids (Saul, personal communication).

Both the molecular weight of 84 000 of the domain 1+2 protein studied here, and that of 118 000 (inclusive of signal sequence) or 115 000 (not including the signal sequence) predicted for the entire protein (domain 1+2+3 protein), are similar to molecular weights reported for other bacterial exoglucanases. Exoglucanase A of *R. flavefaciens* is a dimer of molecular weight 230 000, with subunits of 118 000 (Gardner *et al.*, 1987). *Cl. stercorarium* produces an exoglucanase, cellobiohydrolase I and cellobiohydrolase II having molecular weights of 87 000, 82 000 and 128 000 respectively (Bronnenmeier and Staudenbauer, 1988). The exocellobiohydrolase of *Ce. fimi* has a molecular weight of 47 000 (Gilkes *et al.*, 1984b). Bacterial endoglucanases generally have molecular weights in the ranges 32 000 - 67 000 or 88 000 - 118 000. The molecular weight of the entire protein falls into the upper range for endoglucanases.

Table 6.4 Ultrafiltration of a crude MUCase extract.

Ultrafiltration membrane	MUCase activity (%)	
	permeate	retentate
PM30	11	89
YM100	63	37

6.3.2 Linearity of assay

A trial time course of the MUCase activity was approximately linear up to one hour (Figure 6.3). Approximately 20% of the available MUC was hydrolysed in the first 20 minutes. This time period (20 minutes) was chosen for further assays.

Figure 6.1 Elution profile of MUCase crude protein extract on a TSK Fractogel column.

Wide bars indicate activity in pooled fractions; narrow bars indicate activity in individual fractions (select fractions assayed). Protein (A_{280}) shown, maximum 2.0.

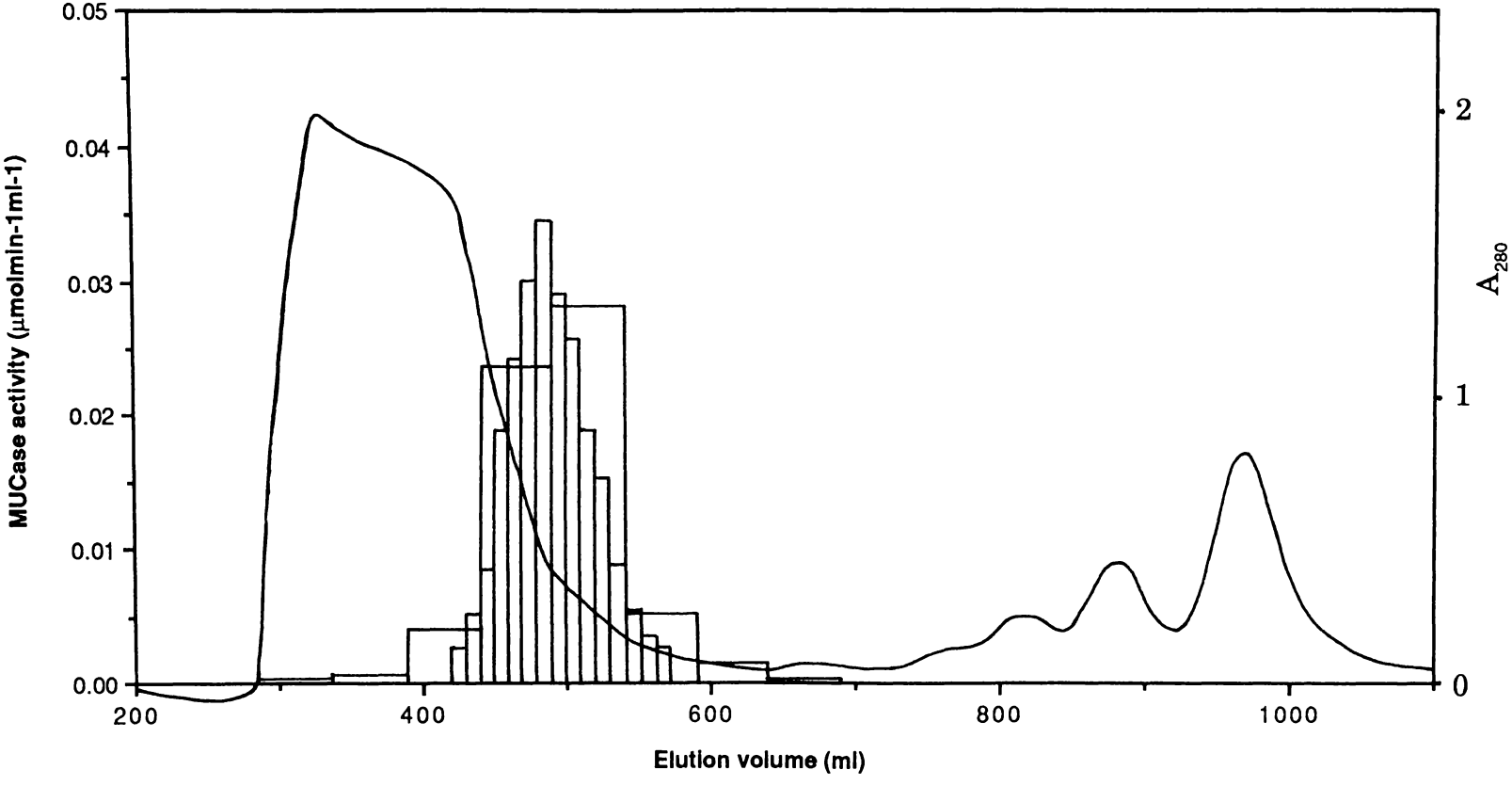


Figure 6.2 Molecular weight standard curve for TSK Fractogel gel filtration of a MUCase crude protein extract.

Molecular weight standards (Sigma):
β-amylase, 200 000; alcohol dehydrogenase, 150 000; albumin, 66 000;
carbonic anhydrase, 29 000 and cytochrome c, 12 400.

