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Involvement of a Lysine Residue in the Active Site of a Thermostable Xylanase from *Thermomonospora* sp.

Sudeep P. George, Absar Ahmad, and Mala B. Rao[1]
Division of Biochemical Sciences, National Chemical Laboratory, Pune 411 008, India

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A highly thermostable xylanase (Xyl I) produced by *Thermomonospora* sp. was purified to homogeneity and was classified as a family 10 xylanase based on its molecular weight (38,000 Da) and isoelectric point (4.1). K2d analysis showed that the secondary structure of Xyl I was made up of 38% α-helix and 10% β-sheet. The optimal temperature for the activity of Xyl I was 80°C. Xyl I was highly thermostable with half-lives of 86, 30, and 15 min at 80, 90, and 100°C respectively. Xyl I was stable in an expansive pH range of 5 to 10 with more than 75% residual activity. Our present investigation using *o*-phthalaldehyde (OPTA) as the chemical initiator for fluorescent chemoaffinity labeling and trinitrobenzenesulphonic acid (TNBS) as chemical modifier have revealed the presence of a single lysine residue in the active site of Xyl I. The high p*K* value for the basic limb of the pH profile reflects the ionization of a lysine residue. The higher K_m values and similar k_{cat} values of the TNBS modified enzyme in comparison to native enzyme and the substrate protection against OPTA and TNBS, suggested the presence of the lysine residue in the substrate-binding site.

Key Words: **lysine; purification; *Thermomonospora;* thermostable; xylanase I.**

Xylan is the main component of hemicellulose and consists of 1,4-glycosidically linked β-D-xylose with branches containing xylose, other pentoses and uronic acids. 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 (1). Xylanases have possible application in waste treatment, fuel and chemical production, paper manufacture and deinking (2). Thermally stable enzymes can be used at high temperatures for efficient hydrolysis of substrates and may be effectively recycled. The major interest of thermostable xylanases in pulp bleaching, is that it helps in reducing the kappa number and increasing the brightness of pulp. The requirement of chlorine as a bleaching agent could be substantially reduced and it could lead to the development of environmentally friendly technologies (3). There are very few reports of xylanases that are active and stable at both alkaline pH and elevated temperatures (4). Most of the commercial xylanases showing activity in the alkaline range are only active at 55°C (5).

Investigations involving chemical modification of an enzyme can potentially yield insights into structure function relationships. In comparison to the extensive studies on the biotechnological applications of xylanases, inadequate information is available concerning the mechanism of action of xylanases. The mechanism of action of xylanases is similar to the double displacement mechanism of lysozyme (6). The role of carboxyl groups (7, 8) in catalysis and aromatic residues such as tryptophan (9) and tyrosine (10) in substrate binding of xylanases are well documented. Crystal structure of family 10 xylanases have shown the presence of histidine in the active site (11, 12). Recently Charnock *et al.* have suggested the possible role of a lysine residue in positioning the substrate in the active site of a xylanase from *Pseudomonas fluorescence* (13). In the present paper, for the first time we have demonstrated the presence of an essential lysine in the active site of a thermostable xylanase from *Thermomonospora* sp. by fluorescent labeling and chemical modification. We have also described the purification and biochemical properties of this remarkably thermostable xylanase.

MATERIALS AND METHODS

Materials. Protein solutions were concentrated in an ultrafiltration cell, with UM-10 membranes (Amicon Corp., Lexington, MA). Oat spelt xylan, TNBS (2,4,6-trinitrobenzenesulfonic acid) and OPTA (*o*-phthalaldehyde) were obtained from Sigma Chemical Co.,

Abbreviations used: OPTA, *o*-phthalaldehyde; TNBS, trinitrobenzenesulphonic acid; Xyl I, xylanase I.

[1] To whom correspondence should be addressed. Fax: 91-20-588 4032. E-mail: malarao@dalton.ncl.res.in.

