Cellulases From Extremely Thermophilic Bacteria

K.R. Sharrock*, C.H. Sissons, R.M. Daniel, H.W. Morgan
Department of Biological Sciences, University of Waikato
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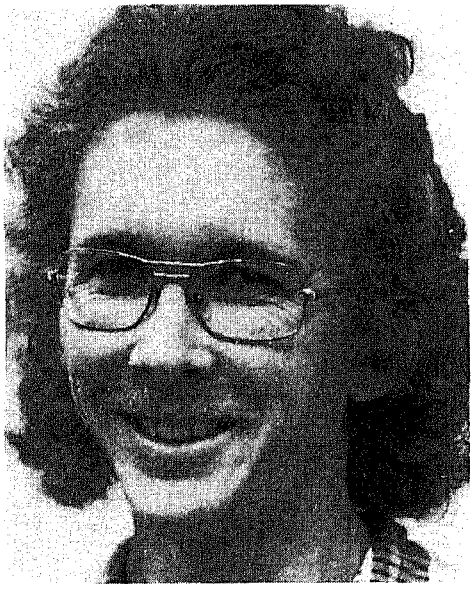

Keith Sharrock, a D.Phil. student of Waikato University, is studying the biochemistry of cellulases from extremely thermophilic bacteria. He graduated B.Sc. from Waikato in 1976 and M.Sc. (Hons) in 1979 at the same university. His M.Sc. research involved a study of nitrogen fixation and translocation in **Coriaria arborea** *(tutu). During 1980 he was a Teaching Fellow in the Botany Department, Canterbury University, and completed his LTCL (Performers) on the violin. In 1981 he returned to Waikato University to join the thermophile research group.*

Roy Daniel is Senior Lecturer in Biochemistry at the University of Waikato. He graduated in Chemistry, B.Sc. (Hons) at the University of Leicester in 1965 and Ph.D. in Biochemistry in 1968 at the same university. After working as a Postdoctoral fellow at the Agriculture Research Unit for Nitrogen Fixation at the University of Sussex and the Division of Plant Industry, CSIRO, Canberra, he took up a lectureship in the Department of Cell Biology, University of Glasgow in 1971. He has been at Waikato since 1975.

Hugh Morgan is Senior Lecturer in Biological Sciences at the University of Waikato. After graduating B.Sc (Hons) in Agricultural Botany at the University of Wales, Aberystwyth in 1965, he completed his Ph.D. in Soil Microbiology at the University of Guelph, Canada in 1970. He was awarded an ICI Postdoctoral fellowship to work at the Macaulay Institute for Soil Research Aberdeen prior to taking up the lectureship in Waikato in 1972.

Chris Sissons joined the Thermophile Group of the University of Waikato in 1981 as a Research Fellow, working on cellulases. He graduated in Biochemistry (M.Sc.) at Victoria University of Wellington in 1965, with a thesis on insecticide detoxification and Ph.D. in Cell Biology at Auckland University in 1971, studying cell free protein synthesis in yeast and including a year lecturing in the Biochemistry Department at Auckland. He then spent six years doing post-doctoral research on enzyme synthesis in the yeast cell cycle in the Zoology Dept. at Edinburgh University and four years at Ruakura as a NRAC Facial Eczema fellow studying mycotoxin detoxification.

INTRODUCTION

Cellulose is the most abundant biopolymer on earth, and is the major component of urban waste. Thus cellulose must be seen as a very significant renewable source of chemical foodstocks when fossil fuels become restricted.

Cellulose is a β (1-4) — linked glucose polymer with a complex secondary and tertiary structure.[1] The glucose molecules exhibit alternating orientation so that the basic repeating unit is cellobiose.

The polyglucose chains align to form microfibrils. These are ordered structures so that about 70% of their length can be termed "crystalline".[1] In these areas the polyglucose chains are cross-linked by one hydrogen bond per glucose. In addition, there are two intra-chain hydrogen bonds per glucose. The gaps between adjacent polyglucose chains are too small to allow enzymes to penetrate the crystal, and even the exposed exterior chains are difficult to break up. This is because several bonds must be broken simultaneously; the β 1-4 glucosidic bond, and also three hydrogen bonds, if a glucose molecule is to be removed to create a "nick" in the chain.

Thus the intervening amorphous regions, where some irregularities opening the structure may already exist, are initially the targets for hydrolytic enzymes.

Most cellulose occurs naturally in a lignin-cellulose — hemicellulose complex which is often extremely resistant to hydrolysis. Lignin is the plant's equivalent of

concrete, and cellulose fibres act as the internal reinforcing. Woody materials generally require some pretreatment to open up the lignin-cellulose-hemicellulose complex sufficiently for effective enzymic hydrolysis of the cellulose. We have centred our studies on the enzymic hydrolysis of pure cellulose as an initial step in understanding cellulose hydrolysis in the natural lignin-cellulose-hemicellulose complex.

