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An extremely thermostable xylanase from the thermophilic eubacterium *Thermotoga*

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Endo-1,4-β-xylanase (EC 3.2.1.8) was isolated from the culture supernatant of *Thermotoga* sp. strain FjSS3-B.1, an extremely thermophilic anaerobic eubacterium which grows optimally at 80 °C. Activity was purified 165-fold by anion-exchange and hydroxyapatite chromatography. The enzyme has an M_r of 31000 as determined by SDS/PAGE and 35000 by analytical gel filtration. The optima for activity and stability for purified xylanase were between pH 5.0 and 5.5. At pH 5.5, which is the optimum pH for thermostability, $t_{\frac{1}{2}}$ (95 °C) is 90 min. The thermostability was improved by immobilization of the xylanase on to porous glass beads; $t_{\frac{1}{2}}$ (105 °C) is 10 min. Several additives, such as sorbitol and xylan, were also found to increase the thermostability. At 130 °C, the half-life of immobilized xylanase in the presence of 90% sorbitol was 1.3 min. At 130 °C in molten sorbitol half of the enzyme denatured rapidly, but the remainder appeared to have a half-life of about 60 min.

INTRODUCTION

Xylan is the main component of hemicellulose which is present in Nature in large amounts and is a major by-product of the farming industry. Xylan consists of 1,4-glycosidically linked β-D-xylose with branches containing xylose and other pentoses, hexoses and uronic acids. Xylan can be degraded by either acid or enzymic catalysis. The enzymic process has the advantages of a highly efficient conversion rate, and the mild conditions required are non-corrosive and non-environmentally hazardous [1]. Although, the complete breakdown of xylan requires the action of several different enzymes, the depolymerizing endo-1,4-β-xylanase (EC 3.2.1.8) is the key enzyme. Consequently, xylanases have possible applications in waste treatment, fuel and chemical production and paper manufacture [2]. Thermostable xylanases may be exploited for xylan digestion processes at elevated temperatures. There have been several reports of high-temperature xylanases produced by various *Bacillus* species, which grow optionally at 65 °C [3–5]. In this paper we describe the purification and partial characterization of an endo-1,4-β-xylanase from the eubacterium *Thermotoga* sp. strain FjSS3-B.1, an anaerobic extreme thermophile which grows optimally at 80 °C.

In this laboratory we have assessed the thermostability of different enzymes from extremely thermophilic bacteria from both the archaebacterial and eubacterial kingdoms to help define the upper limit of thermal stability of enzymes [6]. To date one of the most thermostable enzymes is the xylanase from *Thermotoga* sp. strain FjSS3-B.1. We report on the conditions required to stabilize this enzyme at temperatures up to 130 °C. Since many thermoinactivation reactions are hydrolytic, it is expected that dehydration stabilizes enzymes. Indeed, it has been shown that some enzymes are much more stable in organic solvents under anhydrous conditions than in aqueous solutions [7]. However, we were interested in the potential thermostability of enzymes *in vivo* and focused our investigations on methods of stabilizing the xylanase by the use of various additives and by immobilization.

MATERIALS AND METHODS

Materials

Calibration kits for protein standards to determine M_r values were obtained from Amersham and gel-filtration standards from Bio-Rad. Uncoated controlled-pore glass beads were obtained from Sigma. All other chemicals, except where stated in the text, were analytical grade from Sigma.

Growth of organism

Thermotoga sp. strain FjSS3-B.1 was enriched from an intertidal hot spring on Savu-Savu beach in Fiji [8]. The organism was grown anaerobically in a 600-litre vessel at 80 °C on a mineral salt medium (MSM) containing casamino acids (Difco) (2 g/l) and yeast extract (Merck) (0.1 g/l). The cells were harvested in early stationary phase, 26 h after inoculation with a continuous-flow Sharples centrifuge at approx. 12000 *g*. The supernatant was collected and concentrated to 1800 ml through two Amicon S10 Y10 cartridges in series.

Enzyme purification

All column chromatography was carried out at room temperature. At each stage the active fractions were pooled and the salt was removed by dilution with 20 mM-Mes buffer, pH 6, followed by concentration with Amicon PM10 ultrafiltration membranes.

