Chemical modification of a xylanase from a thermotolerant Streptomyces

Evidence for essential tryptophan and cysteine residues at the active site

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Extracellular xylanase produced in submerged culture by a thermotolerant Streptomyces T, growing at 37–50 °C was purified to homogeneity by chromatography on DEAE-cellulose and gel filtration on Sephadex G-50. The purified enzyme has an $M_r$ of 20463 and a pI of 7.8. The pH and temperature optima for the activity were 4.5–5.5 and 60 °C respectively. The enzyme retained 100% of its original activity on incubation at pH 5.0 for 6 days at 50 °C and for 11 days at 37 °C. The $K_m$ and $V_{max}$ values, as determined with soluble larch-wood xylan, were 10 mg/ml and 7.6 x $10^5$ μmol/min per mg of enzyme respectively. The xylanase was devoid of cellulase activity. It was completely inhibited by Hg$^{2+}$ ($2 \times 10^{-6}$ M). The enzyme degraded xylan, producing xylolbiose, xylo-oligosaccharides and a small amount of xylose as end products, indicating that it is an endoxylanase. Chemical modification of xylanase with N-bromosuccinimide, 2-hydroxy-5-nitrobenzyl bromide and p-hydroxymercuribenzoate (PHMB) revealed that 1 mol each of tryptophan and cysteine per mol of enzyme was essential for the activity. Xylan completely protected the enzyme from inactivation by the above reagents, suggesting the presence of tryptophan and cysteine at the substrate-binding site. Inactivation of xylanase by PHMB could be restored by cysteine.

INTRODUCTION

Hemicellulose is one of the major components of ligno-cellulosic biomass and consists largely of xylan. Xylanases (EC 3.2.1.8) catalyse the random hydrolysis of xylosidic bonds in xylan and related xylo-oligosaccharides. In view of their possible application in the paper and pulp industries, information on microbial xylanases has been increasingly forthcoming in recent years (Jurasek & Paice, 1986). Xylanases have been described from a wide range of micro-organisms, especially fungi (Dekker & Richards, 1976). Among the prokaryotes, the information on actinomycete xylanase is largely derived from Streptomyces spp. (Kusakabe et al., 1977; Sreenath et al., 1978; Ishaque & Kluepfel, 1981). There are comparatively fewer reports on production and purification of xylanases from thermophilic micro-organisms (McCarthy et al., 1985; Kluepfel et al., 1986; Morosoli et al., 1986). Stable enzymes active at high temperatures are favourable for increasing reaction rates and possibly for lessening contamination problems. Generally the xylanases are also associated with other activities, such as cellulase and glucose isomerase (Ishaque & Kluepfel, 1981; Kluepfel et al., 1986). There are no published reports so far on microbial xylanases which are not associated with cellulase activity.

Reports on the inhibition of xylanases by different chemical compounds which are specific to certain amino acids are available (Sreenath & Joseph, 1982; Nakajima et al., 1984; Marui et al., 1985). However, the number of amino acid residues essential for activity and their role in the catalytic site have not been investigated. Large active centres containing several subsites appear to be characteristic of the fungal xylanases in general (Biely et al., 1981; Vrsanska et al., 1982; Meagher, 1984; Meagher et al., 1988). However, there is little data correlating the structure and function of the binding-site region of xylanase.

In the present paper we report the isolation of a high-xylanase-producing thermotolerant Streptomyces T, that is free from cellulase activity. On the basis of the chemical modification of the purified enzyme, our results have provided the first evidence for the involvement of tryptophan and cysteine residues at the active site of xylanase.

