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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_7 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 7.6×10^3 μmol/min per mg of enzyme respectively. The xylanase was devoid of cellulase activity. It was completely inhibited by Hg^{2+} (2×10^{-6} M). The enzyme degraded xylan, producing xylobiose, 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 were 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_7 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, *NNN′N′*-tetramethylethylenediamine (TEMED), Coomassie Blue G-250 and R-250, DEAE-cellulose, *NN′*-methylenebisacrylamide 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, *NNN′N′*-tetramethylethylenediamine.

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tories); yeast extract (Difco); peptone (Biochemicals Unit, New Delhi, India); larch-wood xylan and cysteine (Fluka); ultrafiltration membranes (Amicon); Sephadex G-50 (Pharmacia); Ampholines (LKB). All the other chemicals used were of analytical grade. Xylo-oligosaccharides were kindly given by Professor P. J. Reilly, Department of Chemical Engineering, Iowa State University, Ames, IA 50011, U.S.A. Wheat bran was purchased locally.

Isolation of the strain

From humus-rich soil collected at Nandesari (near Baroda, India) an actinomycete strain was isolated by plating out a soil sample on a medium containing wheat bran (5%) and yeast extract (1%). The wheat bran was autoclaved in water, followed by extraction through muslin cloth to remove most of the starch. When incubated at 50 °C for 48 h, several colonies of bacteria and actinomycetes developed which were screened for xylanase activity in submerged culture. One of them, which produced relatively high xylanase activity and a larger clearance zone on xylan/yeast extract/agar plate, was designated as '*Streptomyces* T_7' and selected for further studies.

Enzyme production

Enzyme was produced in 250 ml Erlenmeyer flasks containing 50 ml of medium (Ishaque & Kluepfel, 1981), except that the amount of $(NH_4)_2SO_4$ was increased five times and 5% (w/v) wheat bran was used as a carbon source. Vegetative inoculum was used to inoculate experimental media. The inoculum was grown in the same medium but containing 1% wheat bran. The flasks were incubated at 50 °C on a rotary shaker at 200 rev./min. The experimental flasks contained 5% wheat bran as carbon source and were cultivated for 72 h. The cells and solid residues were removed from the culture broth by centrifugation and the clear supernatant used as an enzyme source.

Enzyme purification

All steps were carried out at 4 °C unless otherwise mentioned.

The culture filtrate (400 ml) (Step I) was concentrated by precipitating the enzyme with 3 vol. of chilled distilled ethanol. The precipitate was recovered by centrifugation (9000 *g*; 30 min) and dried under vacuum and stored at −10 °C until further use. The precipitate (4 g, 10600 units) was dissolved in 80 ml of 10 mM-sodium phosphate buffer, pH 8.0. The undissolved solid particles from the precipitate were separated by centrifugation and the clear supernatant (Step II) was further purified on DEAE-cellulose in a batchwise treatment. A 260 ml portion of DEAE-cellulose (40 mg/ml, equilibrated with 10 mM-sodium phosphate buffer, pH 8.0) was suspended in 80 ml of enzyme solution (Step II) for 30 min. The slurry was filtered through Whatman no. 1 filter paper. The filtrate was collected and the DEAE-cellulose 'cake' was resuspended twice, each time with 20 ml of the same buffer. The filtrate and washings were pooled and ultrafiltered through an Amicon UM 10 membrane to concentrate the sample to 12 ml (Step III). A 1 ml portion of this enzyme (9 mg, 621 units) was applied to a Sephadex G-50 column (1.5 cm × 110 cm) equilibrated with 50 mM-potassium phosphate buffer, pH 7.2. The fractions were collected at a rate of 2 ml/10 min and 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.

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 (M_r 66000), ovalbumin (45000), pepsin (34700), trypsinogen (24000), β-lactoglobulin (18400) and lysozyme (14300) as reference proteins.

M_r 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. K_m and $V_{max.}$ 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 Trevelyn *et al.* (1950).