Portions (700 ml) of concentrated supernatant were loaded on to a DEAE-Sepharose CL-6B column (16 cm × 10 cm) equilibrated with 20 mM-Mes buffer, pH 6. Xylanase activity was eluted at 0.25 M-NaCl with a 9-litre linear 0–1 M-salt gradient in the same buffer.

A Mono Q 10/10 (Pharmacia) f.p.l.c. anion-exchange column (1 cm × 10 cm), equilibrated with 20 mM-Bistris, pH 6, was used as a second purification step. Activity was eluted at 0.1 M-NaCl with a linear 0–0.3 M-salt gradient. The third step was Bio-Gel HPHT (99 cm × 2.5 cm) hydroxyapatite chromatography. A linear phosphate gradient of 20–300 mM-potassium phosphate, pH 6.8, was applied to the column, and xylanase activity was

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eluted at 200 mM-phosphate. As a final purification step the Mono Q chromatography was repeated.

Enzyme assays and protein determination

Xylanase was assayed by measuring the reducing sugars released as xylose from oatspelts xylan and quantified with *p*-hydroxybenzoic acid hydrazide by using a method based on that of Lever [9]. The reaction mixture (500 μl) contained 0.1 M-citrate buffer, pH 5.5, oatspelts xylan (0.2%, w/v) and enzyme. The reaction was terminated by the addition of 1 ml of ice-cold *p*-hydroxybenzoic acid hydrazide reagent. After 6 min boiling, the A_{420} was monitored. Assays were routinely carried out at 80 °C and pH 5.5 for 10 min. For temperatures above 90 °C, the assay mixtures were incubated in a silicone-oil bath and the buffers used were calibrated at 90 °C. The xylan solution was made up at 0.25% (w/v) in buffer and sonicated for 1–2 min to aid solubilization.

For experiments where xylan was present to stabilize the enzyme, an alternative assay procedure was used to avoid high background values. The release of dye from dyed xylan (Remazol Brilliant Blue R-D-xylan; Sigma) was measured spectrophotometrically [10]. The assay mixture (500 μl) was incubated at 90 °C for 30 min and the reaction stopped by cooling and the addition of 100 μl of 1 M-NaCl and 1 ml of ethanol, which precipitated the dyed xylan. The mixture was then centrifuged at 12800 *g* for 2 min, and the A_{595} of the supernatant was monitored. For assays with immobilized enzyme, agitation was applied. All assays were performed in triplicate.

For pH stability and optima studies the following buffers were substituted for the citrate buffer: 0.1 M-citrate buffer (pH range 3–6.5); 0.1 M-Mes buffer (pH range 5.5–6.5); 0.1 M-Mops buffer (pH range 6.5–9). The stability studies were performed by incubating the enzyme at the selected pH value for 15 min at 115 °C and then titrating back to pH 5.5 and assaying at pH 5.5.

One unit of enzyme activity was defined as the amount of enzyme required to produce 1 μmol of xylose/min. Specific activities are expressed as units per mg of protein.

Protein assays were performed by the method of Lowry *et al.* [11], with BSA as a standard.

Determination of M_r

The M_r of purified xylanase was determined by chromatography on a calibrated molecular-exclusion h.p.l.c. TSK-Gel G3000SW column (600 mm × 7.5 mm).

PAGE was performed by the method of Laemmli [12]. Acrylamide gradient (10–30%, w/v) gels were used. For SDS-containing dissociating gels, a constant current of 10 mA was applied until the Bromophenol Blue marker reached the bottom of the gel. For non-dissociating gels, electrophoresis was carried out in Tris/glycine buffer, pH 9.5, for 2000 V·h. For activity staining of the xylanase, an agar overlay containing oatspelts xylan (0.1%, w/v) was placed over a non-SDS gel and incubated at 80 °C for 30 min. The overlay was stained with aqueous Congo Red (0.1%, w/v). After destaining with 1 M-NaCl, a clear zone indicated xylanase activity [13].

