Biochem. J. (1991) 277, 887–890 (Printed in Great Britain)

Thermostable cellobiohydrolase from the thermophilic eubacterium *Thermotoga* sp. strain FjSS3-B.1

Purification and properties

Leon D. RUTTERSMITH* and Roy M. DANIEL
Thermophile Research Unit, University of Waikato, Hamilton, New Zealand

Exo-1,4-β-cellobiohydrolase (EC 3.2.1.91) was isolated from the culture supernatant of *Thermotoga* sp. strain FjSS3-B.1, an extremely thermophilic eubacterium that grows optimally at 80 °C. The enzyme was purified to homogeneity as determined by SDS/PAGE and has an M_r of 36000. The enzyme is the most thermostable cellulase reported to date, with a half-life at 108 °C of 70 min in buffer. In a 40 min assay, maximal activity was recorded at 105 °C. Cellobiohydrolase from strain FjSS3-B.1 is active against amorphous cellulose and CM-cellulose but only effects limited hydrolysis of filter paper or Sigmacell 20. The only product identified by h.p.l.c. is the disaccharide cellobiose. The enzyme has a pH optimum around neutral and is stabilized by the presence of 0.8 M-NaCl.

INTRODUCTION

Cellulose is an unbranced glucose polymer composed of D-glucose units linked by 1,4-β-D-glucosidic bonds. It is one of the most abundant biopolymers on Earth and represents an important renewable energy source.

Enzyme systems that degrade cellulose have been studied in fungal species, especially in *Trichoderma reesei* (Knowles *et al.*, 1987), and in various bacteria (Robson & Chambliss, 1989). They are composed of essentially three enzyme species: endo-β-1,4-glucanases, exo-β-1,4-glucanases (or cellobiohydrolases) and β-glucosidases. In cellulose fibres, regions of high crystallinity co-exist with less ordered amorphous regions, and the three enzyme groups generally have to work synergistically to effect the hydrolysis of crystalline cellulose.

Most cellulases characterized to date lack thermostability, and this is a major drawback in industrial terms. However, thermostable cellulolytic enzymes have been characterized from thermophilic cellulolytic bacteria such as *Clostridium thermocellum* (Ng *et al.*, 1977), '*Caldocellum saccharolyticum*' (Reynolds *et al.*, 1986) and '*Acidothermus cellulolyticus*' (Tucker *et al.*, 1989). The most stable of these, from '*Acidothermus*', had a half-life of less than 20 min at 85 °C (Tucker *et al.*, 1989).

Thermotoga strain FjSS3-B.1 is an extremely thermophilic eubacterium growing optimally at 80 °C (Huser *et al.*, 1986). This organism is reported to grow on CM-cellulose (Huser *et al.*, 1986) and secretes a cellulolytic enzyme as well as a xylanase and a β-glucosidase (Bragger *et al.*, 1989).

In the present paper we report the purification and characterization of a cellobiohydrolase from *Thermotoga* strain FjSS3-B.1. Data are presented demonstrating the remarkable thermostability of this enzyme and its discovery is discussed with respect to the cellulolytic status of the organism.

EXPERIMENTAL

Materials

All chemicals and others materials used were of analytical grade or of the finest grade commercially available. 4-Methylumbelliferyl β-D-cellobioside, low-viscosity CM-cellulose, *p*-nitrophenyl β-D-cellobioside, Sigmacell 20 and all biological buffers were from Sigma Chemical Co. (St. Louis, MO, U.S.A.). Filter paper was Whatman no. 1. Amorphous cellulose was prepared by an in-house method involving treatment with $ZnCl_2$ and was kindly supplied by F. A. Rainey (Thermophile Research Unit, Hamilton, New Zealand).

Cell culture

Thermotoga strain FjSS3-B.1 was grown anaerobically at 80 °C in a mineral salts medium containing cellobiose (1 g/l), yeast extract (2 g/l) and casein hydrolysate (2 g/l) as described by Huser *et al.* (1986), in crimp-sealed 100 ml bottles (Bellco Glass, Vineland, NY, U.S.A.) or in a 600-litre fermenter.

