DEDICATED TO MY LATE FATHER.....

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DECLARATION

This is to certify that the work incorporated in the thesis entitled **"Studies on xylan degrading enzymes from an alkalophilic thermophilic *Bacillus* sp."** submitted by **Mrs. Devyani S. Nath** was carried out by the candidate under my supervision at Biochemical Sciences Division, National Chemical Laboratory, Pune. Material obtained from other sources has been duly acknowledged in the thesis.

Dr. (Mrs) Mala Rao
(Research Guide)

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DEVYANI NATH

ABSTRACT

The increased interest in the study of extremophilic microorganisms is mainly due to the fact that they are considered as an important biotechnological resource and their specific properties are expected to result in novel process applications. Useful insights gained from the study of extremophilic enzyme systems can extend the understanding of protein chemistry in addition to expanding the potential applications of biocatalysis. Xylan is the major component of hemicellulose, which accounts for 30 % of the dry weight of some plant tissues. Microbial xylanases are the preferred catalysts for xylan hydrolysis due to their high specificity, mild reaction conditions, negligible substrate loss and side product generation. Cellulase-free xylanases have attracted much interest, especially in the paper and pulp industry. Xylanases play an important role in the development of eco-friendly processes by reducing the use of hazardous chlorine chemicals in bleaching processes. They are also important from the point of view fundamental research since, multiple xylanases produced by an organism have diverse structure-function correlation. The commercial and academic importance of extremophilic microorganisms, prompted us to investigate the biochemical and molecular aspects of xylan degrading enzymes from an alkalophilic thermophilic *Bacillus* sp. (NCIM 59). The main features of the work done are:

[A] PURIFICATION AND CHARACTERISATION OF XYLANASE (XYL II) FROM ALKALOPHILIC THERMOPHILIC *BACILLUS* SP. (NCIM 59).

The alkalophilic thermophilic *Bacillus* sp. (NCIM 59) produces cellulase-free xylanase at 50 °C and pH 10.0. The gel filtration chromatographic analysis of the culture filtrate broth showed the presence of two xylanases differing in molecular weight and electrophoretic mobility. In the present studies, Xylanase II (Xyl II) was purified to homogeneity by chromatography on CM-Sephadex followed by Biogel P-10. The molecular weight of the enzyme as determined by SDS-PAGE was 15,800. The optimum pH and temperature of the enzyme were pH 6.0 and 50 °C, respectively. It was found to be stable at 50 °C for 168 h at pH 7.0. It is a basic protein with a pI of 8.0. The Michaelis constant and V_{max} values were estimated to be 3.5 mg/ml and 0.742 μmole min^{-1} mg^{-1}, respectively. Xyl II yielded xylobiose, as the major end product along with xylose,

xylotriose, xylotetraose, and other higher xylooligosaccharides. End product analysis of xylan hydrolysis demonstrated that Xyl II is an endoenzyme. The amino acid analysis showed the predominance of Asp and Glu residues. The N-terminal sequence of Xyl II as determined by Edman degradation method was AVYGGQYATDNL and was distinctly different from other reported xylanases.

[B] STRUCTURE-FUNCTION ANALYSIS OF XYL II.

The pH induced conformational and structural changes of Xyl II have been investigated from the alkalophilic thermophilic *Bacillus* sp. using kinetic, circular dichroism and fluorescence spectroscopy studies. The Trp fluorescence and the kinetic constants were found dependent on the pH. Above pH 8.0, the enzyme exhibited unfolding transitions as revealed by a red shift in the emission maximum as well as decreases in the fluorescence intensity. Circular dichroism studies revealed a decrease in the CD ellipticity at 222 nm at pH 9.0 and 10.0. The reduced catalytic activity of Xyl II at alkaline pH is correlated to the pH induced unfolding and ionization or protonation of key protein residues. The pH profile of Xyl II showed apparent p*k* values of 5.5 and 7.0 for the free enzyme and 5.6 and 6.7 for the enzyme-substrate complex. The abnormally high pk_a of 6.7 indicated the participation of a carboxyl group present in a non-polar environment. The essential amino acid residues for the function of Xyl II activity was resolved through group specific chemical modification studies. Modification of Xyl II with NBS, HNBB, and WRK led to the inactivation of the enzyme suggesting the involvement of a single Trp and carboxyl residue in the active site of the enzyme. Trp residues were located in a relatively electronegative microenvironment as suggested by the quenching studies using charged and neutral quenchers. The proximity of the essential carboxyl residues with high pk_a value of 6.9 probably contributed to the electronegative environment of the Trp residue. Xyl II was highly stable at pH 10, however the enzyme exhibited pH optimum at near neutral values, which can be explained by the ionization and microenvironment of the active site residues. The sequence alignment studies of Xyl II, in combination with kinetic and chemical modification data provide strong evidence for the participation of Trp61 and Asp94 in the catalytic function and may play a role in the structure function of the enzyme.

[C] FOLDING/UNFOLDING STUDIES OF XYL II

SECTION I. A PARTLY FOLDED STATE OF XYL II AT LOW pH.

The conformation and stability of Xyl II at acidic pH was investigated by equilibrium unfolding methods. Using intrinsic fluorescence and CD spectroscopic studies, we have established that Xyl II at pH 1.8 (A-state) retains the helical secondary structure of the native protein at pH 7.0, while the tertiary interactions are much weaker. At variance, from the native species (N-state), Xyl II in the A-state binds 1-anilino-8-sulfonic acid (ANS), a property considered most typical of a protein in the molten globule state. Lower concentration of Gdn HCl are required to unfold the A state. For denaturation by Gdn HCl, the midpoint of the co-operative unfolding transition measured by fluorescence for the N-state is 3.5 M, which is higher than the value (2.2 M) observed for the A-state at pH 1.8. Thus, our results suggest that an equilibrium unfolding intermediate may be present on the unfolding pathway of Xyl II at acid pH.

SECTION II. a -CRYSTALLIN MEDIATED REFOLDING OF XYL II.

Chaperones are catalysts in the sense that they transiently interact with their substrate proteins but are not present in the final folded product, and in that, they increase the yield of folded protein. Crystallins are group of structural proteins, which share both sequence and structural homology with small heat shock proteins. For the functional *in vitro* analysis of α-crystallin, Xyl II from alkalophilic thermophilic *Bacillus* was used as a model system. We have attempted to study the refolding of Gdn HCl denatured Xyl II in the presence of the molecular chaperone, α-crystallin. Our results based on the intrinsic tryptophan fluorescence and the hydrophobic flurophore 8-anilino-1-naphthalene sulfonate binding studies, suggest that α-crystallin forms a complex with a putative molten globule like intermediate in the refolding pathway of Xyl II. The complex exhibited no functional activity. Addition of ATP to the α-crystallin· Xyl II complex initiates the renaturation of Xyl II and 30-35 % activity was recovered. ATP hydrolysis was not a prerequisite for Xyl II release, since the nonhydrolyzable analogue 5'-adenylyl imidodiphosphate (AMP-PNP) was capable of reconstitution of active Xyl II. Although metal ions Ca^{2+} or Mg^{2+} were not required for the *in vitro* refolding of Xyl II, the renaturation yields were enhanced in their

prescence. The ability of refolding was quite specific for α-crystallin since bovine serum albumin and PEG had no effect on the refolding of Xyl II.

SECTION III. ARTIFICIAL CHAPERONE MEDIATED REFOLDING OF XYL II.

To gain insight into the molecular aspects of unfolding/refolding of enzymes from extremophilic organisms, we have used Xyl II from an alkalophilic thermophilic *Bacillus* as the model system. Kinetics of denaturation/renaturation were monitored using intrinsic fluorescence studies. The shift in fluorescence maximum was used to detect the folding intermediate(s). The structural changes during the process showed nonlinear curves. The refolding occurs in at least two phases. The protein fluorescence measurement suggested a state of the protein present in 0.08 M Gdn HCl a species with an emission maximum of 345 nm and was interpreted as a partially folded intermediate state. Far-UV CD spectra revealed that the putative intermediate had a similar content of secondary structure as the native enzyme. Studies with the fluorescent apolar probe 1,8 ANS were consistent with the presence of increased hydrophobic surfaces as compared with the native or fully unfolded protein. The refolding of xylanase (Xyl II) was attempted by a relatively new strategy using an artificial chaperone assisted two-step mechanism. The unfolded xylanase was found to bind to the detergent transiently and the subsequent addition of cyclodextrin helped to strip the detergent and assisted in folding. Our findings suggested that the detergent stabilized an early intermediate in the folding pathway seemingly equivalent to the folding state as molten globule. The reactivation of Xyl II was affected by ionic as well as non-ionic detergents. However, the cationic detergent CTAB provided a maximum reactivation (3-fold) of the enzyme. The delayed detergent addition experiments revealed that the detergent was effective by suppressing the initial aggregate formation but not in dissolving aggregates. The relevance of our findings to the role of artificial chaperones *in vivo* is discussed.

[D] INCREASE IN STABILITY OF XYLANASE FROM AN ALKALOPHILIC THERMOPHILIC BACILLUS (NCIM 59).

The cellulase-free xylanase from the alkalophilic thermophilic *Bacillus* was stable at pH 7.0 to 10.0 at 50 °C for 3 days. At 60 °C, the enzyme showed a decrease in stability

with a half-life of 3 h. The effect of various additives on the stability of the enzyme at 60 °C was studied. Polyhydric alcohols such as glycerol, sorbitol, mannitol, and sugars like glucose, sucrose and xylose did not have any effect on the stability of the enzyme. Similarly, salts such as KCl, $CoCl_2$, $CaCl_2$, and NaCl did not enhance the stability of the enzyme. However, glycine (0.5 M) increased the enzyme half-life by 6-fold in the pH range 7.0-9.0 and at 60 °C and 70 °C. Xylan could offer thermoprotection against inactivation of the xylanase at pH 7.0 and 8.0 at 60 °C and only a marginal increase at pH 9.0 at 70 °C was observed.

ABBREVIATIONS

ANS	8-Anilinonapthalene-1-sulfonic acid
ATP	Adenosine 5'-triphosphate
AMP-PNP	5'-adenylyl imidodiphosphate
CD	Circular dichroism
DEP	Diethyl pyrocarbonate
DNSA	3,5-dinitrosalicylic acid
Gdn/HCl	Guanidine hydrochloride
HNBB	2-Hydroxy-5-nitrobenzyl bromide
NBS	*N*-Bromosuccinimide
NEM	*N*-Ethylmaleimide
PHMB	*p*-Hydroxymercury benzoate
PMSF	Phenylmethylsulfonylfluoride
XYLII	Xylanase II
WRK	Woodward's reagent K

LIST OF PUBLICATIONS

1. **Dey, D**., Hinge, J., Shendye, A., and Rao, M. (1992) Purification and properties of extracellular endoxylanases from alkalophilic thermophilic *Bacillus* sp. *Can. J. Microbiol.* **38**, 436-442.

2. **Nath, D**., and Rao, M. (1995) Increase instability of xylanase from am alkalophilic thermophilic *Bacillus* (NCIM 59). *Biotechnol. Lett*. **17**, 557-560.

3. **Nath, D**., and Rao, M. (1998) Structural and functional role of tryptophan in xylanase from an extremophilic *Bacillus*: Assessment of the active site. *Biochem. Biophys. Res. Commun*. **249**, 207-212.

4. **Nath, D**., and Rao, M. (2001) pH dependent conformational and structural changes of xylanase from an alkalophilic thermophilic *Bacillus* sp. (NCIM 59) *Enzyme Microb. Technol.* (In press).

5. .**Nath, D**., and Rao, M. Chemical modification of the essential carboxyl group of Xyl lI from alkalophilic thermophilic *Bacillus* sp. Abstract accepted for the 67th Annual meeting of SBC, New Delhi, 19-21 December, 1998.

6. **Nath, D**., and Rao, M. Fluorescence and chemical modification studies on the role of tryptophan in xylanase from an extremophilic *Bacillus* sp. Abstract accepted for the 38 th Annual meeting of the Association of Microbiologists of India, New Delhi, 12-14 December, 1997.

CHAPTER 1

GENERAL INTRODUCTION

The utilization of agricultural and forestry residues is currently a topic of tremendous interest. Xylan is one of the most abundant biopolymers and accounts for one third of all renewable organic carbon synthesized in the biosphere. The efficient enzymatic degradation of lignocellulosic materials requires a combination of hydrolyzing enzymes such as cellulases and hemicellulases. Complete enzymic hydrolysis of hemicellulose involves action of a battery of enzymes, of which endo-(1→4)-β-xylanase is one of the most crucial enzyme. Recent years have seen a surge of interest in cellulase free xylanolytic enzymes from microbial sources for its potential application in the paper and pulp industry. For a successful integration of xylanases in biotechnological applications, a detailed understanding of the mechanism of enzyme action is desired.