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TABLE 1

Purification of Xyl I

Steps	Volume (ml)	Activity (IU/ml)	Protein (mg/ml)	Specific activity (IU/mg)	Fold purification
Culture supernatant	200	125	3.6	34.2	1
$NH_4(SO_4)_2$ precipitation	40	1166.6	10.3	112.8	3.3
DEAE Sephadex	18	267.2	1.1	232.5	6.8
Sephacryl S-200	10	118.5	0.26	455	13.3

U.S.A. Sephadex A-50 and Sephacryl S-200 were obtained from Pharmacia (Sweden). All other reagents were of analytical grade.

Microorganism. *Thermomonospora* sp. is an alkalothermophilic actinomycete having optimum growth at pH 9 and 50°C. It was isolated from self-heating compost from the Barabanki district of Uttar Pradesh, India (14, in press).

Enzyme assays. Xylanase assay was carried out by mixing the enzyme with 0.5 ml of xylan (1%) in a final volume of 1 ml and incubating the mixture at 50°C for 30 min. The reducing sugar released was determined by the dinitrosalicylic acid method (15). One unit of xylanase activity was defined as the amount of enzyme that produced 1 μmol of xylose equivalent per minute from xylan, under assay conditions. Protein concentration was determined according to the method of Bradford (16), using bovine serum albumin as standard.

Production and purification of Xyl I. *Thermomonospora* sp. was grown for 96 h in a modified media (17), containing 1% yeast extract, 4% cellulose paper powder and 0.1% Tween 80. The pH of the medium was adjusted after autoclaving, to 9 with 10% Na_2CO_3. The culture and insoluble cellulose paper powder were removed by centrifuging at 10,000 rpm for 10 min. All purification steps were carried out at 4°C unless otherwise stated. The broth was subjected to fractional ammonium sulphate precipitation (35 to 55%). The precipitate was dissolved in minimum amount of 0.05 M sodium phosphate buffer, pH 7 and dialyzed against 100 volumes of the same buffer with several changes for 24 h. The dialyzed fraction was applied to DEAE-Sephadex A-50 column (6 $\times$ 20 cm), previously equilibrated with 0.05 M sodium phosphate buffer at pH 7. The elution was carried out by a linear gradient of sodium chloride (0.25 to 0.50 M) in 0.05 M sodium phosphate buffer, pH 7. The fractions having maximum specific activity were pooled and concentrated by ultrafiltration through Amicon UM-10 membrane. The concentrated sample was applied to a Sephacryl S-200 column (2.5 $\times$ 100 cm), which was equilibrated with 0.05 M, pH 7 sodium phosphate buffer. Elution was carried out by using the same eluant at a flow rate of 10 ml/h, and 2 ml fractions were collected. The fractions having maximum specific activity were pooled and concentrated. The purity of the enzyme was checked by SDS–PAGE (18) followed by staining by Coomassie brilliant blue R-250.

Properties of purified Xyl I. Estimation of xylanase activity at different pH (4 to 10) and temperature (40 to 100°C) values were carried out under standard assay conditions to determine the optimum pH and temperature for enzyme activity. The pH stability of the enzyme was measured by incubating 5 IU of enzyme for 1 h, at 50°C in buffer of desired pH for 16 h. The temperature stability was determined by incubating 5 IU of enzyme for different time intervals and then estimating the residual activity under standard assay conditions.

Determination of molecular weight of Xyl I. The molecular weight of the enzyme was determined by gel filtration on Sephacryl S-200, which was calibrated using the following marker proteins, ovalbumin (45 kDa). Carbonic anhydrase (29 kDa), cytochrome *c* (12.4 kDa) and aprotinin (6.5 kDa). The presence of subunits in the protein was determined by SDS–PAGE.

pI of the enzyme. The isoelectric focusing of the enzyme was carried out in a vertical column, using a glycerol gradient at a constant voltage of 400 V for 8 h at 4°C. The ampholyte range used was 2 to 12 (19).

