0273-2289/1559-0291 (Online)/07/141/273–298/$30.00

Kinetic and Thermodynamic Study of a Chemically Modified Highly Active Xylanase from *Scopulariopsis* sp.

Existence of an Essential Amino Group

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Received May 11, 2006; Revised September 18, 2006; Accepted October 30, 2006

Abstract

The amino groups of purified least acidic xylanase (LAX) isomer and carboxyl groups of purified highly acidic xylanase (HAX) isomer from *Scopulariopsis* sp. were chemically modified, resulting in charge neutralization and reversal. Modification of the second amino group was accompanied by the complete loss of enzyme activity in both the absence and presence of xylose. Multiple alignments of family 10 and 11 xylanases revealed that there is a pair of fully conserved Lys residues only in family 10 members. Xylanase structures from family 10 members showed that one of the conserved Lys residues is found near the active-site cleft that makes an H-bond with the substrate. The LAX and HAX isoenzymes in which one amino and three to four carboxyl groups were modified were subjected to kinetic and thermodynamic characterization. There were no differences in pH optima between the native and modified HAX, but there was a broadening of pH optimum toward the alkaline range for charge-neutralized LAX and a double pH optimum for charge-reversed LAX. The V_{max}/K_m of both modified LAX and HAX decreased relative to the native species. The thermodynamics of xylan hydrolysis showed that the decrease in the catalytic activity of modified LAX enzymes was entropically driven. When compared with native enzyme, the

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thermostabilities of modified LAX enzymes increased in the presence and decreased in the absence of substrate. The thermodynamics of kinetic stability for modified LAX enzymes revealed that this increase in thermolability was owing to the decrease in $\Delta H^{\#}$ with a concomitant increase in $\Delta S^{\#}$ compared with native LAX. The thermostabilities of all the modified HAX species decreased except that of charge-neutralized HAX, whose half-life significantly increased in 50% (v/v) aqueous dioxan. These results suggest that the altered properties of the modified enzymes were a result of the conformational changes brought about by chemical modification.

Index Entries: Fungus; family 10 xylanases; chemical modification; thermostability; structure-function-stability relationship.

Introduction

Endo-1,4-β-xylanase (EC 3.2.1.8) hydrolyzes β-1,4-bonds at random in xylan, the main constituent of the hemicellulosic part of the plant cell wall, to produce xylooligosaccharides of varying lengths *(1)*. Xylanases are a widespread group of enzymes involved in the production of xylose, a major carbon source for cell metabolism. Xylanases are also involved in plant cell infections by a plethora of pathogens including bacteria, algae, and fungi, to name a few *(2)*. In the food industry, xylanases are used to improve maceration and juice clarification as well as to improve elasticity and strength in bread dough. In animal feed, xylanases are used to increase the digestibility and nutritive value of food *(3)*. More important, xylanases are used as prebleaching agents in the paper and pulp industry, which reduces the amount of environment unfriendly chlorine and process energy consumption *(4)*. Recently, xylanases have attracted increasing attention for their potential use in several applications. This is evident from their involvement in a number of patent applications (668) since 2001 (www.uspto.gov/).

The role of carboxyl groups in catalysis and tyrosines/tryptophans in substrate binding is well established through X-ray structure elucidation and chemical modification studies *(1,5,6)*. Carboxyl group modification has been used to identify the essential active-site carboxyls in xylanases and xylosidases *(7–9)*. Recently, work has been carried out on the modification of His residues for their role in the structure-function-stability relationship *(10–12)*. There has been only a single report regarding the role of a lysine residue in xylanases as deduced by chemical modification *(13)*.

Previously the purification and characterization of different xylanase isoenzymes from *Scopulariopsis* sp. was reported *(14)*. In this article, we report the chemical modification of the surface amino groups of purified least acidic xylanase (LAX) isomer and the surface carboxyl groups of highly acidic xylanase (HAX) isomer from *Scopulariopsis* sp. The amino groups of LAX were either acetylated or succinylated for charge neutralization or charge reversal, whereas the carboxyl groups of HAX were activated by 1-ethyl-3-(3-dimethylaminopropyl)-carbodiimide (EDC) in the presence of either methylamine hydrochloride for charge neutralization or

ethylene-diamine dihydrochloride for charge reversal. The native and modified xylanases were kinetically and thermodynamically characterized in order to elucidate the role of amino and carboxyl groups in the activity and stability of these isoforms.

Materials and Methods

Chemicals

All chemicals were purchased from Sigma (St. Louis, MO).

Xylanase Assay

The assay solution contained 0.5% (w/v) oat-spelt xylan as the substrate, whose pH was adjusted to 6.5 with 1% (w/v) MES. Appropriate amounts of purified enzyme (50–100 µL) were added to 1 mL of substrate (xylan) solution. After 30 min of incubation at 40ºC, the reaction was stopped by adding 3 mL of dinitrosalicylic acid (DNS) reagent; the mixture was boiled for 15 min, then cooled in ice; and the optical density was determined at 550 nm. One unit of xylanase activity is defined as 1µmol of xylose equivalents liberated/min *(15,16)*.

Kinetics of Acetylation and Succinylation

Varying amounts of acetic anhydride and succinic anhydride were added in increments to 5 mg/mL of LAX solution in 100 m*M* sodium acetate/NaOH, pH 9.5, in the absence or presence of 50 m*M* xylose (competitive inhibitor). Succinic anhydride (5 mg) or acetic anhydride (5 µL) was added to the enzyme solution to give a final concentration of 50 µ*M* for both reagents. Aliquots (50 µL) were taken at different time intervals (5, 30, and 60 s) and added to 50 µL of 0.25 *M* glycine methyl ester to quench the reaction. The acetylation and succinylation reactions were monitored by a drop in pH. The pH of the reaction mixtures was increased from 5.3 to 9.5 with 12 *M* NaOH, and five modification cycles were repeated. After the seventh cycle, acetylated (LAX-N) and succinylated (LAX-R) xylanase samples were dialyzed against water to remove excess reagents and subjected to xylanase assays and *in situ* native-polyacrylamide gel electrophoresis (PAGE) *(17)* to determine the extent of modification by native enzyme mobility shift assay (NEMSA) *(18)*. For kinetic and thermodynamic characterization of LAX-N and LAX-R, the modification reactions were terminated after the fifth cycle *(19–21)*.

Kinetics of Carboxyl Group Modification

Ethylenediamine Dihydrochloride (HAX-R)– or Methylamine Hydrochloride–Modified (HAX-N) Xylanases

The carboxyl groups of purified HAX from *Scopulariopsis* sp. were activated by EDC in the presence of either ethylenediamine dihydrochloride (1 *M*) or methylamine hydrochloride (1.25 *M*) *(22–25)*. These nucleophiles were

added to 10 mL (25 U/mL) of xylanase solution (pH 5.25) containing 50 m*M* xylose (competitive inhibitor). The reaction was initiated by adding 0.05 g (50 m*M*) of EDC. Aliquots (50 µL) were withdrawn at different time intervals for DNS assay and NEMSA *(26)*. The reaction was stopped by the addition of 50 µL of 0. 25 *M* sodium acetate buffer. After 60 min, the reaction was quenched by adding 5 mL of 0.25 *M* sodium acetate buffer, pH 5.5. The modified xylanase was subjected to fast performance liquid chromatography fast G-10 desalting column chromatography to remove excess reagents.

Determination of Extent of Modification

NEMSA was used to determine the approximate number of carboxyls modified as follows *(18)*:

$$dB_{n+1} - dB_n = \Delta_{n+1}$$

in which dB_n is the distance of the unmodified or native band from the tracking dye front, dB_{n+1} is the distance of the next band in the ladder from the tracking dye front, and Δ_{n+1} is the distance that a band migrates upward in NEMSA when one carboxyl residue is modified. The kinetics of carboxyl group modification is shown as a plot of $E_{n\text{th}}$ (number of carboxyl residues modified) vs time.

Optimum pH and pK_{a2} of Acid Catalytic Residue

Native and modified xylanases were assayed in different buffers ranging in pH from 2.5 to 10.5 with 0.5 pH unit increments for the determination of pH optimum as described previously *(27)*. The pK_{a2} (pK_a of the acid catalytic residue) for native and modified LAX was determined by fitting the data to Dixon plots *(27)*.

