0273-2289/05/120/0051/$30.00

Innovative Kinetic and Thermodynamic Analysis of a Purified Superactive Xylanase From *Scopulariopsis* sp.

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Received January 16, 2004; Revised July 16, 2004; Accepted July 21, 2004

Abstract

Two isoenzymes of endo-1,4-β-xylanase (EC 3.2.1.8) from *Scopulariopsis* sp. were purified by a combination of ammonium sulfate precipitation, hydrophobic interaction, and anion-exchange and gel filtration chromatography. The native mol wts of the least acidic xylanase (LAX) and the highly acidic xylanase (HAX) were 25 and 144 kDa and the subunit mol wts were 25 and 36 kDa, respectively. The k_{cat} values of LAX and HAX for oat-spelt xylan at 40°C, pH 6.5, were 95,000 and 9900 min^{-1} and the K_m values of LAX and HAX were 30 and 3.3 mg/mL. The thermodynamic activation parameters of xylan hydrolysis showed that the high activity of LAX when compared with HAX was not owing to a reduction in $\Delta H^{\#}$ but was entropically driven. High-performance liquid chromatography analysis of the degradation products showed that LAX formed both xylotrioses and xylobioses, but HAX predominantly formed xylotrioses. The half-lives of LAX and HAX at 50°C in 50 m*M* 2-N-morpholino ethanesulfonic acid (MES), pH 6.5 buffer were 267 and 69 min, respectively. Thermodynamic analysis showed that at lower temperatures, the increased thermostability of LAX ($\Delta H^{\#}$ = 306 kJ/mol) compared with HAX ($\Delta H^{\#}$ = 264 kJ/mol) was owing to more noncovalent

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surface interactions. At higher temperatures, LAX (ΔS^* = –232 J/[mol·K]) was more thermostable than HAX (ΔS^* = 490 J/[mol·K]) owing to a more ordered transition-state conformation. An energy-activity diagram was introduced showing that k_{cat}/K_m does not successfully explain the true kinetic behavior of both xylanase isoenzymes. The simultaneously thermostable and highly active LAX could be utilized in biotechnological processes involving xylan hydrolysis.

Index Entries: Charge isomers; enzyme purification; energy-activity diagram; fungus; kinetic characterization; kinetic constant; *Scopulariopsis*; stability-function relationship; superactive xylanase.

Introduction

Endo-1,4-β-xylanase (EC 3.2.1.8) randomly hydrolyzes β-1,4-xylan, the major plant cell-wall component of hemicellulose, to produce xylo-oligomers of different lengths. Xylanases are used to degrade cell walls of fruits and vegetables in juice and animal feed processing. These enzymes are also used to degrade biomass to produce xylose, which could be either fermented or converted into xylitol. Xylanases are increasingly being used as a prebleaching agent in the paper and pulp industry to reduce the amount of environmental unfriendly chlorine used in the process *(1)*. A xylanase having high specific activity and thermostability would therefore be extremely useful. Xylanases have been purified and characterized from a variety of organisms including bacteria, saprophytic yeast, fungi *(2–6)*, and extremophiles *(7)*. *Scopulariopsis* species are important from a medical point of view for they are implicated in onychomycosis *(8)* and other infections *(9)*. Recently, we have reported work on endocellulase from this genus *(10)*, but no work on the purification and characterization of any other enzyme from this genus has been reported so far. In the present study, we purified two isoenzymes of endoxylanase from *Scopulariopsis* sp. The highly active least acidic xylanase (LAX) and highly acidic xylanase (HAX) isoforms were kinetically compared. An energy-activity diagram was introduced to explain the kinetic and thermodynamic behavior of xylanase isoenzymes.

Materials and Methods

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

Xylanase Assay

Endoxylanase was assayed by incubating the enzyme in 1 mL of 50 m*M* 2-N-morpholino ethanesulfonic acid (MES), pH 6.5 buffer containing 0.5% (w/v) oat-spelt xylan at 40°C. After 10 min, 3 mL of dinitrosalicylic acid (DNS) reagent was added, the solution was boiled for 10 min, and the absorbance was measured at 550 nm *(2,11,12)*.

Estimation of Protein

Total proteins were estimated by the Bradford assay using bovine serum albumin as the standard *(13)*.

Production of Xylanase

Scopulariopsis sp. was grown in 300-mL flasks filled with 7.5 g of moist wheat bran at 25°C for 30 d *(14)*. Distilled water (15 mL) was added to the fungal mat, and the crude extracellular extract was centrifuged at 15,600*g* for 30 min. The supernatant was subjected to ammonium sulfate precipitation.

Purification of Xylanase

Xylanases, which precipitated between 55 and 70% ammonium sulfate, were subjected to hydrophobic-interaction chromatography (HIC) on a Phenyl Superose column. Ammonium sulfate-precipitated xylanase fractions were loaded onto a Phenyl Superose column at a flow rate of 1 mL/min employing buffer A (50 m*M* sodium phosphate, pH 7.0 + 2 *M* ammonium sulfate) and buffer B (50 m*M* sodium phosphate, pH 7.0). Two-milliliter fractions were collected. The pooled fractions were subjected to desalting chromatography on a G-25 column. This step was followed by anion-exchange chromatography on a Hi-load column. Pooled and desalted fractions from the previous chromatographic step were loaded onto a Hi-load Q Sepharose column at a flow rate of 2 mL/min employing buffer A (25 m*M* Tris-HCl, pH 7.5) and buffer B (25 m*M* Tris-HCl, pH 7.5 + 1 *M* NaCl). Two-milliliter fractions were collected. The fraction volumes 54–104, 160–192, and 198–222 mL corresponded to LAX, MAX (medium acidic xylanase), and HAX and were pooled separately. This step was followed by gel filtration chromatography. Pooled fractions from the Hi-Load anion-exchange column were loaded onto a Superose column in 20 m*M* Tris-HCl, pH 7.0, at a flow rate of 0.5 mL/min. The distribution coefficient was $K_d = (V_e - V_o)/(V_i - V_o)$, in which V_e is the retention volume of HAX (11 mL) and LAX (12.75 mL), V_o is the retention volume of blue dextran (7.9 mL), and V_i is the retention volume of tyrosine (21.2 mL) *(15)*.

All chromatographic separations were performed on a Pharmacia FPLC System fitted with a detector (280 nm). Additionally, all the fractions were analyzed by Bradford assay for protein estimation *(13)* and DNS assay for xylanase estimation *(11,12)*. The extent of purification was followed by sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE), and the separation of different isoenzymes was tracked by HiRACIN-PAGE *(16)*.

Native and Subunit Molecular Weights

The native molecular weights of isoenzymes were determined by gel filtration chromatography, and the subunit molecular weights were determined on 15% (w/v) SDS-PAGE and 12.5% (w/v) SDS-denaturing-renaturing-PAGE (SDS-DR-PAGE) *(15)*.