Microbial diversity

The microorganisms that produce xylanases and other glycosidases have been found in extremely diverse natural habitats. Under mesophilic growth conditions, xylanolytic activity has been reported in a wide variety of different genera and species of bacteria, fungi, and yeasts (Gilkes *et al*., 1991). For example, xylan degradation occurs in certain strains of *Bacillus polymyxa, Bacillus pumilus, Bacillus subtilis, Cellulomonas fimi, Clostridium acetobutylicum, Streptomyces lividans, Streptomyces flavogriseus, Aspergillus fumigatus, Neurospora crassa, Trichoderma viride, Pichia stipitis,* and *Candida shehatae* (Gosalbes *et al*., 1991; Wong *et al*., 1988). In addition to *meso*-temperature conditions, a host of microorganisms inhabit *extreme* environmental conditions where they thrive and grow at temperatures above 50 °C, at pH values 9 or greater (Uffen, 1997) and/or in high ionic strength aqueous systems containing salt approaching saturating conditions (Wong *et al*., 1988). According to Prof. Adams, highly stable enzymes can be isolated from extreme environments (Gross, 1996). Use of xylanases from microorganisms at temperatures above 50 °C and in alkaline conditions is especially desirable for kraft pulp treatment in the paper industry (Farrell and Skerker, 1992; Nissen *et al.*, 1992). Hyperthermophilic eubacteria have been isolated that grow at temperatures above 80 °C. These microbes include *Thermotoga maritima* (Winterhalter and Liebl, 1995), *Thermotoga* sp. (Saul *et al*., 1995), *Caldocellum saccharolyticum* (Luthi *et al*., 1990), *Dictyoglomus* sp. (Mathrani and Ahring, 1991) and *Rhodothermus marinus* (Dahlberg *et al*., 1993).

Structure of xylans

In plant cells, xylan is one of the three major structural polysaccharides and is localized in the cell wall matrix. The relative distribution of lignocellulosic components in the cell walls is dependent on the plant species and on the stage of growth and development. Xylans are typically polydispersed heteropolysaccharides with a homopolymeric backbone chain of 1,4-linked β-D-xylopyranosyl residues. The xylopyranosyl backbone is substituted at positions C-2, C-3 and C-5 to varying degrees depending upon the plant and the stage of development of the plant when the polymer was obtained (Joseleau *et al*., 1992). In monocots, at the C-2 positions 1→3-linked α-D-glucoronic acid or 4-O-methyl-α-D-glucoronic acid might occur, while at C-3 of xylopyranose, one frequently finds 1→3 linked α-L arabinofuranose. In some xylans, particularly in hardwoods, xylopyranose residues may be O-acetylated at the C-2 or (more commonly) the C-3 positions. Additionally, small amount of phenolic components, such as ferulic and p-coumaric acids (associated with lignin) may be esterified to xylan via their carboxyl groups to C-5 of arabinose branches (Kato and Nevins, 1985).

Xylanolytic enzyme system

Xylan is a complex polysaccharide containing different substituent groups in the backbone and side chains. Due to its heterogeneity, a complex enzyme system is required for the hydrolysis. Some major structural features are summarized in Fig.1. All the enzymes act cooperatively to convert xylan to its constituent sugar. The xylanolytic enzyme system is comprised of the following enzymes:

*Endo-**b**-(1®4)-D-xylanase* [β-(1→4)-D-xylan xylanohydrolase, E.C.3.2.1.8]: These enzymes act randomly on xylan to produce large amounts of xylo-oligosaccharides of various chain lengths. They are grouped into four different categories:

a) *Non-arabinose liberating endoxylanases*: This class of enzymes cannot act on arabinosyl initiated branch points at **b-(1® 4)** linkages and produce only xylobiose and xylose as the major end products. These enzymes can break down oligosaccharides as small as xylobiose.

b) *Non-arabinose liberating endoxylanases:* These are unable to cleave at **a-(1® 2) and a-(1® 3)** branch points produce mainly xylooligosaccharides larger than xylobiose. These xylanases are unable to cleave xylotriose and xylobiose.

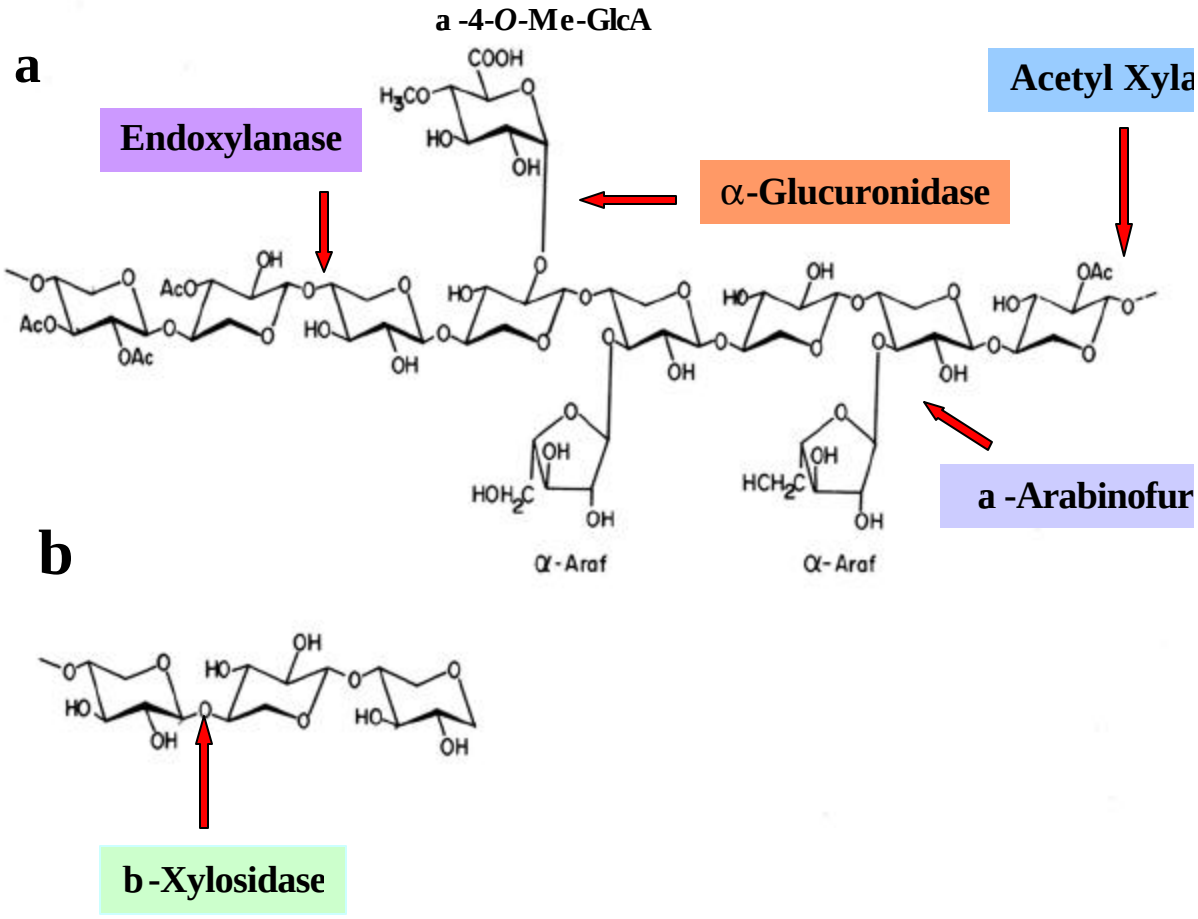


Fig.1 (a) The xylanolytic enzymes involved in the degradation of xylan
Ac: Acetyl group; a -Araf: a -arabinofuranose; a -4-*O*-Me-GlcA: a -4-*O*-me
acid
(b) Hydrolysis of xylooligosaccharide by xylosidase.

c) *Arabinose liberating endoxylanases*: These enzymes can cleave the xylan chain at the branch points and produce mainly **xylobiose, xylose** and **arabinose**.

d) *Arabinose liberating endoxylanases*: These enzymes can hydrolyze the branch points and produce intermediate size **xylooligosaccharides** and **arabinose**.

*Exo-**b**-(1® 4) -D-xylanase* [β-(1→4)-D-xylan xylanohydrolase]: These enzymes remove the single xylose units from the non-reducing end of the xylan chain.

***b**-xylosidase or xylobiase*: These enzymes hydrolyze disaccharides like xylobiose and the higher xylooligosaccharides with decreasing specific affinity.

a-L-arabinofuranosidase (E.C. 3.2.1.55): These enzymes cleave side chain α-1,3 arabinofuranose from the xylan main chain.

***a**-(4-O-methyl)-D-glucuronidase* (E. C.3.2.1.-): These can remove D-glucuronosyl or 4-O-methyl glucoronosyl residues from xylan.

Acetyl xylan esterase (E. C. 3.1.1.6)*:* These enzymes liberate acetyl groups from the xylan backbone.

Xylanase production

Under natural conditions, xylanolytic enzymes appear to be inducible by the products of their own action and are subject to catabolite repression by carbon sources such as glucose or xylose. Xylan, being a high molecular mass polymer, cannot enter the cell. The induction of the enzymes are stimulated by low molecular fragments of xylan namely, xylose, xylobiose, xylooligosaccharides, heterodisaccharides of xylose and glucose and their positional isomers, which are produced in the medium by small amount of constitutively produced enzyme.

Xylan has been shown to be the best inducer of xylanase production in many cases (Simpson, 1956; Fogarty and Ward, 1973; Mishra and Rao, 1984; Kelly *et al*., 1989; Nakamura *et al*., 1992). However, a few organisms show constitutive production of the enzyme (Lyr, 1972; Debeire *et al*., 1990). Cellulose has also been observed to act as an inducer in a few cases (Berenger *et al*., 1985; Deshpande *et al*., 1986; Morosoli *et al*., 1986; Stutzenberger and Bodine, 1992). Induction can also be achieved by various synthetic alkyl, aryl β-D-xylosides (Nakanishi *et al*., 1976) and methyl β-D-xyloside (Nakanishi & Yasui, 1980; Marui *et al*., 1985). These compounds enable the production of xylanolytic enzymes in the absence of xylan and xylooligosaccharides. Cheaper hemicellulosic substrates like corn

cob, wheat bran, rice bran, rice straw, corn stalk and bagasse have been found to be most suitable for the production of xylanase in certain microbes. Wheat bran was found to be the best substrate for xylanase production by alkalophilic *Streptomyces* VP5 (Vyas *et al*., 1990), *Streptomyces* T-7 (Keskar *et al*., 1992) and *Penicillum funiculosum* (Mishra *et al*., 1985). Highest levels of xylanase were formed when *Trichoderma brachiatum* was grown on wood pulp (Royer and Nakas, 1989). Corn cob was the most suitable substrate for the production of xylanase by an alkalothermophilic *Thermomonospora* sp. (George *et al*., 2001). Fungi produce higher levels of xylanase than bacteria or yeasts. However, fungal xylanases are generally associated with cellulases (Steiner *et al*., 1987). Among fungi, the maximum activity reported is 3350 IU ml^{-1} from *Trichoderma reesei* (Haapala *et al*., 1994). However, Haltrich *et al*. (1992) reported maximum xylanase activity (27,000 IU g^{-1}) produced by solid state fermentation from the fungus *Schizophyllum commune*. An increase in xylanase production under solid state fermentation has also been reported from a bacterial strain *B.* licheniformis A99 (Archana and Satyanarayana, 1997).

Cellulase-free xylanases are desirable in the paper and pulp industry for effective bleaching of paper pulp without adversely affecting the quality of the pulp. Another potential application of cellulase-free xylanolytic systems is in the processing of plant fiber sources such as flax and hemp (Sharma, 1987). Earlier the attempts to obtain cellulase-free xylanase were made by treatment of the culture filtrate with mercurial compounds (Bernoud *et al*., 1986; Noe *et al*., 1986), bulk scale purification (Tan *et al*., 1987; Gibson, 1987; Senior *et al*., 1988) or sequencing and cloning of xylanase genes in heterologous non-cellulolytic hosts (Paice *et al*., 1988; Bertrand *et al*., 1989). These efforts have failed to achieve commercial success for practical applications in paper technology. However, search for naturally occurring microrganisms capable of selectively secreting high levels of xylanase have yielded promising results. Cellulase-free xylanase have been isolated from *Chainia* (Srinivasan *et al*., 1992), *Saccharomonospora viridis* (Roberts *et al*., 1990) *Streptomyces roseiscleroticus* (Grabski and Jeffries, 1991) and *Streptomyces* T-7 (Keskar *et al*., 1992).

Xylanase delivery systems

It is important to understand the mechanism of release of xylan-degrading enzyme into the system for developing effective xylan-degrading technology. At present, much of the understanding of xylanolytic enzyme action comes from studies of microorganisms where

xylanases are secreted by the cells. However, in bacteria some cells appear to release the enzymes in the form of 'protein complexes' or 'xylanosomes' (Thomson, 1993). The suggestion that certain bacteria produce structured enzyme aggregates or xylanosomes (Thomson, 1993) is analogous to the formation of cellulosomes in some cellulose-degrading organisms (Lamed and Bayer, 1988). The first report of xylanosome from *B. fibrisolvens* (Lin and Thomson, 1991) appeared to consist of at least 11 xylanolytic active proteins ranging in size from 45 to 180 kDa. In a differential bacterial system, Shao *et al*. (1995) reported the localization of xylanase in the S-layer fraction of the thermophilic organism, *Thermoanaerobacterium* sp. strain JW/SL-YS 485. In *C. xylanolytica* a cell wall, associated xylan degrading system has been reported (Renner and Breznak, 1997).