Activation Energy and Optimum Temperature

The native and modified enzymes were assayed at various temperatures as described under Xylanase Assay. The data were plotted according to Arrhenius *(25)*. The various thermodynamic activation parameters of xylan hydrolysis were calculated by rearranging Eyring's Absolute Rate Equation *(28)*:

$$V_{\max} = (K_B T / h) \cdot \exp(-\Delta H^{\#}/RT) \cdot \exp(\Delta S^{\#}/R) \quad (1)$$

in which h (Planck constant) = 6.63×10^{-34} Js and K_B (Boltzmann constant, $[R/N]$) = $1.38 \times 10^{\text{H23}}$ J/K, in which N (Avogadro's number) = 6.02 H 10^{23} mol^{-1}.

$$\Delta H^{\#} \text{ (enthalpy of activation)} = E_a - RT \quad (2)$$

in which R (gas constant) = 8.314 J/(K·mol).

$$\Delta G^{\#} \text{ (free energy of activation)} = -RT \cdot \ln[(V_{\max} \cdot h)/(K_B \cdot T)] \quad (3)$$

Equation 3 is derived by rearranging Eq. 1 *(29)*:

$$\Delta S^{\#} \text{ (entropy of activation)} = (\Delta H^{\#} - \Delta G^{\#})/T \quad (4)$$

Effect of Substrate

The native and modified LAX and HAX species were assayed in 50 m*M* MES, pH 6.5 buffer containing variable amounts of oat-spelt xylan for determination of $V_{\max}$ and K_m. The data were plotted according to Lineweaver-Burk. In the case of native and modified LAX enzymes, the free energies of substrate binding and transition-state binding are calculated as follows *(29,30)*:

$$\Delta G_s = -RT \ln K_a \quad (5)$$

in which $K_a = 1/K_m$ and ΔG_s is the free energy of substrate binding.

$$\Delta\Delta G_s = \Delta G_{s(\text{native})} - \Delta G_{s(\text{modified})} \quad (6)$$

in which $\Delta\Delta G_s$ is the difference in free energy of substrate binding between the native and modified xylanases.

$$\Delta\Delta G_s^{\#} = -RT \ln[(V_{\max}/K_m)_{\text{native}}]/[(V_{\max}/K_m)_{\text{modified}}] \quad (7)$$

in which $\Delta\Delta G_s^{\#}$ is the difference in free energy of transition-state binding between the native and modified xylanases.

Thermostability

Acetylated xylanase (LAX-N) was incubated in 50 m*M* MES, pH 6.5 buffer at various temperatures. The aliquots were withdrawn at different time intervals, cooled on ice, and assayed for residual xylanase activity at 40°C *(31)* to determine irreversible inactivation. The thermostability of LAX-R was determined only at 60°C. The data were fitted to first-order plots.

Thermal inactivation of native LAX and modified HAX enzymes (HAX-R and HAX-N) was determined by incubating the enzyme solutions in 50 m*M* MES, pH 6.5 buffer at 60°C *(32)*. Furthermore, the thermostability of native (HAX-D)– and methylamine-modified (HAX-N-D) xylanases was also studied in the presence of 50 m*M* MES, pH 6.5 buffer containing 50% (v/v) dioxan *(33)*.

Activation Energy for Denaturation

The first-order rate constants for deactivation (k_d) of LAX-N at different temperatures were determined as described *(32,33)*. The rate constants (k_d) at each temperature were plotted according to Arrhenius and analyzed using Eyring's Absolute Rate Equation (Eq. 1) as described *(32,33)*.

$$\Delta G^{\#} \text{ (free energy of activation)} = -RT \cdot \ln[(k_d \cdot h)/(K_B \cdot T)]$$

$$\Delta H^{\#} \text{ (enthalpy of activation)} = E_a - RT$$

in which R (gas constant) = 8.314 J/(K·mol).

$$\Delta S^{\#} \text{ (entropy of activation)} = (\Delta H^{\#} - \Delta G^{\#})/T$$

Multiple Alignment of Xylanases

The amino acid sequences of selected xylanases from family 10 (*Penicillium chrysogenium* [P29417], *Penicillium simplicissimum* [P56588], *Caldocellum saccharolyticum* [P23557], *Bacillus stearothermophilus* [P40943], *Pseudomonas fluorescens* [P14768], and *Streptomyces lividans* [P26514]) and family 11 (*Aspergillus awamori*; XynC [P33557], *Bacillus circulans*; XynA [P09850], *Bacillus subtilis*; XynA [P18429], *Schizophyllum commune*; XynA [P35809], *Trichoderma reesei*; Xyn I [P36217], and Xyn II [P36218]) were aligned against each other using T-COFFEE, version 2.11 (www.ch.embnet.org/software/TCoffee.html) *(34)*. The shading depicting the degree of conservation of residues was performed by BOXSHADE (www.ch.embnet.org/software/BOX_form.html).

Structure of a Family 10 Xylanase from P. simplicissimum

The structure of a family 10 xylanase from *P. simplicissimum* (PDB = 1B3V_A) was shown using Cn3D (4.1). The degree of conservation of lysine residues and their interaction with the ligand in family 10 xylanases were determined using the PDB-sum database (www.ebi.ac.uk/thornton-srv/databases/cgi-bin/pdbsum/). Structural probing of Lys 51 was done using the on-line protein analysis software Protein Explorer (www.proteinexplorer.org). Ligand-protein interactions were probed using the ligand explorer tool found at the PDB database (www.rcsb.org).

Results and Discussion

Previously we showed that native LAX from *Scopulariopsis* sp. was significantly more active and thermostable than the HAX isoform. In fact, the activity of LAX on oat-spelt xylan (95,000 min^{-1} at 40°C) was greater than that of a psychrophilic xylanase from *Pseudomonas haloplanktis*, and its half-life was more than 4 h at 50°C *(14)*. Although high activity of cold-adapted enzymes is owing to a reduction in activation enthalpy *(35,36)*, in LAX this high activity was shown to be entropically driven *(14)*. In view of LAX's exceedingly elevated activity with concomitant high stability, we decided to study the structure-function-stability relationship of this very important enzyme using chemical modification. Chemical modification of amino (LAX) and carboxyl (HAX) groups was employed to improve the activity/stability profile of both isoenzymes.

Amino Group Modification

The purified LAX was subjected to amino group modification in order to elucidate the structure-function-stability relationship. The kinetics of amino group modification by both acetic anhydride (Fig. 1) and succinic anhydride (result not shown) showed progressive loss in xylanase activity. The results from NEMSA *(18)* showed that a single amino group was modified by acetic anhydride after the second cycle (Fig. 2, lane 4), even though about half of the native LAX still remained unmodified. After the sixth cycle (Fig. 2, lane 6), all of the LAX was found to be in the form in which only a single amino group was modified, whereas after the seventh cycle, very little activity remained (Fig. 1) with concomitant complete disappearance of the xylanase activity band (Fig. 2, lane 7). Moreover, a significant amount of the modified band also disappeared, thereby clearly indicating that acetylation or succinylation of the second amino group resulted in complete loss of activity in both the modified species (LAX-N, Fig. 2; and LAX-R, result not shown). In other words, the modification of only a single lysine is allowed.

Chemical modification of proteins is heterogeneous when amino or carboxyl groups react according to their exposure and charge. If multiple amino groups are modified through acetylation or succinylation, a progressive gel shift pattern will emerge, somewhat similar to what we observed for carboxyl group modification. Therefore, if a number of amino groups were modified at a particular time point, and only one amino group was essential for activity, we would observe protein species with double modification (not involving the essential amino group). This doubly modified active species would be detected as migrating "farther down on the NEMSA activity gel." In that scenario, the gel shift would be the cumulative result of different residues being modified. In our case, though, this was not observed because the enzyme lost activity after the second Lys modification (Fig. 2, lane 7). Hence, it is clear that a gel shift of 1, which corresponds to modification of lysine #1, is allowed (Fig. 2), and modification #2, involving the essential second lysine, results in the loss of xylanase activity. Interestingly, no lysine is present in the active site of xylanases which contain two carboxyls, one acting as an acid/base catalyst and the other as a nucleophile *(37–39)*. The other residues that surround the active-site car-boxyls are tryptophan, tyrosine, glutamine, and asparagine residues *(40,41)*. Moreover, there was no difference in the kinetic pattern of acetyla-tion and succinylation in the presence or absence of xylose, suggesting that the essential lysine may not be in the active-site cleft.