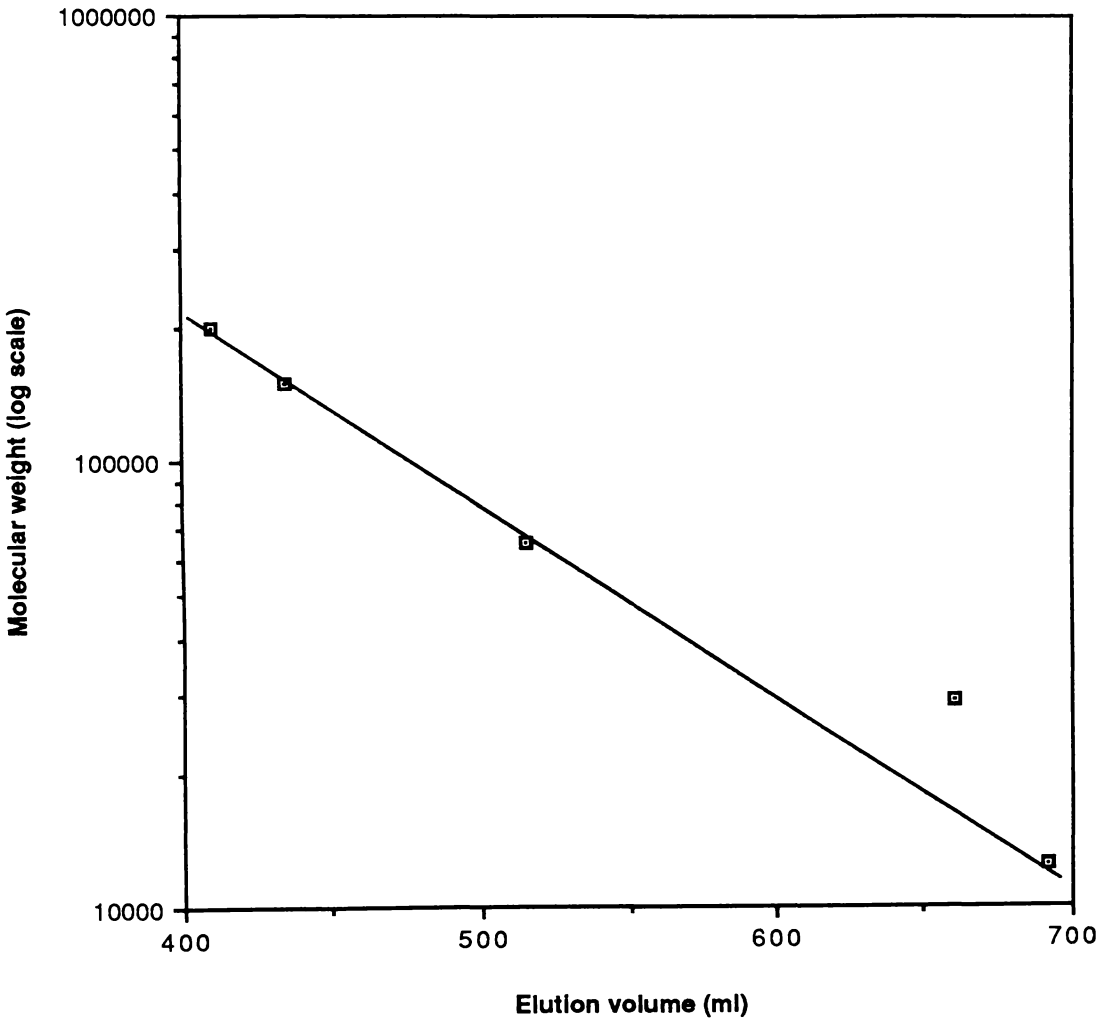
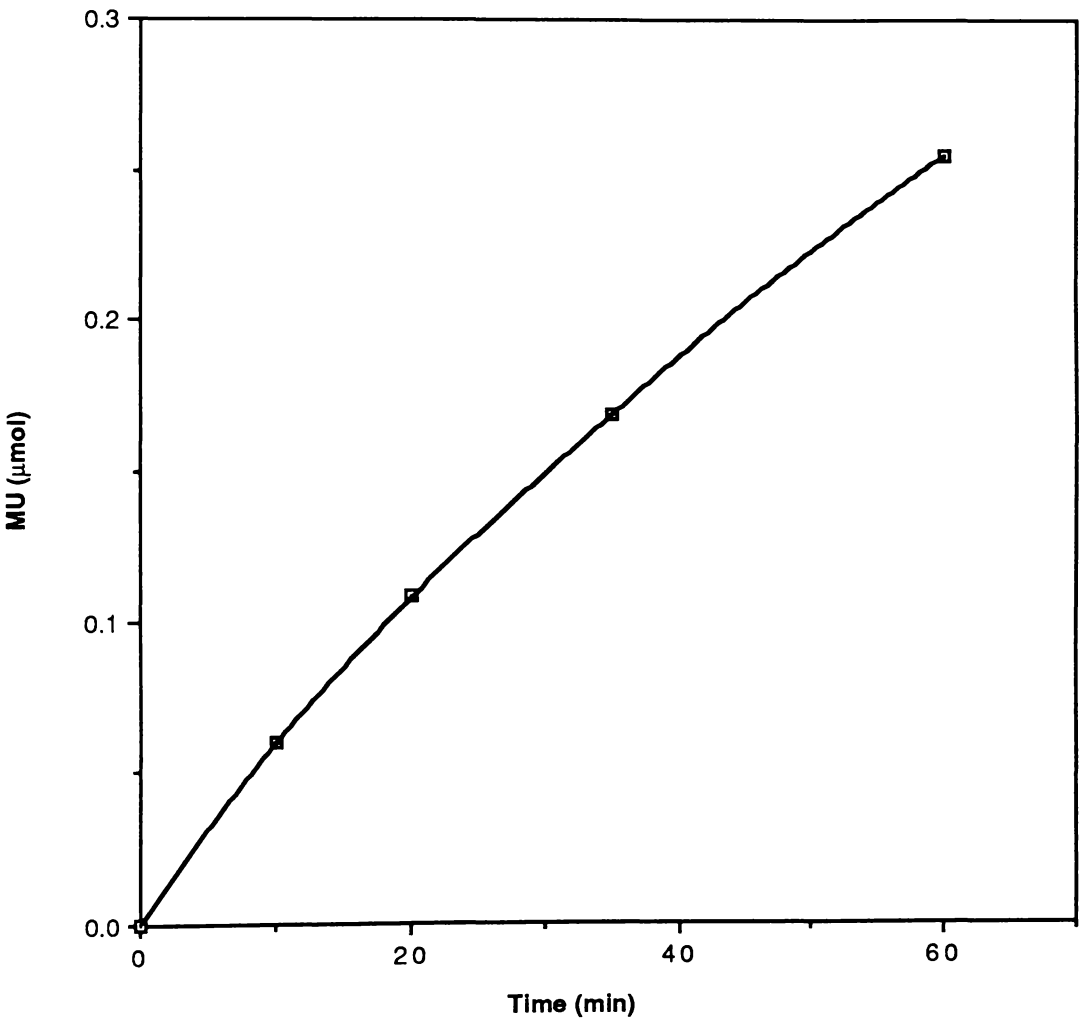


Figure 6.3 Time course of activity on MUC.
Crude protein extract.