Steady-state kinetic studies using xylan. The rate of hydrolysis of xylan was determined at different substrate concentrations (2 to 15 mg). The kinetic parameters Michaelis–Menten constant (K_m) and turnover number (k_{cat}), were obtained from Lineweaver–Burk plots for native and TNBS modified Xyl I. The k_{cat} values for Xyl I hydrolysis were determined from the average of at least three measurements at each substrate concentration and pH.

Modification of Xyl I with o-phthaldehyde. Xyl I (5 μg) in 0.05 M phosphate buffer, pH 7 was incubated with *o*-phthalaldehyde (1 mM) in methanol at 25°C. The formation of Xyl I-isoindole derivative was followed spectrofluorometrically by monitoring the increase in fluorescence at 410 nm with excitation wavelength fixed at 338 nm.

Modification of Xyl I with TNBS. Xyl I (5 μg) was incubated with varying concentrations of 2,4,6-trinitrobenzenesulfonic acid (4 to 10 mM) in the presence of 0.25 ml 4% sodium bicarbonate at 37°C in a reaction volume of 0.5 ml. Aliquots were withdrawn at suitable time intervals and the reaction was terminated by adjusting the pH to 4.5. The degree of inactivation in presence of 1% xylan was also deter-

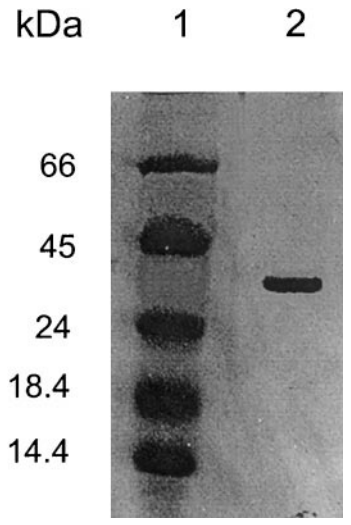

FIG. 1. SDS–PAGE of purified Xyl I from *Thermomonospora* sp. in 10% slab gel. Lane 1 contained standard marker proteins: bovine serum albumin (66 kDa), ovalbumin (45 kDa), trypsinogen (24 kDa), β-lactaglobulin (18.4 kDa), and lysozyme (14.4 kDa). Lane 2 contained purified Xyl I (2 μg).