In the absence of any information regarding the sequence and structure of xylanases from *Scopulariopsis* sp., it will be difficult to assign family status to LAX. However, two separate multiple alignments within members of family 10 (Fig. 3) and family 11 (results not shown) xylanases showed the presence of conserved lysines only in family 10 members *(34)*. Multiple

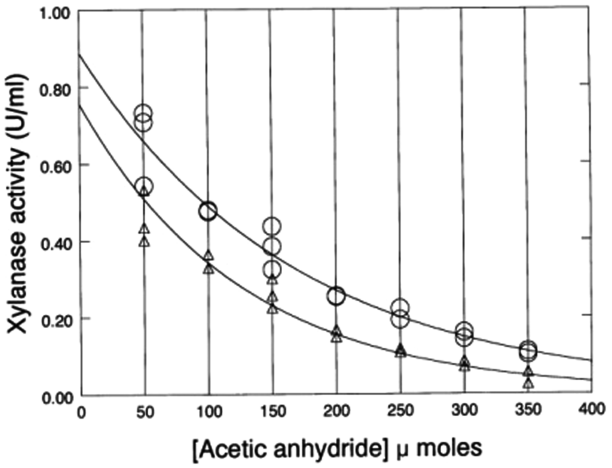

Fig. 1. Effect of acetylation on enzyme activity of LAX at pH 6.0 (○) and 8.5 (△). Aliquots at different time intervals (from 5 to 50 s) were taken after the addition of the first cycle of acetic anhydride to LAX solution and added to the reaction stopper. This was repeated for seven cycles (from 50 to 350 µmol of acetic anhydride). No difference was observed in acetylation in the absence and presence of the competitive inhibitor.

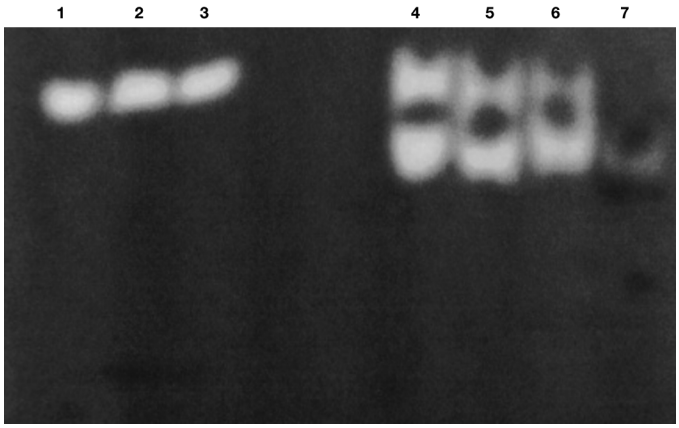

Fig. 2. NEMSA of LAX-N for determination of extent of acetylation. Acetic anhydride was added in increments to native LAX solution and aliquots withdrawn at different time intervals were subjected to *in situ* native-PAGE. The following samples of acetic anhydride were analyzed: lane 1, 0 µL (native LAX); lane 2, 5 µL (30 s); lane 3, 5 µL (60 s); lane 4, 10 µL (40 s); lane 5, 20 µL (40 s); lane 6, 30 µL (40 s); lane 7, 35 µL (40 s). Lanes 2 to 3 comprise the first modification reaction, in which 5 μL of modification agent was used per reaction and reaction time varied. Lane 4 comprises the second modification reaction, and lane 5 comprises the fourth. Lanes 6 and 7 comprise the sixth and seventh modification reactions, respectively.

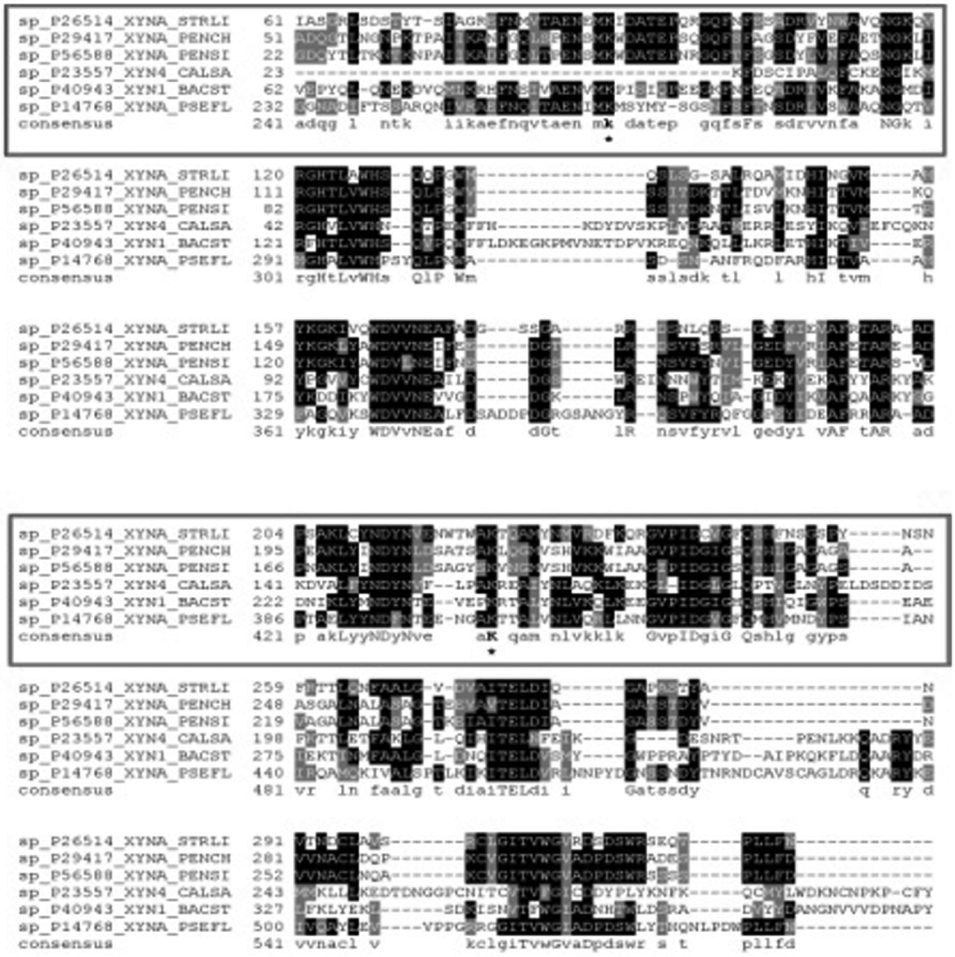

Fig. 3. Multiple alignment of xylanases from family 10 showing two conserved Lys residues using T-COFFEE (www.ch.embnet.org/software/TCoffee.html) and shaded with BOXSHADE Version 3.21 (www.ch.embnet.org/software/BOX_form.html). The relevant part of the sequences has been boxed, with conserved Lys residues shown in bold and marked with an asterisk under the consensus region. The N-terminal K51 (lowercase *k*, upper box) was missing from the xylanase sequence of *C. saccharolyticum*, whereas the other Lys (K184) residue was found in all xylanases from all six organisms. The dark regions show identical residues, and gray regions show amino acid residues with similar properties. The xylanases used for multiple alignment are *P. chrysogenium* (P29417), *P. simplicissimum* (P56588), *C. saccharolyticum* (P23557), *B. stearothermophilus* (P40943), *P. fluorescence* (P14768), and *S. lividans* (P26514). N- and C-terminal sequences from all organisms are not shown.