6.3.3 Influence of temperature on activity

In a preliminary experiment the influence of temperatures from 50°C - 70°C on MUCase activity (Figure 6.4) was studied. Crude extracts of two MUCase clones acted similarly, with increasing temperature causing increasing activity on MUC. However, activation energies calculated from 1/T versus the log of activity plots were different, 39kJmol^{-1} and 55kJmol^{-1} for PB4567 (domain 1+2 protein) and PB4574 (domain 1+2+3 protein) respectively.

6.3.4 Temperature stability

Half of the MUCase activity in a crude extract is lost after a 30 minute heat treatment at 85°C (see Section 3.2.2 (b)). Denaturation curves of the MUCase (domain 1+2 protein) are shown in Figure 6.5. Activities on both MUC and Avicel were stable for hours at 60°C and 75°C after a small initial loss of activity. Each log plot appears to consist of two straight lines (Figure 6.6). One of each of the two slopes may represent denaturation of one of the two domains. Although domain 1 appears to be the only catalytic domain in this protein, it is possible that denaturation of the adjacent domain may have some effect.

6.3.5 Effect of various compounds on activity

Unless well below efficient buffering strength, the concentration of buffer had no effect on MUCase activity in a crude extract (Table 6.5). Decreased activity was seen when the crude extract was assayed in 1mM buffer. Assays were performed with buffers at several pH values. MUCase activity was high at pH 5.6 in either acetate or citrate buffer, but very low at pH 5.0 in acetate buffer (Table 6.5). This may indicate a narrow pH optimum. Exoglucanase A of *R. flavefaciens* has maximal activity at pH 5.0, with less than 20% maximal activity detected below pH 4.0 or above pH 6.5 (Gardner *et al.*, 1987).

At constant ionic strength (using NaCl) the concentration of buffer had no effect on MUCase activity in a crude extract (Table 6.6). Increasing the ionic strength (using NaCl, KCl or KNO_3), at constant buffer concentration also had no effect on the MUCase activity of a protein extract. However, a heat treated extract appeared to show some effect by ionic strength at constant buffer concentration (Table 6.7). Increasing ionic strength appeared to increase MUCase activity.

Figure 6.4 Effect of temperature on activity on MUCase in crude protein extracts from two clones.

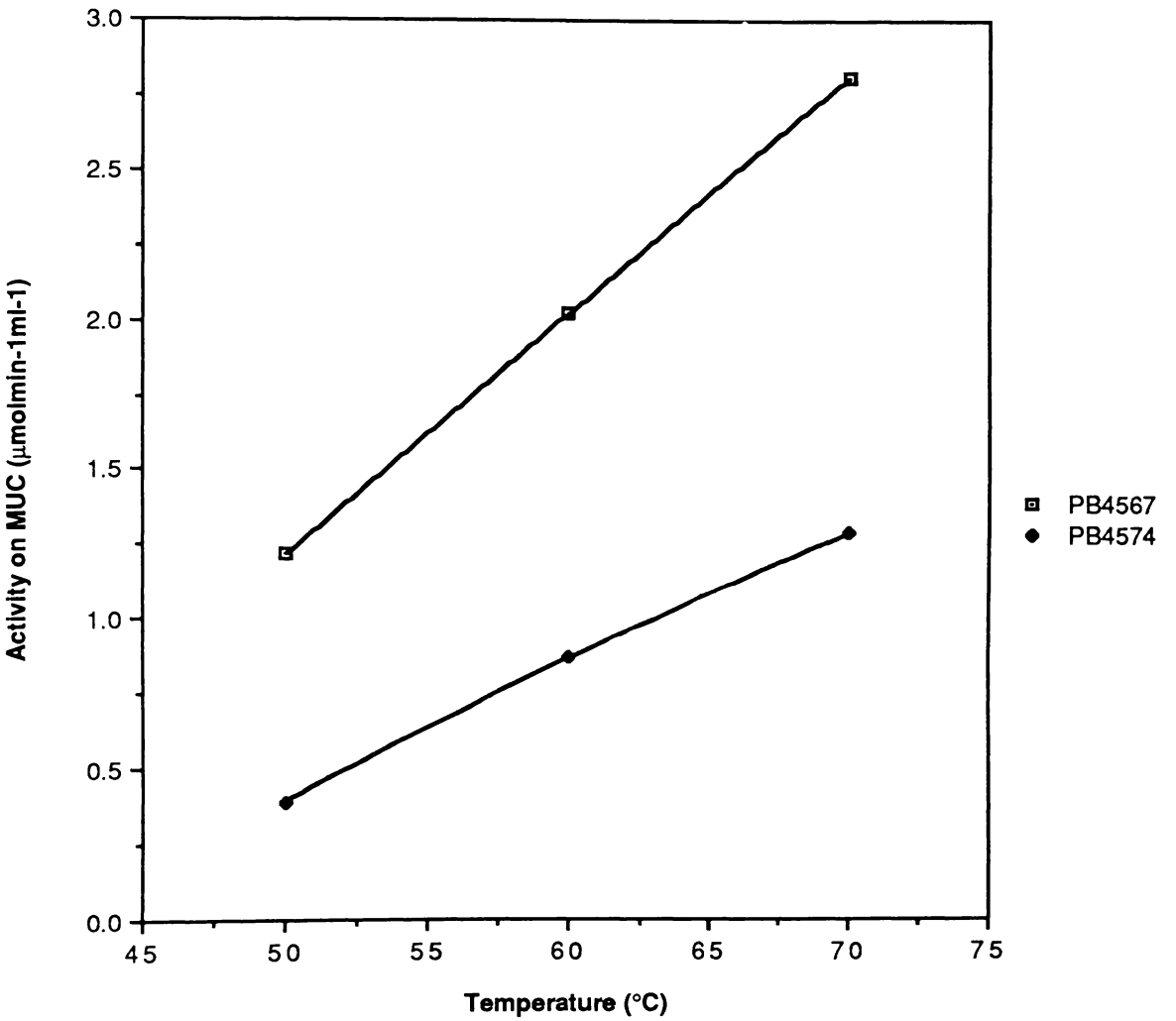


Figure 6.5 Thermostability curves for a MUCase crude protein extract.

Thermostability at 60°C and 75°C, residual activity on MUC and Avicel.