Optimum pH

Optimum pH values of xylanases were determined by assaying the isoenzymes at different pH values ranging from 3.5 to 10.5. The buffers of various pH values were made as described previously *(17)*.

Activation Energies and Optimum Temperatures

The isoenzymes were assayed at various temperatures ranging from 20 to 70°C as described under Xylanase Assay. The data were plotted according to Arrhenius *(18)*.

Effect of Substrate

The isoenzymes 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 *(18)*.

Pattern of Xylan Hydrolysis

Equal amounts (20 µg) of HAX (0.56 nmol) and LAX (0.71 nmol) were added to 0.5% (w/v) xylan in 50 m*M* MES, pH 6.5 buffer. Aliquots (100 µL) were withdrawn at different time intervals, and 25 µL was loaded onto a Bio-Rad Aminex 87 H cation-exchange column for the separation of xylooligomers using a Perkin Elmer Model 200 series high-performance liquid chromatography (HPLC) system connected to a refractive index detector. Sulfuric acid (0.001 *N*) was used as the mobile phase at a flow rate of 0.6 mL/min. The chromatograms were analyzed using the Turbo-chrome System.

Thermostability

The isoenzymes were incubated in 50 m*M* MES, pH 6.5 buffer at various temperatures. The aliquots were withdrawn at different time intervals, cooled in ice, and assayed for residual xylanase activity for determination of irreversible thermal inactivation parameters *(17)*.

Activation Energy for Denaturation (E_a)

The first-order rate constants for deactivation (k_d) of HAX and LAX at different temperatures were determined as described under Thermostability. The rate constants (k_d) for each isoenzyme were plotted and analyzed as described previously *(17,19)*.

Thermodynamic Activation Parameters

The thermodynamic data regarding xylan hydrolysis and enzyme denaturation were calculated by rearranging the Eyring's Absolute Rate Equation derived from Transition State Theory:

$$k_d \text{ or } k_{cat} = (K_B T/h)\exp(\Delta H^{\#}/RT)\cdot\exp(\Delta S^{\#}/R) \quad (1)$$

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

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

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

Rearranging Eq. 1 derives Eq. 3:

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

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

$$Q_{10} = \ln k_{T1}/k_{T2} = -E_a/R(T_2 - T_1)/(T_1 T_2) \quad (5)$$

Results and Discussion

Purification and Molecular Weights of Isoenzymes

Two charge isomers of xylanase were purified for the first time from the extracellular extract of *Scopulariopsis* sp. They were designated HAX and LAX based on their migration pattern on nondenaturing PAGE (Fig. 1A). Among all the xylanases in the crude extract, LAX was the most abundant, whereas HAX was the least abundant isoenzyme. The results of purification showed that we purified HAX threefold and LAX ninefold, and the percentage yield of HAX and LAX were 0.35 and 10, respectively (Table 1). Only HAX and LAX were subjected to kinetic and thermodynamic characterization. The native mol wt of HAX was found to be 144 kDa and that of LAX was 25 kDa (Table 2). The subunit mol wt of LAX was found to be 25 kDa by SDS-DR-PAGE (Fig. 1B, Table 2) and 28 kDa by SDS-PAGE (Fig. 2, Table 2). Similarly, the subunit mol wt of HAX was determined by SDS-DR-PAGE (Fig. 1B) and SDS-PAGE (Fig. 2) and found to be 36 and 37 kDa, respectively. Therefore, HAX is a tetrameric and LAX a monomeric enzyme. The molecular weights and kinetic parameters of HAX and LAX are shown in Table 2.

pH Optima Studies of Isoenzymes

The pH optima of HAX and LAX were the same (Table 2) despite differences in their charge characteristics. In xylanases, the active-site residues consist of two carboxyls, one of which donates protons to the substrate. The pH optimum of this class of enzymes depends on the pK_a of the proton-donating carboxyl, which is strongly influenced by the presence of hydrophobic residues in its immediate vicinity *(20)*. This decrease in the acidic character is apparently unable to influence the pK_a of the proton-donating carboxyl, and, hence, the pH optima of HAX and LAX are similar.

The pH optimum difference between acidic (pI < 5, pH optimum = 3.0 to 4.0) and basic xylanases (pI > 7, pH optimum = 4.0 to 5.0) from *Trichoderma reesei* is owing to the fact that in alkaline enzymes the proton-donating carboxyl makes a hydrogen bond with asparagine whereas in acidic isoenzymes it makes a hydrogen bond with aspartic acid *(21)*. The H-bond