Purification of Xylanases

Xylanase purification schemes have generally utilized standard column chromatographic techniques, mainly ion exchange and gel filtration, but also hydrophobic interactions. A xylanase from fungal maize pathogen *Cochiobolus carbonum* has been purified by hydrophobic interaction chromatography (Holden and Walson, 1992). Paul and Varma (1992) used Concavalin A-Sepharose chromatography to purify a xylanase probably having a carbohydrate moiety. Immunoaffinity chromatography has been used to purify xylanase from *Trichoderma reesei* (Lappalainen, 1986). Xylanases have also been purified using various techniques like isoelectric focussing, chromatofocussing, PAGE (Dey *et al*., 1992; Pereira *et al*., 2000) and FPLC (Simpson *et al*., 1991; Wong and Saddler, 1988). Purification of xylanases from crude culture filtrate using affinity precipitation with a commercial polymer Eudragit S-100 has also been reported (Breccia *et al*., 1998; Gawande and Kamat, 1999). Recently xylanases from an alkalophilic *Bacillus* sp. strain K-1 have been purified to homogeneity by affinity adsorption-desorption on insoluble xylan (Ratankhanokchai *et al*., 1999).

Properties of xylanases

Recent comprehensive reviews (Warren, 1996; Antranikian *et al*., 1997; Kulkarni *et al*., 1999) have described characterization of xylanases from microbial systems. The present section is limited to the properties of xylanases from extremophilic organisms. Studies of xylanases from alkalophilic and/or thermophilic organisms have led to the discovery of enzymes, which exhibit some unique properties. The molecular weights of the xylanases vary

Table. 1. Physicochemical properties of xylanases from extremophilic organisms.

Source	Growth		Optimum		pI	MW [kD]	K_m [mg/ml]	
	Temp [°C]	pH	pH	Temp [°C]				
Thermophilic bacteria								
***Bacillus* acidocaldarius**	3.5-4	65	4	80	-	56	1.68	Uc 19
***Bacillus stearothermophilus* T-6**	60	7.0-7.3	6.5	75	9	43	1.63	Kh
***Bacillus* sp SPS-0**	60	8.0	6.0-7.0	75	-	99	0.7	Ba
Bacillus amyloliquefaciens	45	7.0	6.8-7.0	80	10.1	9.6	-	Br
Thermophilic *Bacillus* sp. XE	55	7.0	6.0	75	7.8	22	0.60	De *al.*
***Bacillus stearothermophilus* 21**	55	7.0	7.0	60	4.8	39.5	3.80	Na
Clostridium stercorarium	65	7.0	5.5-7.0	75	4.5	44	3.2	Be
			5.5-7.0	75	4.4	72	2.9	
			5.5-7.0	75	4.3	62	3.7	
***Clostridium stercorarium* HX-1**	60	6.0-7.0	6.5	75	4.5	53	1.4	Sa

Clostridium thermolacticum	65	6.0-	6.5	80	4.4	39	0.40	De
TC 21		7.0						*al.*
			6.5	80	4.5	55	0.53	
			6.5	80	4.6	65	0.48	
Streptomyces **T_7**	50	7.0	4.5-5.5	60	7.8	21.8	10.0	Ke
Streptomyces **sp B-12-2**	45		6.0	55	7.5	26.4	5.80	Ele
	50		6.0	60	8.3	23.8	3.40	
			7.0	60	5.4	36.2	3	
			7.0	60	5	36.2	1.20	
			6.0	60	4.8			
							-	
Streptomyces	50	7.0	7.0	70	4.2	54		Tsu
thermoviolaceus **OPC520**								
			7.0	60	8.0	33	-	
Thermoanaerobacterium **sp.**	60	6.0	6.2	80	4.3	350	3	Sh
JW/SL-YS485								
Thermonospora curvata	55	6.0-	7.8	75	4.2	36	2.50	Stu
		7.0						Bo
			7.2	75	7.1	19	1.40	
			6.8	75	8.4	15	2.00	
Thermotoga **sp. Fj SS3 B1**	80	6.8-	5.3	105	-	31	0.07	Ru
		7.0						
Thermotoga maritima **MSB8**	80	7.0	6.2	92	-	120	1.10	Wi

								Lie
			5.4	105	-	40	0.29	
Thermotoga thermarum	77	6.0	6.0	80	-	266	0.36	Su
			7.0	90-100	-	35	0.24	
Alkaliphilic bacteria								
Alkalophilic *Bacillus* 41M-1	37	10.3	9.0	50	5.5	36	-	Na
***Bacillus* sp. C-125**	37	10.5	6.0-	70	--	43	-	Ho
			10.0					
						16		
***Bacillus* C-59-2**	37	8.0	5.0-5.5	60	6.3	10.4	--	Oh
***Bacillus* sp. strain K-1**	37	10.3	5.5	60	-	23	-	Ra
								al.
***Bacillus* NCL-87-6-10**	28	9.5	8.0	60	-	29		Ba
						10.4		19
***Aeromonas* sp. 212**	37	10.0	5.0-7.0	60	-	23.0	-	Ku
			6.0-8.0	50	-	37.0	-	
			7.0-8.0	50	-	145.0	-	
Alkalophilic thermophilic bacteria								
***Bacillus* sp. TAR-1**	50	10.5	Broad	70	>9.3	23.0	-	Ta
***Bacillus* sp**	45-	9-10	6.0	65	8.5	21.5	4.5	Ok

	50							
			7.0-9.0	70	3.6	49.5	3.6	
			6.0	65	8.3	22.5	8.3	
			7.0-9.5	70	3.7	50.0	3.7	
Thermophilic fungi								
Talaromyces	*50*	6.2	5.5	75	4.3	76.0	N.D.	Yo
byssochlamydoides YH-50			4.5	70	3.8	54.0	N.D.	
			5.0	70	476.0	45.0	N.D.	
***Talaromyces emersonii* CBS**	45	4.5	4.2	78	74.8	5.3	N.D.	Tu
814.70			3.5	67	54.2	4.2	N.D.	19
Thermoascus aurantiacus	45	6.0	5.1	80	32.0	7.1	1.70	Ta
C436								

from 8-145 kDa (Sunna and Antranikian, 1997). The properties of some of the purified xylanases from extremophiles are described in Table 1. However, xylanases from *Thermoanaerobacterium sp*. (Shao *et al*., 1995) and *Thermotoga thermarum* (Sunna and Antranikian, 1996) showed higher molecular weights of 350 and 266 kDa respectively. Horikoshi and Atsukawa (1973) were the first to report xylanase production from an alkalophilic bacteria. The *Bacillus* sp. C-59-2 secreted two xylanases of molecular weight 43 and 17 kDa respectively. The purified xylanases exhibited a pH optimum of 6.0-8.0. Many of the xylanases produced by alkalophilic microorganisms such as *Bacillus* sp. (Okazaki *et al.*, 1984) and *Aeromonas* sp. (Ohkushi *et al.*, 1985) showed remarkable stability at pH 9-10 but were not active above pH 8.0. An alkalophilic fungus having activity at pH 6.0-9.0 has been reported by Bansod *et al*. (1993). Recently an alkali tolerant xylanase from *Aspergillus fischeri* (Chandra and Chandra, 1996) was reported to exhibit remarkable stability at pH 9.0.

A small number of bacterial and fungal xylanases show maximal activities at temperatures 60-80 °C (McCarthy *et al*., 1985; Gruninger and Feichter, 1986; Khasin *et al*., 1993). The purified endoxylanases from various species belonging to the genus *Thermotoga* are optimally active at temperatures between 80 and 105 °C (Simpson *et al.*, 1991; Sunna *et al*., 1996; Winterhalter and Liebl, 1995). Xylanase from *Dictyoglomus* sp. exhibited a half-life of 80 min at 90 °C. (Mathrani and Ahring, 1991). *C. stercorarium* xylanase exhibited a temperature optimum of 70 °C and a half-life of 90 min at 80 °C. The thermophilic fungi include *Thermoascus aurantiacus* (Yu *et al*., 1987) which produces a thermostable xylanase reported to be stable at 70 °C for 24 h, *P. variota* (Krishnamurthy and Vithayathil, 1989) and *T. byssochlamydoides* (Yoshika *et al*., 1981) with temperature optimum of 65-75 °C at pH 5-6.5.

Mode of action

Xylanases are classified as debranching (arabinose liberating) or non-debranching enzymes depending on whether or not they catalyze removal of arabinose side-chain substituents in addition to cleaving main-chain linkages (Dekker, and Richards, 1976). Xylanases from *N. crassa* and *A. niger* are found to liberate arabinose from arabinoxylan (Mishra *et al.*, 1984; Takenishi and Tsujisaka, 1973). Majority of the xylanases known are endotype enzymes which usually show preferences for internal glycosidic linkages in xylans

and xylooligosaccharides and which act by a random attack mechanism (Bérenger *et al*., 1985; John *et al*., 1979; Panbangred *et al*., 1983). However a few xylanases, such as those from *B. polymyxa* (Morales *et al*., 1993) and *Chaetomium thermophile* (Ganju *et al*., 1989) are known to hydrolyze xylans and to produce mainly xylobiose and traces of xylose and/or xylotriose by an exotype mechanism. Xylanase V from *Aeromonas caviae* ME-1 (Kubata *et al*., 1994) is reported to be an unusual xylanase, which produced xylobiose as the only low molecular weight oligosaccharide from xylan by an exotype mechanism.

Multiplicity of xylanases

It has become apparent that both fungal and bacterial cells produce a multiplicity of enzymes that belong to the same functional class and which sometimes exhibit broad plant polymer specificity (Gilkes *et al.,* 1991; Wong *et al*., 1988). These enzymes can be grouped into families based on conserved amino acid sequences in the catalytic domains and by hydrophobic cluster analysis. Thus, all high molecular weight xylanases belong to the family F/10, whereas low molecular weight xylanases belong to the family G/11. (Wong, *et al*., 1988; Gilkes *et al*., 1991; Henrissat, 1992). Enzyme multiplicity has been studied in the fungus, *T. reesei* and *T. harzianum* (Wong *et al*, 1988). Results suggested that *T. reesei* produced four xylanases, each with different MW and pI values. Törönnen *et al*, (1992) cloned two *T. reesei* genes, xyn1 and xyn2 that appeared to encode separate products, XYL1 and XYL2 exhibiting similar molecular weights 19 and 21 kDa respectively but had pI values of 5.2 and 9.0. Similarly, *T. harzianum* produced three distinct xylanases (Wong *et al*., 1986). In analogous studies, Shareck *et al.* (1991) reported that *Streptomyces lividans*, produces three xylanases encoded by three different genes, *xlnA* belongs to the family F, while *xlnB* and *xlnC* are members of the family 11. Thomson (1993) suggested various mechanisms that could account for the multiplicity of function and specificity of the xylan degrading enzymes. Electrophoretically distinct xylanases could arise from posttranslational modification of a gene product such as differential glycosylation or proteolysis. The detection of minor xylanases may also be an artifact of the growth and /or purification conditions or these enzymes may have functions, which are not required in large amounts, e.g. hydrolysis of linkages not found frequently (Wong and Saddler, 1988). Multiple xylanases can also be produced from different alleles of the same gene (Wong *et al*., 1988) or may be a result of independent genes (Hazlewood and Gilbert, 1993).

Stereochemistry and mechanism of action

Glycosyl hydrolases are classified as retaining or inverting enzymes depending on the stereochemistry of the released product. Retaining enzymes liberate products with the same anomeric configuration as the substrate by a double-displacement mechanism involving a glycosyl enzyme intermediate (Fig. 2a). Inverting enzymes form products with inverted configuration mediated by a single displacement mechanism (Fig. 2b). The stereochemistry of the reaction products released as shown by NMR studies for both family 10 and 11 indicated that catalysis occurs through double displacement mechanism with the retention of the anomeric configuration in both the families (Gebler *et al.*, 1992; Biely *et al*., 1994). Several glycosyl hydrolases have been characterized, and their mechanism of hydrolysis is found to resemble that of lysozyme through an acid-base mechanism involving two residues (Sinnot *et al*., 1990). The first residue acts as a general catalyst and protonates the oxygen of the osidic bond. The second residue acts as a nucleophile, which in the case of retaining enzymes, interacts with the oxocarbonium intermediate or in case of inverting enzymes promotes the formation of an OH^- ion from a water molecule. In retaining glycosidases, distances between the nucleophile and the acid base catalyst are 5.4-5.5 Å (McCarter and Withers, 1994) whereas in inverting glycosidases, the corresponding distances are greater (9-9.5 Å) because for inversion to take place, water molecule has to be accommodated between the aglycone and the enzyme.