MATERIALS AND METHODS

Materials

N-Bromosuccinimide (NBS), N-ethylmaleimide, diethyl pyrocarbonate, 2-hydroxy-5-nitrobenzyl bromide (HNBB), p-hydroxymercuribenzoate (PHMB), 5,5′-dithiobis-(2-nitrobenzoic acid) (DTNB), phenylglyoxal, butane-2,3-dione, iodoacetamide, N-acetylimidazole, β-alanine, 3,5-dinitrosalicylic acid (DNS), p-nitrophenyl β-D-xyloside, molecular-mass markers, NNNN′-tetramethylethylene diamine (TEMED), Coomassie Blue G-250 and R-250, DEAE-cellulose, NNN′-methylenebis-acrylamide and CM-cellulose C8758 were purchased from Sigma. The suppliers of the following chemicals are indicated in parentheses: SDS (Koch–Light Labora-

Abbreviations used: NBS, N-bromosuccinimide; HNBB, 2-hydroxy-5-nitrobenzyl bromide; PHMB, p-hydroxymercuribenzoate; DTNB, 5,5′-dithiobis-(2-nitrobenzoic acid); DNS, 3,5-dinitrosalicylic acid; TEMED, NNNN′-tetramethylethylene diamine.

* To whom correspondence and reprint requests should be sent.
Chemicals, yeast of the New Unit, G-50 were produced at 50 °C for 48 h, incubated actinomycetes at 50 °C for 48 h, incubated in Baroda, India; the New Unit, 50 produces cloth autoclaved (5 °C for 48 h, incubated actinomycetes Baroda, India; designated was Enzyme production containing further that the source. Vegetative enzyme source. Purification Enzyme (9000 g; buffer, 10600 units) was through filtered. The filtrate was buffer. The filtrate and enzyme source. Whatamn no. 1 were determined by incubating 1 ml of reaction mixture, containing suitably diluted enzyme, with 0.5 ml of CM-cellulose (1 %) or filter paper (25 mg) in 50 mm-acetate buffer, pH 5.0, at 50 °C for 30 or 60 min respectively. The reducing sugar formed was determined by the DNS method described above. Proteinase activity was determined by Kunitz's (1947) method.

Protein was determined by the method of Lowry et al. (1951), with bovine serum albumin as standard. Electrophoresis at pH 4.3 was performed as described by Maurer (1971), with 7.5% (w/v) acrylamide gel.

SDS/polyacrylamide-gel electrophoresis was carried out as described by Laemmli (1970), with albumin (Mₐ, 66000), ovalbumin (45000), pepsin (34700), trypsinogen (24000), β-lactoglobulin (18400) and lysozyme (14300) as reference proteins.

Mₐ values were also determined by gel filtration on Sephadex G-50 and Bio-Gel P-10.

Suitably diluted xylanase was incubated with different amounts (3–15 mg) of xylan under the assay conditions given. Kₘ and Vₘₐₓ were determined from Lineweaver–Burk plots.

Isoelectric focusing in thin polyacrylamide gels was carried out by the method of Vesterberg (1972) over the pH range 3.5–10.0.

Free thiol groups in the enzyme were determined by titrating enzyme against PHMB (Boyer, 1954) and DTNB (Ellman, 1959).

Xylan degradation products

Xylanase (1.0 unit) was incubated with xylan (10 mg) in 20 mm-acetate buffer, pH 5.0, for different periods of time. The end products formed were analysed by paper chromatography in the solvent system butanol/acetic acid/water (3:1:1, by vol.) by the method of Trevelyan et al. (1950).

Synergism with β-xilosidas

Xylanase (1.0 unit) and β-xilosidase from Aspergillus niger (0.07 unit) were mixed with 10 mg of xylan in estimated for xylanase activity. The active fractions were pooled, dialysed against water and concentrated by freeze-drying (Step IV).

Analytical methods

A 2 g portion of xylan was suspended in 100 ml of 50 mm-sodium acetate buffer, pH 5.0, and was stirred for 12–16 h. The insoluble fraction (about 50 %) was removed by centrifugation and the soluble fraction was used for xylanase assay.

Xylanase was assayed by mixing a 0.5 ml aliquot of appropriately diluted enzyme with 0.5 ml of 1%, xylan and incubating at 60 °C for 30 min (Mandels & Weber, 1969). The reducing sugar released was determined by the DNS method with D-xylose as standard (Miller, 1959).