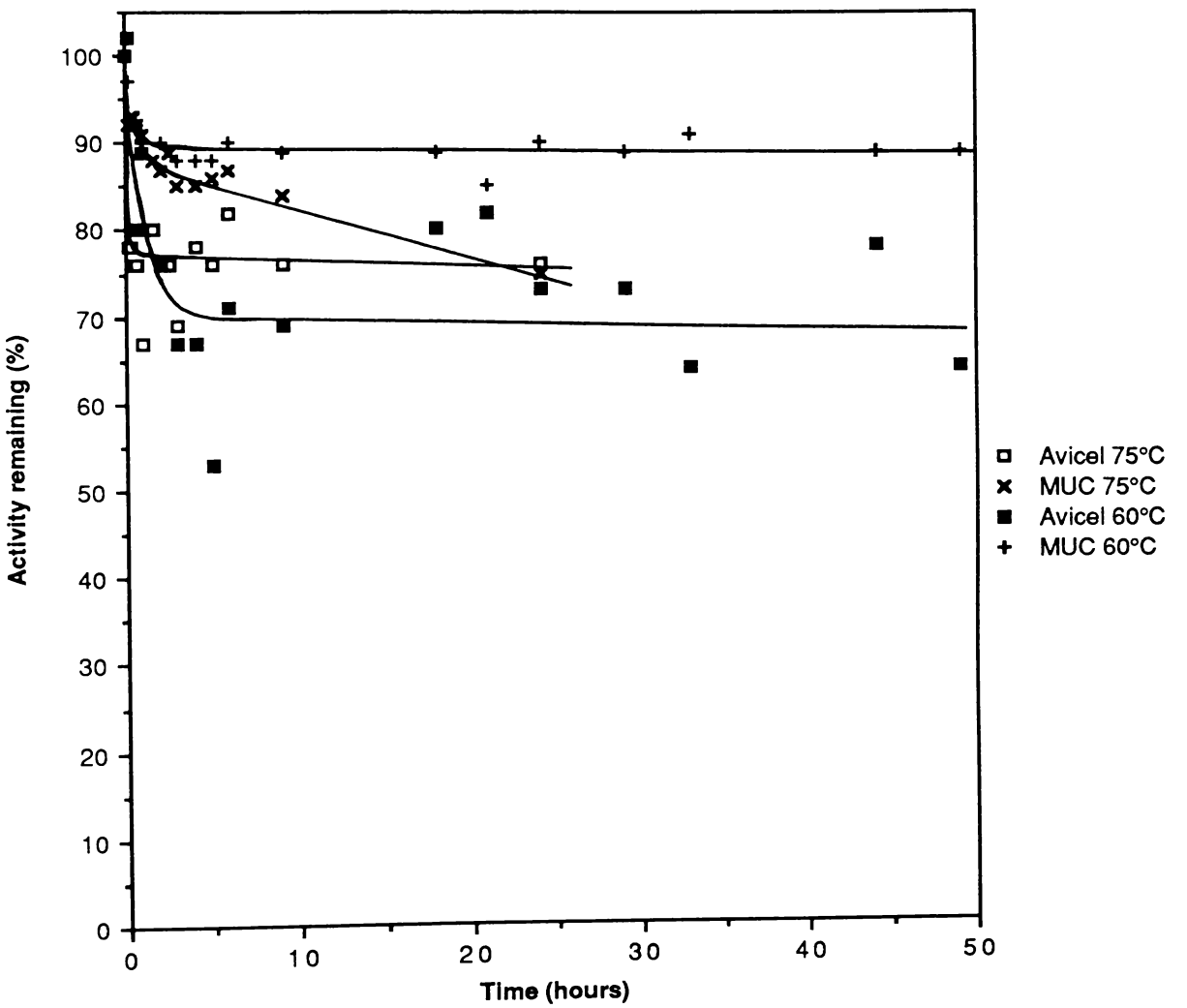


Figure 6.6 Time versus the log of residual activity for a MUCase crude protein extract.
Thermostability at 60°C and 75°C,
residual activity on MUC and Avicel.

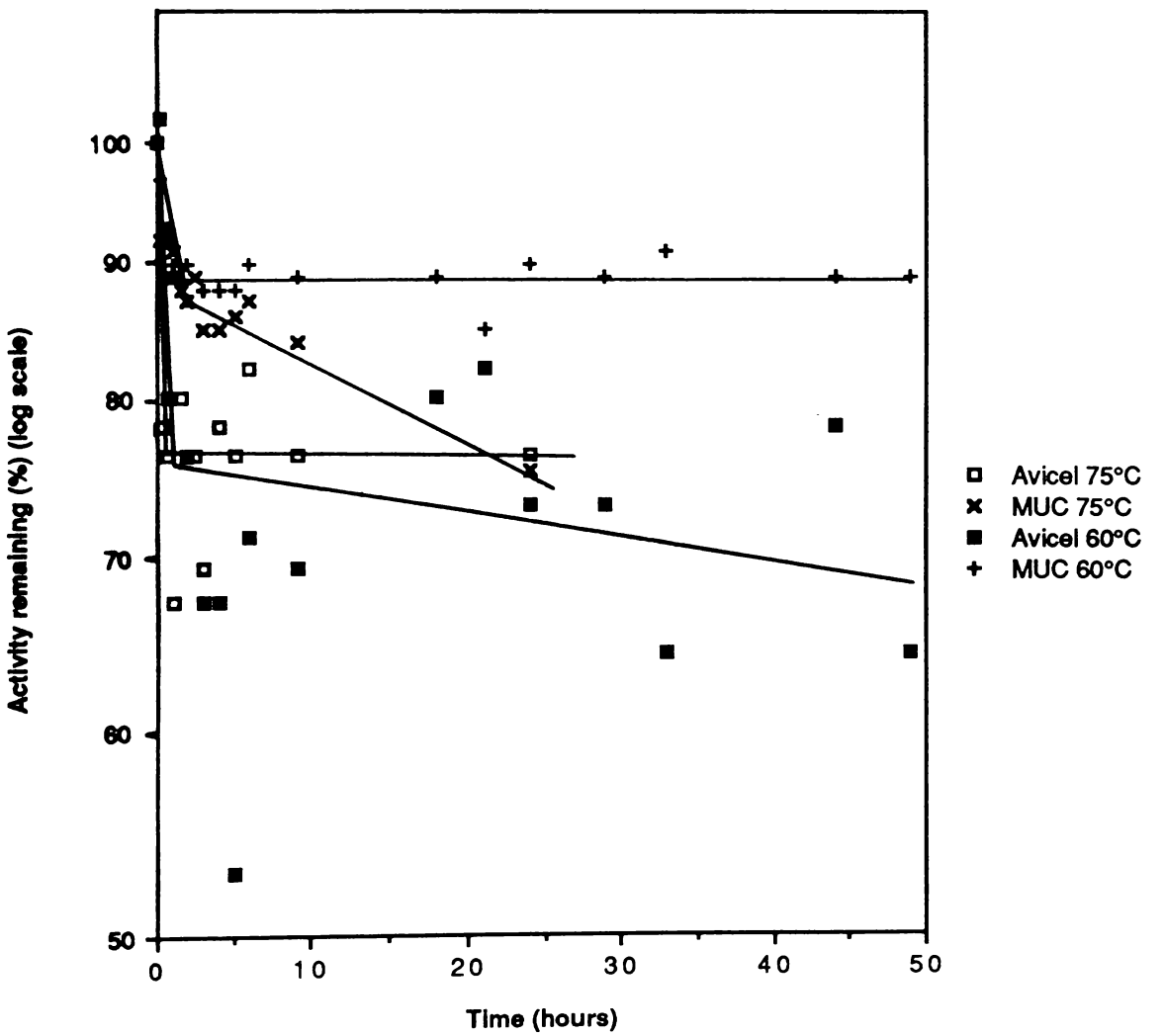


Table 6.5 Effect of buffer concentration and pH on MUCase activity in a crude extract.