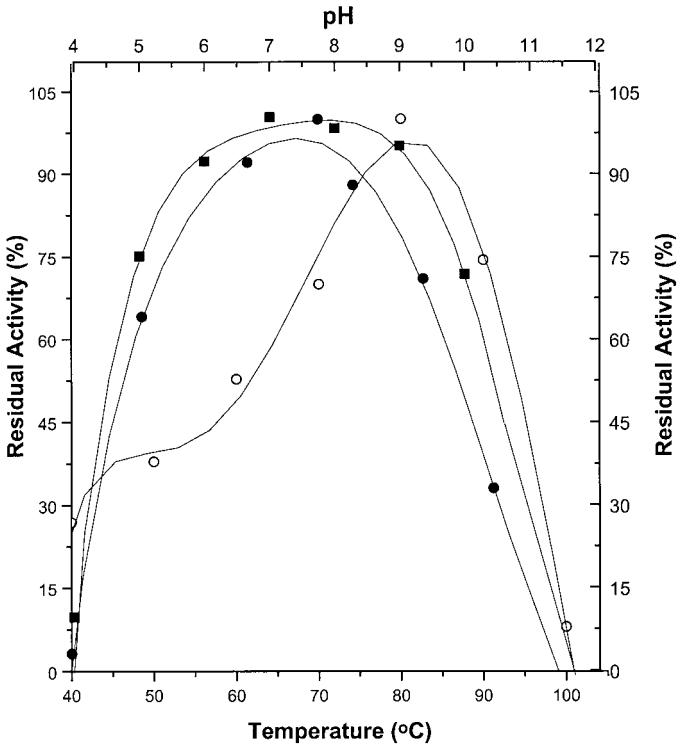

FIG. 2. The pH optimum (●) and pH stability (■) of Xyl I. The pH stability of the enzyme was measured by incubating 5 IU of enzyme for 1 h at 50°C in buffer of desired pH. The following buffer systems were used: 0.05 M acetate buffer (pH 4–5), 0.05 M phosphate buffer (pH 6–7), 0.05 M Tris–HCl buffer (pH 8), 0.05 M carbonate–bicarbonate buffer (pH 9–10). The optimum temperature (○) of Xyl I in 0.05 M phosphate buffer, pH 7. The activity of the enzyme was determined in the range (40–100°C).

mined. Control tubes with only enzyme, only inhibitor and inhibitor/substrate were incubated under identical conditions.

CD measurements. CD spectra were recorded in a Jasco-J715 spectropolarimeter at ambient temperature using a cell of 1 mm path length. Replicate scans were obtained at 0.1 nm resolution, 0.1 nm bandwidth and a scan speed of 50 nm/min. Spectra were average of 6 scans with the baseline subtracted spanning from 260 to 190 nm in 0.1-nm increments. The CD spectrum of the Xyl I (25 μg/ml) was recorded in 50 mM sodium phosphate buffer (pH 7).

RESULTS AND DISCUSSION

Purification of Xyl I

Xyl I was purified by fractional ammonium sulphate precipitation (35 to 55%) followed by sequential chromatography on DEAE Sephadex ion exchange column and Sephacryl S-200 gel filtration column. Xyl I from *Thermomonospora* sp. was purified to homogeneity with thirteen fold purification and specific activity of 455 IU/mg (Table 1). The purity of Xyl I was analyzed by SDS-PAGE (Fig. 1).

Characterization of Xyl I

Xyl I was active in a wide range of pH from 5 to 10, with optimum activity at pH 7.5. It was stable in an expansive range of 5 to 10 with more than 75% residual activity. The temperature optima of the purified Xyl I was 80°C and it showed up to 75% relative activity at 90°C (Fig. 2). The enzyme was extremely thermostable, retaining complete activity at 50°C for up to 72 h. Xyl I had a half-life of 24 h and 12 h at 60 and 70°C respectively. While at 80, 90, and 100°C it had half-lives of 86, 30, and 15 min, respectively (Fig. 3). Xyl I was more thermostable when compared to most of the other reported thermostable xylanases from wild strains. It was more stable than the xylanase isolated from *Thermotoga* sp. strain FjSS-B.1, which had a half-life of 8 min at 100°C (20). However, a recombinant xylanase whose gene has been isolated from *Thermotoga neopalitina* and expressed in *E. coli* had a half-life of 30 min at 100°C (21). The xylanase isolated from *Thermus thermophilus* was also highly thermostable with 61% residual activity after incubation at 90°C for 48 h. However, this xylanase was cell bound and was solubilized into the medium by sonication (22). The molecular weight of the purified Xyl I as determined by gel filtration (37,400 Da) and SDS–PAGE (38,000 Da) were found comparable, indicating the absence of subunits in the protein. The isoelectric point of Xyl I was