Synergism with β-xylosidase

Xylanase (1.0 unit) and β-xylosidase from *Aspergillus niger* (0.07 unit) were mixed with 10 mg of xylan in

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^{-5} 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 T_7

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 clearance was observed, indicating the hydrolysis of xylan and extracellular secretion of xylanase. The optimum temperature for the growth of the *Streptomyces* T_7 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-xylosidase activity, extracellular activity against CM-cellulose or filter paper, or proteinase activity at pH 7.0 or 10.0.

The *Streptomyces* T_7 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* T_7 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

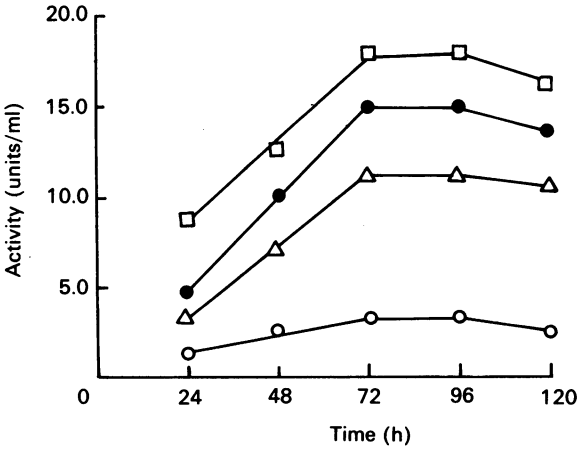

Fig. 1. Effect of temperature on xylanase production

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. ○, 28 °C; △, 37 °C; ●, 45 °C; □, 50 °C.

Table 1. Purification of *Streptomyces* xylanase

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

Table 2. Properties of purified xylanase

Parameter	Value
Optimum pH	4.5–5.5
Optimum temperature	60 °C
K_m	10 mg/ml
$V_{max.}$	7.6 mmol/min per mg
pI	7.8
M_r by:	
SDS/PAGE*	21880
Bio-Gel P-10	19230
Sephadex G-50	20180

* 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.

Titration 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 xylo-oligosaccharides (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-xylosidase, the end product was mainly xylose, and the hydrolysis increased from 43 to 60%, owing to the synergistic action of xylanase and β-xylosidase.

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

The enzyme was incubated with the inhibitor at 25 °C for 10 min before the addition of substrate.

Chemical	[Inhibitor] (mM)	Inhibition (%)	Incubation buffer (50 mM)
NBS	1.0	100	Sodium acetate buffer, pH 4.5
HNBB	10.0	100	Sodium acetate buffer, pH 4.5
Diethyl pyrocarbonate	10.0	0	Potassium phosphate buffer, pH 7.0
N-Ethylmaleimide	2.0	50	Sodium acetate buffer, pH 6.0
Iodoacetamide	5.0	30	Sodium acetate buffer, pH 6.0
Phenylglyoxal	10.0	0	Tris/HCl, pH 8.0
PHMB	1.0	100	Sodium acetate buffer, pH 6.0
Phenylmethanesulphonyl fluoride	10.0	0	Potassium phosphate buffer, pH 7.0
N-Acetylimidazole	10.0	0	Potassium phosphate buffer, pH 7.0
Butane-2,3-dione	10.0	0	Sodium borate buffer, pH 8.0