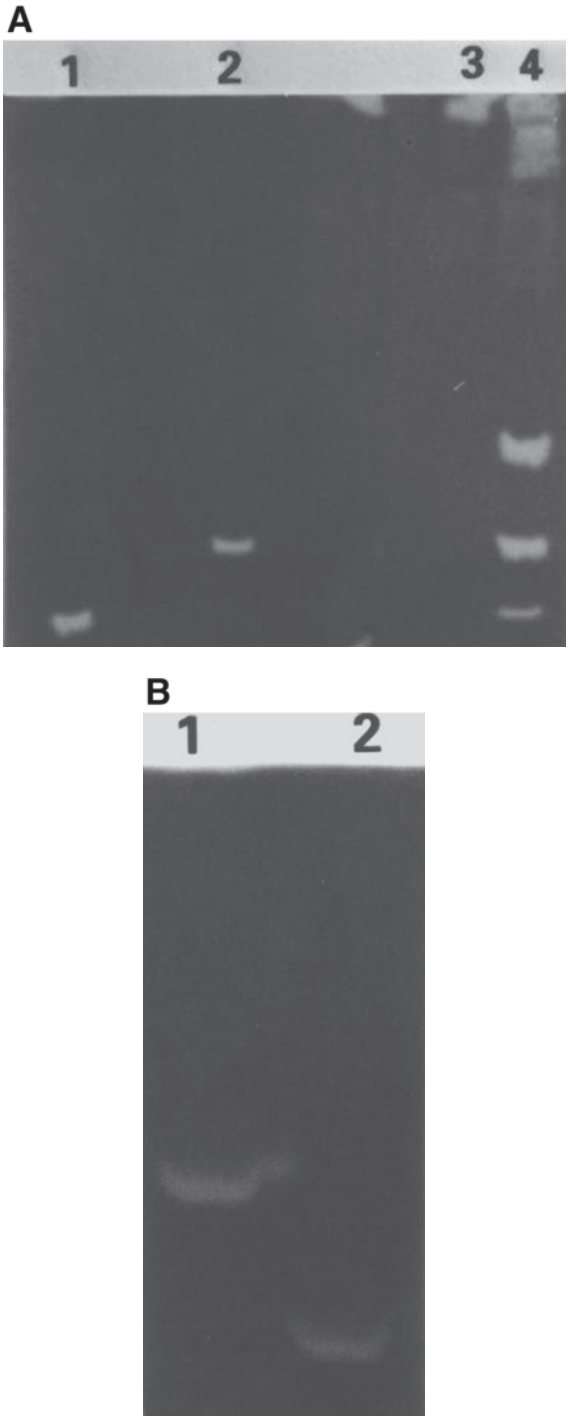


Fig. 1. **(A)** Native-PAGE (7.0 %) of HAX, MAX and LAX from *Scopulariopsis* sp. stained for xylanase activity. *Lane 1*: highly acidic xylanase (HAX), *lane 2*: medium acidic xylanase (MAX), *lane 3*: least acidic xylanase (LAX) and *lane 4*: crude extract. **(B)** 12.5% (w/v) SDS-denaturing-renaturing-PAGE of purified charge isomers stained for xylanase activity. From left to right, *lane 1*: HAX and *lane 2*: LAX. The molecular weight markers stained for protein are not shown.

Table 1
Summary of Purification Steps of Isoenzymes of Xylanase From *Scopulariopsis* sp.

Treatment	Enzyme[a]	Total units	Total protein (mg)	Specific activity (U/mg)	Purification factor	Recovery (%)
Crude extract	—	13,350	3926	3.4	1	100
70% Ammonium sulfate precipitation	—	3360	763.6	4.4	1.29	25
Phenyl Superose: HIC	—	2952	508.9	5.8	1.71	22
Q Sepharose:	HAX	57	10.5	5.4	1.60	0.43
Hi-load anion-exchange	MAX	156	26	6.0	1.76	1.17
chromatography	LAX	1635	125.76	13	3.82	12.24
Superose:	HAX	47.5	4.75	10	2.94	0.35
gel filtration chromatography	LAX	1356	43.74	31	9.11	10.16

[a]Only HAX and LAX were purified to homogeneity and further kinetically and thermodynamically characterized. HIC, hydrophobic interaction chromatography.

Table 2
Properties of Two Isoenzymes of Xylanase From *Scopulariopsis* sp.

Property	LAX	HAX
Native mol wt (kDa)[a]	25,000	144,000
Subunit mol wts (kDa)	28,000[f]/25,000[g]	37,000[f]/36,000[g]
k_{cat} (min^{-1})[b]	95,000	9900
V_{max} (μmol/[min·mg])[b]	5096	328
Turnover time, T_t (ms/mol)[b]	0.63	6.06
K_m (% [w/v])[b]	3.0	0.33
Specificity constant (k_{cat}/K_m)[b]	32,000	30,000
T_t/K_m (ms/[substrate mol·%])	0.20	18.18
pH optimum[c]	6.5	6.5
Temperature optimum (°C)[d]	50	50
Activation energies (kJ/mol)[e]	69, –14	23, –74
Q_{10}[d]	2.40	1.34

[a]Determined by gel filtration chromatography on fast protein liquid chromatography (FPLC).
[b]Determined at 40°C, pH 6.5, using oat-spelt xylan.
[c]Determined at 40°C using 0.5% (w/v) xylan.
[d]Determined at pH 6.5 using 0.5% xylan and 10-min incubation time.
[e]Determined at pH 6.5 using 0.5% xylan and 10-min incubation time. The positive values of E_a show the progressive increase in the xylanase rate up to the inflexion point (optimum temperature), whereas the negative values of E_a show the progressive decrease in the enzymatic rate owing to the thermal inactivation of the enzyme.
[f]Determined by 15% SDS-PAGE.
[g]Determined by 12.5% (w/v) SDS-DR-PAGE.

between carboxyl and asparagine residues in alkaline isoenzymes raises the pK_a of the catalytic residue with a concomitant increase in pH optimum. Interestingly, the xylanase from *T. reesei* has an acidic p*I* and an alkaline pH optimum, because an asparagine rather than an aspartic residue is H-bonded to the catalytic carboxyl *(21)*. This explains why both acidic HAX and basic LAX have identical pH optima (Table 2). The same evidence could possibly lend support to the view that LAX and HAX might not be distinct gene products but that LAX could have been derived from HAX by proteolytic cleavage *(22)*. In the case of different gene products, both LAX and HAX would have different pH optima corresponding to a different p*I*, as in the case of XYNI and XYNII (family G) of isoenzymes from *T. reesei (21)*, although it must be stressed that the present results are not sufficient enough to assign with certainty the family status (F or G) to either HAX or LAX.

Catalytic Efficiency of Isoenzymes

Significant differences in the values of V_{max} and K_m were found in the case of LAX and HAX (Table 2). The V_{max} (5096 μmol/[min·mg of protein]) and k_{cat} (95,000 min^{-1}) values of LAX at 40°C were abnormally high for a mesophilic polymer-degrading glucanase; they are usually in the range of 15–85 U/mg for xylanase from *Bacilluss polymyxa (23)*, 5–50 U/mg for xylanase from *Chaetomium thermophile (24)*, and 588 U/mg for xylanase

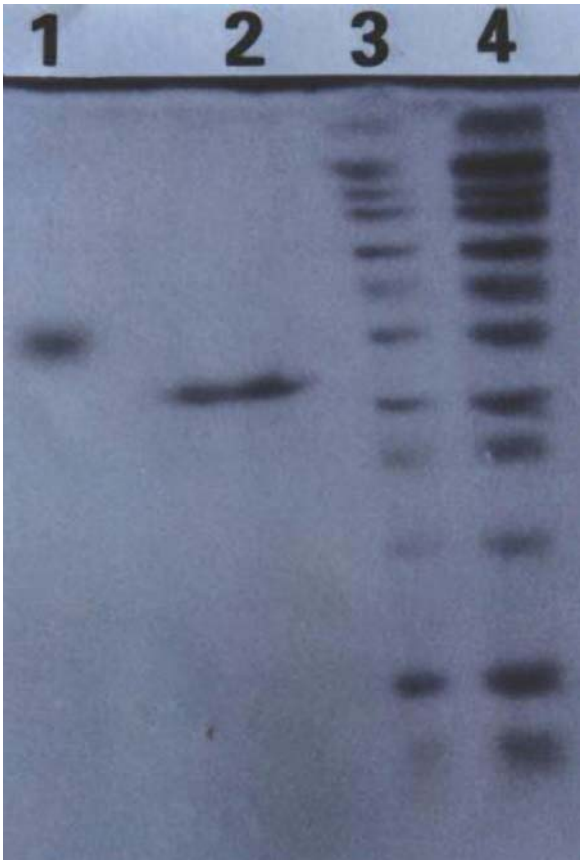


Fig. 2. 15% SDS-PAGE of purified xylanases stained for protein for the determination of subunit molecular weight. From left to right, *lane 1*: HAX, *lane 2*: LAX, *lanes 3* and *4*: wide range molecular weight (kDa) markers (top to bottom, α-galactosidase; 116, phosphorylase b; 97, fructose-6-phosphate kinase; 84, BSA; 66, glutamate dehydrogenase; 55, ovalbumin; 45, glyceraldehyde-3-phosphate dehydrogenase; 36, carbonic anhydrase; 29, trypsinogen; 24, trypsin inhibitor; 20, α-lactalbumin; 14.2 and aprotin; 6.5)