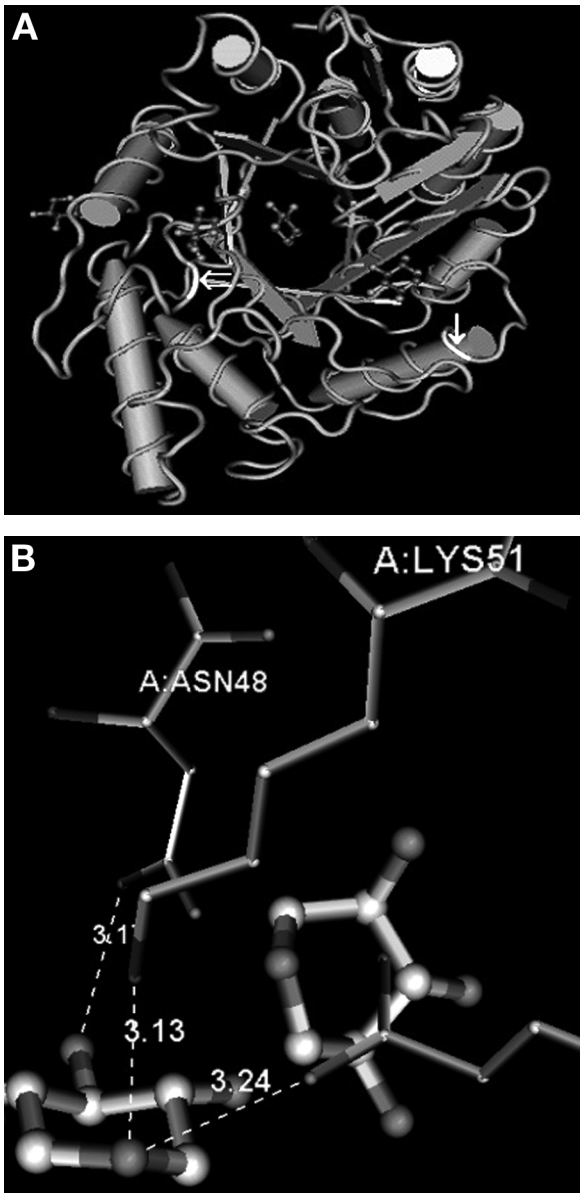

Fig. 4. **(A)** Structure of a family 10 xylanase from *P. simplicissimum* (PDB 1B3V) showing a xylose residue in active site made up of $(\alpha/\beta)_8$ barrel. The conserved K51 (⇐) and K184 (↓) residues were revealed by multiple alignment of xylanases from family 10 including xylanase from *P. simplicissimum* (P56588). Note that K184 does not form any interaction with the ligand, whereas K51 is located at the edge of the substrate-binding rim and forms an H-bond with the ligand (C). This may explain why modification of this conserved lysine residue in LAX resulted in loss of activity. **(B)** Interactions of *P. simplicissimum* xylanase (P56588) Lys51 and Asn48 residues with xylopentose substrate (PDB 1B3Z) modeled using ligand explorer at the Protein Data Bank (www.rcsb.org/pdb/). Hydrogen bonds between amino acid residues and the ligand are shown as dotted lines. Both Lys51 and Asn48 form hydrogen bonds with the ligand. Bond lengths are shown in (A).

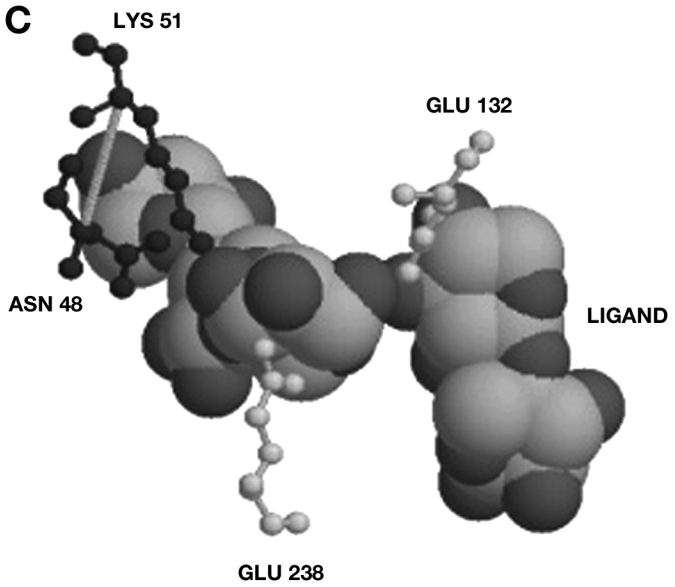

Fig. 4. *(continued)* **(C)** Xylanse Lys51 and Asn48 (PDB 1B3Z) from *P. simplicissimum* form an interchain hydrogen bond (www.proteinexplorer.org). Lys51 imparts stability to the ligand (space-filled) both indirectly, via hydrogen bonding with Asn48, and through direct contact (B). Catalytic residues Glu132 and Glu238 are also shown in contact with the ligand.

alignment between family 10 xylanases (*P. chrysogenium*, *P. simplicissimum*, *C. saccharolyticum*, *B. stearothermophilus*, *P. fluorescence*, *S. lividans*) revealed that there are at least two highly conserved Lys residues (*k and *K, boxed sequences in Fig. 3) in all aligned sequences. The same residues (K51 and K184) are also shown in a family 10 xylanase from *P. simplicissimum* (Fig. 4). However, no highly conserved Lys was found anywhere in the sequences from family 11 xylanases (*A. awamori*, XynC; *B. circulans*, XynA; *B. subtilis*, XynA; *S. commune*, XynA; *T. reesei*, Xyn I and Xyn II). This implies that LAX might belong to family 10, rather than family 11, xylanases.

By querying PBDsum (www.biochem.ucl.ac.uk/bsm/pdbsum/) for family 10 members, we acquired sequence and structural information on the xylanases present in the database. The residue conservation scale showed K51, K184, and K292 as the most conserved lysines when all family 10 xylanases were aligned. In addition, although K51 and K123 can make contacts with the ligand, K123 is only moderately conserved in family 10 xylanases. Conversely, the structure of xylanase from *P. simplicissimum* revealed that highly conserved K292 lies too far away from the active site to be of any functional consequence. The highly conserved K184 lies in an exposed helix where it does not appear to make H-bonds with the ligand. This leaves us with Lys K51, which is located at the edge of the surface rim and can potentially form a hydrogen bond with the ligand (Figs. 3 and 4A). We therefore propose that in LAX a residue equivalent to K51 of *P. simplicissimum*

is the lysine essential for activity (Figs. 2 and 3). Our hypothesis is strengthened by the observation that a family 10 xylanase isoenzyme from *Thermomonospora* sp. also contains a Lys residue in the substrate-binding pocket *(13)*. Moreover, Charnock et al. *(42)* and Moreau et al. *(43)* have theorized about the possible role of a Lys residue in the activity of family 10 xylanases. They showed that the nitrogen atom of K51 can potentially form an H-bond with an oxygen atom of the substrate. We have shown through *in silico* analysis that Lys51 does in fact form a hydrogen bond with the substrate (Fig. 4B). Analysis of *P. simplicissimum* xylanase crystal structure complexed with substrate (1B3Z) has revealed that the substrate structure is stabilized owing to this electrostatic interaction. Furthermore, structure probing has revealed that Lys51 interacts with Asn48 (Fig. 4C), which is also involved in substrate binding (Fig. 4B). Hence, the role of Lys51 in substrate stabilization is both direct and indirect; That is, ligand substrate stabilization is a consequence of direct interaction of Lys51 with the ligand and to a lesser extent owing to Lys51-Asn48 interaction. The results herein are consistent with the loss in activity on modification of a second lysine. Future work will involve the cloning and site-directed mutagenesis of this essential non-active-site lysine to confirm that this Lys is in fact the essential Lys implicated in loss of activity. Moreover, matrix-assisted laser desorption ionization time-of-flight mass spectrometry studies will be carried out to confirm our chemical modification and computational (bioinformatics) analysis.

pH Optima and pK_{a2} of Modified Xylanases

One of the foremost applications of xylanases is in the bleaching process employed in the pulp and paper industry. The industrial application of this enzyme requires it to be active at high pH and temperature *(43,44)*. The pH optimum of xylanases depends on the pK_a of the active-site proton-donating carboxyl group *(5,45)*. The microenvironment in the vicinity of this carboxyl controls its pK_a *(5)*. The variation in the pH optimum among different xylanases depends on the residues that surround the acid/base catalytic residue *(39)*. This change in the microenvironment is brought about not only by the presence of different nonconserved residues *per se* but also by the conformational changes that place this carboxyl in a different neighborhood *(39)*. We modified amino groups of lysines present in LAX with both acetic anhydride for charge neutralization (LAX-N) and succinic anhydride for charge reversal (LAX-R). Under these conditions, only one lysine was chemically altered (Fig. 2) without loss of enzyme activity (Fig. 1).