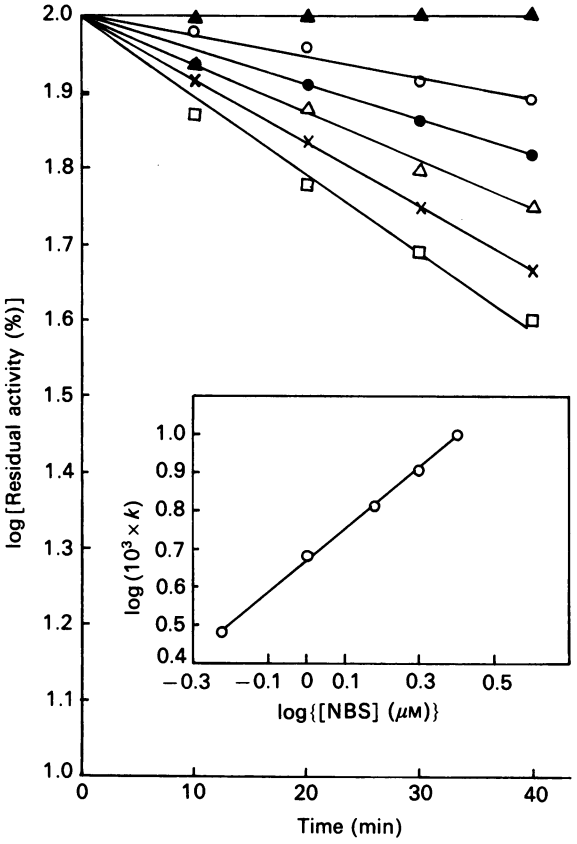

Fig. 2. Kinetics of inactivation of xylanase by NBS

The enzyme (5 μg) was incubated with NBS (▲, 0 μM; ○, 0.6 μM; ●, 1.0 μM; △, 1.5 μM; ×, 2.0 μM; □, 2.5 μM). The inset shows the apparent order of reaction with respect to reagent concentration. The logarithm of observed pseudo-first-order rate constants (k) calculated from Fig. 2 was plotted against the logarithm of the inhibitor concentration.

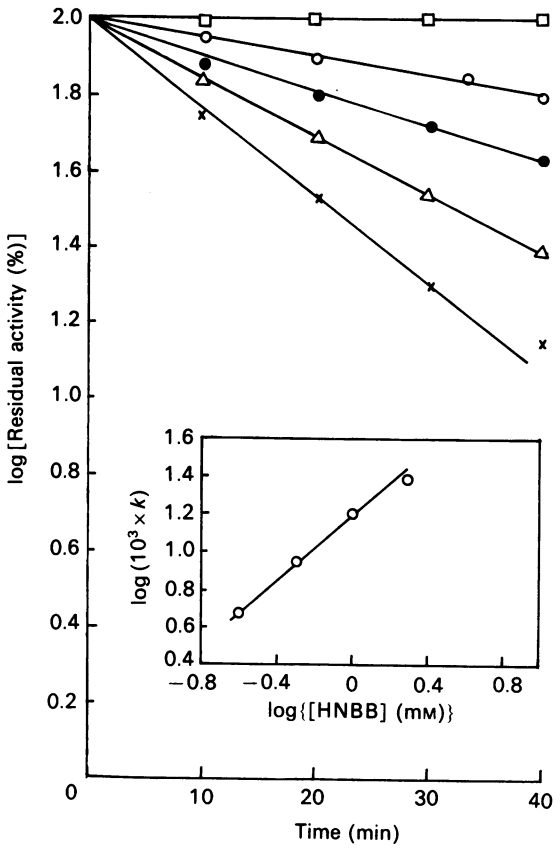

Fig. 3. Inactivation of xylanase by HNBB

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

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 ($-CH_2-S^-$), which is the reactive nucleophile. Inhibition by Hg^{2+}, 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 again confirmed 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