from *Aspergillus fischeri (25)*. Our values were found to be only second to those published for xylanase from *Aureobasidium pullulans* Y-2311-1 *(4)* after normalizing V_{max} values using the Q_{10} formula (Eq. 5). Li et al. *(4)* reported a V_{max} of 2650 µmol/(min·mg of protein) at 28°C for xylanase from *A. pullulans*. The V_{max} of xylanase from *Trichoderma longibrachiatum (26)* was also found to be high (4025 U/mg of protein at 45°C). Interestingly, when the k_{cat} of LAX and cold-active xylanases were calculated at various temperatures by using the Q_{10} equation, it was found that the k_{cat} of LAX (79,000 min^{-1}; Q_{10} = 2.4) was nearly comparable with that of cold-adapted xylanase from *Pseudoalteromonas haloplanktis* (99,000 min^{-1}; Q_{10} = 1.32) at 35°C *(7)*. At their respective T_{opt}, LAX (228,000 min^{-1} at 50°C) was more than twice as active compared with cold-adapted xylanase (79,000 min^{-1} at 35°C). It should be

Table 3
Thermodynamics of Xylan Hydrolysis
by HAX and LAX From *Scopulariopsis* sp.

Parameter[a]	LAX	HAX
ΔG^* (kJ/mol)	57.6	63.5
ΔH^* (kJ/mol)	66.4	20.4
ΔS^* (J/[mol·K])	28.1	–137.7

[a]The parameters are defined as follows: $\Delta G^* = -RT \ln(k_{cat} \cdot h)/(K_B \cdot T)$
$\Delta H^* = E_a - RT$
$\Delta S^* = (\Delta H^* - \Delta G^*)/T$

kept in mind that cold-adapted enzymes are highly active compared to their mesophilic and thermophilic homologs owing to their decreased $\Delta H^{\#}$ *(27,28)*. On the contrary, the $\Delta H^{\#}$ of LAX (66 kJ/mol) was very high compared with that of both HAX (20 kJ/mol) (Table 3) and cold-adapted xylanase (21 kJ/mol) *(29)*. As shown in Table 3, high activity in LAX was achieved by increasing its $\Delta S^{\#}$ (28 J/[mol·K]) as compared with HAX (–137 J/[mol·K]) and cold-active xylanase (–116 J/[mol·K]). This entropically driven high activity of LAX could possibly be explained as follows: In many xylanases, such as from *Pseudomonas fluorescens*, there is a chain of about 20 well-ordered water molecules, which carpet the length of the active site *(30)*. All or some water molecules are displaced when the substrate or transition state binds with the active site *(30)*. There is a gain of 40 J/K in entropy for every mole of water released *(31,32)*. If more molecules of water are released on binding of the transition state (sofa form of xylose residue) to enzyme than on binding of the ground-state substrate (chair form of xylose residue) to the active site, then there will be considerable entropic benefit for the formation of enzyme-transition-state complex *(33)*, thus resulting in high activity. Another example of positive $\Delta S^{\#}$ (35.5 J/[K·mol]) is the hydrolysis of chymotrypsinogen by trypsin, whereas replacement of the substrate with the smaller benzoyl-L-arginine amide resulted in $\Delta S^{\#}$ of –26 J/(K·mol) *(34)*.

It has been found that in the case of tyrosyl-tRNA synthetase, the binding of tyrosine displaces a bound water molecule whereas phenylalanine probably does not *(31)*. Recently, it has been found that more active guanosine 5'-triphosphatase (GTPase) from *Methanosarcina thermophila* has positive $\Delta S^{\#}$ compared with less-active enzyme from psychotolerant *Methanococcoides burtonii* whose $\Delta S^{\#}$ was negative. In GTPases the binding of guanosine di- and triphosphates has been shown to involve interactions of the nucleotide with water molecules. The displacement of these water molecules on substrate or transition-state binding is supposed to affect the $\Delta S^{\#}$ values of these GTPases *(35)*. The high value of $\Delta H^{\#}$ in the case of LAX compared with HAX is indicative of more energy required to distort the xylose ring from the chair to sofa conformation *(36)*.

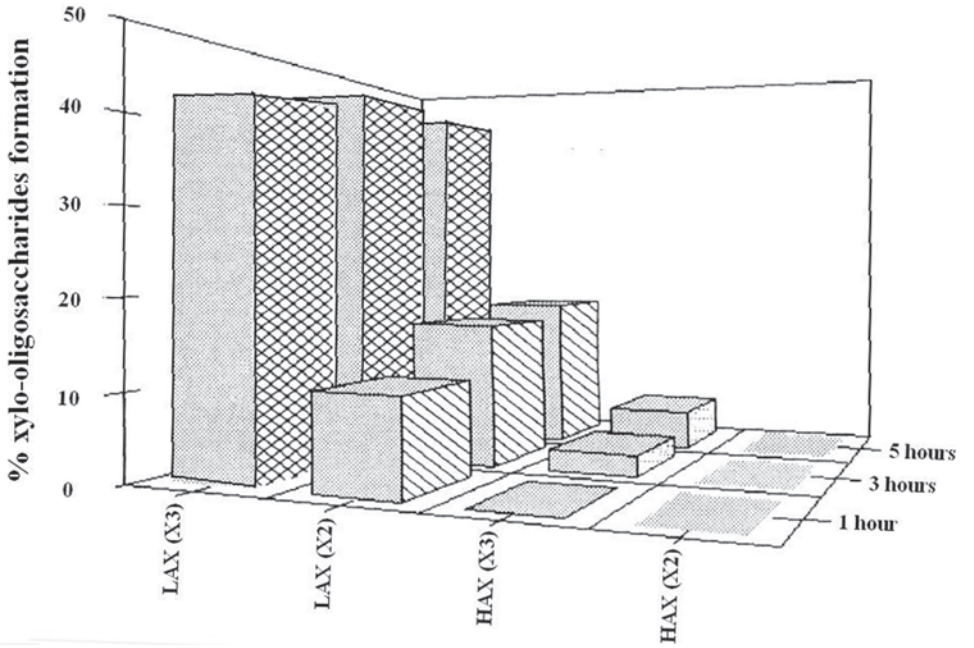


Fig. 3. 3D Histogram of xylan hydrolysis analyzed on HPLC showing xylo-oligosaccharide formation by 0.56 nmol of HAX and 0.72 nmol of LAX for 1, 3, and 5 h respectively in 1 mL of 50 m*M* MES (pH 6.5) containing 0.5% (w/v) xylan at 40°C.