Buffer present during assay	MUCase activity ($\mu molmin^{-1}ml^{-1}$)
200mM acetate pH 5.6	0.448
100mM acetate pH 5.6	0.536
20mM acetate pH 5.6	0.525
10mM acetate pH 5.6	0.475
1mM acetate pH 5.6	0.126
20mM acetate pH 5.0	0.027
20mM citrate pH 5.6	0.541
20mM citrate pH 6.2	0.459

Table 6.6 Effect of buffer components on MUCase activity in a crude extract.

Buffer components present during assay (mM)			MUCase activity ($\mu molmin^{-1}ml^{-1}$)
acetate	NaCl	MES	
0	780	20	0.710
125	675	0	0.705
800	0	0	0.760
20	10	0	0.672
20	30	0	0.738
20	105	0	0.702
20	180	0	0.757
20	780	0	0.675
20	1980	0	0.639

Table 6.7 Effect of buffer components on MUCase activity of a heat treated MUCase extract.

Buffer components present during assay (mM)		MUCase activity ($\mu molmin^{-1}ml^{-1}$)
acetate	NaCl	
10	0	0.361
10	10	0.410
10	90	0.530
10	190	0.536

A range of compounds were tested for their effect on MUCase activity. 1.5mM ammonium persulphate was the only compound to have a significant effect on MUCase activity, decreasing activity to 69%. There was no significant effect by the reducing agents β-mercaptoethanol (1.5mM) and DTT (1.5mM), or by Ca^{2+} (1.5mM), EDTA (1.5mM), Tris (1.5mM), MOPS (1.5mM), cellobiose (1.5mM), L-histidine (1.5mM), glycine (1.5mM), triethanolamine (1.5mM), Pepstatin A ($0.3\mu gml^{-1}$), sodium azide (0.008% w/v), PMSF ($0.015mgml^{-1}$), glycerol (1.5% w/v), DMF (0.04% v/v), TEMED (0.02% v/v), SDS (0.02% v/v) and various concentrations of Triton X-100 (0.001% - 1% w/v).

6.3.6 Effect of various compounds on stability

There was no difference in storage at 4°C and -20°C of crude protein extracts and heat treated extracts. The crude and heat treated MUCase, and activity on Avicel in the heat treated extract, were also stable to prolonged dialysis. In one experiment the samples were dialysed against a concentration of urea slowly increasing to 4M and decreasing to 0M. This attempt to renature any denatured activity was unsuccessful (Table 6.8).

Table 6.8 Effect of various treatments on the stability of two MUCase extracts: crude and heat treated.

Protein extract	Treatment	Activity on MUC ($\mu molmin^{-1}ml^{-1}$)	Activity on Avicel ($\mu molmin^{-1}ml^{-1}$)
crude	storage at -20°C	0.585	nd
	storage at 4°C	0.569	nd
	dialysis	0.472	nd
	dialysis with urea	0.455	nd
heat treated	storage at -20°C	0.530	0.0085
	storage at 4°C	0.529	0.0081
	dialysis	0.440	0.0070
	dialysis with urea	0.428	0.0080

nd = not determined

Different buffer concentrations (at constant ionic strength) and different ionic strengths (at constant buffer concentration) seemed to have no effect on the stability of the MUCase during heat treatment (Table 6.9). A range of concentrations (0.001 - 1% w/v) of Triton X-100 also had no effect on the stability of the MUCase during heat treatment. Various compounds were included in extracts for heat treatment. Only ammonium persulphate affected the stability of the MUCase during heat treatment causing a marked decrease in stability. Those that had no effect were: β-mercaptoethanol (10mM), DTT (10mM), Ca^{2+} (10mM), EDTA (10mM), Tris (10mM), MOPS (10mM), cellobiose (10mM), L-histidine (10mM), glycine (10mM), triethanolamine (10mM), Pepstatin A ($2\mu gml^{-1}$), sodium azide (0.05% w/v), PMSF ($0.1mgml^{-1}$), glycerol (10% w/v), DMF (0.25% v/v), TEMED (0.1% v/v) and SDS (0.1% v/v).

Table 6.9 Effect of buffer components on the stability of a MUCase extract heat treated at 70°C for 30 minutes.

Buffer components present during heat treatment (mM)			MUCase activity remaining (%)
acetate	NaCl	MES	
0	780	20	76
20	780	0	87
125	675	0	82
800	0	0	79
20	0	0	74
20	105	0	82

6.3.7 Substrate specificity

See Section 4.5.

6.3.8 Active site

Domain 1 belongs to cellulase family F, the active site residues of which have been predicted by Henrissat *et al.* (1989). This study has already shown that family F contains further members (see Section 4.5). The HCA plots of family F, including domain 1, are shown in Figure 4.3. Aspartate, histidine and glutamate residues of domain 1 that are conserved in all the HCA plots are listed in Table 6.10. The number of catalytic residues can be reduced by making several assumptions, as outlined in Section 5.3.9 (the resulting four residues are **bold**ed in Table 6.10). If this is the case, one of the catalytic residues of domain 1 may be Glu 83 and the other, one of His 124 or His 129.

Although the entire protein (domain 1+2+3) was not studied in detail, the two potentially catalytic residues of domain 3 can be predicted. Domain 3 is a member of family A. The two potentially catalytic residues predicted by Henrissat *et al.* (1989) for family A correspond to His 743 and Glu 792 of domain 3.

Table 6.10 List of potentially catalytic residues of domain 1 of the MUCase.