The pH optimum of LAX-N seemed to envelop a broader pH range, whereas for LAX-R, there were double pH optima (Fig. 5, Table 1). A concomitant increase in pK_as of the proton-donating carboxyl from 8 to 8.6 and 8.4 in the case of native LAX, LAX-N, and LAX-R was also observed (Table 1). The X-ray structure of basic xylanase isoenzyme from *T. reesei* has

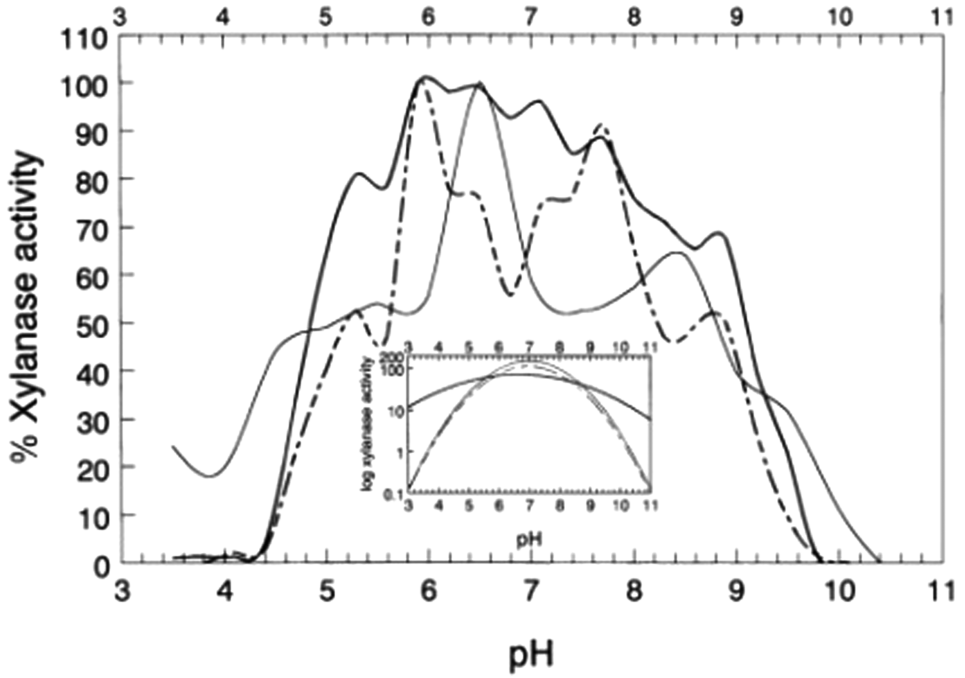

Fig. 5. pH optima of native LAX (thin line), LAX-N (thick line), and LAX-R (dashed/dotted line). The inset shows the Dixon plot for the determination of pK_a values of the active-site carboxyls.

established that the catalytic carboxyl can flip. The result is a 100° torsion angle change that can put the carboxyl in a more hydrophobic neighborhood by making a hydrogen bond with the active-site tyrosine, hence increasing its pK_a *(45,46)*. The structural rearrangement in the position of the catalytic carboxyl can be brought about by small conformational changes induced by pH, ligand binding, or acetylation and succinylation (as in our case). The increase in the pH optimum of both LAX-N and LAX-R may result from the charge inductive effect directly influencing the pK_a of the catalytic carboxyl. The results of site-directed mutagenesis on xylanase from *S. lividans* showed that the conserved Asn to Asp mutation was accompanied by a decrease in pH optimum with a concomitant decrease in the pK_a of both of the active-site carboxyls. Surprisingly, the p*I* of the acidic mutant also increased, indicating a gross conformational change affecting electrostatic interactions throughout the molecule *(47)*. This was also found in the case of xylose isomerase in which modification of 30 of the 57 surface carboxyls was unable to influence the pK_a of the active-site histidines *(24)*. The results regarding the broadening of the LAX pH range by modification of amino groups are very significant and could be used to enhance the pH optimum of highly active xylanases by protein engineering.

Table 1
Kinetic and Thermodynamic Parameters for Xylan Hydrolysis by Native LAX, Acetylated LAX (LAX-N), and Succinylated LAX (LAX-R)

Parameter[a]	LAX	LAX-N	LAX-R
V_{max}(μmol/min)	9.53	0.435	0.343
K_m(% [w/v])	3.00	0.393	0.328
V_{max}/K_m	3.18	1.106	1.045
K_a(% [w/v])	0.33	1.30	3.33
E_a (kJ/mol)	66.4	36.4	36.4
T_{opt}	50	55	55
$\Delta G^{\#}$ (kJ/mol)	81	88	90
$\Delta H^{\#}$ (kJ/mol)	66	36	36
$\Delta S^{\#}$ (J/[mol·K])	–50	–167	–172
ΔG_s (kJ/mol)	2.88	–0.68	–3.13
$\Delta\Delta G_s$ (kJ/mol)	—	3.56	6
$\Delta\Delta G_s^{\#}$ (kJ/mol)	—	–2.83	–2.98
pH optimum	6.5	6–8	6.5 and 7.8
pK_{a2}	8	8.6	8.4

[a]The parameters D$G^{\#}$ through pK_{a2} are defined as follows:
$DG^{\#} = -RT \ln[(V_{max} \cdot h)/(K_B \cdot T)]$
$DH^{\#} = E_a - RT$
$DS^{\#} = (DH^{\#} - DG^{\#})/T$
DG_s (free energy of substrate binding)$= -RT \ln K_a$
$DDG_s = DG_{s(native)} - DG_{s(modified)}$
$DDG_s^{\#}$ (free energy difference of transition state binding) =
$-RT \ln[(V_{max}/K_m)_{native}]/[(V_{max}/K_m)_{modified}]$
pK_{a2} = acid/base catalytic carboxyl

Amino Group Modification as a Structure-Stability Relationship Probe

The modified xylanase (LAX-N) was subjected to kinetic and thermodynamic analysis. Interestingly, it was found that the modification of a single amino group resulted in a drastic decrease in thermostability (Fig. 6, Table 2). This result can be explained if one invokes Wyman's theory. The theory states that a buried acidic group such as a carboxyl having a high pK_a makes the structure unstable and prone to thermolability. This theory was successfully applied to explain results from chicken egg lysozyme, which is structurally and functionally similar to xylanases *(48)*. Our results also show that the increase in pK_a of the proton-donating carboxyl of LAX-N is accompanied by a decrease in its thermostability owing to structural destabilization (Tables 1 and 2). (The plots regarding native LAX and native HAX are not included herein, but their values are given for comparison purposes.)

The kinetic and thermodynamic values for thermostability (Table 2) showed that at 65°C LAX-N is an order of magnitude more thermolabile than native LAX owing to a decrease in both enthalpy ($\Delta H^{\#}$) and entropy

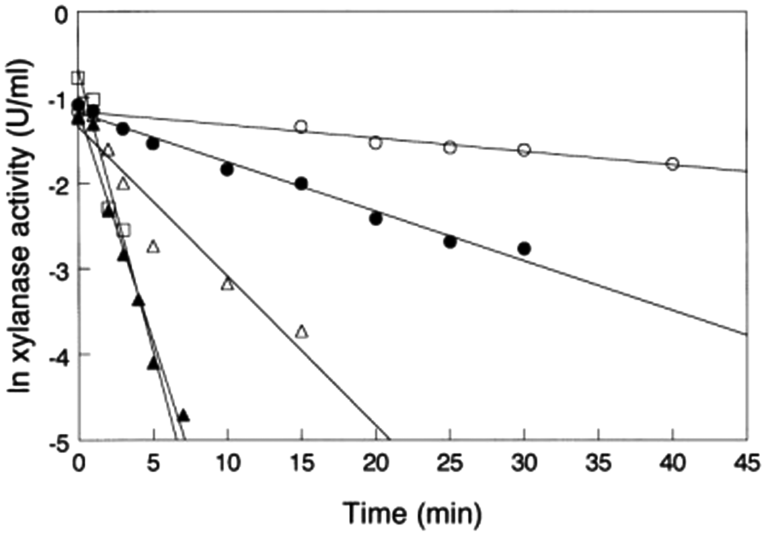

Fig. 6. First-order plots of irreversible thermal inactivation of LAX-N. Samples were incubated at 45°C (○), 50°C (●), 55°C (△), 60°C (▲), and 66°C (□). Aliquots taken at different time intervals were cooled in ice before measuring residual xylanase activity at 40°C.