Assay for cellobiohydrolase

The enzyme was routinely assayed in 25 mM-Mops/NaOH buffer, pH 7.0 (at 80 °C), containing 3.3 mM-4-methylumbelliferyl β-D-cellobioside. A 0.15 ml volume was incubated at 80 °C, typically for 20 min, and the reaction was stopped with 1 ml of 0.5 M-glycine, pH 10.4. The absorbance was measured at 365 nm to determine the extent of hydrolysis (ϵ 18000 $M^{-1} \cdot cm^{-1}$ for 4-methylumbelliferone).

Buffers used for the determination of the pH profile were: pH 3.5–5.5, 0.1 M-sodium acetate buffer; pH 5.5–6.6, 0.1 M-Mes/NaOH buffer; pH 6.6–7.7, 0.1 M-Mops/NaOH buffer; pH 7.7–8.8, 0.1 M-Bicine/NaOH buffer; pH 8.8–10.1, 0.1 M-Ches [2-(cyclohexylamino)ethanesulphonic acid]/NaOH buffer. The pH values were adjusted at 80 °C.

Purification of cellobiohydrolase

All operations were carried out at room temperature. Enzyme was stored at 4 °C between steps.

Cells from 20 litres of medium were first removed by concentration with the use of an Amicon Hollow Fibre cartridge (Amicon, Danvers, MA, U.S.A.) followed by centrifugation at 13000 *g* ($r_{av.}$ 11.9 cm) for 15 min. The 20 litres of cell-free medium were then concentrated with the use of an Amicon spiral

* To whom correspondence should be addressed.

ultrafiltration cartridge S1Y10 to 1 litre and further concentrated to 40 ml with the use of YM10 membranes in Amicon ultrafiltration cells.

The concentrated supernatant was then dialysed against 20 mM-Bistris/NaOH buffer, pH 7.0, and applied to a 40 ml column (24 mm × 880 mm) of Q-Sepharose Fast Flow pre-equilibrated in the same buffer. Protein was eluted with a linear gradient of 0–0.7 M-NaCl in the same buffer. Fractions containing methylumbelliferyl β-D-cellobioside-hydrolysing activity were pooled and dialysed against 20 mM-Bistris/NaOH buffer, pH 6.5.

Enzyme was then subjected in two batches to chromatofocusing on a Pharmacia (Uppsala, Sweden) Mono P (HR 5/20) f.p.l.c. column pre-equilibrated in 20 mM-Bistris/NaOH buffer, pH 6.5; protein was eluted with Polybuffer 74, pH 3.6, i.e. with a decreasing pH gradient of pH 6.5 to pH 3.6. Pooled fractions containing enzyme were concentrated by ultrafiltration and then gel-filtered on a TSK G3000SW gel-filtration column (0.75 cm × 60 cm) in 20 mM-Bistris/NaOH buffer, pH 6.5, containing 0.2 M-NaCl.

A fraction was prepared for *N*-terminal sequencing by dialysis against Milli Q water and freeze-drying. Protein concentrations were determined by using the method of Bradford (1976), with BSA as standard.

SDS/PAGE

Proteins were subjected to SDS/PAGE by using the Pharmacia Phast system with 10–15 % gradient gels; this method is based on the method of Laemmli (1970).

Determination of thermal stability

Thermal-stability experiments below 96 °C were carried out in 1.5 ml Eppendorf tubes immersed in a water bath at the required temperature. Above 96 °C samples were incubated in sealed 3.5 ml screw-topped vials (Wheaton, NJ, U.S.A.) completely immersed in an oil bath at the required temperature. Experiments with a thermistor probe (Omega, Stamford, CT, U.S.A.) sealed into a vial had shown that liquid in the vials reached the required temperature within 5 min.

Assay for reducing sugars

Enzyme activities on CM-cellulose, amorphous cellulose, filter paper and Sigmacell 20 were measured by determining the rate of release of reducing sugars from the substrate with the use of *p*-hydroxybenzoic acid hydrazide (Lever, 1973).

Product analysis

Oligosaccharides released from cellulosic substrates were analysed by h.p.l.c. on a Bio-Rad Aminex HPX-42A oligosaccharide column. This column resolves oligosaccharides up to a degree of polymerization of 11.