Glu 83
His 124
His 129
Asp 173
Glu 177
Asp 221
Glu 225
His 255
Glu 285
Asp 287
Asp 338
Asp 359

Chapter Seven

Conclusion

Five xylanolytic and cellulolytic enzymes cloned from an extremely thermophilic bacterium were studied. A systematic study of heat treatment purification of these enzymes was made, in an attempt to define the parameters under which heat treatment can most effectively be carried out. Heat treatment is easy and requires no special equipment. It is especially useful for the first step of a large scale purification. A single time and temperature (30 minutes, 70°C) were able to be found for effective purification of all the enzymes, as the protein composition of the source material was essentially the same for all enzymes. Although further improvement may be gained by varying the conditions, a good compromise between purification and recovery was achieved. A higher degree of purification may be gained by heat treating at an acidic pH. At pH 5.4 or lower, approximately 5-fold more protein is precipitated than at neutrality. However, this will only be successful for enzymes that are stable at the pH of heat treatment. High molecular weight enzymes should be easier to purify after heat treatment, as heat treatment seems to preferentially remove high molecular weight *E. coli* proteins. Heat treatment also serves to remove any contaminating activities produced by *E. coli*. Such interfering activity is apparent in reducing sugar-producing assays and may be significant when expression of the enzyme is low.

Heat treatment was a successful purification step for all five enzymes. It is especially useful in cases where a thermophilic enzyme is cloned into *E. coli* (or a mesophile) and/or where there is a complex system of enzymes which are difficult to separate by conventional methods, such as thermophilic hemicellulases and cellulases.

The substrates specificities of the five enzymes were investigated. The β-xylosidase had a very narrow substrate specificity. It only showed high activity on aryl substrates containing pentoses and was not active on xylobiose. It was also found to have some transferase activity. It may be best described as an aryl β-xylosidase or possibly exoxylanase.

The xylanase had high activity on xylan, releasing xylobiose and xylotriose. Xylose was only released from the hydrolysis of xylotriose. The

xylanase had preference for the second xylosidic linkage over the third in xylan. As it could not hydrolyse xylobiose, the smallest xylooligosaccharide it could hydrolyse was xylotriose. The xylanase showed some transferase activity. It had a broad substrate specificity, with endoxylanase activity on substrates containing pentoses or hexoses, but transferase activity only on substrates containing pentoses. Overall it was found to act in a similar way to most endoxylanases.

The β-glucosidase also had a broad substrate specificity. It had activity on cellobiose and aryl glucosides and although it had activity on aryl substrates containing pentoses, it did not have activity on xylobiose. The activity on aryl substrates containing pentoses may have been due to aryl activation.

The CMCase had a very narrow substrate specificity, with high activity on CMC only. This enzyme can be considered to be an endoglucanase.

The MUCase appeared to have two active sites. The active site in domain 1 acted as an exoglycobiohydrolase. It cleaved disaccharides from hexose- or pentose-containing substrates. Domain 3 acted as an endoglucanase, with activity on CMC. Domain 2 may act as a binding domain. The entire protein shows higher activity on Avicel than the sum of the active domains. However, the activity on Avicel is much lower than the activities on xylan, CMC or MUC.

Dyed substrates were made, but assays using these substrates were unsatisfactory. The assays were found to be inherently much less sensitive than the existing assay methods. Other problems were possibly due to inhibition or destabilization of the enzyme by the dye, the adsorption of enzymatically cleaved dyed fragments to the substrate polymer and the effect of protein, temperature and ionic strength on the solubilization of the dyed fragments.

Most xylanolytic and cellulolytic enzymes are classified on the basis of their substrate specificity, but the range of substrates tested is often not broad enough to give a clear idea of the mode of action of the enzyme. It is also important to identify both the products and their configuration. The possibility of transferase activity must always be considered.

Two of the five enzymes were studied in more detail. The xylanase was purified and despite appearing non-homogeneous by silver staining after SDS-PAGE, it was shown to contain only one active enzyme. The heat treatment step resulted in a good level of purification. The xylanase was found to fit into the acidic (pI)/high molecular weight group of bacterial xylanases. Signal sequence processing in three places by *E. coli* could account for the appearance of three active bands on the IEF gel. The xylanase was monomeric, had a typical pH optimum and pH stability range, was very thermostable and had a very low K_m. There was no evidence of xylose inhibition but there may have been inhibition by xylobiose. The active site residues were predicted by comparison of the HCA plots of family F. The most likely catalytic residues are positioned at the N-terminal end of the enzyme.

The MUCase was not purified but several purification methods were attempted. Heat treatment proved successful and gel filtration would be successful as a later purification step. The MUCase was thermostable and its molecular weight was similar to those seen for other exoglucanases. The catalytic residues of domain 1 were predicted to be the same as those of the xylanase, that is, close to the N-terminal end of the enzyme. The catalytic residues of domain 3 were also predicted and are in the middle of domain 3.

It is unusual for a glucanase to have three domains, two of them catalytic domains with completely different activities. It is also unusual for a single enzyme to have activity on Avicel. These two unusual properties may be related. One of the most stringent requirements for a whole cellulase complex is activity on crystalline substrates. Such activity may only occur in multi-active site enzymes or enzyme complexes. In this way the various complementary active sites could be brought into close proximity and into the correct juxtaposition with respect to the substrate to allow synergy among the components. *Cl. thermocellum* produces cellulosomes or multisubunit protein complexes that contain multiple cellulase activities and cellulose binding ability. So far only endoglucanases and no exocellobiohydrolases have been found to be produced by *Cl. thermocellum*. The hydrolysis of crystalline substrates is thought to occur by simultaneous multicutting events by endoglucanases. In contrast, the cellulolytic system of *Ce. fimi* appears to consist of three components, an exocellobiohydrolase and two endoglucanases. *R. flavefaciens* also produces a high molecular weight cellulase complex containing cellulase enzymes including an exoglucanase. In these cases

endoglucanases and exoglucanases appear to work in synergy to produce activity on crystalline substrates. Endoglucanases act initially but require exocellobiohydrolases to prevent the cleaved bonds from reforming. '*C. saccharolyticum*' does not appear to produce cellulosome-like structures. Activity on crystalline substrates may be due to the multidomain enzyme MUCase, or to other similar enzymes. The MUCase may have a similar mode of action to the systems of *Ce. fimi* and *R. flavefaciens*. The endoglucanase domain may act initially and the exoglycobiohydrolase domain may then act to prevent the cleaved bonds from reforming. If the endoglucanase and exoglycobiohydrolase domains act synergistically and the endoglucanase acts on one of the two types of glucosidic bonds in cellulose, then the exoglycobiohydrolase must act on the one type of non-reducing endgroup generated (see Section 1.3.2 (b)). However, this enzyme could still be part of a larger complex, or could act in synergy with other components. The MUCase is also unusual in retaining activity, including that on Avicel, after the removal of one catalytic domain.