Analysis of degradation products

Samples taken from assay mixtures were injected on to a h.p.l.c. Bio-Rad Aminex HPX-42A column. Filtered vacuum-degassed water formed the mobile phase. The flow rate was 0.6 ml/min, the column temperature was 85 °C and the products were monitored with a refractive-index detector. Standards used were 20 mM-xylose and a mixture of 20 mM-glucose, -maltose, -maltotriose, -maltotetraose and -maltohexaose.

Immobilization

Xylanase was immobilized on to three different solid supports. They were: uncoated controlled-pore glass beads [pore diameter 24 nm (240 Å), particle size 37–74 μm]; long-chain alkylamine-coated glass beads [pore diameter 50 nm (500 Å), particle size 125–177 μm]; and oxirane acrylic beads (containing approx. 800 μmol of oxirane groups per g of solid). Purified xylanase (50 μg) was immobilized on to glass beads (0.5 g) by the silane/glutaraldehyde coupling method [14]. Purified xylanase (100 μg) was immobilized on to oxirane acrylic beads by the method of Solomon *et al.* [15]. After immobilization, the beads were dried by filtration and stored at 4 °C.

Thermostability measurements

For the experiments at temperatures over 100 °C, sealed Mininert vials (Pierce Chemical Co.) were fully submerged in an oil bath. The vials containing the specified additives in 300 μl of 0.1 M-citrate buffer, pH 5.5, were pre-equilibrated at the temperature stated. A suspension of the immobilized enzyme (200 μl) was injected through the Mininert valve, which was immediately closed, and the vial was submerged in the oil bath. After specified lengths of time at the required temperature, the vials were placed on ice. The samples were subsequently assayed with agitation either by spectrophotometrically measuring the release of dye from dyed xylan [10] or by washing the beads and assaying by the *p*-hydroxybenzoic acid hydrazide method [9]. The activity of control samples in which the enzyme was added after the vial was placed on ice (i.e. the enzyme solution was not preincubated at the elevated temperature) was taken as full activity. A thermistor (Omega) was sealed into the lid of a control vial to check the temperature of the buffer inside the vials during the period of incubation.

RESULTS AND DISCUSSION

Xylanase purification

The enzyme was purified 165-fold resulting in a 9% yield (Table 1). The purification factor may have been underestimated, since the concentration of the culture supernatant by ultrafiltration with a 10000-M_r-cut-off membrane could have removed small proteins. Analysis of 15 μg of protein from the purified xylanase preparation by SDS/10–30% (w/v) polyacrylamide gradient-gel electrophoresis resulted in the detection of one protein band when stained with Coomassie Blue.

M_r

Both non-SDS/PAGE and SDS/PAGE revealed a single protein band corresponding to a M_r of 31000, indicating that the xylanase was a monomer. The protein band corresponded to a single zone of clearing on a xylan-containing overlay indicating xylanase activity. The M_r as determined by h.p.l.c. TSK size-exclusion chromatography was 35000. This value is in agreement with other xylanases from bacterial and fungal sources, which have been reported to have M_r values ranging between 20000 and 50000 [16–21].

Degradation products

The end products of a 60 min hydrolysis of oatspelts xylan have an h.p.l.c. mobility corresponding to xylobiose, xylotriose and medium-sized oligomers. Further incubation (120–240 min) produces in addition a peak corresponding to xylose, and the proportion of X_4–X_7 oligomers decreases. The simplest explanation is that the xylanase under study is an endoxylanase. However, since oatspelts xylan contains arabinose and glucuronic acid attached to the xylose backbone, hydrolysis may have

Table 1. Purification of xylanase from *Thermotoga* sp. strain FjSS3-B.1

Purification step	Total activity (units)	Total protein (mg)	Specific activity (units/mg)	Purification (fold)	Recovery (%)
Concentrated supernatant	378	490	0.77	1	100
1. DEAE-Sepharose	167	19.8	8.45	11	44
2. Mono Q f.p.l.c.	125	3.7	32.9	44	33
3. HPHT hydroxyapatite	57	0.7	84.2	110	15
4. Mono Q f.p.l.c.	34	0.28	126.3	165	9

Table 2. Summary of immobilization

Xylanase (50 μg) was immobilized on to the various matrices. After the immobilization procedure, the beads were washed and filtered and xylanase assays were performed to calculate the percentage of activity bound.