The V_{max} or k_{cat} values of LAX are almost an order of magnitude higher than that of HAX. As shown by HiRACIN-PAGE *(16)* (Fig. 1A), LAX is a much more basic molecule than HAX. The acidic (HAX) and basic (LAX) isoenzymes roughly correspond to p*I* 5.5 and 9 xylanase isoenzymes from *T. reesei (37)*. The more basic isoenzyme (p*I* 9) is also an order of magnitude more active than the acidic isoenzyme *(37)*, as found in the case of isoenzymes from *Scopulariopsis* sp. Similarly, the two isoenzymes from *C. thermophile* have an order-of-magnitude difference in V_{max} values *(24)*. Moreover, LAX does not follow a normal Lineweaver-Burk plot, because the intercept on the *x*-axis is positive while the intercept on the *y*-axis is negative (graph not shown). Similar results were obtained in the case of carboxymethylcellulase (CMCase) from *A. niger (18)*.

Degradation Products of Xylan Hydrolysis

HPLC analysis was done utilizing 20 µg of each isoenzyme, which corresponds to 0.56 nmol of HAX and 0.72 nmol of LAX after applying the correction factor and keeping in mind their respective subunit molecular weights. The HPLC results showed that LAX was 15 times more efficient in hydrolyzing the substrate than HAX (Fig. 3), proving conclusively that the former is at least an order of magnitude more efficient in xylan hydrolysis than the latter. Another important finding regarding HPLC analysis was that HAX formed insignificant amounts of xylobiose when compared with

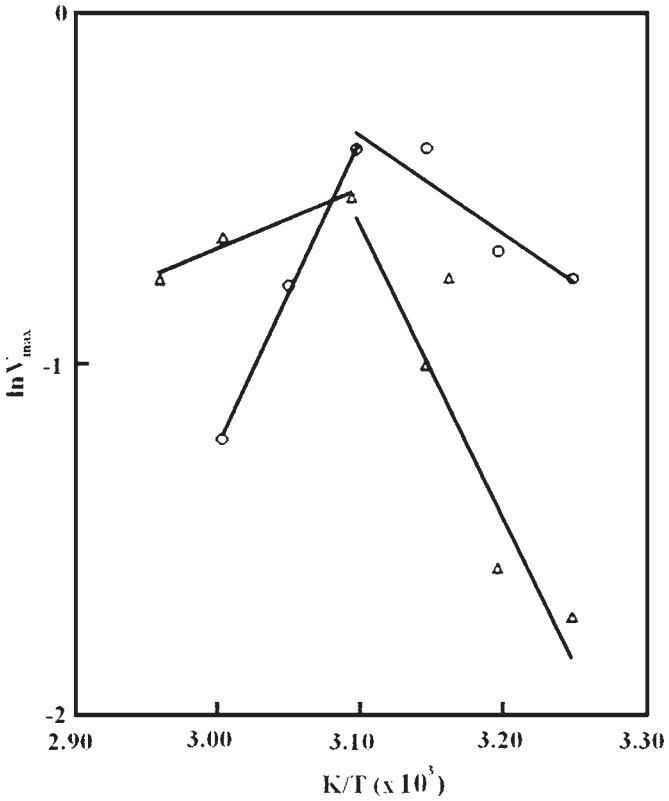
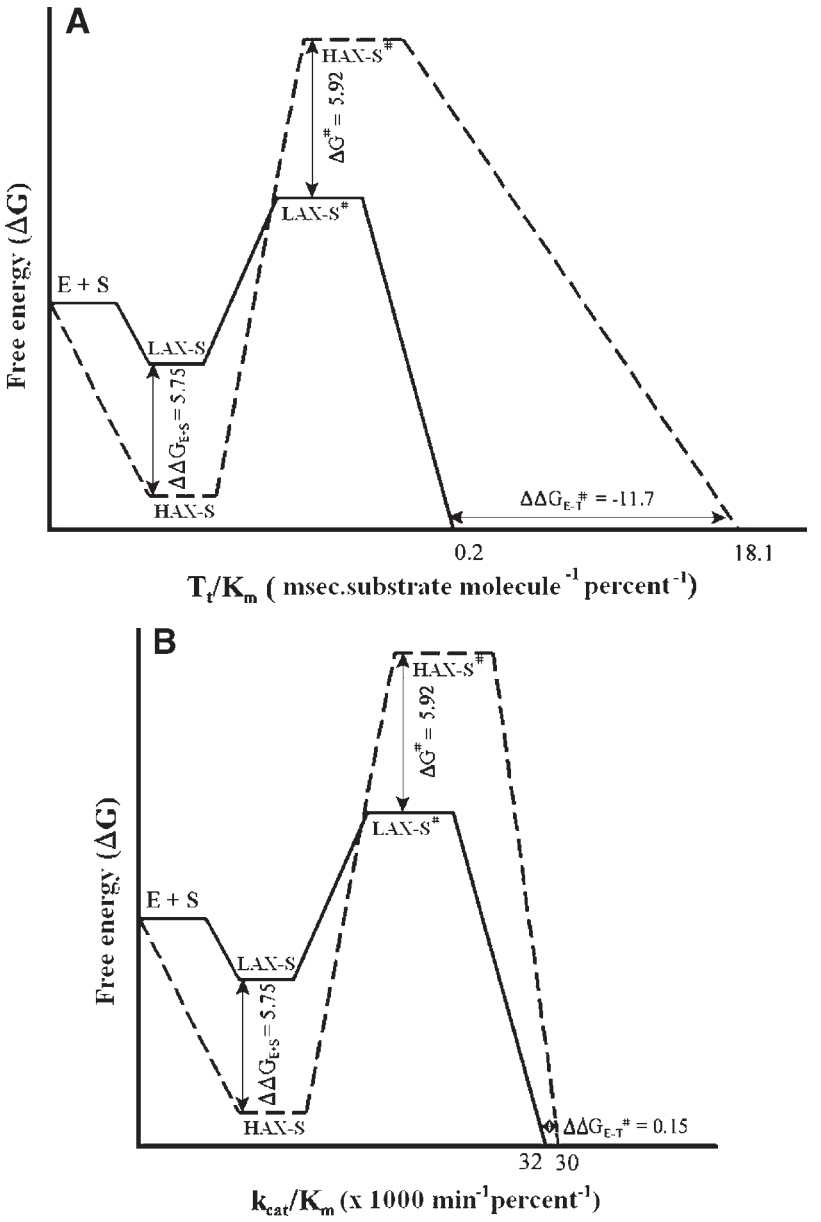


Fig. 4. Arrhenius plot for the determination of activation energy for the hydrolysis of xylan by different isoenzymes. HAX (○) and LAX (△). Activation energy (E_a) = Slope·R, where R = 8.314 JK^{-1}/mol.