β-Xylosidase was estimated as described by Kluepfel & Ishaque (1982) with p-nitrophenyl β-D-xyloside as substrate by determining the p-nitrophenol liberated by the enzyme action at 40 °C after 30 min.

The unit of xylanase or xylosidase was defined as that amount of enzyme which produces 1 μmol of xylose or p-nitrophenol/min from xylan or p-nitrophenyl β-D-xyloside respectively under the given assay conditions. Activity towards CM-cellulose and filter paper (Whatman no. 1) were determined by incubating 1 ml of reaction mixture, containing suitably diluted enzyme, with 0.5 ml of CM-cellulose (1 %) or filter paper (25 mg) in 50 mm-acetate buffer, pH 5.0, at 50 °C for 30 or 60 min respectively. The reducing sugar formed was determined by the DNS method described above. Proteinase activity was determined by Kunitz's (1947) method.

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0.5 ml of 50 mM-acetate buffer, pH 5.0. The reaction was carried out at 37 °C for 20 h. The control with only xylanase was also run under identical conditions. The percentage hydrolysis was determined by measuring the reducing sugar.

**Reaction with different chemical modifiers**

A 0.5 ml portion of enzyme (10 μg/ml) was incubated in a total volume of 2.5 ml with various concentrations of modifier in appropriate buffer. Control tubes containing enzyme only or inhibitor only were incubated under identical conditions. Aliquots (0.5 ml) were removed at 10, 20, 30 and 40 min for measurement of residual enzyme activity.

**Titration with NBS**

Oxidation of tryptophan residues by NBS was carried out in two cuvettes: one containing xylanase (2.72 × 10^{-4} M) in 50 mM-acetate buffer, pH 4.5, and another containing buffer. Successive 10 μl aliquots of NBS (1 × 10^{-4} M) were added to the sample as well as to the reference cuvette and absorbance at 280 nm was measured. After each addition of NBS, the number of tryptophan residues oxidized (Δn) per mol of enzyme was calculated from the equation (Witkop, 1961; Spande & Witkop, 1967):

\[
\Delta n = \frac{1.31 \Delta A_{280}}{5500 \times \text{molarity of enzyme}}
\]

where ΔA_{280} is the decrease in absorbance at 280 nm, 1.31 is an empirical factor based upon oxidation of model tryptophan peptides by NBS (Patchornik et al., 1958) and 5500 is the molar absorption coefficient for tryptophan at 280 nm. Simultaneously portions of the reaction mixture were assayed for xylanase activity.

**Substrate-protection studies**

A 0.1 ml portion of enzyme (1 μg) in buffer was added to different amounts of xylan (0.1–5 mg) in a total volume of 0.5 ml before the addition of NBS (4 μM) or PHMB (5 μM). The reaction mixture was incubated at 25 °C for 10 min. The activity of the enzyme was determined as usual by adding the remaining amount of xylan.

For different concentrations of HNBB (2.5 mM, 4 mM and 8 mM), percentage inhibition of xylanase was determined. For every HNBB concentration the amount of xylan needed to give 100% protection was determined by the above procedure.

**Re-activation of xylanase after modification by PHMB**

The enzyme (1 μg) was incubated with PHMB (2 μM) in 50 mM-acetate buffer, pH 6.0, in a volume of 0.25 ml at 25 °C. At different time intervals, residual activity was determined. Simultaneously the incubation mixtures (0.25 ml) were also transferred to 0.25 ml of 50 mM-cysteine and incubated at 25 °C for 20 min. The enzyme activity was determined in the usual way by adding the substrate and incubating at 60 °C for 30 min.

Alternatively, regain of xylanase activity completely inactivated by PHMB (5 μM) was determined by transferring aliquots of inactivated enzyme in different concentrations of cysteine and incubating at 25 °C for 20 min.