Matrix	Activity bound (%)
Oxirane acrylic beads	13
Glass beads, uncoated	85
Glass beads, long-chain alkylamine-coated	27

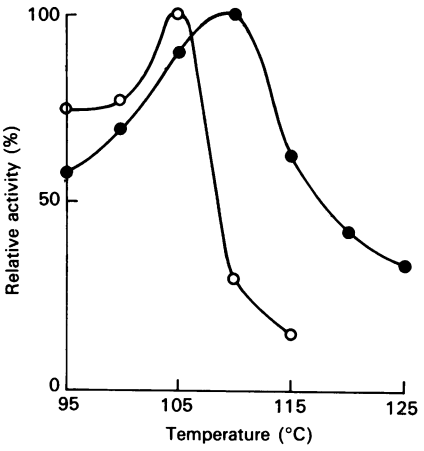

Fig. 1. Temperature–activity profile for free (○) and immobilized (●) xylanase over a 20 min assay

generated a more diverse and complex range of hetero-oligosaccharides than could be resolved with the standards available in this study. In these circumstances it is difficult to be unequivocal in respect of the specificity for naturally occurring substrates.

Immobilization

The uncoated controlled-pore glass beads were the most efficient carrier (Table 2) and were used for all the experiments. Glass beads are a useful support because they are rigid inert structures which can tolerate high temperatures and pressures. Sepharose CL-6B was found to be unsuitable as an immobilization matrix, because reducing sugars were released during incubation at high temperatures.

Michaelis constants

The K_m of purified xylanase for oatspelts xylan was 0.007% (w/v), as determined by means of a double-reciprocal plot. The apparent K_m of the immobilized xylanase for oatspelts xylan was 0.02% (w/v), suggesting that immobilization caused diffusional restrictions due to the size of the substrate molecules, steric hindrance or electrostatic repulsion. Immobilization had little effect on the $V_{max.}$, which was 0.1 and 0.08 unit/ml for free and immobilized xylanase respectively.

pH optimum and pH stability

The pH optimum of the purified xylanase was 5.4, with 50% activity limits at pH 4.2 and pH 6.7. The immobilized enzyme exhibited an optimum pH of 5.0, with 50% activity limits at pH 3.8 and pH 6.6. The shift of pH optimum on immobilization could indicate that functional groups on the enzyme surface were affected by the coupling reaction, or may simply reflect electrostatic charges on the surface of the glass beads.

When immobilized enzyme was incubated at 115 °C for 15 min at pH 4.5, 5.0 and 5.5, and then assayed for activity at pH 5.0 at 80 °C, little effect on thermostability was observed. However, beyond this range, activity was rapidly lost until, at pH 3.0, the immobilized enzyme lost 87% of activity in 15 min. The immobilized xylanase was most stable at pH 5.5, losing only 10% activity after 15 min at 115 °C.

Effects of temperature on activity

Activity of purified free and immobilized xylanase was assayed over the temperature range 60–125 °C. Over a 20 min assay at pH 5.5, maximum activity was observed at 105 and 110 °C for free and immobilized enzyme respectively (Fig. 1). Arrhenius-plot data were determined for the free enzyme from 75 to 105 °C for 5 min assays, during which denaturation was insignificant. The Arrhenius plot was linear up to 95 °C and the activation energy (E_a) of the reaction was calculated to be 68 kJ/mol. Above 95 °C the slope decreased, suggesting some type of conformational change which lowered the E_a to 12 kJ/mol. There have been several reports in the literature that a conformational change occurs in thermostable enzymes well below the temperature at which the catalytic site is destroyed [22–24].

Xylanase thermostability

Initial experiments with concentrated crude supernatant from the growth medium of *Thermotoga* sp. strain FjSS3-B.1 indicated that the xylanase had a half-life of more than 20 min at 100 °C. However, the purified enzyme preparation exhibited half-lives of 90 min, 8 min and < 2 min at 95 °C, 100 °C and 105 °C respectively. The loss of thermostability during purification suggested that the xylanase may be stabilized by effectors present in the crude supernatant or by high protein concentrations.