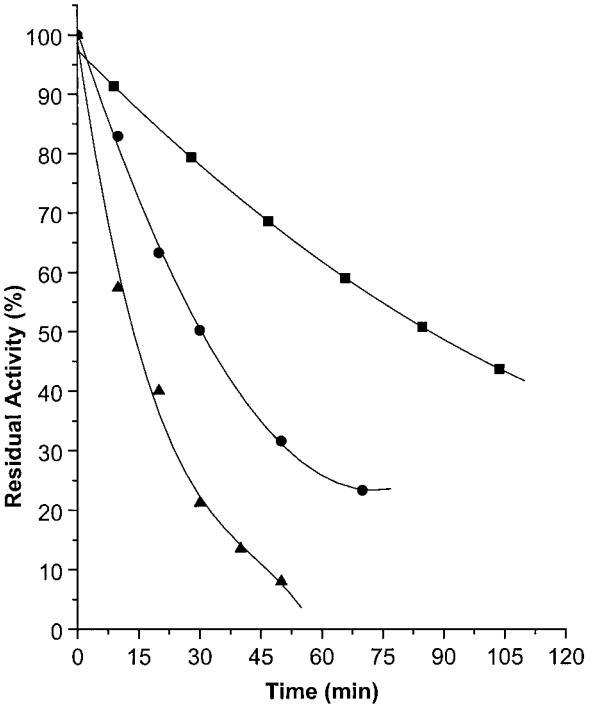

FIG. 3. Thermal stability of Xyl I: 5 IU of xylanase was incubated in 0.05 M phosphate buffer (pH 7) at 80°C (■), 90°C (●), and 100°C (▲) for different intervals and residual activity was determined.

TABLE 2

Comparison of the Secondary Structure of Xyl I with Other Known Family 10 Xylanases

	α-Helix (%)	β-Sheet (%)	Random coil (%)	Molecular weight (Da)
Penicillium simplicissimum	39.74	13.9	46.36	32,408
Pseudomonas fluorescens	32.85	14.7	52.45	38,411
Thermoascus aurantiacus	40.4	13.91	45.69	32,626
Thermomonospora sp. (Xyl I)	38	10	52	38,000

determined to be 4.1 by isoelectric focusing, indicating that it is an acidic protein. Based on its molecular weight and p*I*, Xyl I can be classified as a family 10 xylanase.

Secondary Structure of Xyl I

The CD spectrum of the native Xyl I was analyzed using the algorithm in K2d program (23, 24), in order to determine its secondary structure. The outcome from the K2d analysis showed that the secondary structure of Xyl I is comparable with those of previously studied family 10 xylanases (Table 2). Based on crystallographic studies, Derewenda *et al.* have shown that the family 10 xylanase isolated from *Streptomyces lividans* has a TIM barrel structure, containing eightfold α/β barrels (25). The similarity in the contents of α and β structures in Xyl I with other family 10 xylanases suggests that it could have a tertiary structure similar to the eightfold α/β barrel structure of family 10 xylanases. Xylanases belonging to family 11 exist as a single domain that contains two mostly antiparallel β-sheets, which are packed against each other (26).

pH Dependence of the Enzyme Activity

The ionizable groups essential for the function of Xyl I activity was determined through the effects of pH on k_{cat} and $k_{cat} \cdot K_m^{-1}$ values. The plots of k_{cat} and $k_{cat} \cdot K_m^{-1}$ against pH are shown in Fig. 4. The dependence of initial velocity upon substrate concentration was hyperbolic at each pH investigated and all Lineweaver-Burk plots were linear. The plots indicate the dependence of Xyl I activity on the ionization of at least two groups. The log k_{cat} versus pH plot gave p*K* values of 5 and 10.1 for the enzyme substrate complex. Values of 5.7 and 8.1 were obtained from the log $k_{cat} \cdot K_m^{-1}$ versus