Unlike the multisubunit cellulase complexes possessing activity on microcrystalline substrates described to date, the single protein possessing activity on Avicel described here, offers a good opportunity to study the hydrolysis of microcrystalline substrates much more easily. This enzyme may also offer new opportunities to biotechnologists.

Proteins with separate domains having possibly different functions can be studied by the deletion or exchange of specific domains or by site-specific mutagenesis experiments. Further studies on the MUCase could include deletions of each domain to ascertain more clearly the function of each domain.

The overall xylanolytic and cellulolytic system of '*C. saccharolyticum*' so far consists of an aryl β-xylosidase, an endoxylanase, a β-glucosidase, an endoglucanase and a bifunctional exoglycobiohydrolase/endoglucanase. The xylanase and the β-xylosidase seem unlikely to be able to act in synergism for the complete hydrolysis of xylan. The major product released by activity of the xylanase on xylan is xylobiose, but the β-xylosidase has been shown to have no activity on xylobiose. It therefore seems likely that further xylanolytic enzymes will be found to be produced by the original organism '*C. saccharolyticum*'. Among them may be a second xylanase, of low molecular weight and basic isoelectric point, and a β-xylosidase having activity on xylooligosaccharides

such as xylobiose. Unlike the xylanolytic enzymes investigated to date, synergism between the cellulolytic enzymes of '*C. saccharolyticum*' appears likely. The MUCase shows activity on insoluble (microcrystalline) cellulose, while the CMCase shows activity on soluble cellulose. The products released by the action of the MUCase could easily be hydrolysed by the β-glucosidase. Synergism experiments using these three enzymes would prove interesting. By comparison with other, more well studied systems, further components are sure to be found. Among them may be further endoglucanase(s) and exocellobiohydrolase(s).

Cloned enzymes may differ slightly from native enzymes, for example in the presence of additional amino acids, proteolytic degradation or lack of glycosylation. This may give a distorted impression of xylan and cellulose saccharification as carried out by the native organism. It could also affect the mechanistic action of any complex assembled from the components. Furthermore, selecting cloned genes simply on the basis of activity will not detect products which play an activating but non-catalytic role in the cellulase complex. Despite these problems, cloning is the most promising route to understanding xylan and cellulose degradation.

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Appendix

Heat treatment purification of thermostable cellulase and hemicellulase enzymes expressed in *E. coli*

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Four enzymes implicated in the breakdown of cellulose and hemicellulose by the extremely thermophilic anaerobe Caldocellum saccharolyticum *have been purified 7–19-fold in a single heat-treatment step. In earlier studies,* C. saccharolyticum *genes expressing either carboxymethyl cellulase (CMCase), β-glucosidase (E.C.3.2.1.21), xylanase (E.C.3.2.1.8), or β-xylosidase (E.C.3.2.1.37) activity had been cloned into* Escherichia coli, *and these clones served as enzyme sources for purification experiments. After cell lysis by a nonionic detergent-osmotic shock-lysozyme method and/or sonication, the lysate was heated to 70°C for 30–65 min, resulting in the denaturation and precipitation of most* E. coli *proteins. Centrifugation of the heat-treated lysates produced supernatants containing at least 50% of the original enzyme activity, with a 7–23-fold decrease in protein concentration. The method has general application as a means of obtaining thermostable enzymes free of contaminating activities, and also achieves a rapid and simple partial purification.*

Keywords: Thermostable cellulases; carboxymethyl cellulase; β-glucosidase; xylanase; β-xylosidase; heat treatment; protein purification; denaturation; *Caldocellum saccharolyticum*

Introduction

We have been studying the enzymology and microbial physiology of cellulose degradation carried out by *Caldocellum saccharolyticum* (isolate TP8.T), an extremely thermophilic anaerobic bacterium isolated from a thermal spring near Lake Taupo, New Zealand.[1,2] This organism has an optimum growth temperature of 68°C, and produces a very thermostable cellulase complex.[2,3] Because of difficulties encountered in purifying extracellular components from the cellulase complex, several genes coding for *C. saccharolyticum* cellulolytic and hemicellulolytic enzymes had been cloned into *E. coli*[4–6] with the aim of enhancing the yield and simplifying the isolation of each enzyme.

The use of heat treatment to purify enzymes by selective denaturation and subsequent precipitation of denatured protein is a simple, rapid, and well established procedure. Successful applications are limited to those few enzymes that possess a thermostability considerably higher than the majority of cell proteins. The introduction of thermostable enzymes into the protein population of a mesophile by cloning offers a clear opportunity to use a heat-treatment method of purification to its full advantage. This paper describes the heat-treatment purification of four thermostable *C. saccharolyticum* cellulase and hemicellulase components cloned into *E. coli*.

Materials and methods

Bacterial strains, media, and harvesting

E. coli strains kindly provided by P. L. Bergquist were pNZ1001/PB2481 (PB4551) β-glucosidase^{+}[4], pNZ1012/PB2477 (PB4563)CMCase^{+}[5], and pNZ1076/PB2477 (PB4716) β-xylosidase^{+} xylanase^{+}[6]. The *C. saccharolyticum* genes had been identified from a genomic library constructed in λ1059[4] and had been cloned into host *E. coli* strains using pBR322 (conferring ampicillin resistance) as described in earlier studies[4–6]. *E. coli* strains were grown with vigorous agitation and aeration at 37°C in Luria broth (tryptone peptone, 5 g l^{-1}; yeast extract, 5 g l^{-1}; NaCl, 5 g

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l^{-1}) at pH 7 in the presence of not less than 25 mg l^{-1} active ampicillin. Cells were harvested in early stationary phase of growth.

Cell lysis and heat treatment

Cell-free extracts of β-xylosidase were prepared by sonication on ice in 1.5 volumes (per wet weight of cells) of 0.1 M 2-(*N*-morpholino) propane sulphonic acid (MOPS)/KOH buffer, pH 6.7 (20°C), followed by centrifugation to remove cell debris.