SOURCES OF CELLULASE

Most of the cellulolytic enzymes available for commercial purposes are of fungal origin. More recently a thermophilic anaerobic bacterium *Clostridium thermocellum* (growth optimum 60-65°C) has been shown by overseas groups [2-6] to produce cellulases capable of completely hydrolysing crystalline cellulose. Since these enzymes possess specific activities which are similar to those of the best fungal cellulases but are more heat-stable, [6,7] they are potentially of commercial interest. This being so, it seemed worthwhile to look for a source of even more thermostable cellulases, in extreme thermophiles (i.e. organisms with growth optima above 65°C).

ENZYMIC DEGRADATION OF CELLULOSE

No single enzyme has yet been discovered that alone is capable of hydrolysing crystalline cellulose to any great extent. Fungal systems have been most intensively studied, and they are often based upon the concerted action of two or three types of enzyme, endocellulase, exocellulase (probably physically associated) and β glucosidase. [8-10] Many fungi have been shown to produce several different enzymes within each of these basic types, [9,10] so the term "cellulase" refers to an enzyme complex, not a single enzyme.

HOT POOLS SCREENED FOR CELLULOLYTIC BACTERIA

The first step in our study was to find extremely thermophilic bacteria that produce cellulases. We were particularly interested in cellulases with high thermal stability and high specific activity. The level of cellulase production was not considered to be so important at this stage since the yield of enzyme by the bacterium can be genetically raised. Thermal stability and specific activity, being characteristics of the enzyme itself, will be extremely difficult to improve.

Water and sediments were collected from about 50 natural thermal sites and bacteria from them were grown at 75°C in cellulose-containing enrichment media, both aerobically and anaerobically. After six and 21 days growth we subjected the cultures to the first screening step. This involved assay of the cell-free supernatants for endocellulases. Only eight cultures fulfilled this requirement. All were anaerobic.

The second screening tested for the ability to degrade crystalline cellulose, since the ultimate objective was to find bacteria capable of degrading natural cellulosic materials with a minimum of pretreatment. Two of the cultures, TP8 and TP10, could be seen to consume crystalline cellulose from their growth media, and assays on the cell-free culture supernatants confirmed that they were producing cellulases capable of hydrolysing crystalline cellulose to reducing sugars.

Specific activities of the cellulases of both crude culture supernatants compared well with that of the cellulase we obtained from *C. thermocellum*. We included *C. thermocellum* as a reference in most of our comparisons, although it had to be grown at 60°C whereas our bacteria grew at 75°C.

Apart from TP8 and TP10, none of the other cultures produced enzymes that would release significant quantities of reducing sugar from crystalline cellulose.

Stage 3 of the screening involved a comparison of the thermal stabilities of the supernatant endocellulase activities (Figs 1.). These exhibited half-lives at 85°C of five hours, 20 minutes and less than five minutes from TP8, TP10 and *C. thermocellum* respectively. Thus TP8 was selected for further study.

Some of the bacteria which we rejected after stage 2 of the screen subsequently proved to have extremely stable endocellulases, with half-lives at 85°C in excess of 20 hours. Unfortunately these bacteria are not able to attack crystalline cellulose by themselves.

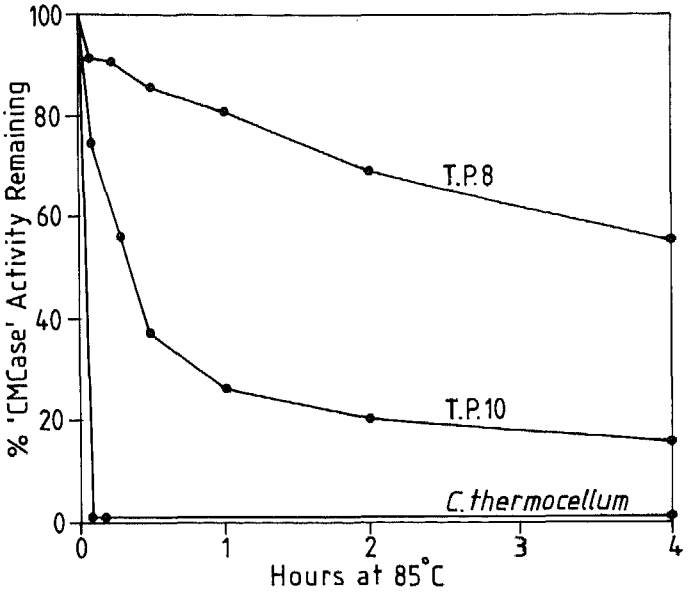

Fig 1 Thermal stabilities of endocellulases
Culture supernatants were incubated at 85°C and samples taken at the times indicated were assayed at 75°C for remaining endocellulase (CMCase).[13]

ISOLATION OF BACTERIA IN PURE CULTURE

In order to study the cellulases of TP8 further, it was necessary to isolate the celluloytic bacteria in pure culture. Cellulolytic anaerobes are renowned for the difficulties they present in the face of any effort made to isolate them [11], and those from the TP8 culture proved to be no exceptions. We had additional problems in dealing with extreme thermophiles in that agar dries out or melts at their growth temperatures. Eventually we developed a system which allowed isolation and cultivation from individual colonies, each of which originated from a single bacterium. Two different celluloytic strains were isolated from TP8. The more stable endocellulase was produced by a strain which didn't attack crystalline cellulose to any extent. The other strain was considered more worthy of further study since it produced enzymes capable of hydrolysing crystalline cellulose to reducing sugars.