**RESULTS AND DISCUSSION**

**Characteristics of strain T7**

The isolate is an aerobic *Streptomyces* which forms greyish sporulating colonies on wheat bran/yeast extract/agar after incubation for 3 days at 50 °C. When grown on agar plates containing 1% xylan, a distinct clearing was observed, indicating the hydrolysis of xylan and extracellular secretion of xylanase. The optimum temperature for the growth of the *Streptomyces T7* was over the range 45–50 °C; the strain was also able to grow at 37 °C, but it did not grow above 50 °C, indicating that it is a thermotolerant culture. Fig. 1 shows the xylanase production at different incubation temperatures. The production of enzyme was maximum (70 units/ml) at 50 °C after 72 h of cultivation when 5% wheat bran was used. The strain did not show detectable intra-or extra-cellular β-xylanase activity, extracellular activity against CM-cellulose or filter paper, or proteinase activity at pH 7.0 or 10.0.

The *Streptomyces T7* is similar to *Streptomyces lividans*, which was reported by Kluepfel et al. (1986) to grow over the temperature range 18–46 °C. The optimum temperatures for growth and enzyme production were 29 and 40 °C respectively. Xylanases from *S. lividans* (Kluepfel et al., 1986) and *S. flavogriseus* (Ishaque & Kluepfel, 1981) were reported to be associated with cellulase and glucose isomerase activities respectively.

**Enzyme purification**

Table 1 summarizes the results for the purification of xylanase. The enzyme was purified 41-fold over the culture supernatant. The xylanase from *S. lividans* has been purified 33-fold over the culture supernatant (Morosoli et al., 1986), whereas that from *S. xylophagus* was purified 276-fold (specific activity 581 units/mg of protein) (Iizuka & Kawaminami, 1965). The *Streptomyces T7* contains a single component of xylanase. The purified enzyme showed a single band on gel electrophoresis in the presence or absence of SDS. The xylanases from mesophilic *Streptomyces* have also been

![Fig. 1. Effect of temperature on xylanase production](image-url)

The organism was grown on the basal medium containing 2% wheat bran. The samples were removed periodically and the culture filtrate was examined for xylanase activity.

- O, 28 °C; Δ, 37 °C; ●, 45 °C; □, 50 °C.
Table 1. Purification of *Streptomyces* xylanase

<table>
<thead>
<tr>
<th>Step</th>
<th>Fraction</th>
<th>Total volume (ml)</th>
<th>Total protein (mg)</th>
<th>Total activity (units)</th>
<th>Specific activity (units/mg)</th>
<th>Purification (fold)</th>
</tr>
</thead>
<tbody>
<tr>
<td>I</td>
<td>Culture filtrate</td>
<td>400</td>
<td>1200</td>
<td>12000</td>
<td>10.0</td>
<td>1.00</td>
</tr>
<tr>
<td>II</td>
<td>Ethanol precipitation</td>
<td>80</td>
<td>256</td>
<td>10600</td>
<td>41.4</td>
<td>4.14</td>
</tr>
<tr>
<td>III</td>
<td>DEAE-cellulose chromatography</td>
<td>12</td>
<td>116</td>
<td>7462</td>
<td>64.4</td>
<td>6.44</td>
</tr>
<tr>
<td>IV</td>
<td>Sephadex G-50</td>
<td>3</td>
<td>1.95</td>
<td>805</td>
<td>412.8</td>
<td>41.30</td>
</tr>
</tbody>
</table>