Active site of xylanases

Useful information on the nature of the groups at the active site can be obtained by investigating the effects of chemical modification on binding and /or catalysis. (Glazer *et al*., 1975). Since, substrates and competitive inhibitors, bind to the active site, they frequently protect against modification/inactivation and can provide confirmatory evidence of involvement of particular residues in activity. The reports on the involvement of Trp residues in the reactions catalyzed by xylanases have all been based on inactivation by N-bromosuccinimide (NBS). The participation of Trp in the active site of xylanases from various organisms have been reported (Kubackova *et al*., 1978; Hoebler and Brillouet, 1984; Keskar *et al*., 1989; Deshpande *et al*., 1990; Khasin *et al*., 1993). The role of Trp residues in substrate binding to catalytic domains of xylanase C from Fibrobacter succinogenes S85 has been shown (McAllister *et* al., 2000). The only report on the identification of an essential Tyr

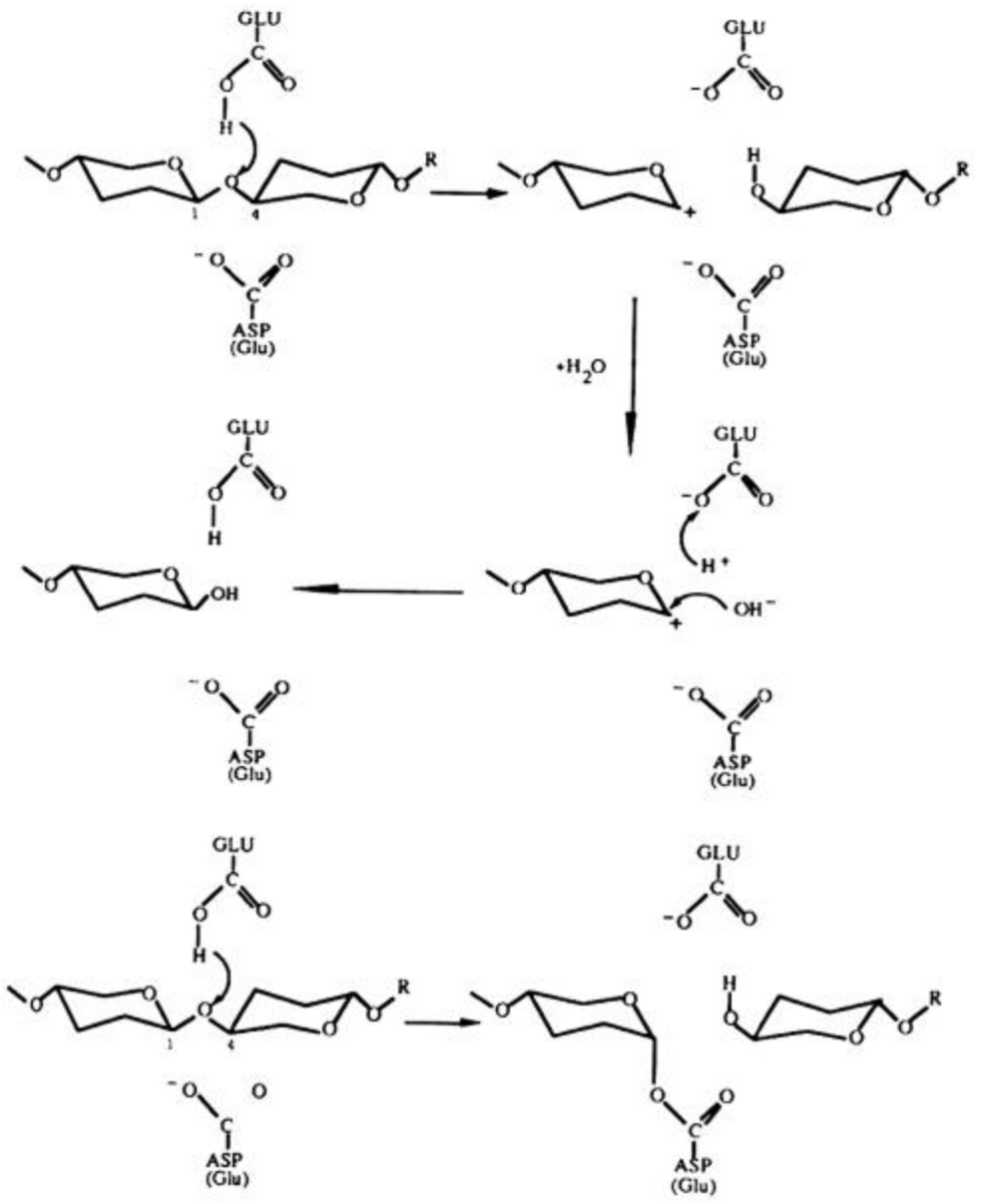


Fig.2A Reaction mechanism of the xylanases

(a) Double displacement reaction (a) involving stabilization of an oxocarbonium ion by electrostatic interaction with the carboxylate of an Asp (or Glu)at the active site or (b) involving formation of a covalent intermediate by nucleophilic attack of the Asp (or Glu) onthe incipient sugar (based on Hardy and Poteete, 1991)

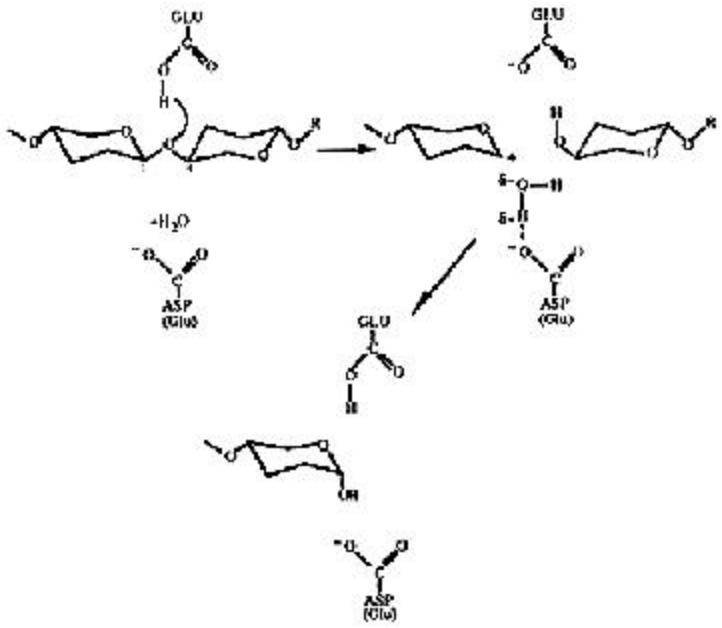


Fig.2B Single displacement reaction. Involvement of a general acid (Glu), a general base (Asp or Glu) and attack by a nucleophilic water molecule is shown [(based on Sinnot, (1990)].

residue in a xylanase from *Schizophyllum commune* Xylanase A been furnished by Bray and Clarke (1995). Chemical modification of xylanases from the fungus *S. commune* (Bray and Clarke, 1990) indicated the involvement of carboxyl groups in the catalysis. The involvement of Cys residue in the active site of a few bacterial xylanases has also been reported (Deshpande *et al.*, 1990; Keskar *et al*., 1989). Several specific reagents were used to identify reactive residues by competitive labelling in the presence or absence of substrate, in conjunction with kinetic analysis. Bray and Clarke (1994) using [^{14}C] EAC labelling of the enzyme followed by proteolytic cleavage, showed that the labeled reagent interacts with one Glu residue. The characterization and sequencing of the Cys containing active site peptide of the xylanase from *Streptomyces* T-7 (Keskar *et al*., 1989) and *Chainia* (Rao *et al*., 1996; Hegde *et al*., 1998) have been reported. The peptides showed the presence of a conserved Asp residue. The Cys residue in some xylanases may participate in covalent glycosyl-enzyme intermediate formation as has been proposed for the mutant T4 lysozyme in which Asp20 was replaced by a Cys residue (Hardy and Poteete, 1991).

Site directed mutagenesis

In recent years, several xylanases were subjected to site directed mutagenesis either in an attempt to improve their biochemical properties or to gain insight into structure-function relationships. Among the xylanases belonging to family 11, two *Bacillus* enzymes were mutated. In the xylanase from *Bacillus pumilus*, mutation of Glu93 and Glu182 resulted in decreased enzymatic activity, indicating that these residues were important for catalytic activity (Ko *et al*., 1992). Similarly, for *Bacillus circulans* xylanase Glu78 or Glu172 represented the catalytic residues of the enzyme (Wakarchuk *et al*., 1994). Family 10 mutated xylanases included *T. saccharolyticum* xylanase at positions Asp 537, Asp602 and Glu600 (Lee *et al*., 1993), *Streptomyces lividans* xylanase (XynA) at positions Glu128 and Glu236 (Moreau *et al*, 1994 and *C. fimi* (Cex) at position Glu127 (Macleod *et al*., 1994), all residues being replaced by their corresponding 'isoteric form'. Kinetic studies of all these mutants showed decreased activities towards the substrate consistent with the replacement of a catalytic residue. Roberge *et al*. (1997) showed that two His residues (His81 and His207) out of three were present in the active site of xylanase A from *S. lividans* and were found to be completely conserved in family 10 glycanases. The structural analysis revealed that they were involved in a network of hydrogen bonds which were responsible in maintaining the

ionization states of the two catalytic residues (Glu128 and Glu236). Recently, it has been shown in xylanase from *B. circulans* (Joshi *et al*., 2000) that the substitution of an Asn residue with an Asp residue (N35D BCX) shifts its pH optimum from 5.7 to 4.6, with ~20% increase in activity. Engineering of proteins by *in vitro* mutagenesis has become a process that allows almost any desired modification to be constructed in the laboratory. For example, introduction of several Cys residues into a xylanase from *Bacillus circulans* and the spontaneous formation of disulfide bridges resulted in increased thermostability of the enzyme by 15 °C (Wakarchuk *et al*., 1994). In the case of xylanase from *S. lividans* (Moreau *et al*., 1994) replacement of Arg156 with a Glu residue showed a higher temperature optimum of 5 °C and the half-life of the mutant was 6 min longer than the wild type. Similarly, site directed mutagenesis of a xylanase gene from *C. saccharolyticum* (Luthi *et al*., 1992) yielded mutant xylanases with altered temperature stability and temperature optimum, whereas no change was observed in the pH optimum. Arase *et al*. (1993) reported stabilization of xylanases by random mutagenesis of the cloned gene fragment. However, reports on xylanase to date suggest that it has not been fully exploited to improve properties of the enzyme.

Three-dimensional structures of xylanases

The resolution of the three-dimensional structures of the enzymes is indispensable for understanding the catalytic mechanism, the difference in substrate specificities among the enzymes belonging to the same family and for further improving their functions through protein engineering. In this context, the number of xylanases whose three-dimensional structure have been solved is increasing rapidly.

X-ray crystallographic structures of xylanases of family 10 and family 11 have been determined. While xylanases of both families act upon the same substrate, there are significant differences in their structures. The three-dimensional structures of the family 10 catalytic domains are reported for *Streptomyces lividans* Xln A (Derewenda *et al*., 1994), *C. fimi* Cex (White *et al.*, 1994), *Pseudomonas fluorescens* Xyn A (Harris *et al*., 1994), *C. thermocellum* Xyn Z (Domínguez, *et al*., 1995) and *T. auranticus* xylanase I (Leggio *et al.*, 1999). They all exhibit a tertiary fold of a typical $(\alpha/\beta)_8$ barrel motif. Seen from the side, the molecule has a general 'salad bowl' shape. The active site is formed by an acidic cleft on the carboxyl-terminal side of the β-barrel and this cleft is well exposed to solvents (Ohmiya *et al*., 1997; Dupont *et al*., 1996). A long loop between β-strands 7 and 8 were observed for *P.*

fluorescens xylanase (Harris *et al*., 1994) while the corresponding loops in *S. lividans* XlnA and *C. fimi* Cex were significantly shorter. This loop in Xyn A of *P. fluorescens* has been recently identified as the binding site for the calcium ion playing a role in stabilization of the enzyme. The authors have suggested that the occupation of Ca^{2+} binding loop with its ligand protects the enzyme from thermal inactivation, thermal unfolding and proteolysis. Site directed mutagenesis studies revealed that Asp residues at positions Asp256, Asp261 and Asp262 were pivotal for calcium binding (Spurway *et al*., 1997). Among the family 10 xylanases, *T. auranticus* and *C. thermocellum* are from thermophilic organisms. A comparison with other mesophilic organisms from family 10 suggests that thermostability is affected mainly by improvement of the hydrophobic packing, favourable interactions of charged side-chains with the helix dipoles and introduction of Pro residues at the N-terminus of helices. *P. simplicissimum* xylanase is similar to other family 10 xylanase, but its active site cleft is much shallower and wider. This probably accounts for the difference in catalysis and mode of action of the enzyme (Schmidt *et al*., 1998).