LAX. HPLC analysis showed a lack of xylose formation, thus indicating the absence of β-xylosidase activity in our purified samples.

Effect of Temperature on Xylan Hydrolysis

The curves to the left of T_{opt} of the Arrhenius plots show that LAX is more thermostable than HAX (Fig. 4). The slope of the plot (Fig. 4) on the right side of the T_{opt} corresponds to the activation energy of xylanase activity, while that on the left side of the temperature optimum corresponds to the activation energy of xylanase inactivation owing to enzyme denaturation *(18,38)*. The activation energies of xylan hydrolysis of HAX and LAX were 23 and 69 kJ/mol, respectively (Fig. 4). The results for the activation energies of HAX and LAX for xylan hydrolysis are surprising because the activation energy for highly active LAX is more than HAX. Thus, for LAX to be more active than HAX, the higher E_a for LAX has to be compensated by the entropy term. The increase in activation energy for LAX is more than compensated by an increase in entropy for LAX, resulting in a lower free energy of xylan hydrolysis for LAX compared with HAX (Table 3, Scheme 1).



Scheme 1. Energy-activity diagram of LAX and HAX depicting that T_t/K_m **(A)** is a more meaningful constant than k_{cat}/K_m **(B)**. $\Delta\Delta G_{E-S} = \Delta G_{E-S(HAX)} - \Delta G_{E-S(LAX)}$, where $\Delta G_{E-S} = -RT \ln K_a$ ($K_a = 1/K_m$), $\Delta\Delta G^{\#} = \Delta G^{\#}_{(HAX)} - \Delta G^{\#}_{(LAX)}$, where $\Delta G^{\#} = -RT \ln (k_{cat} \cdot h)/(K_B \cdot T)$ and $\Delta\Delta G^{\#}_{E-T} = \Delta G^{\#}_{E-T(HAX)} - \Delta G^{\#}_{E-T(LAX)}$, where $\Delta G^{\#}_{E-T} = -RT \ln k_{cat}/K_m$ in case **A** and $\Delta G^{\#}_{E-T} = -RT \ln T_t/K_m$ in case **B**. The sum of $\Delta\Delta G_{E-S} + \Delta\Delta G^{\#} = 11.7$ kJ/mol in both **A** and **B**, whereas $\Delta\Delta G^{\#}_{E-T} = -11.7$ and -0.15 kJ/mol in case **A** and **B** respectively. Therefore, $\Delta\Delta G^{\#}_{E-T}$ (using T_t/K_m) $= \Delta\Delta G_{E-S} + \Delta\Delta G^{\#}$ **(A)** but $\Delta\Delta G^{\#}_{E-T}$ (using k_{cat}/K_m) $- \Delta\Delta G_{E-S} + \Delta\Delta G^{\#}$ **(B)**, hence proving that T_t/K_m is more meaningful constant than k_{cat}/K_m. Note that $\Delta\Delta G_{E-S} + \Delta\Delta G^{\#}$ is positive (11.7 kJ/mol), which means that this amount of energy is needed to form transition state (LAX–$S^{\#}$ and HAX–$S^{\#}$) from the ground state enzyme-substrate complex (LAX–S and HAX–S). Similarly, $\Delta\Delta G^{\#}_{E-T}$ (free energy of transition state binding) is negative (–11.7 kJ/mol), which means that this amount of energy is released as a result of binding of LAX and HAX with the transition state of xylan backbone.

Thermodynamics of Xylan Hydrolysis

The thermodynamic parameters of xylan hydrolysis by xylanases are shown and explained in Scheme 1. LAX has both k_{cat} and K_m values ninefold higher than HAX (Table 2), which corresponds to xylanase transition-state complementarity. This could mean that the chair conformation of the substrate (xylose polymer) binds less strongly to the active-site pocket of LAX than the transition state (sofa conformation of positively charged oxocarbonium ion). According to Fersht *(31)*, the principle of enzyme transition-state complementarily states that both the k_{cat} and K_m will be increased because the active site will bind the transition state more strongly than the original substrate, resulting in concomitant enhancement in the binding energy, hence increasing the rate of reaction. This principle does not support the widely held view that low K_m or tight binding of substrate with the active site is the key to efficient catalysis, but, on the contrary, high K_m maximizes the enzymatic rate. The K_m of highly active LAX is ~10 times less than the less active HAX (Table 2). It is now becoming obvious that in many cold-active enzymes, high k_{cat} is accompanied by high K_m compared with their mesophilic and thermophilic homologs *(28)*. For example, it was found that all the α-amylase mutants from cold-active *P. haloplanktis* showed a close association between k_{cat} and K_m. High k_{cat} is always accompanied by high K_m and low k_{cat} is accompanied by low K_m *(28)*. Moreover, the K_m of both LAX (30 mg/mL) and cold-active xylanase (28 mg/mL) is also comparable *(7)*. It is therefore more reasonable to use T_t/K_m (T_t = turnover time = $1/k_{cat}$) instead of usual k_{cat}/K_m values for comparisons (Table 2, Scheme 1). If we keep in mind that the T_t/K_m value of LAX (0.2 ms of substrate/[mol·%]) is significantly lower than that of HAX (18 ms of substrate/[mol·%]), then according to the complementarity principle *(31)*, we can conclude that LAX is catalytically much more efficient than HAX. This line of reasoning is depicted in the energy-activity diagram in Scheme 1A, which shows that the total energy (11.7 kJ/mol) needed to form the transition state from the enzyme-substrate complex is exactly balanced by the release of 11.7 kJ/mol as a result of transition-state binding if T_t/K_m is employed as a constant. By contrast, employing k_{cat}/K_m as a constant gives only 0.15 kJ/mol of transition-state binding energy (Scheme 1B).

Thermodynamics of Irreversible Inactivation of Isoenzymes

Although work on the thermodynamic stability of a xylanase from *Streptomyces halstedii* has been reported *(39)*, no work has been published regarding the kinetic stability of xylanases. We recently reported the kinetic stability of native and chemically modified CMCases from *A. niger (40)*. The kinetic stability of an enzyme could be enhanced by either increasing $\Delta H^{\#}$ (favoring more ionic or hydrophobic interactions) or decreasing $\Delta S^{\#}$ (increasing the order of transition state) *(40–44)*.