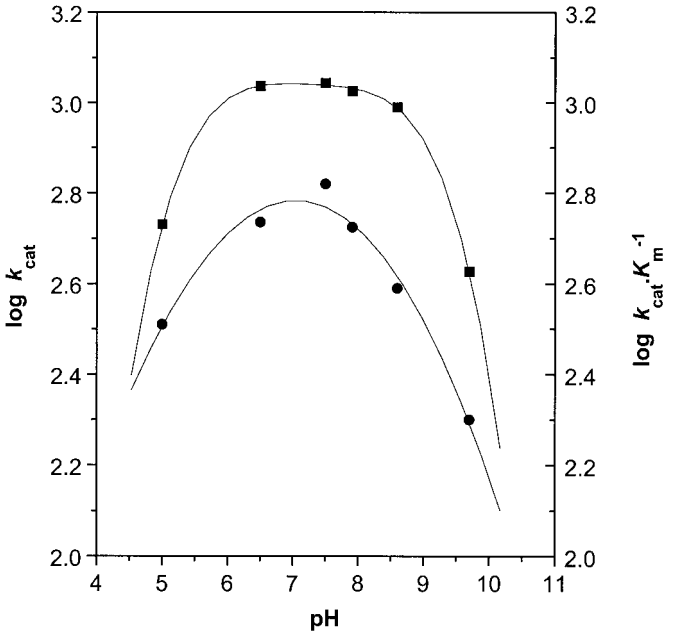

FIG. 4. Dependence of kinetic parameters on pH: Xyl I (0.5–1 μg) was reacted with different concentrations of substrate (1–20 mg, of soluble xylan) in 50 mM buffers of pH ranging from 5 to 10. K_m and k_{cat} values were derived from Lineweaver–Burk plots. The effect of pH on log k_{cat} (■) and log $k_{cat} \cdot K_m^{-1}$ (●).

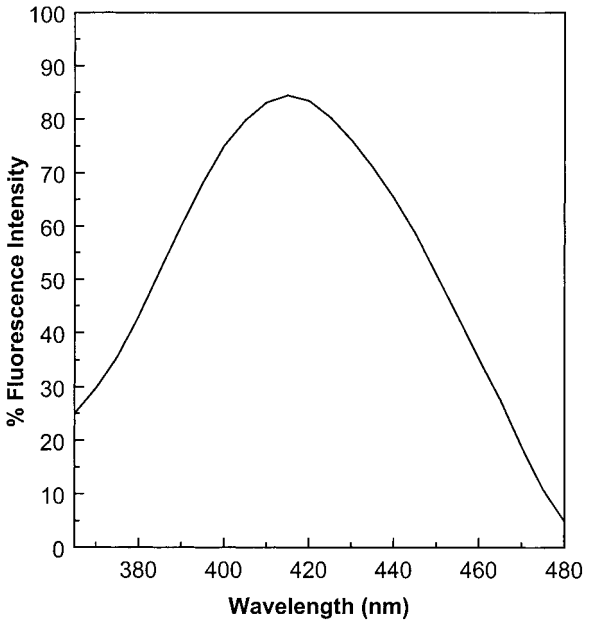

FIG. 5. Isoindole fluorescence of Xyl I modified by OPTA. Xyl I sample (5 μg) was incubated with 1 mM of OPTA for 10 min at 25°C. The isoindole fluorescence was monitored at λ_{ex} 338 and λ_{em} 415.