The three-dimensional structures of family 11 catalytic domains are reported for *Bacillus pumilus* XynA (Katsube *et al*., 1990), *B. circulans* xylanase (Wakarchuk *et al*., 1994) *Trichoderma harzianum* xylanase (Campbell *et al*., 1993), thermophilic *Bacillus* sp. (Pickersgill *et al*., 1993), *B. stearothermophilus* xylanase (Anna *et al*., 1997), *T. reesei* XynI (Törönnen and Rouvinen, 1994) and XynII (Törönnen *et al*., 1994) and *Aspergillus kawachii* xylanase C (Fushinobu *et al*., 1998). Family 11 xylanases fold into a β sandwich' consisting of two-pleated antiparallel β-sheets, which are folded against each other, in a parallel manner, forming a cleft on one side of the protein structure. For *B. circulans* xylanase, an enzyme tetrasaccaharide complex was crystallized and the xylotetraose was found in the cleft, confirming the cleft's role as the active site of this xylanase (Wakarchuk, *et al*., 1994). The crystal structure of *B. pumilus* (Moriyama *et al*., 1987) was of ellipsoidal shape while two major xylanases from *T. reesei* (Törönnen *et al*., 1994) were reported to be monoclinic and those of *T. harzianum* (Rose *et al*., 1987) were orthorhombic. In case of *B. pumilus* Xyn A (Katsube *et al*, 1990), although the structure was predominantly characterized as three large β- sheets, it was actually similar to the structures of *T. reesei* xylanases. Recently the crystal structure of xylanase (PVX) from a thermophilic fungus *Paecilomyces varioti Bainer* has

been solved. This fungus has been attracting attention as a pathogen causing post-surgical infections (Kumar *et al*., 2000).

Thermal denaturation/renaturation studies

Thermal denaturation studies of xylanase from *Streptomyces halstedii* by using differential scanning calorimetric (DSC) studies has been reported (Arribas *et al.,* 1994). *S. halstedii* produced two xylanases Xys 1L (45 kDa) and Xys1S (35 kDa). Thermal denaturation of Xys 1L revealed three thermodynamically independent domains, and that of Xys1S, which is a proteolytic fragment of Xys 1L (without a C-terminal part), revealed two thermodynamically independent domains, each of which follows a two-state transition. The thermodynamic parameters of unfolding for each domain did not fit some of the correlations obtained for most compact globular proteins. In another study, catalytic activity measurements as a function of temperature, complemented with DSC data, were used to characterize the thermostability of the exoglucanase/xylanase Cex from C*ellulomonas fimi* (Nikolova *et al*., 1997). The melting temperature (T_m) of Cex as measured by DSC was 64.2 °C and that of the isolated catalytic domain was 64 °C, suggesting that the binding and catalytic domains of the protein fold independently.

Xylanase (XynA) from the thermophilic bacterium *Thermotoga maritima* (Wassenberg *et al*., 1997) was studied to characterize the domain organization and stability of the recombinant enzyme and its isolated cellulose-binding domain (CBD). XynA and CBD were monomers with 116 and 22 kDa molecular masses. Denaturation/renaturation was used to gain insight into the folding mechanism of the complex multidomain protein. Guanidine induced unfolding of XynA leads to biphasic transitions. The shift of the transition of the unfolding to higher guanidine concentration at acid pH was attributed to the CBD.

Cloning and expression of extremophilic xylanases

Recombinant DNA techniques offer opportunities for the construction of microbial strains with selected enzyme machinery. From the biotechnological point of view, the main objectives of recombinant DNA technology would involve the construction of producers of xylanolytic systems free of cellulolytic enzymes, improvement of fermentation characteristics of industrially important organisms by introduction of xylanase and xylosidase genes for direct fermentation of xylan and for hyperproduction of the enzyme. Xylanase genes have been cloned from different microbial organisms into various suitable hosts (Kulkarni *et al*.,

Rao, 1999). In this section, cloning of xylanase from extremophilic microorganisms will be discussed. Thermophilic organisms, such as *Bacillus stearothermophilus* T-6 xylanase gene has been cloned, and the recombinant enzyme was found to be optimally active at pH 9.0 and 65 °C (Gat *et al.*, 1994). Cloning and expression of a xylanase gene from the extreme thermophile *Dictyoglomus thermophilum* Rt46B.1 in *E. coli* has been reported (Gibbs *et al*., 1995) and the enzyme was found to have temperature optimum of 85 °C. Similarly, *Thermotoga maritima* recombinant enzyme showed an optimum temperature of 90 °C at pH 5.5 and was stable up to 100 °C (Chen *et al*., 1997). Studies on the cloned xylanase from *Thermotoga neapolitana* was found to have temperature optimum of 102 °C at pH 5.5 and was stable at 90 °C for 4 h with a half life of 2 h at 100 °C (Veoikodvorskaya *et al*., 1997). The cloned xylanase from *Clostridium thermocellum* F1 revealed that it was optimally active at 80 °C and was stable upto 70 °C at neutral pH (Hayashi, 1997). Cloning of xylanase genes from a few alkalophilic organisms like alkaliphilic *Aeromonas* (Kudo *et al*, 1985), alkaliphilic *Bacillus* (Honda *et al*., 1985), alkalophilic thermophilic *Bacillus* (Shendye and Rao, 1993) and *Cellulomonas* sp. (Bhalerao *et al*., 1990) have been reported.

Biotechnological potentials of xylanases.

The potential biotechnological application of xylan and xylanases has been of tremendous importance to researchers. Commercial applications of xylanases involve conversion of xylan, which is present in wastes from agricultural and food industry, to xylose (Biely, 1985). In the pharmaceutical industry, xylan is used as an agent for direct tableting and in combination with other components; for sustain release tablet construction. Xylose and xylooligosaccharides have possible applications in the food industry as thickeners or as fat substitutes and as an anti-freeze food additive. The hydrolysis products of xylan can be subsequently converted to liquid fuel, single cell proteins, solvents, and artificial low calorie sweetners (Wong and Saddler, 1992).

Xylanases are of great importance in the paper and pulp industry as they replace toxic chemicals such as elemental chlorine and chlorine-dioxide for developing environment friendly processes. They play an important role in the debarking, deinking of recycled fibers and in the purification of cellulose for the preparation of dissolving pulps (Jager *et al*., 1992). Enzyme aided prebleaching was found to increase the brightness of the paper. Various organisms have been explored for producing xylanase enzymes for treatment of kraft pulps.

The first commercial xylanase preparation available for pulp bleaching was marketed by Novo Nordisk under the name 'Pulpzyme HA' which was produced by a strain of *Trichoderma reesei*, subsequently Pulpzyme HB and HC from bacterial sources were also marketed (Pederson, 1989). Xylanases can be used in the production of dissolving pulps (purified cellulose) for making viscose rayons, cellulose esters and cellulose ethers (Paice and Jurasek, 1984) and to remove undesired hemicellulose content (Hinki *et al*., 1985; Viikari *et al*., 1993)

The use of xylanases has also been proposed in clarification of juices and wines (Biely, 1985), maceration of vegetable matters (Beck and Scoot, 1974), liquefaction of coffee mucilage for making liquid coffee, recovery of oil from subterranean mines, extraction of flavors and pigments, plant oils and starch (McCleary, 1986) and to improve the efficiency of agricultural silage production (Wong and Saddler, 1992). The use of xylanases in bakery has been suggested (Maat *et al*., 1992) and they were found to increase the specific volume, textural properties and shelf life of the bread. The application of xylanases in poultry diets has also been investigated (Bedford and Classen, 1992). Poultry are incapable of efficiently digesting cereals, the major ingredient in their diets. The addition of xylanases to the feed results in an improved nutritive value of wheat based diets for broilers. Xylanases with low pH optimum and broad pH stability would be most suitable for application in animal feed, where activity and stability at low pH is crucial. These biotechnological potentials of xylanases have prompted the search for suitable enzymes and technologies for large- scale economic production.

The present investigation relates to the study of a cellulase-free xylanase from an alkalophilic thermophilic *Bacillus* sp. (NCIM 59) and includes the following aspects:

Chapter 1: General Introduction

Chapter 2: Purification and characterization of an extracellular xylanase (Xyl II).

Chapter 3: Determination of active site residues and structure function analysis of Xyl II.

Chapter 4: Folding/Unfolding studies of Xyl II comprised of

a) α-crystallin mediated refolding of Xyl II

b) Artificial chaperone assisted folding of Xyl II

c) pH induced unfolding of Xyl II

Chapter 5: Increase in stability of xylanase from alkalophilic thermophilic *Bacillus* sp.

CHAPTER 2

PURIFICATION AND CHARACTERISATION OF XYLANASE (XYL II) FROM ALKALOPHILIC THERMOPHILIC BACILLUS SP. (NCIM 59)

SUMMARY

The alkalophilic thermophilic *Bacillus* sp. (NCIM 59) produces cellulase-free xylanase at 50 °C and pH 10.0. The gel filtration chromatographic analysis of the culture filtrate broth showed the presence of two xylanases differing in molecular weight and electrophoretic mobility. In the present studies, Xylanase II (Xyl II) was purified to homogeneity by chromatography on CM-Sephadex followed by Biogel P-10. The molecular weight of the enzyme as determined by SDS-PAGE was 15,800. The optimum pH and temperature of the enzyme were pH 6.0 and 50 °C, respectively. It was found to be stable at 50 °C for 168 h at pH 7.0. It is a basic protein with a pI of 8.0. The Michaelis constant and V_{max} values were estimated to be 3.5 mg/ml and 0.742 µmole min^{-1} mg^{-1}, respectively. Xyl II yielded xylobiose, as the major end product along with xylose, xylotriose, xylotetraose, and other higher xylooligosaccharides. End product analysis of xylan hydrolysis demonstrated that Xyl II is an endoenzyme. The amino acid analysis showed the predominance of Asp and Glu residues. The N-terminal sequence of Xyl II as determined by Edman degradation method was AVYGQYATDNL and was distinctly different from other reported xylanases.

INTRODUCTION

Xylanase, the major component of an enzymatic consortium, acts in nature by depolymerizing xylan molecules into monomeric pentosan units that are used by bacterial and fungal populations as a primary carbon source. Cellulase–free xylanases are currently a topic of considerable interest worldwide, following the realization of their potential impact in paper industry applications (Viikari *et al*., 1994; Bajpai *et al*., 1994). Search for xylanases active and stable at highly alkaline pH conditions have intensified in more recent years in keeping with the requirements of the pulping operations, which are carried out at relatively high pH and temperatures (Zamost, *et al*., 1991). Thermostable xylanases could be useful in such processes, in order to obtain higher reaction rates and greater solubility of the reactants. One of the ways to identify the industrially suitable enzymes active and stable at high temperatures preparations is to look for the enzymes from extremophilic microorganisms (Gross, 1996). Bacilli are used in the industry for the production of enzymes of commercial significance. The enzymes are normally secreted into the extracellular medium facilitating their recovery. Alkalophilic thermophilic *Bacillus* sp. (NCIM 59) was isolated in our laboratory and the potential application of the enzyme in the paper and pulp industry has been reported (Kulkarni and Rao, 1996). The organism produces two xylanases and the purification and characterization of Xyl I has been reported (Dey *et al*., 1992; Chauthaiwale and Rao, 1993). The present chapter deals with the purification and characterization of the extracellular low molecular weight xylanase (Xyl II).

MATERIALS AND METHODS

MATERIALS

CM-Sephadex C-50, 3,5-dinitrosalicylic acid (DNSA), SDS-PAGE and gel filtration molecular weight markers, BSA, standard amino acid mixture and xylan were obtained from Sigma Chemical Co., USA. Ampholytes were from Pharmacia, Sweden. Media components, yeast extract, agar-agar were obtained from Difco Laboratories, USA. Wheat bran was purchased locally. Biogel P-10 was obtained from BioRad Laboratories, USA. All other chemicals were of analytical grade.

METHODS

Maintenance of culture

Alkalophilic thermophilic *Bacillus* was routinely maintained on wheat bran 10 % w/v, yeast extract 0.1 % w/v and agar slants. Culture was grown at 50 °C for 48 h and slants were preserved at 4 °C, 15 °C and room temperature. For long storage, 15 % glycerol suspension of the 16 h grown culture was kept frozen at -70 °C.

Xylanase production

The inoculum was developed in washed wheat bran 10 %, yeast extract 0.5 %, all w/v and was incubated at 50 °C for 16 h. The fermentation was carried out by transferring 5 ml of the inoculum into 250 ml Erlenmeyer flasks containing 50 ml of the medium (10 % w/v wheat bran and 0.5 % w/v yeast extract) and incubating under shaking conditions (200 rpm) at 50 °C for 48 h. Filtering the culture broth through muslin cloth separated the residual wheat bran. Cells were removed by centrifugation (10,000 **g**, 20 min) and the clear supernatant was used as the enzyme.