Very interesting and significant results were obtained on the thermodynamics of inactivation (Fig. 5A,B; Table 4) that clearly showed that LAX

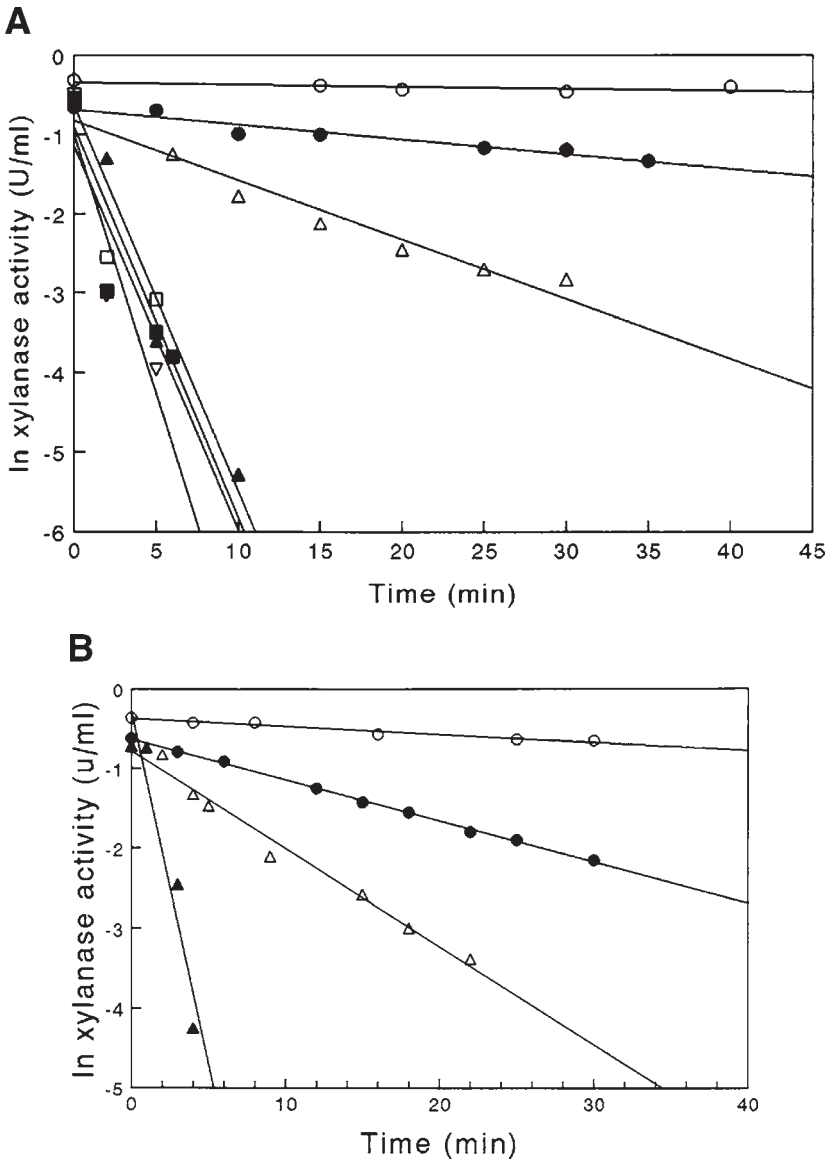


Fig. 5. **(A)** First-order plots of the effect of thermal inactivation on LAX. Samples were incubated at 55°C (○), 60°C (●), 65°C (△), 70°C (▲), 75°C (□), 80°C (■) and 85°C (▽) in 50 m*M* MES buffer, pH 6.5 and aliquots withdrawn at different time intervals were cooled on ice before assaying for residual enzyme activity at 40°C. **(B)** First-order plots of the effect of thermal inactivation on HAX. Samples were incubated at 55°C (○), 60°C (●), 65°C (△) and 70°C (▲).

is fourfold more thermostable than HAX at 50°C. To explain the enhanced resistance of LAX to irreversible inactivation compared with HAX, the thermostability of both isomers was determined at various temperatures (Fig. 6) and the data were analyzed by applying the Transition State Theory.

Table 4
Kinetic and Thermodynamic Parameters
for Irreversible Thermal Inactivation of LAX and HAX

Enzyme	T (K)	k_d (s^{-1})	$t_{1/2}$ (min)	$\Delta H^{\#}$ (kJ/mol)	$\Delta G^{\#}$ (kJ/mol)	$\Delta S^{\#}$ (J/[mol·K])
LAX	328	4.33×10^{-5}	267	306.30	306.30	604.67
	333	0.31×10^{-3}	37	306.27	104.20	606.80
	338	1.25×10^{-3}	9.0	306.22	101.90	604.49
	343	8.10×10^{-3}	1.43	306.18	98.13	606.55
	343	8.10×10^{-3}	1.43	18.77	98.13	–231.50
	348	8.19×10^{-3}	1.41	18.72	99.57	–232.32
	358	10.91×10^{-3}	1.00	18.64	101.66	–231.89
HAX	328	0.16×10^{-3}	69	264.73	104.40	488.88
	333	0.85×10^{-3}	13	264.69	101.41	490.30
	338	2.04×10^{-3}	5	264.64	100.53	485.53
	343	14.6×10^{-3}	1	264.60	96.45	490.23

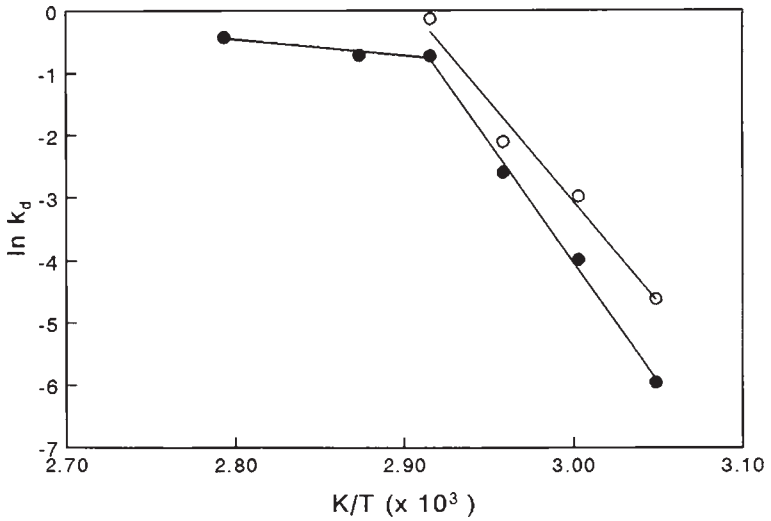


Fig. 6. Arrhenius plot for the determination of activation energy for denaturation of HAX (○) and LAX (●).