Table 2
Thermodynamic and Kinetic Parameters for Irreversible Thermal Inactivation of Native LAX, LAX-N, and LAX-R from *Scopulariopsis* sp.[a]

T (°K)	k_d (s^{-1})	$t_{1/2;}$(min)	$\Delta H^{\#}$ (kJ/mol)	$\Delta G^{\#}$(kJ/mol)	$\Delta S^{\#}$(J/[mol·K])
LAX-N					
318	2.58×10^{-4}	45	204.36	99.89	328.54
323	9.61×10^{-4}	12	204.32	97.97	329.27
328	2.90×10^{-3}	4	204.28	96.52	328.54
333	9.02×10^{-3}	1.3	204.24	94.89	328.28
338	0.01098	1	28.08	96.10	–200.64
LAX					
328	4.33×10^{-5}	267	306.30	306.30	604.67
338	1.25×10^{-3}	9.00	306.22	101.90	604.49
LAX-R					
338	0.0164	0.70	ND	ND	ND

[a]ND, not determined. k_d (first-order rate constants of denaturation) of LAX-N were determined from Fig. 4. E_a (activation energies of denaturation) of LAX-N were calculated from Fig. 5. The other parameters are defined as follows:

$t_{1/2}$ (half-life) = $(\ln 2/k_d)/60$

$\Delta H^{\#} = E_a$ (207.01 kJ/mol) – RT

$\Delta G^{\#} = -RT \ln[(k_d \cdot h)/K_B \cdot T)]$

$\Delta S^{\#} = (\Delta H^{\#} - \Delta G^{\#})/T$

The graphs regarding LAX and LAX-R are not shown.

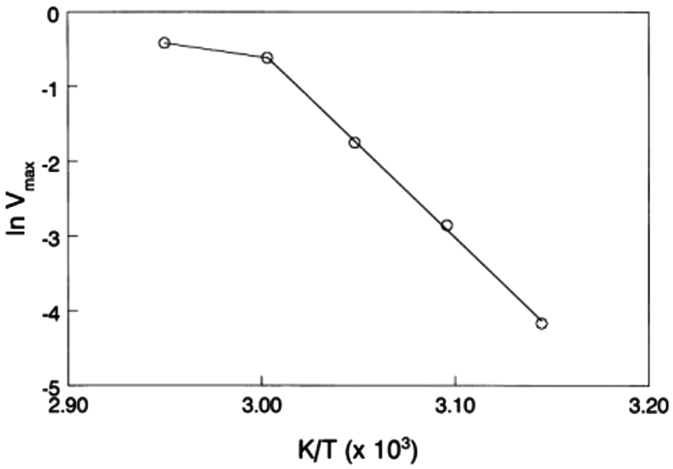

Fig. 7. Arrhenius plots for determination of activation energy of denaturation of LAX-N. Activation energy of denaturation = –slope × *R*, in which *R* = 8.314 J/(K·mol).

($\Delta S^{\#}$) of activation. Compared to native LAX, the decrease in $\Delta H^{\#}$ of LAX-N (Fig. 7) signifies the loss of ionic and hydrophobic interactions, whereas the decrease in $\Delta S^{\#}$could mean that the LAX-N transition state is more ordered. Another factor that could account for the observed decrease in entropy is the loss and gain of bound water/ions on chemical modification of LAX. For example, at 55°C, $\Delta\Delta H^{\#}$($\Delta H^{\#}_{LAX} - \Delta H^{\#}_{LAX\text{-}N}$) is 102 kJ/mol and $\Delta\Delta S^{\#}$ ($\Delta S^{\#}_{LAX} - \Delta S^{\#}_{LAX\text{-}N}$) is 276 J/(mol·K). At 65°C, the difference in $\Delta\Delta H^{\#}$ (278 kJ/mol) and $\Delta\Delta S^{\#}$ (807 J/[mol·K]) becomes more pronounced; that is, at higher temperatures, there is a total breakdown of LAX-N structure, as shown by a very low $\Delta H^{\#}$ (Table 2), accompanied by hydrophobic-hydrophobic interlocking, as shown by the negative $\Delta S^{\#}$ (Table 2). Similarly, kinetic stabilization of xylanase mutants from *Bacillus pumilus* showed that Gly38-Ser/Arg48-Lys and Ser26-Cys mutants were more thermostable than the wild-type enzyme owing to an increase in $\Delta H^{\#}$ and $\Delta S^{\#}$, respectively *(49)*. Similar to our findings, it has been found that the thermostabilities of charged neutralized and charged reversed β-glucosidases were significantly reduced compared to the native enzyme with concomitant reduction in bothenthalpy and entropy of activation *(31)*. Moreover, Clarke and Fersht *(50)* showed that acetylation and succinylation of surface amino groups resulted in a decrease in the kinetic stability of modified enzymes *(50)*.

Structure-Function Relationship Between Native and Modified Xylanase

The kinetic and thermodynamic parameters of xylan hydrolysis of native and modified xylanases are given in Table 1. The activation parameters ($\Delta G^{\#}$) of xylan hydrolysis were calculated by putting the value of V_{max} in Eyring's equation (Eq. 3) as described *(29)*. We emphasize that the values obtained by transforming Eyring's equation are not absolute, although relative values are useful for studying comparative structural/functional

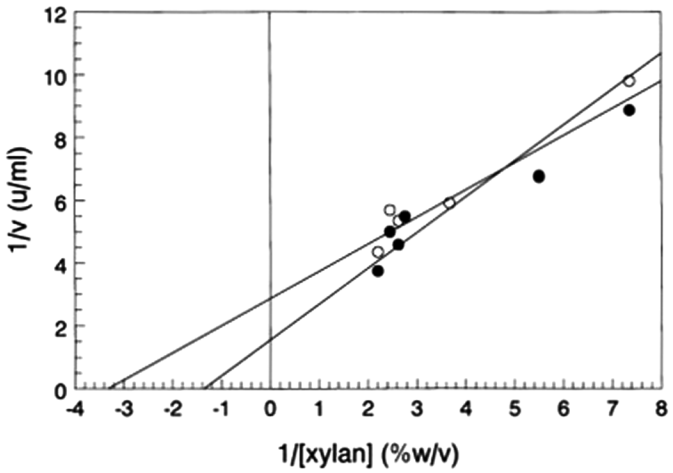

Fig. 8. Log-Log (Hill plot) for determination of V_{max} and K_m of LAX-R (■) and LAX-N (▲).

relationships. Similarly, the substrate binding energy (ΔG_s) is normally calculated by putting the value of K_a or K_m in molar units as in Eq. 5, but we used % (w/v) units because the molecular weight of the xylan polymer is not known *(47)*.

Relative to native LAX, the V_{max} of modified xylanases decreased significantly, with LAX-R being less active than LAX-N, with a concomitant decrease in K_m values (Fig. 8, Table 1). This indicates stabilization of substrate binding in the ground state with concomitant destabilization of the transition state for modified xylanases (Table 1) *(47)*. Table 1 reveals that the very low catalytic efficiency of the modified xylanases is owing to very low $\Delta H^{\#}$ and $\Delta S^{\#}$ values. Very low negative $\Delta S^{\#}$ is indicative of the fact that the enzyme-transition state complex (sofa form of oxo-carbonium ion) is more ordered in relation to the ground enzyme-substrate complex (chair form of xylan backbone). On the other hand, a very low $\Delta H^{\#}$ means that less heat is required to distort the chair form of the substrate to the planer sofa form of the transition state. From the apparent comparative values of $\Delta\Delta G_s$ and $\Delta\Delta G_s{}^{\#}$ (Table 1), we know that the active-site clefts of modified enzymes have significantly more affinity with the substrate than the transition state relative to the native enzyme. According to X-ray structure, the active site of xylanases contains a carpet of water molecules, which are normally displaced as the substrate binds and, therefore, contribute positively toward enzyme entropy *(51,52)*. Previous calculations have shown that there is a gain of 40 J/(K·mol) in entropy as bound water molecules are released to the bulk solvent *(30)*. Owing to the tight affinity of substrate with the active site of modified xylanases (ΔG_s), some water molecules are displaced. These water molecules are regained when the substrate is converted to the transition state because of low enzyme-transition state affinity (positive $\Delta\Delta G_s{}^{\#}$), resulting in the drop in $\Delta S^{\#}$ (Table 1). On the contrary, the reverse is true for

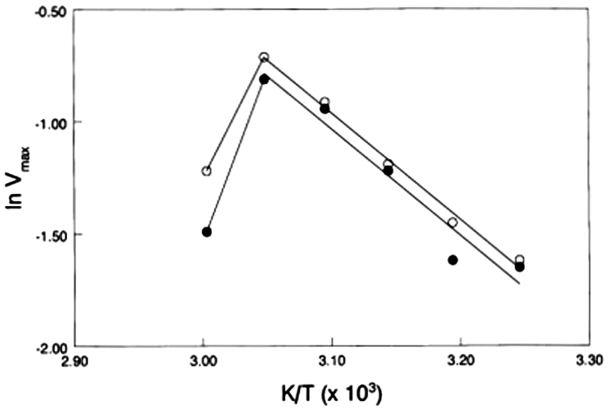

Fig. 9. Arrhenius plots for determination of activation energy of xylan hydrolysis by LAX-R (○) and LAX-N (●). The point of inflexion corresponds to optimum temperature (T_{opt}).

the native enzyme, which binds substrate less tightly but shows high affinity for the transition state, resulting in displacement of ordered water molecules, hence the high entropy of activation. This means that the low catalytic activity of acetylated (LAX-N) and succinylated (LAX-R) xylanases is entropically driven.