Estimation of xylanase activity

Oat spelt xylan (2 g) was suspended in 50 mM potassium phosphate buffer pH 7.0 and was stirred for 16 h at 28 °C. The insoluble fractions were separated by centrifugation and the soluble fraction was used for the estimation of xylanase activity. The enzyme properly diluted in 0.5 ml of 50 mM potassium phosphate pH 6.0 was mixed with 0.5 ml of

xylan (1 %). The reaction mixture was incubated at 50 °C for 30 min and the reaction was terminated by the addition of 1 ml of DNSA reagent. The reducing sugars liberated were estimated spectrophotometrically at 540 nm according to Miller (1959) using D-xylose as a standard. One unit (U) of xylanase activity was defined as the amount of enzyme that produced 1 *m*mol of xylose equivalent per minute under the assay conditions. Protein concentration was determined by the method of Bradford (1976) using bovine serum albumin as a standard.

Purification of xylanase

All steps were carried out at 4 °C unless otherwise mentioned. The culture filtrate was brought to 80 % saturation by adding solid ammonium sulfate under constant stirring and left for 30 min. The precipitate was recovered by centrifugation at 10,000 ***g***, for 20 min dissolved in minimum volume of 0.05 M potassium phosphate buffer, pH 6.0, and dialyzed for 24 h against several changes of the same buffer. The resultant insoluble materials were removed by centrifugation (10,000 **g**, 20 min).

Ion-exchange chromatography: The supernatant (15 mg) was applied to CM-Sephadex C-50 column (45 x 2.5 cm) equilibrated at pH 6.0 with the same buffer. The column was then washed with the same buffer at a flow rate of 15 ml. h^{-1}, until the flow through fractions did not show any activity. The bound enzyme was eluted with a linear gradient of NaCl (0-1 M) in potassium phosphate buffer (0.05 mM), pH 6.0. Fractions of 5 ml were collected at a flow rate of 30 ml h^{1} and the active fractions were pooled, dialyzed and concentrated using amicon ultrafiltration unit having YM-3 membrane.

Gel filtration chromatography: The concentrated enzyme (2 ml) was passed through a gel filtration column Biogel P-10 column (1.5. x 90 cm). The column was pre-equilibrated with 0.05 M potassium phosphate buffer pH 6.0 and then eluted with the same buffer at a flow rate of 15 ml. h^{-1}. Fractions (1.5 ml) were analysed for enzyme activity. The active fractions were pooled, concentrated by ultrafiltration (3000-molecular weight cut off membrane, Amicon) and stored at 20 °C for further use.

Determination of molecular weight

The molecular weight of the enzyme was determined by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) according to the method of Laemmli (1970) using 12 % gel followed by silver staining (Rabilloud, 1988). M_r was also determined by gel filtration on Biogel P-10 (2.5 x 100 cm) equilibrated with 0.05 M potassium phosphate buffer, pH 6.0. Aprotinin (6,500), cytochrome c (12,400), carbonic anhydrase (29,000) and bovine serum albumin (66,000) were used as standard molecular weight markers. The molecular weight of Xyl II was calculated by regression analysis.

Carbohydrate content

The carbohydrate content was determined both for the native and for the denatured enzyme. The enzyme (250 µg) was denatured with 8 M urea for 4 h and dialyzed against distilled water to remove non-covalently adsorbed sugars. Xyl II (10 µg) was incubated with 400 µl of 5 % (w/v) phenol for 10 min at room temperature. 2 ml of H_2SO_4 was allowed to cool for 20 min. The total carbohydrate was then estimated at 440 nm, using D-mannose as standard (Dubois *et al*., 1956).

Effect of pH on the activity and stability

The optimum pH of the enzyme was determined by assaying the enzyme at different pH values ranging from pH 4-10. 0.05 M sodium acetate (pH 4-5), potassium phosphate (pH 6-8), sodium hydroxide-glycine buffer (pH 9-10) were used. The influence of pH on the stability of the enzyme was determined by incubating 5 U at 50 °C for 24 h at different pH and the residual activities were measured.

Effect of temperature on activity and stability

To determine the optimum temperature of the enzyme, the estimations were carried out at 37-80 °C. The thermal stability of the enzyme was measured by incubating 5 U of the enzyme in 0.05 M phosphate buffer, pH 7.0 at 50 and 60 °C for different time intervals.

Xylan hydrolysis

The hydrolysis of xylan (25 mg) was carried out in stoppered tubes with 1 U of enzyme in 0.05 M phosphate buffer, pH 7in a reaction mixture volume of 0.5 ml at 50 °C. The aliquots were removed at different time intervals, and the reducing sugars were assayed by the dinitrosalicylic acid method, using xylose as a standard. Percentage hydrolysis was calculated as shown below and it was multiplied by a factor of 0.9 to account for the addition of water molecule during hydrolysis.

$$\% \text{ hydrolysis} = \frac{\text{reducing power as xylose}}{\text{weight of xylan}} \text{x100 x 0.9}$$

Determination of xylan degradation products

Xylanase (1 U) was incubated with xylan (5 mg) in 0.05 M phosphate buffer, pH 7.0, at 50 °C for 24 h in a volume of 0.1 ml. The aliquots at different time intervals were analyzed for the sugar products formed by paper chromatography in a solvent system containing butanol-acetic acid-water (3:1:1). The paper chromatograms were sprayed as described by Travelyan *et al*. (1950).

Isoelectric focussing

Analytical isoelectric focussing was carried out by the method discussed by Pawar *et al*. (1988).

Amino acid analysis and N-terminal sequence

Xyl II was hydrolyzed with 6 M HCl in sealed tubes in oxygen free environment for 24, 48 and 72 h at 110 °C. The analysis was performed in Spinco model 120 B automatic analyser by the method of Spackman *et al*., (1968). Total cysteine content was determined spectrophotometrically, according to Habeeb (1972) while tryptophan content was determined according to Goodwin and Morton (1946). The N-terminal amino acid sequence of the purified pepetide was analysed with a protein sequencer (Applied Biosystems model 476A).

RESULTS AND DISCUSSION

Purification of Xyl II

The xylanase (Xyl II) from alkalophilic thermophilic *Bacillus* was purified to homogeneity as analyzed by SDS-PAGE (Fig. 1). The purification steps are summarized in Table 1. The specific activity of the purified enzyme was 300 U/mg of protein.

Table 1. Purification of Xyl II from alkalophilic thermophilic Bacillus

Purification step	Volume (ml)	Protein (mg. ml^{-1})	Activity ($U.mL^{-1}$)	Specific activity (U. mg^{-1})	Fold purification
Culture broth	1000	2.0	30	15	1.0
Ammonium sulfate fractionation	50.0	3.0	275	91	6.0
CM-Sephadex	10	0.6	160	267	18.0
Biogel P-10	20	0.1	30	300	20.0

Physicochemical properties of Xyl II

Molecular weight of Xyl II

The M_r of purified Xyl II as determined by Biogel P-10 column was 8,849 (Fig.1). The corresponding molecular weight as determined by SDS-PAGE was 15,800 (Fig. 2). The difference in the molecular weights observed by the gel filtration and the SDS method may be due to the glycoprotein nature of the enzymes (Pitt-Rivers and Impiombato, 1968).

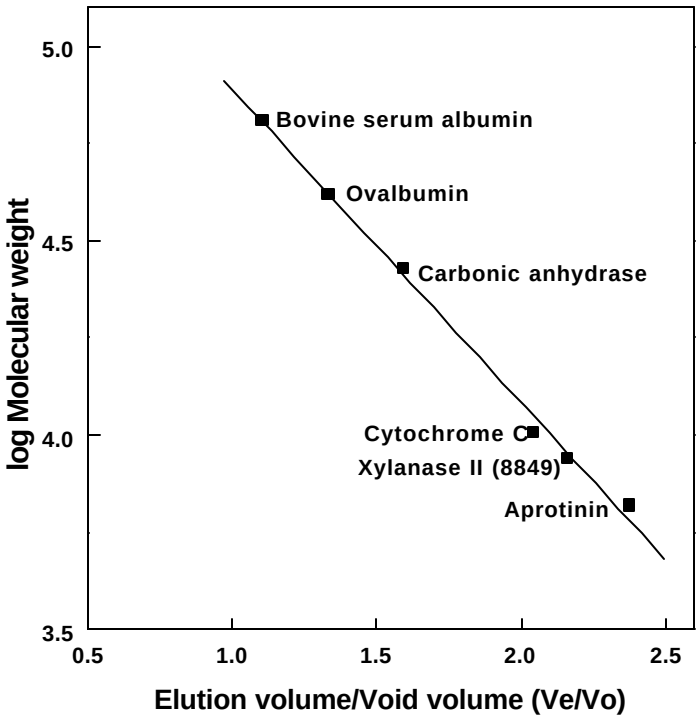


Fig. 1 Molecular weight determination of Xyl II by gel filtration

A Biogel P-10 column was equilibrated with potassium phosphate buffer, pH 7.0 and calibrated with the marker proteins.

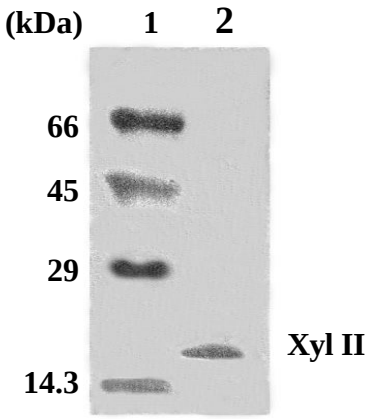


Fig. 2 SDS-PAGE of Xyl II

Lane 1, Protein standards (from the top): bovine serum albumin (66,000), ovalbumin (45,000), carbonic anhydrase (29,000), lysozyme (14,300)**. 2**, **Lane 2**, Purified Xyl II (10 μg).

Effect of pH on activity and stability

Xyl II exhibited pH optimum of 6.0, however, the enzyme showed 40 % and 25 % activity at pH 9.0 and 10.0, respectively (Fig. 3). The enzyme exhibited remarkable stability at pH 9.0 and 10.0 by retaining 75 % and 60 % activity for 24 h. The activity and stability of Xyl II resemble those of xylanases of several alkalophilic *Bacillus* species. (Nakamura *et al*., 1993; Blanco *et al*., 1995). Most of the xylanases from alkaliphiles described earlier had pH optima in the near neutral range although some of them showed relatively high activities at alkaline pH (Horikoshi and Atsukawa, 1973; Honda *et al*., 1985, Tsujibo *et al*., 1990; Nakamura *et al*., 1993, 1995).

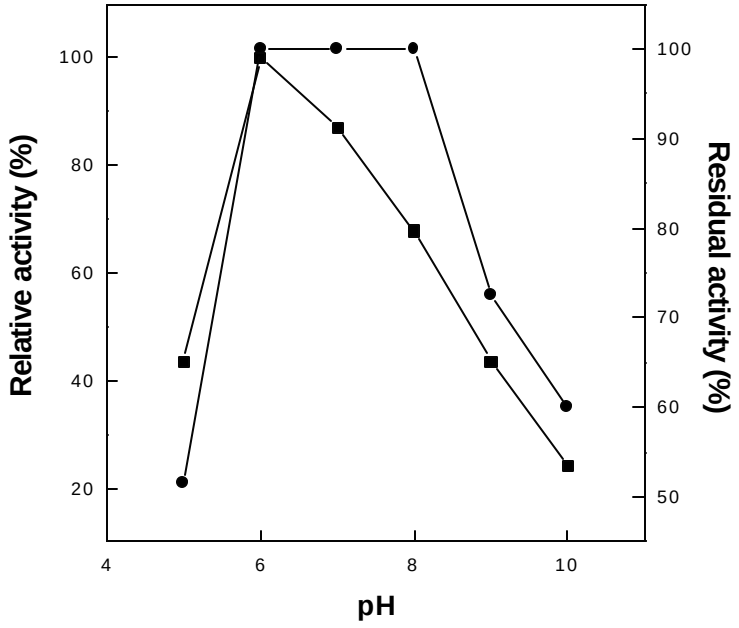


Fig. 3 Effect of pH on activity and stability of Xyl II.

Optimum pH (■): Xyl II was assayed in a series of pH (5-10) at 50 °C. The activity of 5U at 50 °C was taken as 100 %.

pH stability (●) **:** Xyl II (5 U) was incubated in a series of pH (5-10) at 50 °C for 24 h, and the residual activities were measured. The activity of 5 U at 50 °C and pH 7.0 was taken as 100 %.

Effect of temperature on activity and stability

The optimum temperature for xylanase activity was found to be 50 °C (Fig. 4A) and showed 95 % activity at 60 °C. The study of thermostability showed that the enzyme was highly stable at 50 °C for 96 h. At 60 °C, Xyl II showed a half-life of 4 h (Fig. 4B). Few xylanases with stability at higher temperatures have been reported. These include enzymes from a few *Bacillus* sp. (Khasin *et al*., 1993; Nakamura *et al*., 1993, 1995), *Thermotoga* sp. (Sunna *et al*., 1996) and thermophilic fungi (Cesar and Mrsa, 1996).