The thermodynamic parameters revealed quite interesting results (Table 4). At lower temperatures, the enhanced thermostability of LAX was owing to its higher value of $\Delta H^{\#}$ compared with HAX (Table 4), which signifies that more ionic interactions are present on the surface of LAX than HAX. The results regarding xylanase mutant *(45)*, native β-glucosidase *(46)*, and thermophilic GTPase *(35)* also showed that their increased thermostabilities were owing to elevated $\Delta H^{\#}$. On the contrary, cold-active xylanase showed that its thermolability is entropically driven *(29)*.

More dramatic results were obtained when the thermodynamic parameters regarding LAX and HAX inactivation were compared at higher temperatures (Table 4). HAX completely lost its activity around 70°C, whereas LAX was still active with a concomitant decrease in the entropy of activation ($\Delta S^{\#}$) to negative values. This is a very significant finding; it means that the partially unfolded transition state of LAX is more ordered than its native state. This finding has been reported in the case of native CMCase from *A. niger (47)* and α-amylase from *Bacillus licheniformis (48)*. The reason for this behavior is presumably twofold. First, hydrophobic interactions are weakened at lower temperatures but become stronger as the temperature is increased, whereas the reverse is true in the case of ionic interactions *(44)*. Second, enhanced hydrophobic interactions compensate for the increased thermal agitations at higher temperatures, thus preventing thermal unfolding *(49)*. It seems that if the hydrophobic side chains of an enzyme are in close proximity, a negative entropy effect will be seen at higher temperatures provided that the active site of the enzyme retains its structure. The more thermostable LAX undergoes conformational change at higher temperatures, which rearranges its structure in such a way as to enhance its hydrophobic interactions *(44)*, whereas HAX is completely denatured under similar conditions. At lower temperatures, the transition state of HAX ($\Delta S^{\#}$ = 488 J/[mol·deg]) is more ordered than that of LAX ($\Delta S^{\#}$ = 604–606 J/[mol·deg]; Table 4), probably owing to lesser number of nonpolar amino acid side chains. Another reason for the enhanced thermostability of LAX could be the fact that at pH 6.5, HAX is predominantly negatively charged, whereas LAX is neutral. The excess negative charge on HAX will result in charge repulsion, thereby making the overall structure more flexible and, hence, more thermolabile *(50)*.

Compensation Effect

If a linear variation of change in $\Delta H^{\#}$ is accompanied by a change in $\Delta S^{\#}$, a compensation effect is said to have taken place *(51)*. Recently, it has been proposed that entropy–enthalpy compensation is caused by an ordering of water molecules around exposed hydrophobic residues with concomitant reduction of entropy of the transition state *(52)*. Thermal inactivation processes exhibiting a similar mechanism of reaction or nature of the transition state show identical temperature of compensation or isokinetic temperature *(40,52)*. In the case of native HAX, native LAX, and acetylated LAX (our unpublished results), the temperature of compensation was found to be 70°C (Fig. 7), which means that the mechanism of unfolding is different for all xylanases above and below the transition temperature of 70°C.

Activity and Thermostability

Previous studies have suggested that stability at high temperatures is incompatible with high catalytic activity *(53,54)*. It has been reasoned that enzymes cannot be both highly active and highly thermostable at the same

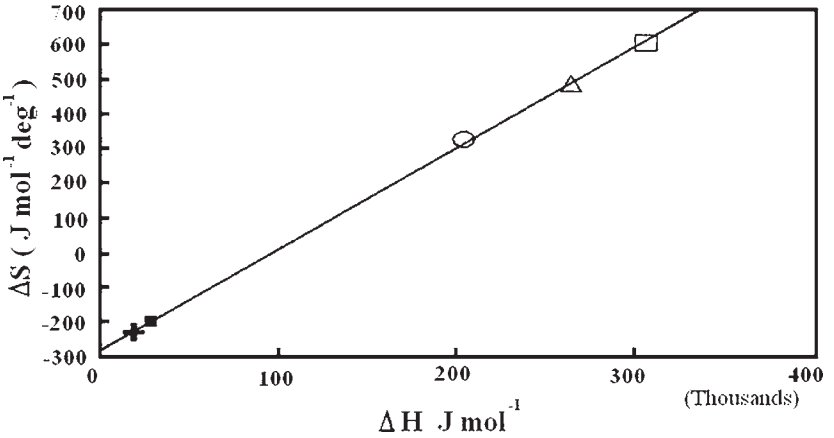


Fig. 7. Compensation plot between $\Delta H^{\#}$ and $\Delta S^{\#}$ for the determination of transition temperature. Native LAX at 328 K (□), native HAX at 328 K (△), native LAX at 343 K (+), acetylated LAX at 328 K (○) and acetylated LAX at 339 K (■). Transition temperature = 1/slope.

time. For example, there is a trade-off between high activity and thermostability in the case of cold-adapted enzymes, which is owing to the fact that high activity requires flexible structure and thermostable enzymes need to be rigid *(27,28)*. It was hypothesized that stability at high temperatures is incompatible with high rates of catalysis at lower temperatures through mutually exclusive demands on enzyme flexibility. Recently, though, it has been shown in the case of thermolysin-like protease from *Bacillus* sp. *(55)* and thermostable esterase *(56)* that enzymes can be thermostable and catalytically active at the same time. Our results are also in line with this observation; LAX was simultaneously more active and thermostable than HAX. It is now believed that thermostability and catalysis do not make mutually exclusive demands on enzyme flexibility but that natural selection exerts pressure on one property and not both *(56)*. Furthermore, analysis of the stabilities and activities of a large number of random mutations in thermolysin like protease (TLP) showed that the two properties are not inversely related *(57)*. However, mutations that increase thermostability while maintaining low-temperature activity are extremely rare *(58)*.

Conclusion

A correlation between protein stability and protein activity has been reported *(53)*, which showed that an increase in activity is accompanied by decreased thermostability. The correlation of activity and stability has recently become a subject of debate, and there is mounting evidence that enzymes can be active and thermostable simultaneously *(55,56)*. Our results showed that LAX is simultaneously more active and thermostable than HAX. The high catalytic activity of such thermostable enzymes at mesophilic temperatures suggests that these enzymes combine local flexibility

of the active site with overall structural rigidity *(59)*. The V_{max} and k_{cat} of LAX were of the highest reported for polymer-degrading glucanases within the mesophilic group of enzymes, and, unlike cold-active enzymes, this attainment of high activity is not owing to a reduction in $\Delta H^{\#}$. For the first time, an energy–activity diagram showed T_t/K_m to be a more consequential constant than k_{cat}/K_m for depiction of the high enzyme activity of LAX.

Acknowledgments

We wish to acknowledge Dr. David Lightfoot for meaningful discussions and help with the manuscript; and Sikander Ali, director NIBGE, for providing research facilities. The research work was part of the M.Phil (Biotechnology) of AJA and supervised by KSS.

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