SEPARATION AND CHARACTERISATION OF CELLULASE COMPONENTS

Purification and study of the cellulases from this isolate has only just begun, and so far we have preliminary information only.

Enzymes from the supernatant of 80 litres of culture

were concentrated by ammonium sulphate precipitation, redissolved and washed over a 10,000 M.W. cut-off ultra-filter. The concentrate was then applied to a G-100 Sephadex column and the fractions collected were subjected to four different assays (Fig. 2).

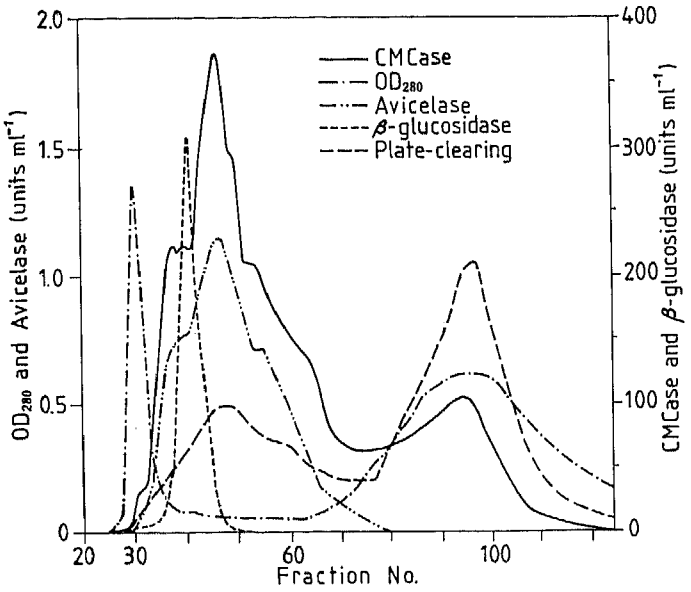

Fig 2 Elution profile for cellulases and β-glucosidase on a Sephadex G-100 column

All assays at 75°C, with units as follows:

"CMCase" and "Avicelase": One nmol.glucose equivalents.min.$^{-1}$

B-glucosidase: 130 pmol. p-nitrophenol.min^{-1}

Plate-clearing assay: Units arbitrary, based on antilog. of cleared diameter around wells in CMC-agar.[14]

Optical density at 280 nm was measured to roughly monitor protein concentration, and showed two major peaks. The first contained proteins of the void volume, and the second appeared much later and tailed considerably.

β-glucosidase[12] eluted as a single sharp peak of activity. Endocellulase activity was measured by two methods. The first, denoted the "CMCase" assay, involved incubation of column fractions with sodium carboxymethyl cellulose and measurement of the reducing sugars produced.[13] The second was a plate-clearing assay, presumably dependent upon shortening of the carboxymethyl cellulose molecules[14]. The plate-clearing assay was therefore more sensitive to centrally-acting endocellulase than was the "CMCase" reducing sugar assay. Both assays produced two broad peaks of activity, but the relative magnitudes of the first and second peaks were reversed, suggesting that the endocellulase in the second peak was the more centrally-acting.

The fractions were also incubated with Avicel, which is crystalline cellulose, and the reducing sugars produced were measured.[15] This resulted in a single peak. There was "Avicelase" activity only in the first "CMCase" peak.

Thus it appears that there are at least three different enzymes involved in cellulose hydrolysis by this bacterium. One has β-glucosidase activity, and has a molecular weight of about 55,000.

There may be a single enzyme or group of enzymes, each exhibiting both exo- and endocellulase activity, since crystalline cellulose and CMC hydrolysis peaked in the same fractions. Alternatively, each of these activities may be due to separate enzymes of similar molecular weights.

The second protein peak had endocellulase but no exocellulase activity. Its late appearance from the column could suggest an extremely low molecular weight (< 2000), but more probably it was retarded by binding to Sephadex. Such binding was expected since at 75°C the enzyme concentrate had been shown to hydrolyse Sephadex G-100 (which is a dextran) almost as efficiently as it hydrolysed crystalline cellulose. However, no detectable release of reducing sugars from Sephadex occurred during incubation at room temperature overnight. In preliminary work with a Biogel acrylamide-packed column the second endocellulase peak was absent, which may suggest that its molecular weight is actually quite similar to that of the first endocellulase, and that their differing affinities for Sephadex allowed their separation.

FUTURE WORK

Further fractionation techniques such as affinity and ion exchange chromatography and isoelectric focussing should allow purification of the individual components of the cellulase complex. The components will then be characterized independently and investigated for their possible co-operative action.

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