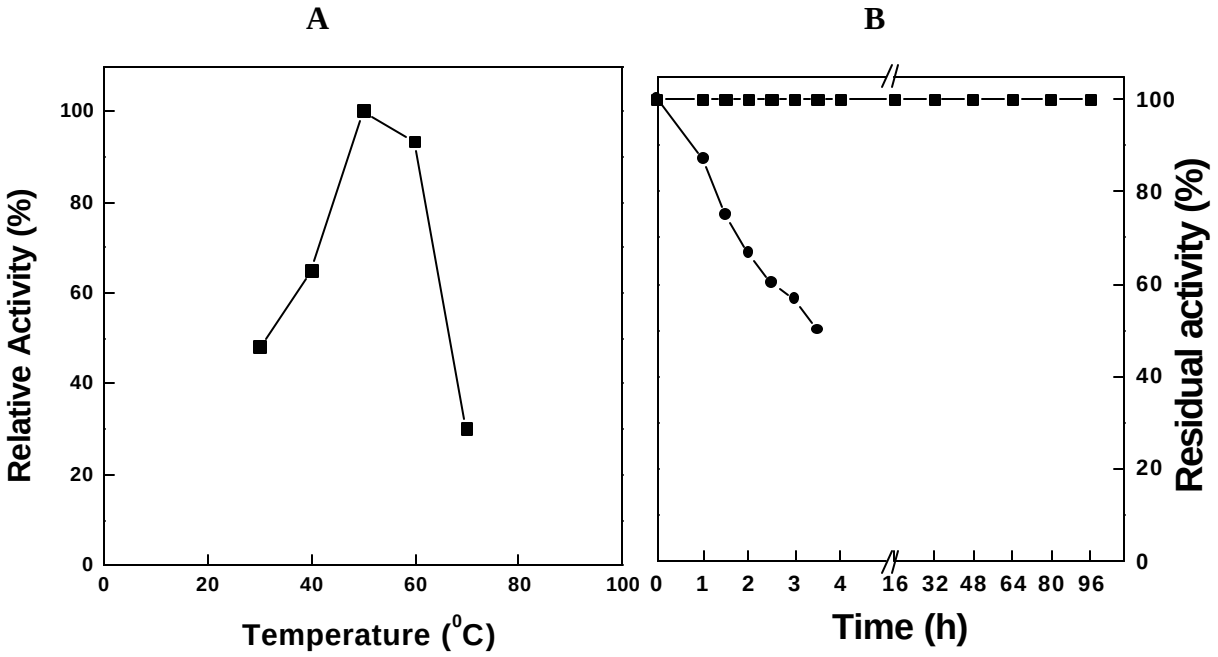


Fig. 4 Optimum temperature and thermal stability

A. Optimum temperature: Xyl II was assayed at various temperatures from 30-75 °C at pH 7.0. The activity of 5 U at 50 °C was taken as 100 %.

B. Thermal stability: Xyl II (5 U) was incubated at 50 °C (■) and at 60°C (●) in 0.05 M phosphate buffer pH 7.0. Aliquots were removed at regular time intervals and assayed for activity under standard conditions.

Substrate specificity and kinetic parameters of Xyl II

The enzyme did not hydrolyze p-nitrophenyl-β-D xylopyranoside, p-nitrophenyl-glucopyranoside or p-nitrophenyl-β-D arabinofuranoside and had no effect on carboxymethyl cellulose or starch. The apparent K_m value of purified Xyl II was 3.5 mg/ml and V_{max} value was calculated to be 0.742 $\mu mol.min^{-1}$ $.mg^{-1}$. The isoelectric point of the enzyme was 8 and the carbohydrate content of the enzyme was approximately 10 %. Some xylanases from *Bacillus* sp. have been reported to be glycoproteins (Bernier *et al*., 1983; Paul and Varma, 1992; Bastawde, 1992; Breccia, 1998).

Influence of metal ions on Xyl II

The activity of Xyl II was measured under the standard assay conditions in the presence of metal ions and other agents (Table 2). 5 mM Ca^{2+}, Mn^{2+}, Mg^{2+}, Zn^{2+} did not influence the activity whereas, Hg^{2+} and Cu^{2+} (1 mM) completely inactivated the enzyme. EDTA did not have any significant effect on the enzyme activity.

Table 2 Effect of metal ions on xylanase activity

Metal ion	Relative activity (%)
None	100
$CaCl_2$	100
$MgCl_2$	100
$MnCl_2$	100
$HgCl_2$	30
$CuCl_2$	35
LiCl	100
NaCl	100

Hydrolysis of xylan and determination of end products

The maximum degree of hydrolysis of xylan by Xyl II was 28 %. (Fig. 5A). The products of xylan hydrolysis were analyzed by paper chromatography. Xyl II produced xylobiose as the major product, along with xylotriose, xylotetraose and higher oligosaccharides. Xylose was liberated after 16 h. (Fig. 5B). Xyl II did not release arabinose from the xylan tested. Our data suggest that Xyl II is an endoxylanase without debranching activity.

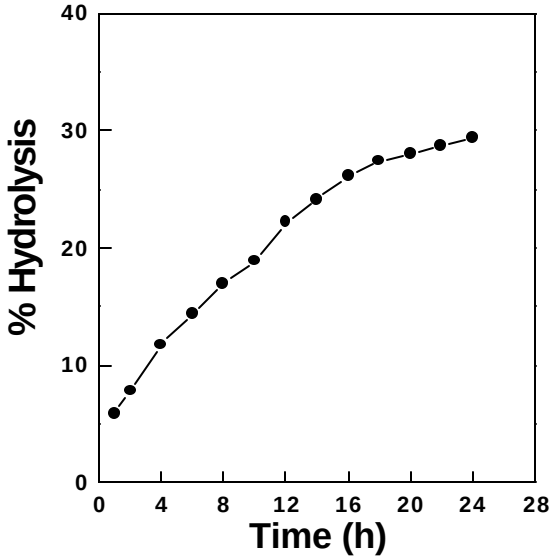


Fig. 5A **Time course of hydrolysis of xylan**

Xylan (25 mg) was incubated with 1 U of Xyl II at pH 7.0 and 50 °C

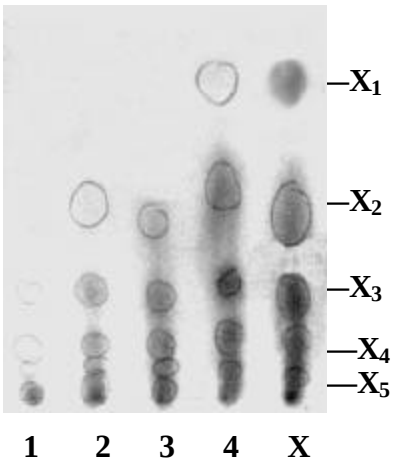


Fig. 5B Paper chromatogram of hydrolysis of xylan

Products from 5 mg xylan obtained using 1 U of Xyl II. **Lane X**, standard mixture of 10 µg each of xylose (X_1), xylobiose (X_2), xylotriose (X_3), xylotetraose (X_4), and xylopentose (X_5); **Lanes 1-4**, 1-, 4-, 16-, and 24-h samples of hydrolysis products.

Amino acid composition and N-terminal sequence

The amino acid composition of the purified Xyl II and other *Bacillus* sp. is shown in Table 3. Xyl II showed high content of Asp and Glu acid residues. In the genus, *Bacillus* xylanases with high concentrations of Asx-Glx (Khasin *et al.*, 1993; Nakamura *et al.*, 1995; Blanco *et al*., 1995) or Asx-Gly (Panbangred *et al.*, 1983; Bernier *et al*., 1983) have been found. Alkalophilic thermophilic *Bacillus* Xyl I and Xyl II seemed to fall into the first category, while xylanase from *Bacillus amyloliquefaciens* MIR 32 belongs to the second category (Breccia *et al*., 1998). Significant amounts of the hydroxy amino acids, threonine and serine were also seen to be present in Xyl II. These amino acid residues have been presumed to provide flexibility in the structure of polysaccharide hydrolases (Coughlan & Hazlewood, 1993).

The N-terminal amino acid sequence of Xyl II was found to be **AVYGQYATDNL** and was distinctly different from other reported xylanases. The N-terminal sequence of xylanases from *Trichoderma viride* (Yaguchi *et al*., 1992), are homologous to xylanases from *Clostridium acetobutylicum* (Zappe *et al*., 1990), *Bacillus subtilis* (Paice *et al*., 1986) *Bacillus pumilus* (Fukusaki *et al*., 1984) and *Streptomyces* sp. (Nagashima *et al*., 1989). However, the reports on xylanases from the tomato vascular pathogen *Fusarium*

oxysporum f. sp. lycopersici (Ruiz *et al*., 1997) and *Thermococcus zilligii* (Uhl, *et al.*, 1999) strain AN1 showed no N-terminal region homology with other known xylanases.

Table 3 Percent amino acid composition of Xyl I, Xyl II from alkalophilic thermophilic (AT) *Bacillus* and xylanases from other *Bacillus* sp.

Amino acid	AT *Bacillus* Xyl II	AT *Bacillus*[a] Xyl I	*Bacillus* sp. Strain BP-23[b] Xylanase A	*Bacillus amyloliquefaciens*[c]
Aspartic acid	**16.07**	18.9	14.3	13.8
Threonine	**10.32**	0.44	4.4	12.1
Serine	**9.57**	0.46	6.0	8.4
Glutamic acid	**13.08**	45.0	12.5	7.8
Proline	**4.28**	0.17	4.9	3.4
Glycine	**4.20**	27.8	9.7	14.5
Alanine	**6.6**	1.0	8.2	4.1
Cysteine	**nil**	0.23	nil	nil
Valine	**9.44**	0.23	4.9	8.6
Methionine	**0.74**	0.76	1.4	0.2
Isoleucine	**5.31**	0.43	5.3	3.8
Leucine	**5.49**	0.44	6.5	3.4
Tyrosine	**4.72**	0.43	3.5	8.2
Phenylalanine	**2.58**	0.15	3.9	2.4
Lysine	**2.5**	0.51	4.9	1.5
Histidine	**1.65**	0.18	1.6	1.8
Tryptophan	**3**	2.72	4.7	nd
Arginine	**5.94**	0.21	3.3	6.0

[a] Chauthaiwale and Rao, 1994; [b] Blanco *et al.*,1995; [c] Breccia *et al*., 1998

A multiplicity of xylanases has been documented in various bacteria (Wong *et al*., 1988; Bérenger *et al*., 1985; Flint, *et al*., 1989; Gilbert *et al*., 1988). Our studies have revealed that alkalophilic thermophilic *Bacillus* produces two enzymes exhibiting xylanase activity. Xyl I has been purified and characterized (Dey *et al*., 1992; Hinge *et al*., 1989; Chauthaiwale and Rao 1993). Based on the pI (4) and molecular mass (35,000), XyI belongs to the high molecular mass enzyme group (Family 10) of glycosyl hydrolases, as proposed by (Gilkes *et al*., 1991; Henrissat and Bairoch, 1993). Xyl II belongs to the basic low molecular mass enzyme group. The M_r of Xyl II molecular weight of 15,800. Few xylanases from other microrganisms having low molecular mass have been summarized in Table 4.

Table 4 Low molecular weight xylanases (<20 kDa).

Organism	Xylanase	Molecular mass (kDa)	Reference
***Bacillus sp.* C-125**	N	16	Honda *et al*., 1985
Cellulomonas fimi	A	13.2	Khanna and Gauri, 1993
Thermomonospora curvata	3	15	Stutzenberger, 1992
Humicola insolens	Xyl1	6	Dusterhofter, 1997
Humicola grisea var. thermoidea	2	13	Thakur *et al*., 1992
Aspergillus tamarii	-	14	Gouda, 2000
Aspergillus tamarii	I	12.5	Souza *et al*., 1998
***Myrothecium verrucaria* CMI 45541**	-	15.9	Filho *et al*., 1993

A comparison of K_m values suggests that Xyl I has higher affinity for xylan than Xyl II. The optimum temperature and pH of the two xylanases did not show any striking difference. Xyl I was more stable than Xyl II at 60 °C. As suggested by Fontes *et al*., 1995), family 11 xylanases are generally more thermolabile than family 10 enzymes

probably because their conformation is sensitive to sequence modifications. To date only one thermostable xylanase from family 11, exhibits an optimum temperature of 80 °C (Morris *et al*., 1998), whereas six family 10 xylanases having optima above 80 °C (Saul *et al*., 1995; Winterhalter and Liebl, 1995; Zverlov *et al*., 1996) have been reported.