The thermostability was improved when the purified xylanase was immobilized on to porous glass beads, and the half-life at 105 °C was 10 min. This finding suggests that immobilization results in a more rigid structure of the protein, which is less easily

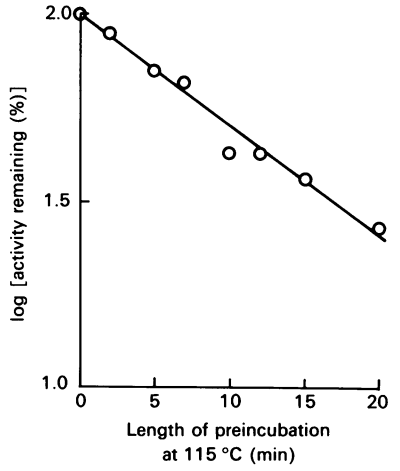

Fig. 2. Stability at 115 °C in the presence of 0.25% (w/v) xylan plus 41% (w/v) sorbitol of immobilized xylanase from *Thermotoga* sp. strain FjSS3-B.1

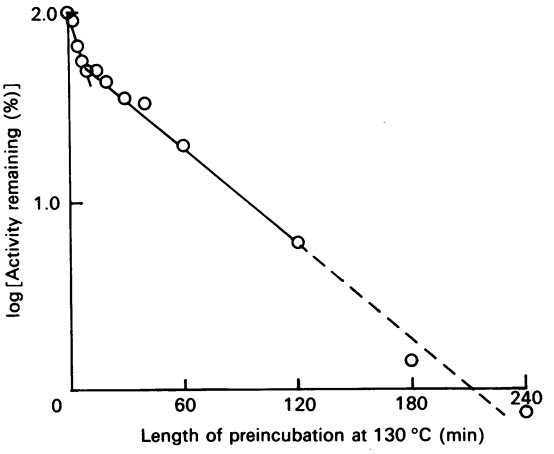

Fig. 3. Stability at 130 °C of immobilized xylanase in molten sorbitol

Table 3. Effect of various additives on the thermostability of immobilized xylanase at 120 °C

Additive	Activity remaining after 10 min at 120 °C (%)
None (0.1 M-citrate buffer, pH 5.5)	1
0.5% (w/v) xylan	1.4
10% (w/v) sorbitol	2
50% (w/v) sorbitol	7
90% (w/v) sorbitol	59
90% (w/v) sorbitol + 0.2% BSA	56
90% (w/v) sorbitol + 0.5% (w/v) xylan	80
100% (w/v) sorbitol	85
100% (w/v) glycerol	21

unfolded and, consequently, more resistant to thermal inactivation. There have been many reports of data obtained by both physical methods and enzyme activity [25–27] or stability assays [28] which illustrated that immobilization resulted in a more rigid structure of the protein. There is also evidence that more rigid proteins are less susceptible to irreversible inactivation processes [29].

Several additives were found to improve the thermostability of both the free and the immobilized xylanase. Incubation of free enzyme in the presence of 0.25% (w/v) oatspelts xylan alone or 0.25% xylan plus 0.2% (w/v) BSA resulted in half-lives at 105 °C of 9 min and 11 min respectively. Immobilized enzyme exhibited a half-life of 2 min at 115 °C, and the thermostability was increased by the addition of 0.25% oatspelts xylan with 0.2% BSA to give a half-life of 5 min at 115 °C. When a combination of 0.25% xylan, 0.2% BSA and 41% (w/v) sorbitol was added to a suspension of immobilized enzyme, the half-life was 10 min at 115 °C. The same half-life was observed with xylan and sorbitol (Fig. 2). Overall, the stabilizing effect of BSA and xylan was cumulative, but that of BSA and sorbitol was not.