RESULTS

Purification of cellobiohydrolase

A summary of the purification of the cellobiohydrolase is given in Table 1. A 34-fold increase in the purity yielded enzyme that was found to be homogeneous as judged by SDS/PAGE and silver staining; comparison of the electrophoretic mobility with those of standard proteins run simultaneously gave a polypeptide chain M_r of 36000. When determined by gel filtration on the TSK G3000SW column, an M_r of 22000 was obtained. It is likely that hydrophobic interactions of the enzyme with the column caused this low value.

Substrate-specificity

The activity of the purified enzyme was tested against a variety of cellulase substrates. The specific activities for these substrates are given in Table 2. Filter paper, crystalline cellulose (Avicel) and Sigmacell 20 were only degraded to the extent of 1–2 % even after prolonged incubation. Amorphous cellulose could be totally digested if the products were repeatedly removed. CM-cellulose was degraded to the extent of at least 6 %.

Analysis of the products from these substrates by h.p.l.c. on the Bio-Rad HPX-42A oligosaccharide column identified cellobiose as the only product from the carbohydrate substrates.

Catalytic properties

The enzyme was catalytically active with several substrates (shown in Table 2) and showed a hyperbolic dependence of rate on the concentrations of CM-cellulose, 4-methylumbelliferyl β-D-cellobioside and *p*-nitrophenyl β-D-cellobioside. These data were analysed by the direct-linear-plot method of Eisenthal & Cornish-Bowden (1974), giving K_m values of 0.04 %, 0.23 mM and 0.9 mM respectively.

Enzyme inhibition by cellobiose was studied by assaying the cellobiohydrolase activity with 4-methylumbelliferyl β-D-cellobioside in the presence of cellobiose. Cellobiose was found to be a competitive inhibitor with a K_i of 0.2 mM.

pH optimum and pH stability

A symmetrical bell-shaped pH–activity profile with an optimum at pH 6.8–7.8 was found. The enzyme retained 50 % of its maximal activity at pH 5.1 and pH 9.0. The enzyme showed high thermostability between pH 4.8 and pH 8.1. At pH 4.2 and

Table 1. Purification of *Thermotoga* strain FjSS3-B.1 cellobiohydrolase

For experimental details see the text. Activity units are expressed as μmol of 4-methylumbelliferone produced/min.

Purification step	Volume (ml)	Total enzyme (units)	Total protein (mg)	Specific activity (units/mg)	Recovery (%)	Overall purification (fold)
Supernatant concentration	40	7.4	53	0.14	100	1
Q-Sepharose Fast Flow	35	5.1	6.4	0.8	68	5.6
Mono P chromatofocusing	8	1.5	1.4	1.1	20	7.7
TSK G3000SW gel filtration	30	0.59	0.12	4.9	8	34.3

Table 2. Substrate-specificity of *Thermotoga* strain FjSS3-B.1 cellobiohydrolase

Specific activities are expressed as μmol of product produced/min per mg of protein and were measured at 80 °C. Product measured was reducing sugar (cellobiose equivalent), *p*-nitrophenol or 4-methylumbelliferone.

Substrate	Specific activity (μmol/min per mg)
Amorphous cellulose	7.5
CM-cellulose	38
Filter paper (Whatman no. 1)	3.1
Sigmacell 20	1.4
4-Methylumbelliferyl β-D-cellobioside	3.6
p-Nitrophenyl β-D-cellobioside	2.3
Xylan	0.0
p-Nitrophenyl β-D-glucoside	0.0
p-Nitrophenyl β-D-lactoside	0.0

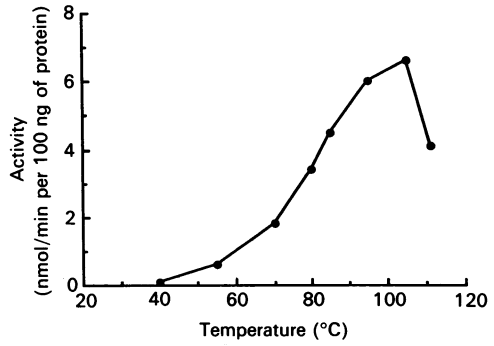

Fig. 1. Temperature–activity profile for *Thermotoga* strain FjSS3-B.1 cellobiohydrolase (assayed with CM-cellulose) over a 40 min assay

pH 8.9 half-lives at 95 °C of 45 min and 57 min respectively were found.