Table 2. Properties of purified xylanase

<table>
<thead>
<tr>
<th>Parameter</th>
<th>Value</th>
</tr>
</thead>
<tbody>
<tr>
<td>Optimum pH</td>
<td>4.5–5.5</td>
</tr>
<tr>
<td>Optimum temperature</td>
<td>60 °C</td>
</tr>
<tr>
<td>$K_m$</td>
<td>10 mg/ml</td>
</tr>
<tr>
<td>$V_{max}$</td>
<td>7.6 mmol/min per mg</td>
</tr>
<tr>
<td>pl</td>
<td>7.8</td>
</tr>
<tr>
<td>$M_r$ by: SDS/PAGE*</td>
<td>21880</td>
</tr>
<tr>
<td>Bio-Gel P-10</td>
<td>19230</td>
</tr>
<tr>
<td>Sephadex G-50</td>
<td>20180</td>
</tr>
</tbody>
</table>

* Abbreviation: PAGE, polyacrylamide-gel electrophoresis.

shown to consist of only one xylanase component (Kusakabe *et al.*, 1977; Nakajima *et al.*, 1984).

Properties

Table 2 shows the physico-chemical properties of the purified enzyme. The activity was maximum at 60 °C over the pH range 4.5–5.5. At 60 °C the half-life of the enzyme was 30 min. The enzyme retained full activity on incubation at 50 °C for 6 days and at 37 °C for 11 days. Morosoli *et al.* (1986) reported a xylanase from *S. lividans* which was stable at pH 6.0 for 24 h at 37 °C.

Titrations with PHMB and DTNB showed the presence of 3.5 thiol groups per molecule of enzyme. Among the metal ions tested, Hg$^{2+}$ completely inhibited the enzyme activity, indicating that thiol-containing amino acids may be involved in the activity.

Degradation of xylan

The major end products of xylan hydrolysis by the *Streptomyces* T$_7$ xylanase were xylobiose and xyloligosaccharides (Xyl$_3$–Xyl$_6$). Very little xylose was produced even after 16 h, indicating that it is an endoxylanase. When xylanase was mixed with β-xylanosidase, the end product was mainly xylose, and the hydrolysis increased from 43 to 60%, owing to the synergistic action of xylanase and β-xylanosidase.

Effect of chemical modifiers

Table 3 indicates the effect of various modifiers on xylanase activity. Complete inhibition by NBS, HNBB and PHMB showed that tryptophan and cysteine residues are modified. Complete inhibition by PHMB but less

Table 3. Effect of chemical inhibitors on xylanase

<table>
<thead>
<tr>
<th>Chemical</th>
<th>[Inhibitor] (mm)</th>
<th>Inhibition (%)</th>
<th>Incubation buffer (50 mm)</th>
</tr>
</thead>
<tbody>
<tr>
<td>NBS</td>
<td>1.0</td>
<td>100</td>
<td>Sodium acetate buffer, pH 4.5</td>
</tr>
<tr>
<td>HNBB</td>
<td>10.0</td>
<td>100</td>
<td>Sodium acetate buffer, pH 4.5</td>
</tr>
<tr>
<td>Diethyl pyrocarbonate N-Ethylmaleimide</td>
<td>10.0</td>
<td>0</td>
<td>Potassium phosphate buffer, pH 7.0</td>
</tr>
<tr>
<td>Iodoacetamide</td>
<td>5.0</td>
<td>30</td>
<td>Sodium acetate buffer, pH 6.0</td>
</tr>
<tr>
<td>Phenylglyoxal</td>
<td>10.0</td>
<td>0</td>
<td>Tris/HCl, pH 8.0</td>
</tr>
<tr>
<td>PHMB</td>
<td>1.0</td>
<td>100</td>
<td>Sodium acetate buffer, pH 6.0</td>
</tr>
<tr>
<td>Phenylmethanesulphonyl fluoride N-Acetylimidazole</td>
<td>10.0</td>
<td>0</td>
<td>Potassium phosphate buffer, pH 7.0</td>
</tr>
<tr>
<td>Butane-2,3-dione</td>
<td>10.0</td>
<td>0</td>
<td>Sodium borate buffer, pH 8.0</td>
</tr>
</tbody>
</table>
inhibition by iodoacetamide may be due to the fact that, at pH 6.0, there is a very small proportion of the ionized form of cysteine (−CH₂S⁻), which is the reactive nucleophile. Inhibition by Hg²⁺, NBS and PHMB has been reported for xylanases from several Streptomyces species (Sreenath & Joseph, 1982; Nakajima et al., 1984; Marui et al., 1985).