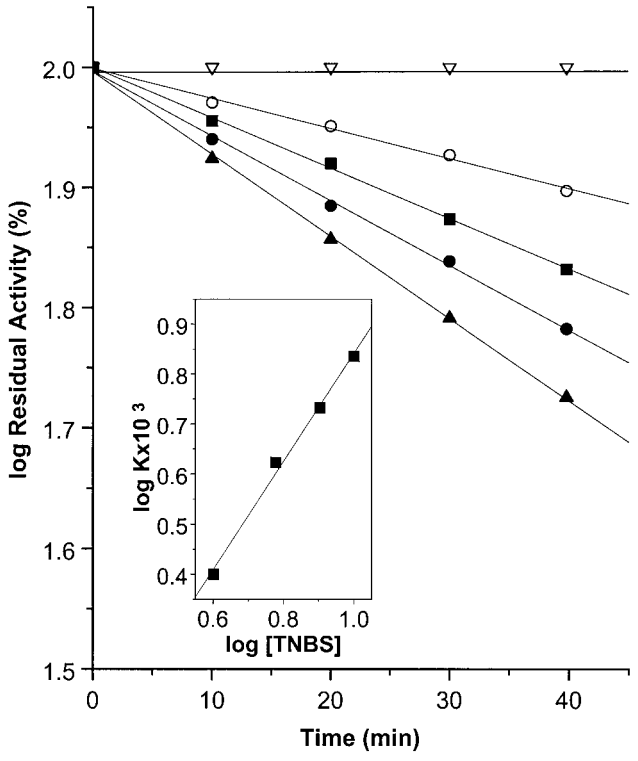

FIG. 6. Kinetics of inactivation of Xyl I by TNBS. Pseudo first order plots for the inactivation of Xyl I by TNBS. Xyl I (2 μM) was incubated with 4 mM (○), 6 mM (■), 8 mM (●), 10 mM (▲) TNBS and control (▽) at 25°C. Aliquots were removed at indicated time intervals and the reaction terminated by adjusting the pH to 4.5. Inset: Double logarithmic plots of pseudo first order rate constants as a function of TNBS concentration.

pH plot for the free enzyme. The values obtained from the acidic limb of the curves are consistent with the participation of a carboxylate in the Xyl I catalysis which is in agreement with the proposed mechanism of action. However, the basic limb of the rate profile may reflect the ionization of a lysine residue since lysine groups in proteins have pK values usually in the range of 9–10 (27). Slopes obtained from the log $k_{cat} \cdot K_m^{-1}$ versus pH and log k_{cat} versus pH plots were +0.41, −0.28 and +0.69, −0.89 respectively. The slopes of the acidic and basic limb of the curves are less than unity, indicating that the simple model of the pH dependence of enzyme action does not describe adequately the Xyl I enzyme system. The divergence from unit slopes may arise from electrostatic perturbation of ionization constants, multiple intermediates or conformational effects. This may indicate the interaction of two ionizable groups on the enzyme suggesting a complex mechanistic pathway for the Xyl I system (28). The nature of the essential residues for the function of Xyl I activity was resolved through specific chemical modification of the enzyme.

Inactivation of Xyl I by OPTA

Fluorescence excitation and emission spectroscopic data showed that an isoindole derivative was formed following the reaction between Xyl I and OPTA. The inactivation of Xyl I by OPTA resulted in concomitant increase in fluorescence at 415 nm (excitation wavelength, 338 nm), which is characteristic for the formation of an isoindole derivative (Fig. 5). Therefore indicating that the SH and NH_2 groups of Xyl I involved in the reaction with OPTA are situated at the active site of Xyl I. OPTA forms a fluorescent isoindole derivative by cross-linking the proximal thiol and amino groups of the protein. OPTA has been used as a fluorescent probe and a modifier of cysteine and lysine residues (29, 30).

Modification of Lysine Residue by TNBS

Incubation of Xyl I (5 μg) with different concentrations of TNBS resulted in a time and concentration dependent loss of enzyme activity as shown in Fig. 6. The reaction followed pseudo first order kinetics. The pseudo first order rate constants (K) were linearly related to the concentrations of the reagent, suggesting that no reversible complex was formed during the inactivation process. Furthermore a reaction order of 1.05 with respect to the modifier was determined from the slope of the double logarithmic plots (Fig. 6, inset),