Temperature–activity profile

The relative enzyme activity measured in a 40 min assay at different temperatures is shown in Fig. 1. The highest activity was found to be at 105 °C. It is possible that the higher temperatures caused some disruption in the structure of the CM-cellulose, making the molecule more susceptible to enzymic attack.

Thermostability

Initial experiments with concentrated crude supernatant from the growth medium of this organism indicated that the 'cellulase' had a half-life of 15 min at 105 °C (Bragger *et al.*, 1989). On purification, the cellobiohydrolase exhibited half-lives of 70 min, 7 min and < 2 min at 108 °C, 113 °C and 117 °C respectively in aqueous solutions of 0.1 M-Mops buffer, pH 7.0.

The presence of various additions had no effect on thermostability. Thus 10% (v/v) glycerol, 10% (w/v) sorbitol, 10 mM-$CaCl_2$ or 0.005% CM-cellulose did not alter the measured half-lives. However, the inclusion of 0.8 M-NaCl in the sample did have a stabilizing effect, and under these conditions the half-lives at 108 °C, 113 °C and 117 °C were 120 min, 14 min and < 2 min respectively.

Calculation of these half-lives was carried out assuming no loss of activity during the first 5 min of the incubation, required for temperature equilibration. The estimation of half-lives was thus conservative but imprecise for short half-lives.

DISCUSSION

This paper reports the purification and characterization of the most thermostable cellulase enzyme studied to date. The xylanase from the same organism has also been studied (Simpson *et al.*, 1991) and in the free form is slightly less stable, showing a half-life of 8 min at 100 °C. *Thermotoga* strain FjSS3-B.1 is evidently a source of very stable enzymes.

It may be noted that there are no reports in the literature of the Thermotogales being cellulolytic organisms, although the growth of *Fervidobacterium islandicum* was stimulated by the presence of cellulose (Huber *et al.*, 1990). In our laboratory it has not been possible to show that *Thermotoga* strain FjSS3-B.1 grows on insoluble or amorphous preparations of cellulose despite its ability to grown on CM-cellulose (Huser *et al.*, 1986). The fact that it possesses an active cellobiohydrolase is therefore somewhat puzzling. Studies of culture supernatants from *Thermotoga maritima* (DSM 3109) and *Thermotoga neopolitana* (DM 4359) have shown that these organisms also possess CM-cellulose- and 4-methylumbelliferyl β-D-cellobioside-hydrolysing enzymes (results not shown).

The data reported here suggest that this cellobiohydrolase attacks amorphous regions of cellulose, liberating cellobiose units from non-reducing ends of the polysaccharide chain. The three-dimensional structure of a cellobiohydrolase (cellobiohydrolase II) from the cellulolytic fungus *Trichoderma reesei* has been solved (Rouvinen *et al.*, 1990) and shows that the active site is in an enclosed tunnel through which the cellulose threads. This tunnel may well be a common feature among cellobiohydrolase enzymes, although the fact that the enzyme from strain FjSS3-B.1 is active against CM-cellulose suggests that any tunnel in the structure of this enzyme does not preclude entry to carboxymethyl side chains. It is widely reported in the literature that ability to degrade CM-cellulose is exclusively a characteristic of endocellulases. The strain FjSS3-B.1 cellobiohydrolase is evidently an exception to that rule.

The thermostability of the cellobiohydrolase from strain FjSS3-B.1 is considerably higher than that of any cellulase previously reported (Tucker *et al.*, 1989). Indeed, its stability in aqueous conditions ranks it among the most thermostable enzymes known. Reliably determined half-lives for enzymes of more than a minute or so above 110 °C under conditions where activity is possible are very rare. Measurable half-lives above 100 °C have been reported for a few thermophilic enzymes: an α-glucosidase from *Pyrococcus furiosus* had a half-life of 1 h at 105 °C (Brown *et al.*, 1990), a hydrogenase from the same organism had a half-life of 2 h at 100 °C (Bryant & Adams, 1989), and proteinases from *Pyrococcus furiosus* and a *Desulfurococcus* strain had half-lives of 33 h at 98 °C and 8 min at 105 °C respectively (Blumentals *et al.*, 1990; Cowan *et al.*, 1987).