The effect of various additives on the thermostability of the immobilized enzyme at 120 °C was also investigated (Table 3). The stabilizing effect of sorbitol increased with concentration, and at 120 °C the solubility of sorbitol is not a limiting factor, because at this temperature sorbitol is molten. The stabilizing effect of high sorbitol concentrations does not seem to be entirely due to a decrease in the water concentration, since 100% glycerol was relatively ineffective. Furthermore, the reaction mixtures using molten sorbitol may have contained up to 1 M residual water. It has been suggested that polyhydric alcohols, such as glycerol, stabilize proteins by decreasing the water activity by forming strong hydrogen bonds with the water. The protein molecules preferentially bind water, and the structure so formed is less able to unfold against the structured glycerol solvent than it would be in water alone [30]. However, it has recently been reported that the interaction of polyols with the active site of the enzyme is another factor in enzyme stabilization [31]. A linear relation was observed between the affinity for the active site of α-amylase and the effect at high concentration of various polyols [32]. Clearly, the mechanism of enzyme stabilization by polyols depends on several factors. Polyols may, via hydrogen bonds, both bind to the enzyme and improve the degree of order of the solvent.

At 130 °C the half-life of the immobilized enzyme in the presence of 90% sorbitol was 1.3 min. In molten sorbitol, 50% activity was lost after 10 min at 130 °C, but the inactivation curve was biphasic and 25% activity remained after 60 min (Fig. 3). The immobilization of the xylanase on to glass beads is essentially a random process where binding to the matrix occurs at a variety of different points on the surface of the enzyme. Consequently, there may be some binding orientations that give rise to both an active enzyme and one that retains stability at 130 °C. In this case about 50% of the enzyme appears to have a half-life of the order of 50 min at 130 °C. Although below 100 °C conformational unfolding leading to loss of the tertiary structure of proteins is one of the main causes of thermoinactivation, there is no obvious theoretical reason why proteins should not be conformationally stable at temperatures well above 100 °C. The upper limit for the thermostability of proteins at neutral pH may be determined by irreversible inactivation processes such as the deamidation of asparagine residues, hydrolysis of peptide bonds at aspartic acid residues, destruction of S–S bonds and formation of incorrect (scrambled) structures [32,34,35]. However, although the above processes are of a general nature, it is possible that enzymes from extreme thermophiles have evolved to have fewer aspartic acid residues and fewer disulphide bonds. Furthermore, the deamidation by heat of asparagine residues is dependent on the nature of the adjacent amino acid and polypeptide-chain flexibility [29].

The xylanase enzyme described here is an extracellular enzyme. However, it does appear that the intracellular enzymes from extreme thermophiles are also very thermostable. Lactate de-

hydrogenase, an intracellular enzyme from *Thermotoga maritima*, exhibited a half-life of 150 min at 90 °C [36]. The u.v.–visible absorption spectrum and electron carrier activity to *Pyrococcus furiosus* hydrogenase was unaffected by 12 h incubation at 95 °C under anaerobic conditions [37]. A thermostable hydrogenase has also been isolated from *Methanococcus jannaschii*, a barophilic extremely thermophilic archaebacterium (D. S. Clark, personal communication). A crude preparation exhibited some residual hydrogenase activity after 1 min at 120 °C when assayed with 50 % (v/w) glycerol and at a pressure of 2 atm. A proteinase purified from a *Desulfurococcus* strain had a half-life of 0.65 min at 115 °C [38]. A half-life of 13.5 min at 100 °C was reported for a pullulanase from *Thermus aquaticus* YT-1 when incubated at pH 7 in the presence of Ca^{2+} ions [39]. Since the stability of these enzymes is not markedly lower than that found for the *Thermotoga* xylanase, it does not seem unreasonable to expect that these enzymes are also capable of being stabilized still further and of displaying activity above 120 °C.

It is not clear what are the upper limits for the thermal stability of proteins. Studies arising from reports of microbial growth at 250 °C [40] (not since confirmed) suggested that at these temperatures the hydrolysis of peptide bonds will be very rapid [41–43]. However, there is a paucity of information on protein stability work carried out at 100–150 °C. A study of enzymes from extremely thermophilic bacteria, such as *Pyrodictium*, which grows at 110 °C [44], may reveal the existence of enzymes with still greater thermostability. This has implications for enzyme applications at high temperatures, as well as suggesting that enzyme stability need not confine the existence of life to 110 °C or below.

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