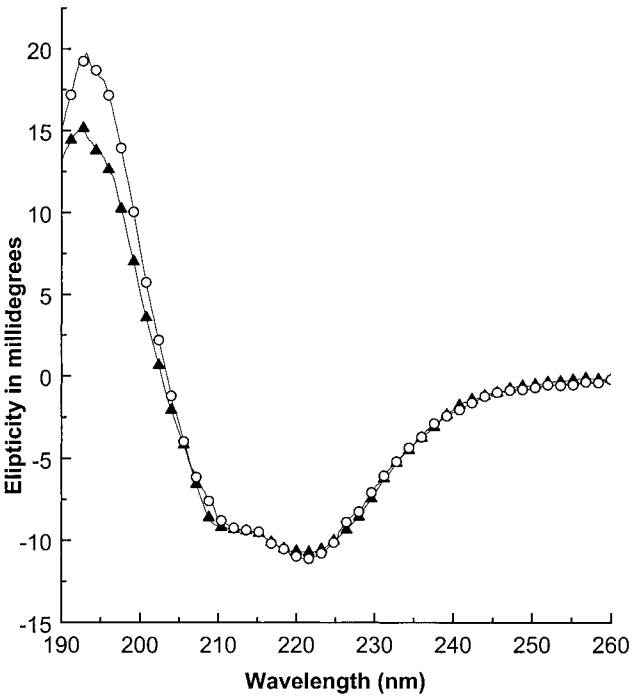

FIG. 7. CD spectra of native and TNBS modified Xyl I. Far-UV CD spectra were recorded for native (○) and TNBS modified (▲) Xyl I from 260 to 190 nm at 25°C. Each spectrum represents the average of six scans.

indicating that 1 mol of TNBS inactivated 1 mol of enzyme. The CD measurements revealed no effect of the modifier on the α-helix and β-sheet content of Xyl I. Hence the TNBS induced inactivation of Xyl I is a result of direct chemical modification of an essential lysine residue and cannot be attributed to the disruption of the enzyme structure (Fig. 7). There was no change in the k_{cat} values of native and modified enzyme, while the K_m values were 3 mg/ml and 7 mg/ml respectively (Table 3). The comparable k_{cat} values suggested that lysine was not present in the catalytic site, while the significant increase in the K_m indicated the presence of lysine at the substrate-binding site. The complete protection of Xyl I against inactivation by TNBS and OPTA, by the substrate, confirms the presence of lysine at the substrate-binding site (Table 4). The three dimensional structure of family 10 xylanases have revealed several highly conserved residues that are on the surface of the active site. Charnock *et al.* have suggested that Lys-47 plays an important role in positioning the substrate into the active site of Xylanase A from *Pseudomonas fluoresens* (13).

In the present paper, we have described the purification of a highly thermostable xylanase produced by *Thermomonospora* sp. This is significant because moderately thermophilic organisms such as *Thermomonospora* sp. have dual advantage over hyperthermophilic organisms. It is capable of growing at 50°C, thereby preventing contamination during upstream processing. However, in comparison to hyperthermophilic organism (80°C) it grows at lower temperature and therefore has lower consumption of energy for its growth and enzyme production. Moreover, the thermostability of Xyl I is comparable to that of xylanases produced by hyperthermophilic organisms like *Thermotoga* sp. A plethora of xylanases has been reported which contain carboxyl and tryptophan residues in the active site and there are few reports of histidine. To the best of our knowledge there is only a single report of the presence of lysine in the active site of a xylanase from *Pseudomonas fluoresens*. Our studies demonstrate the presence of an essential lysine residue in the active site of Xyl I by fluorescent chemoaffinity labeling and group specific modification.

TABLE 3

Kinetic Parameters of Native and TNBS Modified Xylanase I

Xylanase I	Residual activity (%)	K_m (mg/ml)	k_{cat} (min^{-1})
Native	100	3	13400
TNBS modified	60	4.2	13500
	52	6.1	13250
	35	7	13300

Note. TNBS modified Xyl I was prepared by incubating 5 μM of Xyl I with 5 mM TNBS for different time intervals in a total a volume of 1 ml at 25 ± 1°C. The reaction was terminated by adjusting the pH to 4.5. The K_m and k_{cat} values were determined from Lineweaver–Burk plots. The substrate concentrations used for the determination of K_m and k_{cat} were in the range of 1–10 mg.

TABLE 4

Substrate Protection of Xylanase I against Inhibition by OPTA and TNBS

Reaction	Residual activity (%)
None (Control)	100
OPTA (1 mM)	0
Xylan (1 mg) + OPTA	65
Xylan (2 mg) + OPTA	80
Xylan (5 mg) + OPTA	98
TNBS (10 mM)	0
Xylan (1 mg) + TNBS	70
Xylan (2 mg) + TNBS	85
Xylan (5 mg) + TNBS	100

Note. Enzyme samples (5 μg each) were incubated with different concentrations of xylan for 10 min before addition of inhibitors.

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