The stability of native and modified enzymes is opposite in the absence and presence of the substrate. The optimum temperature that was measured in the presence of xylan was 5°C higher for modified xylanases than the native enzyme (Fig. 9, Table 1). Conversely, the results of irreversible denaturation measured in the absence of a substrate clearly showed that the modified xylanases were very thermolabile compared with the native enzyme (Table 2). The modified enzymes had high affinity with xylan (Fig. 8, Table 1), compared with the native enzyme (low K_m). Moreover substrate binding may protect the modified xylanases from denaturation and increase the T_{opt} (Fig. 9, Table 1). On the other hand, no substrate is present to protect the modified enzymes from irreversible thermal inactivation (Table 2).

Carboxyl Group Modification

Extent of Modification

The purified HAX was subjected to carboxyl group modification for charge reversal (HAX-R) and neutralization (HAX-N). Indicative of carboxyl group modification, NEMSA *(18)* showed that progressive charge neutralization (Fig. 10A) and reversal (Fig. 10B) resulted in HAX-N and HAX-R native bands shifting upward. The kinetics of chemical modification (Fig. 11) showed that in both cases (HAX-N and HAX-R) the first carboxyl was modified very quickly, whereas modification of the second carboxyl required 15 more min. The third carboxyl was modified in 40 and 45 min for HAX-N and HAX-R, respectively (Fig. 11). This implies that the

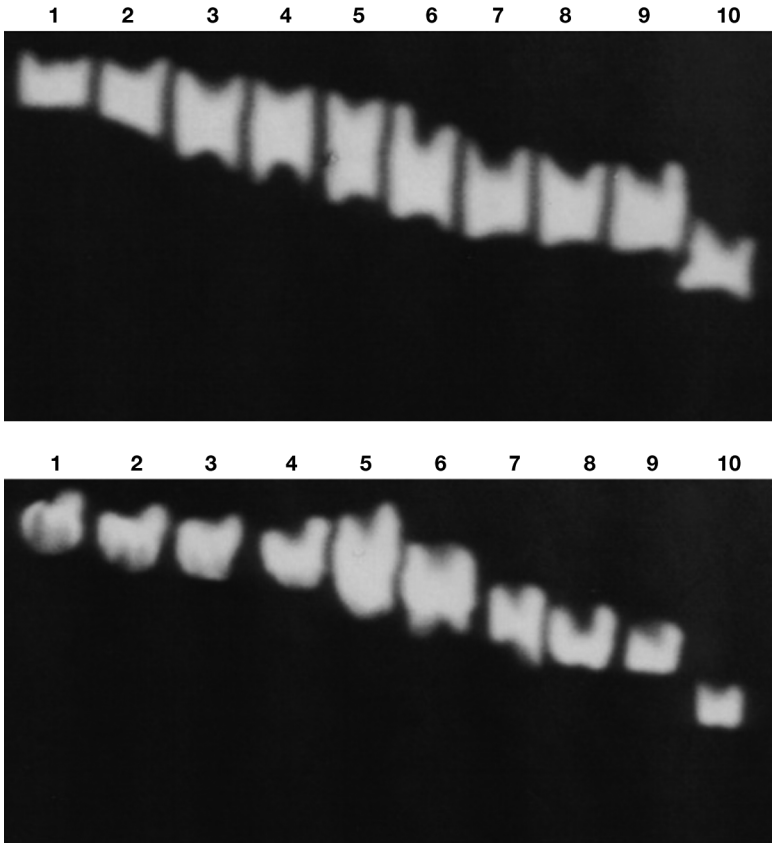

Fig. 10. NEMSA of **(A)** HAX-N and **(B)** HAX-R. The native HAX was activated by EDC in the presence of methylamine hydrochloride (HAX-N) and ethylenediamine dihydrochloride (HAX-R), respectively. The aliquots taken at different time intervals were subjected to 7% *in situ* native-PAGE, and the gels were stained for xylanase activity. The lanes from right to left show the time of modification: 0, 1, 5, 10, 15, 20, 30, 40, 50, and 60 min.

first carboxyl in HAX is fast reacting and exposed. The second and third carboxyls seem to be either partly or fully buried and are exposed only owing to a conformational change as a result of the modification of the preceding carboxyls. Amino acid sequence analysis of low molecular weight acidic xylanases from different prokaryotes and fungi shows a total of 9 to 10 acidic residues, of which four to five carboxyls are conserved according to multiple sequence alignment *(53)*. Two fully conserved carboxyls are involved in catalysis and act as an acid/base catalytic residue and a nucleophile, respectively *(45)*.

Effect of Modification on Activity

The kinetics of chemical modification of HAX showed that both charge neutralization (HAX-N) and reversal (HAX-R) resulted in a significant

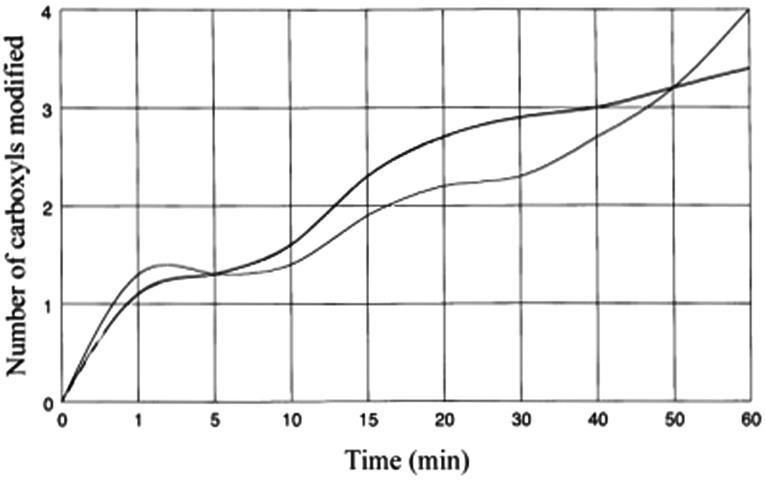

Fig. 11. Graph of number of carboxyls modified vs time of HAX-N (thick line) and HAX-R (thin line). The data of this plot were generated from the respective NEMSA gels (Fig. 10).

reduction in V_{max} (Fig. 12, Table 3). Compared with the K_m for native HAX, the K_m for HAX-N remained the same, whereas in the case of HAX-R, it increased (Table 3). The chemical modification of carboxyl groups of a xylanase from *Bacillus* sp. by Woodward's reagent also resulted in a three-fold decrease in k_{cat} without affecting K_m*(8)*. Because the active-site carboxyls are fully protected by competitive inhibitor, significant conformational changes may occur owing to the modification reaction. The large enthalpies of activation in the case of HAX-R and HAX-N in 50% dioxan (Fig. 13, Table 3) suggest that the ground-state xylan substrate (chair form) is under considerable conformational strain to form the transition state (sofa form of oxo-carbonium ion); that is, more heat is required to break bonds to form the transition state. The pH optimum of all carboxyl group–modified xylanases was unaffected by chemical modification and was found to be 6.4 ± 0.1 (results not shown).