Various attempts were made to increase the thermostability of this cellobiohydrolase. The presence of NaCl did have a stabilizing effect. Salt binds to protein charged groups or dipoles ('salting-in') or decreases the solubility of protein hydrophobic groups ('salting-out'): essentially it excludes water from the protein molecule.

A number of studies of thermoinactivation in model proteins (Ahern & Klibanov, 1985; Zale & Klibanov, 1986) have demonstrated the role of water in processes such as hydrolysis of disulphides, hydrolysis of Asp–Xaa bonds and deamidation of asparagine residues. Clearly the thermal stability of proteins can be greatly increased in low-water environments (Zaks &

Klibanov, 1984; Ayala *et al.*, 1986). Ahern & Klibanov (1986) reported a half-life of 200 h at 100 °C for lysozyme as a dry powder. It would seem likely that the thermal stability of hydrolytic enzymes is limited by their resistance to attack by one of their substrates, water.

The award of a New Zealand University Grants Committee postdoctoral fellowship to L. D. R. and financial support from Pacific Enzymes are gratefully acknowledged.

REFERENCES

Ahern, T. J. & Klibanov, A. M. (1985) Science **228**, 1280–1283
Ahern, T. J. & Klibanov, A. M. (1986) in Protein Structure, Folding and Design (Oxender, D. L., ed.), pp. 283–289, Alan R. Liss, New York
Ayala, G., de Gomez Puyou, M. T., de Gomez Puyou, A. & Darszon, A. (1986) FEBS Lett. **203**, 41–43
Blumentals, I. I., Robinson, A. S. & Kelly, R. M. (1990) Appl. Environ. Microbiol. **56**, 1992–1998
Bradford, M. M. (1976) Anal. Biochem. **72**, 248–254
Bragger, J. M., Daniel, R. M., Coolbear, T. & Morgan, H. W. (1989) Appl. Microbiol. Biotechnol. **31**, 556–561
Brown, S. H., Costantino, H. R. & Kelly, R. M. (1990) Appl. Environ. Microbiol. **56**, 1985–1991
Bryant, F. O. & Adams, M. W. W. (1989) J. Biol. Chem. **264**, 5070–5079
Cowan, D. A., Smolenski, K. A., Daniel, R. M. & Morgan, H. W. (1987) Biochem. J. **247**, 121–133
Eisenthal, R. S. & Cornish-Bowden, A. (1974) Biochem. J. **139**, 715–720
Huber, R., Woese. C. R., Langworthy, T. A., Kristjansson, J. K. & Stetter, K. O. (1990) Arch. Microbiol. **154**, 105–111
Huser, B. A., Patel, B. K. C., Daniel, R. M. & Morgan, H. W. (1986) FEMS Microbiol. Lett. **37**, 121–127
Knowles, J., Lehtovaara, P. & Teeri, T. (1987) Trends Biotechnol. **5**, 255–260
Laemmli, U. K. (1970) Nature (London) **227**, 680–685
Lever, M. (1973) Biochem. Med. **7**, 274–281
Ng, T. K., Weimer, P. J. & Zeikus, J. G. (1977) Arch. Microbiol. **114**, 1–7
Reynolds, P. H. S., Sissons, C. H., Daniel, R. M. & Morgan, H. W. (1986) Appl. Environ. Microbiol. **51**, 12–17
Robson, L. M. & Chambliss, G. H. (1989) Enzyme Microb. Technol. **11**, 626–644
Rouvinen, J., Bergfors, T., Teeri, T., Knowles, J. K. C. & Jones, T. A. (1990) Science **249**, 380–386
Simpson, H. D., Haufler, U. R. & Daniel, R. M. (1991) Biochem. J. **277**, 413–417
Tucker, M. P., Mohagheghi, A., Grohmann, K. & Himmel, H. E. (1989) Bio/Technology **7**, 817–820
Zaks, A., & Klibanov, A. M. (1984) Science **224**, 1249–1251
Zale, S. E. & Klibanov, A. M. (1986) Biochemistry **25**, 5432–5444

Received 9 April 1991/28 May 1991; accepted 4 June 1991