Plots of percentage residual activity as a function of time at various concentrations of NBS (Fig. 2), HNBB (Fig. 3) and PHMB (Fig. 4) indicate that the inactivation process exhibits pseudo-first-order kinetics with respect to time at any fixed concentration of the inhibitor. Applying the analysis described by Levy et al. (1963), the pseudo-first-order rate constants were calculated from the slope of the plots of logarithm of the residual activity against the time of reaction. The order of the reaction was estimated from the slopes of the plots of log(pseudo-first-order rate constant) against log(inhibitor concentration). These graphs (insets to Figs. 2, 3 and 4) indicated that the loss of enzyme activity resulted from reaction of only one tryptophan (Figs. 2 and 3) or cysteine residue (Fig. 4) per molecule of enzyme.

**Titration with NBS**

Fig. 5 shows the effect of NBS on enzyme activity.

After each addition of NBS, there was a progressive decrease in absorption at 280 nm. For complete inactivation of the enzyme, 3.6 mol of NBS were required per mol of enzyme. The number of tryptophan residues oxidized per molecule of enzyme were found to be 2.2 (Fig. 6). This usually gives the number of residues modified but not the number of residues essential for activity.

**Protection by substrate against inactivation**

A 1 mg portion of substrate was needed to give 100% protection against inactivation by NBS and PHMB. Protection by the substrate indicated the presence of essential tryptophan and cysteine residues at the substrate-binding site.

The xylanase was incubated with different concentrations of HNBB (2.5–8 mm). The amount of xylan needed to give 100% protection increased from 2.5 to 7.5 mg as the extent of inhibition increased. This clearly showed the presence of essential tryptophan residue at the substrate-binding region of the xylanase. It is surprising that 100% protection by substrate is obtained at a concentration less than the $K_m$. This may be attributed to the heterogeneity of xylan, which results in an 'apparent' value for the $K_m$.

The catalytic mechanism of two other hydrolytic...
Fig. 4. Effect of PHMB on xylanase activity

Enzyme (5 μg) was incubated with PHMB (□, 0 μM; ○, 0.25 μM; ●, 0.5 μM; △, 1.0 μM; ×, 2.0 μM). The inset shows a plot of the logarithm of the pseudo-first-order rate constant of PHMB inactivation against the logarithm of PHMB concentration.

Fig. 5. Activity and absorbance changes of xylanase as a function of molar excess of NBS

Aliquots (10 μl) of NBS (1×10^{-4} M) were added successively to the enzyme (2.72×10^{-4} M). After each addition the residual activity (○) and the decrease in absorption at 280 nm (△) were measured.

Fig. 6. Titration of NBS with xylanase

Oxidation of tryptophan residues in xylanase was carried out with stepwise addition of NBS to the enzyme, as described in Fig. 5. The number of tryptophan residues oxidized was determined as described in the Materials and methods section.

Enzymes, namely lysozyme and cellulase, which are functionally related to xylanase, have been delineated in structural detail at the molecular level (Imoto et al., 1972; Yaguchi et al., 1983). Tryptophan residues have been shown to be involved in the binding of substrate to these enzymes (Hurst et al., 1977; Clarke, 1987). The present results add further support to these earlier findings and suggest a relationship between xylanase, lysozyme and cellulase.

Re-activation of xylanase after modification by PHMB

The progressive loss of activity as a function of time when the enzyme was treated with PHMB and its re-activation by cysteine were studied. The enzyme, which was completely inactivated by PHMB, was re-activated fully with 50 mm-cysteine, indicating a competitive displacement of PHMB by the high concentration of thiol (Means & Feeney, 1971).

Involvement of cysteine residues at the active site of cellulase or lysozyme has not hitherto been reported.

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