Enhancement of Stability of Charge-Neutralized HAX in a Water-Miscible Organic Solvent

The half-lives of native and modified HAX enzymes in an aqueous and water-miscible organic solvent (50% [v/v] aqueous dioxan) at 60°C are shown in Fig. 14 and Table 3. Charge reversal and neutralization had a drastic effect on the thermostability of the modified enzymes when water was used as a solvent. However, the thermostability of HAX-N increased significantly in 50% (v/v) aqueous dioxin compared with native HAX in organic solvent and modified enzyme (HAX-N) in 100% (v/v) aqueous solvent (Table 3). Slight hydrophobization of the surface of an enzyme owing to the addition of methyl groups may result in the enhancement of stability in a water-miscible organic solvent *(54)* but leads to a decrease in

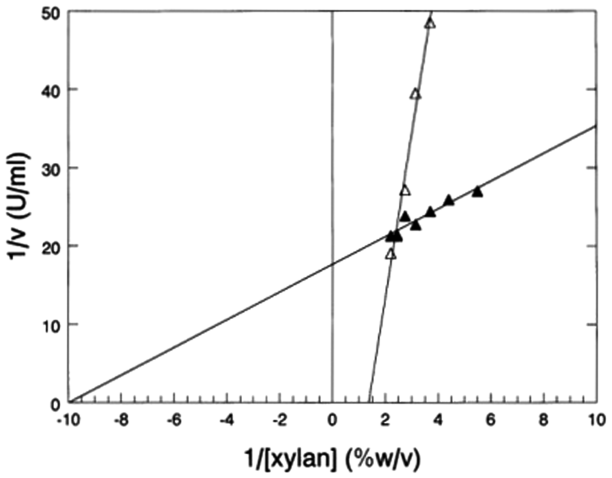

Fig. 12. Lineweaver-Burk plot for determination of V_{max} and K_m of HAX-R (△) and HAX-N (▲).

Table 3
Kinetic Parameters and Thermostability of Native HAX, HAX-N, and HAX-R Xylanases in Aqueous and 50% (v/v) Dioxan[a]

Xylanase	V_{max} (mmol/min)	K_m (%[w/v])	V_{max}/K_m	$\Delta H^{\#}$ (kJ/mol)	$t_{1/2}$ (min)	T_{opt} (°C)
HAX	0.399	0.143	2.7	34	48.9	55
HAX-D	ND	ND	ND	ND	0.60	ND
HAX-R	0.035	0.714	0.049	170	3.85	27
HAX-N	0.056	0.100	0.566	55	0.88	45
HAX-N-D	ND	ND	ND	144	4.23	30

[a]ND, not determined. V_{max}, K_m, and V_{max}/K_m were determined at 40°C, pH 6.5 using oat-spelt xylan. $t_{1/2}$ (half-life) at 60°C = ln $2/k_d$. $\Delta H^{\#} = E_a - RT$; E_a is determined from Fig. 11.

thermostability in aqueous solvent, as reported by Urabe et al. *(55)* and by us in this study (Table 3). A number of groups have speculated that strengthening hydrophobic interactions in the interior of an enzyme will lead to enhanced thermostability *(56–59)*, but we believe that an increase in the surface hydrophobicity may result in enhanced resistance to thermal unfolding as the interior of an enzyme is already optimally packed *(60)*. This becomes more evident when the thermostability of enzymes in water-miscible organic solvents is considered. Polar solvents strip bound water molecules from an enzyme, thereby weakening the surface polar electrostatic interactions *(61)*. One way to get around this problem is to eliminate surface-charged residues altogether and replace them with non-polar ones. When carboxymethylcellulase (CMCase) from *Aspergillus niger* was chemically modified by totally neutralizing the surface negative charges owing to carboxyl as well as positive charges owing to amino groups into

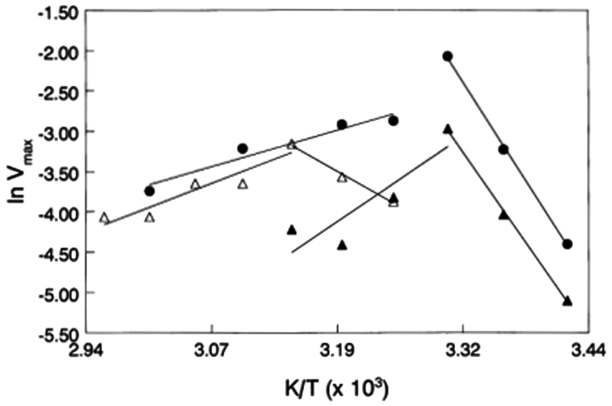

Fig. 13. Arrhenius plot for determination of activation evergy of xylan hydrolysis by HAX-N (△), HAX-R (●), and HAX-N-D (▲). The point of inflexion corresponds to optimum temperature. Activation energy (E_a) = slope × R.

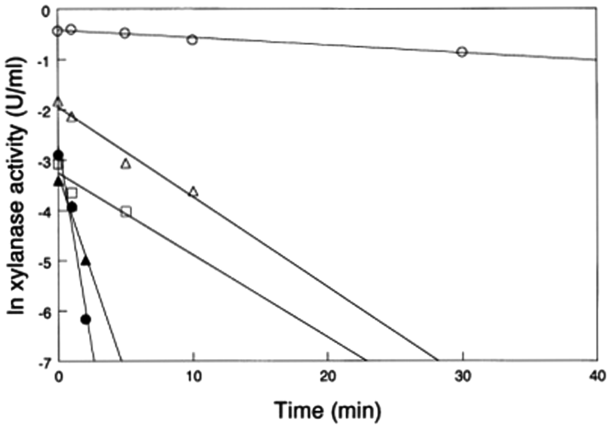

Fig. 14. First-order plots of irreversible thermal inactivation of native HAX (○), HAX-D (●), HAX-R (△), HAX-N (▲), and HAX-N-D (□). All samples were incubated at 60°C, and aliquots taken at different time intervals were cooled in ice before measuring residual xylanase activity at 40°C.

hydrophobic ones *(33)*, the doubly modified CMCase became superthermostable in a water-miscible organic solvent. Herein, HAX-N was stabilized in 50% aqueous dioxin (Table 3).

In the absence of substrate, HAX-R has a half-life of 3.85 min; HAX-N, 0.88 min; and native HAX, 49 min. Optimum temperatures are measured in the presence of xylan, and, therefore, the xylanase with the lowest K_m will have the highest temperature optima owing to substrate protection. Indeed, HAX-R has the lowest temperature optimum (27°C) and the highest K_m (0.71%), whereas HAX-N and native HAX have temperature optima of 45 and 55°C corresponding to K_m values of 0.1 and 0.14%, respectively

(Table 3). A low K_m means a very good fit between the substrate and the active-site cleft, resulting in displacement of bound water molecules from the hydrophobic xylan-binding subsites of xylanases *(51)*. The trapped water molecules in the hydrophobic region of the cleft make the enzyme unstable *(62)*. Therefore, the displacement of water molecules from the active-site by xylan in the case of native HAX and HAX-N most probably resulted in their higher temperature optima (Table 3).

Conclusion

Highly active and stable, LAX has a lysine residue essential for activity and presumably belongs to family 10, although further experiments must be undertaken to confirm this deduction. Charge neutralization and reversal of surface amino and carboxyl groups bring about gross conformational changes in different proteins, thereby affecting their stability, pH optimum, and enzymatic activity. The kinetic and thermodynamic characterization of native and modified isoenzymes (LAX and HAX) showed that the function and stability of xylanases is conformation dependent, brought about by the movement of different structural elements owing to either charge neutralization or charge reversal of surface side chains. It would be very exciting to work out the sequence and structure of LAX from *Scopulariopsis* sp. in order to learn more about its structure-function relationship in general and its very high activity in particular.

Acknowledgments

We wish to acknowledge Drs. Hassan Ghazal and Hany El Shemy for manuscript review and Dr. David Lightfoot for meaningful discussions and help with the manuscript. We are also thankful to Dr. Sikander Ali, director of the National Institute for Biotechnology and Genetic Engineering (NIBGE), for providing research facilities.

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Nomenclature

HAX = native highly acidic xylanase
HAX-D = HAX whose thermostability is determined in 50% (v/v) aqueous dioxan
HAX-N = HAX activated by EDC in presence of methylamine hydrochloride (charge neutralized, – → neutral)
HAX-N-D = HAX-N whose thermostability is determined in 50% (v/v) aqueous dioxan
HAX-R = HAX activated by EDC in presence of ethylenediamine dihydrochloride (charge reversed, – → +)
LAX = native least acidic xylanase
LAX-N = acetylated LAX (charge neutralized, + → neutral)
LAX-R = succinylated LAX (charge reversed, + → –)