Kilogram (wet cell weight) quantities of *E. coli* containing cloned β-glucosidase, CMCase, and xylanase enzymes were lysed using a slight modification of Schwinghamer's[7] non-ionic detergent-osmotic shock-lysozyme lysis method. Cells were mixed with one-third of their wet weight of glycerol, and when the slurry was sufficiently liquid, 1% (v/wet cell weight) of 10% (w/v) Triton X-100 and 0.1% (v/wet cell weight) 2-mercaptoethanol were added and stirred for 1–3 h at 5°C. Several volumes (per wet weight of cells) of the cold extraction buffer appropriate for each enzyme were then added rapidly. Buffers used were 40 mM sodium phosphate, pH 7.2 (20°C) (6 volumes), 0.1 M Tris/HCl, pH 7.5 (20°C) (1.5 volumes), and 50 mM MOPS/KOH buffer, pH 7.0 (20°C) (6 volumes), for β-glucosidase, CMCase, and xylanase respectively. For these three enzymes, the extraction buffer also contained 7.5 mM 2-mercaptoethanol, 7.5 mM disodium EDTA, 0.02% NaN_3, and 0.02% lysozyme (catalogue number L2879, Sigma). The CMCase lysate was also sonicated briefly before heat treatment. The temperature was raised to 35°C and 0.01% (w/wet cell weight) of DNAase (catalogue number D0876, Sigma) added. With the exception of the CMCase experiment, 0.06% (w/wet cell weight) of phenylmethanesulphonyl fluoride [as a 1% (w/v) solution in acetone] was also added at this stage. The slurry was then stirred for 1–2 h.

Heat treatment for all four enzymes was carried out by raising the temperature of the extracts to a specified temperature, at which it was maintained for a given time (see *Table 1* for details). The lysate was then cooled on ice and centrifuged to produce a supernatant that was assayed for activity and protein concentration.

Enzyme assays and protein determination

β-Glucosidase activity was assayed at 70°C in 0.1 M sodium phosphate buffer, pH 6.2 (70°C) with 30 mM *p*-nitrophenyl β-D-glucopyranoside.[8] β-Xylosidase activity was determined at 70°C in 0.1 M-MOPS/KOH buffer, pH 6.3 (70°C) with 2 mM *p*-nitrophenyl β-D-xylopyranoside. CMCase activity was determined at 70°C in 0.09 M MOPS/NaOH buffer, pH 7.0 (25°C), containing 5 mM dithiothreitol and 5 mM EDTA, with 1.8% (w/v) CMC as a substrate. Xylanase activity was assayed at 70°C in 0.1 M sodium citrate buffer, pH 6.0 (20°C) with 0.25% (w/v) oatspelts xylan as substrate. All substrates were supplied by Sigma. Reducing sugars produced were determined by the *p*-hydroxybenzoic acid hydrazide procedure.[9] One unit of enzyme activity was defined as the amount of enzyme producing 1 μmol of product (reducing sugar expressed as glucose for CMCase and xylose for xylanase activity) per minute. Protein was determined by the dye-binding method of Bradford[10] using bovine serum albumin as a standard.

Results and discussion

Cloned enzyme activities in *E. coli* culture supernatants were typically less than 3% of total activity, and cell-associated activities increased to a maximum in early stationary phase. Denatured protein began to precipitate from *E. coli* lysates at about 50°C during heating. By use of appropriate *E. coli* strains, it may be possible to streamline the procedure and simply heat-treat whole cells with vigorous stirring to avoid entrapment of the desired enzyme.[11] The heat treatments performed on *E. coli* lysates resulted in a 7- to 19-fold purification for the four enzymes (*Table 1*). A temperature of 70°C was a good compromise between greater protein precipitation and greater loss of activity. Both thermal denaturation and co-precipitation are possible causes of the loss of soluble enzyme activity during heat treatment and subsequent centrifugation. At least some of the activity losses seem incompatible with

Table 1 Purification of cellulase and hemicellulase components of *C. saccharolyticum* by heat treatment

		Soluble protein (mg ml^{-1})		Specific activity (units mg^{-1})			
	Treatment	Before	After	Before	After	Purification	Recovery %
CMCase pNZ1012/PB2477	70°C, 60 min	47.8	2.1	0.0460	0.53	11.5	51
β-Glucosidase pNZ1001/PB2481	70°C, 65 min	14.4	0.68	2.26	42	18.6	88
Xylanase pNZ1076/PB2477	70°C, 30 min	5.24	0.80	3.01	22	7.2	109
β-Xylosidase pNZ1076/PB2477	70°C, 30 min	31.0	3.5	0.0835	0.66	7.9	89
β-Xylosidase pNZ1076/PB2477	80°C, 30 min	31.6	2.1	0.0734	0.72	9.8	65

thermal denaturation. The half-life of the CMCase at 70°C, for example, is 7.5 h. As the protein composition of source material is essentially the same for all enzymes, the variation in degree of purification suggests that further optimization of the heat-treatment step is possible for some of the enzymes. Inclusion of specific stabilizing substances such as substrates[12] to protect against thermal denaturation, and selection of a protein concentration, pH, and ionic strength that minimize losses due to co-precipitation are both possible approaches.

A heat-treatment step in the purification procedure for products of genes from thermophiles cloned into mesophiles has been used by other workers to simplify the purification of thermostable proteins expressed in *E. coli*.[13–20] After heat treatment, *Bacillus stearothermophilus* α-amylase[16] and β-galactosidase[17] cloned into *E. coli* were isolated to near homogeneity in a single chromatographic step, and a *Clostridium thermocellum* endoglucanase was purified to homogeneity from cytoplasmic granules in *E. coli* without the need for any column chromatography.[20] After the heat treatment described in this report, DEAE-sepharose and molecular exclusion chromatography steps produced a homogeneous cloned *C. saccharolyticum* β-glucosidase. Differences in the physical properties of soluble denatured *E. coli* proteins and native thermostable enzymes probably contribute to the success of these subsequent purification steps. The number of *E. coli* proteins that remain soluble due to their stability towards heat treatment is likely to be small, facilitating their separation from the desired enzyme in a few steps.

Differences in molecular weights,[18,19] electrophoretic mobilities,[21] and thermostability properties[13,14,16] that have been observed between some native and cloned gene products indicate that caution is required in inferring the properties of native proteins from the characteristics of a cloned protein. These differences may be due to dissimilarities between the post-translational modifications performed by host and donor organisms. Nevertheless, owing to problems with the purification of bacterial cellulases by conventional methods,[22–25] and the multiplicity of enzymes involved in an active complex (e.g., ref. 26), gene cloning is a promising approach to understanding the enzymology of bacterial cellulose saccharification.

High purification costs are a major factor in restricting the industrial and analytical use of intracellular enzymes, two difficulties being the low yields and the need to remove contaminating activities. Gene cloning using an appropriate expression vector can improve enzyme yield,[27] and subsequent purification of cloned thermostable enzymes by heat treatment will also inactivate contaminating activities produced by the host organism. Work done to date suggests that both (high-level) expression of thermophilic genes in mesophilic hosts and qualitative retention of product thermostability are the rule rather than an exception.[4,14,16,17,28–32] When relatively pure enzymes are required for industrial or scientific use, we believe the heat-treatment method will be an incentive to select enzymes from extreme thermophiles.[33]

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