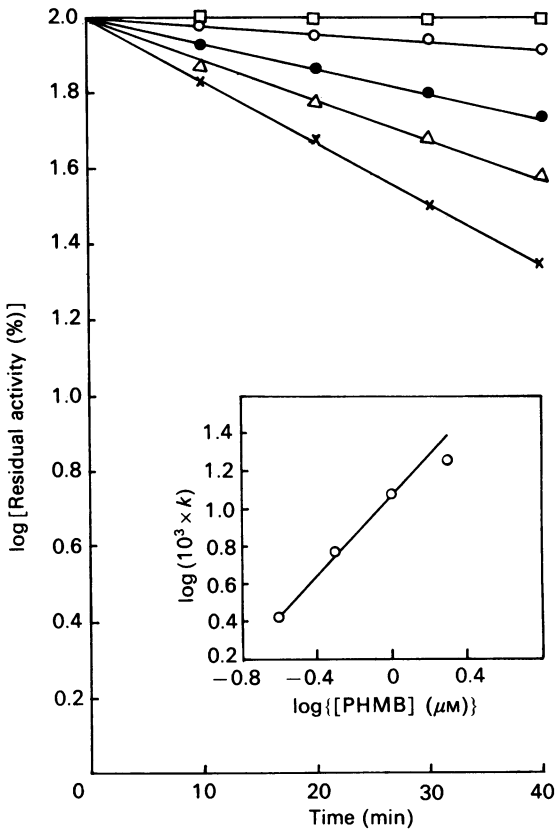

Fig. 4. Effect of PHMB on xylanase activity

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 psuedo-first-order rate constant of PHMB inactivation against the logarithm of PHMB concentration.

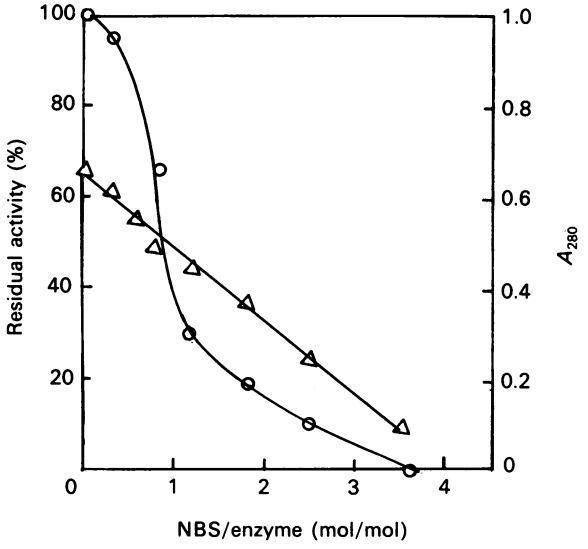

Fig. 5. Activity and absorbance changes of xylanase as a function of molar excess of NBS

Aliquots (10 μl) of NBS (1×10^{-4} M) were added successively to the enzyme (2.72×10^{-5} M). After each addition the residual activity (○) and the decrease in absorption at 280 nm (△) were measured.

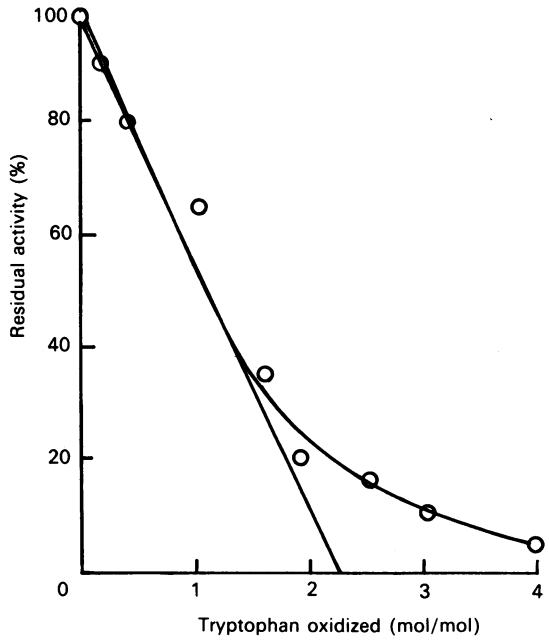

Fig. 6. Titration of NBS with xylanase

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, has 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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REFERENCES

Biely, P., Kratky, Z. & Vrsanska, M. (1981) Eur. J. Biochem. **119**, 559–564
Boyer, P. D. (1954) J. Am. Chem. Soc. **76**, 4331–4337
Clarke, A. J. (1987) Biochim. Biophys. Acta **912**, 424–431
Dekker, R. F. H. & Richards, G. N. (1976) Adv. Carbohydr. Chem. Biochem. **32**, 278–352
Ellman, G. L. (1959) Arch. Biochem. Biophys. **82**, 72–77

Hurst, P. L., Sullivan, P. A. & Shepherd, M. G. (1977) Biochem. J. **167**, 549–556
Iizuka, H. & Kawaminami, T. (1965) Agric. Biol. Chem. **29**, 520–524
Imoto, T., Johnson, L. N., North, A. C. T., Phillips, D. C. & Ruplex, J. A. (1972) Enzymes 3rd Ed. **7**, 666–868
Ishaque, M. & Kluepfel, D. (1981) Biotechnol. Lett. **3**, 481–486
Jurasek, L. & Paice, M. (1986) Chemtech **16**, 360–365
Kluepfel, D. & Ishaque, M. (1982) Dev. Ind. Microbiol. **23**, 389–395
Kluepfel, D., Shareck, F., Mondou, F. & Morosoli, R. (1986) Appl. Microbiol. Biotechnol. **24**, 230–234
Kunitz, M. (1947) J. Gen. Physiol. **30**, 291–310
Kusakabe, I., Yasui, T. & Kobayashi, T. (1977) Nippon Nogei Kagaku Kaishi **51**, 669–679
Laemmli, U. K. (1970) Nature (London) **227**, 680–685
Levy, H. M., Leber, P. D. & Ryan, E. M. (1963) J. Biol. Chem. **238**, 3654–3659
Lowry, O. H., Rosebrough, N. J., Farr, A. L. & Randall, R. J. (1951) J. Biol. Chem. **193**, 265–275
Mandels, M. & Weber, J. (1969) Adv. Chem. Ser. **95**, 391–414
Marui, M., Nakanishi, K. & Yasui, T. (1985) Agric. Biol. Chem. **49**, 3399–3407
Maurer, H. R. (1971) in Disc Electrophoresis and Related Techniques of Polyacrylamide Gel Electrophoresis (Maurer, H. R., ed.), pp. 32–110, Walter de Gruyter, Berlin and New York
McCarthy, A. J., Peace, E. & Broda, P. (1985) Appl. Microbiol. Biotechnol. **21**, 238–244
Meagher, M. M. (1984) M.S. Thesis, Iowa State University, Ames
Meagher, M. M., Tao, B. Y., Chow, J. M. & Reilly, P. J. (1988) Carbohydr. Res. **173**, 273–283
Means, G. E. & Feeney, R. E. (1971) in Chemical Modification of Proteins (Means, G. E. & Feeney, R. E., eds.), pp. 198–204, Holden–Day, San Francisco
Miller, G. L. (1959) Anal. Chem. **31**, 426–428
Morosoli, R., Betrand, J. L., Mondou, F., Shareck, F. & Kluepfel, D. (1986) Biochem. J. **239**, 587–592
Nakajima, T., Tsukamota, K. I., Watanabe, T., Kainuma, K. & Matsuda, K. (1984) J. Ferment. Technol. **62**, 269–276
Patchornik, A., Lawson, W. B. & Witkop, B. (1958) J. Am. Chem. Soc. **80**, 4747–4748
Spande, T. F. & Witkop, B. (1967) Methods Enzymol. **11**, 498–506
Sreenath, H. K. & Joseph, R. (1982) Folia Microbiol. (Prague) **27**, 107–115
Sreenath, H. K., Joseph, R. & Murthy, V. S. (1978) Folia Microbiol. (Prague) **23**, 299–303
Trevelyn, W. E., Procter, P. D. & Harrison, J. S. (1950) Nature (London) **166**, 444–445
Vesterberg, O. (1972) Biochim. Biophys. Acta **257**, 11–19
Vrsanska, M., Gorbacheva, I. V., Kratky, Z. & Biely, P. (1982) Biochim. Biophys. Acta **704**, 114–122
Witkop, B. (1961) Adv. Protein Chem. **16**, 221–314
Yaguchi, M., Roy, C., Rollin, C. F., Paice, M. G. & Jurasek, L. (1983) Biochem. Biophys. Res. Commun. **116**, 408–411

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