CHAPTER 3

STRUCTURE-FUNCTION ANALYSIS OF XYL II

SUMMARY

The pH induced conformational and structural changes of Xyl II have been investigated from the alkalophilic thermophilic *Bacillus* sp. using kinetic, circular dichroism and fluorescence spectroscopy studies. The Trp fluorescence and the kinetic constants were found dependent on the pH. Above pH 8.0, the enzyme exhibited unfolding transitions as revealed by a red shift in the emission maximum as well as decreases in the fluorescence intensity. Circular dichroism studies revealed a decrease in the CD ellipticity at 222 nm at pH 9.0 and 10.0. The reduced catalytic activity of Xyl II at alkaline pH is correlated to the pH induced unfolding and ionization or protonation of key protein residues. The pH profile of Xyl II showed apparent p*k* values of 5.5 and 7.0 for the free enzyme and 5.6 and 6.7 for the enzyme-substrate complex. The abnormally high pk_a of 6.7 indicated the participation of a carboxyl group present in a non-polar environment. The essential amino acid residues for the function of Xyl II activity was resolved through group specific chemical modification studies. Modification of Xyl II with NBS, HNBB, and WRK led to the inactivation of the enzyme suggesting the involvement of a single Trp and carboxyl residue in the active site of the enzyme. Trp residues were located in a relatively electronegative microenvironment as suggested by the quenching studies using charged and neutral quenchers. The proximity of the essential carboxyl residues with high pk_a value of 6.9 probably contributed to the electronegative environment of the Trp residue. Xyl II was highly stable at pH 10, however the enzyme exhibited pH optimum at near neutral values, which can be explained by the ionization and microenvironment of the active site residues. The sequence alignment studies of Xyl II, in combination with kinetic and chemical modification data provide strong evidence for the participation of Trp61 and Asp94 in the catalytic function and may play a role in the structure function of the enzyme.

INTRODUCTION

Studies on the structure-function relationship have always been one of the central issues in the investigations of biological macromolecules. Attempts to correlate structural changes in proteins to alterations in their biological activities have been hitherto largely confined to the modification of the side-chain functional groups. Chemical modification using group specific reagents is one of the approaches to study the role of amino acids present at the active site (Eyzaguirre, 1986; Means and Feeney 1971). The utility of chemical modification is greatly extended by its use in conjunction with X-ray crystallographic studies, which mutually supplement one another.

Significant progress has been made in the recent years in the understanding of the sequence of events that take place at the catalytic center of xylanases. The initial drive originated from the observation that xylanases not only share amino acid similarities with lysozyme (Morosoli *et al.*, 1986) but also show a similar pattern of action (Biely *et al*., 1981). The studies on sequence similarities of xylanase, cellulase and lysozyme have supported the possibility that catalytic mechanism of these enzyme follows the same pathway. The difference in function among the glycosidases reflects the great diversity of molecular architecture of these enzymes and reinforces the need for simpler unambiguous methods to define functional residues in enzyme active sites (Legler, 1990). Despite the biotechnological importance of xylanases from extremophilic organisms, very few reports characterizing the structure-function relationship of these enzymes are documented.

In the present chapter, we have attempted to correlate the loss in activity at high pH of Xyl II by studying (i) the pH dependence of the enzyme conformation, which may account for the pH dependence of the catalytic activity, and (ii) the effect of pH on ionization or protonation of carboxyl residues.

The kinetic and chemical modification studies delineating the presence of Trp residues in the catalytic site are described. The microenvironment of the Trp residues using various solute quenchers has also been investigated.

MATERIALS AND METHODS

MATERIALS

Xylan (oat spelt) was obtained from Aldrich. NBS (*N*-bromosuccinimide), HNBB (2-hydroxy-5-nitrobenzyl bromide), DEPC (diethylpyro-carbonate), NEM (N-ethyl maleimide), Woodward's reagent K (2-ethyl-5-phenyl isoxalium-3-sulfonate) (WRK), Phenyl glyoxal, dithionitrobenzoic acid (DTNB) and PHMB (p-hydroxy mercury benzoate) were obtained from Sigma Chemical Co., USA. All other chemicals were of analytical grade.

METHODS

Steady state Kinetic studies using xylan.

Hydrolysis rates for xylan were determined at 6-8 different substrate concentrations. The k_{cat} values for Xyl II hydrolysis were determined from the average of at least three substrate measurements at each substrate concentration and pH. For experimental convenience, the enzyme concentration was increased for determinations made with substrate at pH values above 8.0 and below 6.0. The k_{cat}/K_m were calculated from the slope of the Lineweaver-Burk plot.

Reaction of Xyl II with chemical modifiers

The enzyme solution (0.5 ml) containing 2 mM protein in buffer (50 mM acetate buffer, pH 5.0) was incubated in the presence of various amounts of modifier for different time periods. Termination of chemical modification as well as initiation of enzyme reaction was made by adding 5 mg of xylan and respective residual activities were measured. Control tubes having only enzyme or only inhibitor or inhibitor and substrate were incubated under identical conditions.

Reaction of Xyl II with WRK

Xyl II (10 µg) in 50 mM potassium phosphate buffer (pH 6.0) was incubated with different concentrations of WRK (5-30 mM) at 25 °C. Aliquots of the reaction mixture were withdrawn at indicated time intervals and the excess reagent was quenched with a final concentration of sodium acetate buffer (pH 5.0; final concentration 250 mM). The

residual xylanase activity was measured and expressed as percentage of a control. Purified xylanase (10 µg) was incubated with 10 mM diethyl pyrocarbonate in 50 mM sodium phosphate buffer pH 7.0 for 10 min. Residual activity was determined under standard assay conditions. The number of free sulfhydryl groups in Xyl II was assessed by titration with dithiobis (2-nitrobenzoic acid) according to the method of Ellman (1959) and Habeeb (1972).

Titration of enzyme with NBS

Oxidation of the Trp residues by NBS was carried out into cuvettes, one containing Xyl II ($1x10^{-5}$ M) in 50 mM acetate buffer pH 5.0 and the other containing only buffer. Successive 10 µl aliquots of NBS ($1x10^{-5}$ M) were added to the sample and the reference cuvette and the progress of the oxidation reaction was monitored at 280 nm. A mixture of xylan and NBS solution showed an optical density equivalent to that of xylan alone, indicating that NBS does not react with xylan. The number of tryptophan residues oxidized per mol of enzyme was determined by the method of Spande and Witkop (1967). Simultaneously, aliquots (10 µl) were withdrawn, diluted with 15 mM tryptophan in 50 mM sodium acetate buffer (pH 6.0) to destroy any residual NBS, and assayed for xylanase activity. Substrate protection studies were performed by preincubating the enzyme with the substrate xylan for 10 min prior to the addition of the modifier.

Kinetics of inactivation

The apparent first-order constant of inactivation depends on the concentration of the modifier and can be expressed as

$$K_{app} = K\,(M)^n$$

where, K_{app} is the apparent first order rate constant, M is the concentration of the modifier and n is the number equal to the average order of reaction with respect to the concentration of modifier

$$\log K_{app} = \log K + n \log (M)$$

K_{app} can be calculated as a slope from a semi-logarithmic plot of residual enzyme activity as a function of time. The second order rate constant for inactivation was determined from the slope of the plot of pseudo-first order rate constant against inhibitor concentration. The order of the reaction (n) can be experimentally estimated by determining the K_{app} at

different concentrations of the modifier. A plot of log K_{app} against log (M) gives a straight line with a slope equal to *n*, where *n* is the number of the molecules of the modifier reacting with each active unit of the enzyme to produce an enzyme inhibitor complex (Levy *et al*., 1963).

Fluorescence measurements and quenching experiments

Tryptophan fluorescence spectra were measured on an Aminco SPF-500 spectofluorometer. The excitation wavelength was fixed at 295 nm and emission ranged between 300-400 nm. Concentration of enzyme was 100 µg/ml. The bandwidth of excitation and emission were 5 nm and 10 nm respectively. Quenching experiments were performed by titrating the solution of 5 M acrylamide, KI and CsCl. Fluorescence intensities were measured at the wavelength corresponding to the emission maximum of each protein and were corrected for dilution, blanks, and the inner filter effect (Lakowicz, 1983). The results of the quenching reactions were analyzed according to the Stern-Volmer equation

$$F_o/F = 1+K_{sv}[Q]$$

where, F_o and F are the fluorescence intensities at appropriate emission wavelength in the absence and presence of quencher respectively, K_{sv} is the Stern-Volmer quenching constant and [Q] is the molar quencher concentration (Stern-Volmer, 1919). For proteins containing more than one fluorescing tryptophan residues differing in their accessibility to the quencher, the Stern-Volmer plots will be non linear and hence a modified Stern-Volmer equation has been applied

$$F_o/F = 1/f_a + 1/f_a\ K_{sv}[Q]$$

$$\Delta F = F_o - F$$

f_a is fraction of the quenchable fluorescence was obtained from the ordinate intercept of the linear portion of the linear portion of the $F_o/\Delta F$ vs 1/Q plot (Lehrer,1971).

Circular dichroism (CD) measurements

The CD spectra of enzyme samples (6.5 µM) were recorded on a Jasco-500 spectropolarimeter from 200- 250 nm using quartz cells with a path length of 1mm.

RESULTS AND DISCUSSION

pH dependent conformational and structural changes of Xyl II

Influence of pH on the conformation of Xyl II

Xyl II exhibited maximum activity at pH 6 to 7.5 and decreased on either side of the optimum. Figure 1 shows the effect of alkaline pH on the spectral parameters of Xyl II. In the alkaline range of pH 8-10, the fluorescence intensity decreases with a concomitant red shift (4 nm). This is probably caused by the deprotonation of the amino, guanidine, and the phenol groups of lysine, arginine and tyrosine residues respectively, which quench the fluorescence of the Trp residues in the deprotonated form (Bushueva and Tonvitsky, 1987). Deprotonation of these residues could give rise to several changes unfavorable to the native state, including electrostatic repulsions, destruction of salt bridges and the formation of buried isolated charges, eventually leading to functional loss of activity.

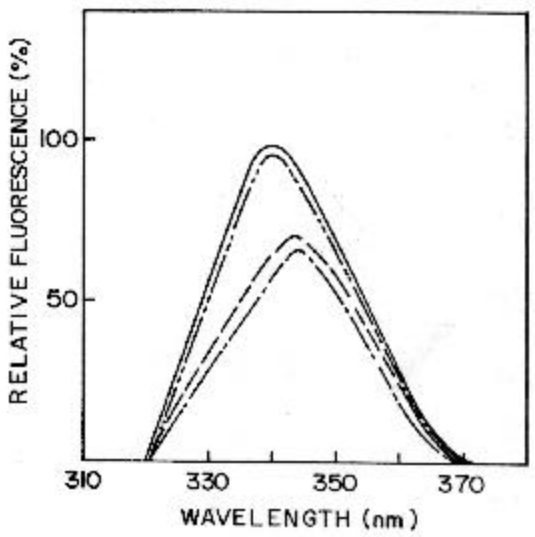


Fig. 1 pH dependent conformational changes of Xyl II

Tryptophanyl fluorescence of Xyl II (0.5-1 μM) in 50 mM potassium phosphate buffer, pH 7.0 (—), Tris HCl buffer, pH 8.0 (- · · -), 9.0 (----) and sodium carbonate-bicarbonate buffer, pH 10 (- · -).

The structural alterations in the conformation of the protein were further corroborated by changes in the CD spectra. The CD intensity at 222 nm was unchanged at pH range 7.0 and 8.0, however at pH 9.0 and 10.0 there was a decrease in magnitude of the mean residue ellipticity, suggesting conformational changes in the enzyme (Fig. 2). The reduced catalytic activity of Xyl II at alkaline pH may have resulted from the pH induced unfolding of the enzyme.

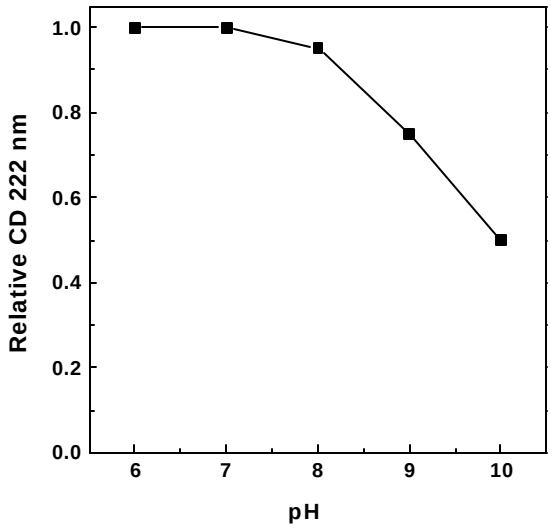


Fig 2 **Effect of pH on CD of Xyl II**
CD spectra of Xyl II (6.5µM) were recorded from 200 to 250 nm using a 1mm path length at 25 °C, in 50 mM buffers of pH ranging from 6-10.

pH dependence of the enzyme activity

The nature of the ionizable groups essential for the function of Xyl II activity was resolved through the effects of pH on k_{cat} values. The variation of K_m over the pH range 5-10 is shown in Table I. Decreased saturation of the enzyme with substrate at alkaline pH led to the decrease in affinity, which may be due to alteration in the conformation. The plots of k_{cat} and $k_{cat}.K_m^{-1}$ against pH are shown in Figure 3. 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 II activity on the ionization of at least two groups.

Table 1: Kinetic parameters for soluble xylan with Xyl II from alkalophilic thermophilic *Bacillus*

pH	K_m (mg/ml)	k_{cat} (min^{-1})
5 .0	4.16	7.30×10^5
6.0	4.10	4.21×10^6
7.0	4.0	3.69×10^6
8.0	7.69	7.80×10^5
9.0	8.33	4.55×10^5
10.0	62.50	9.10×10^4

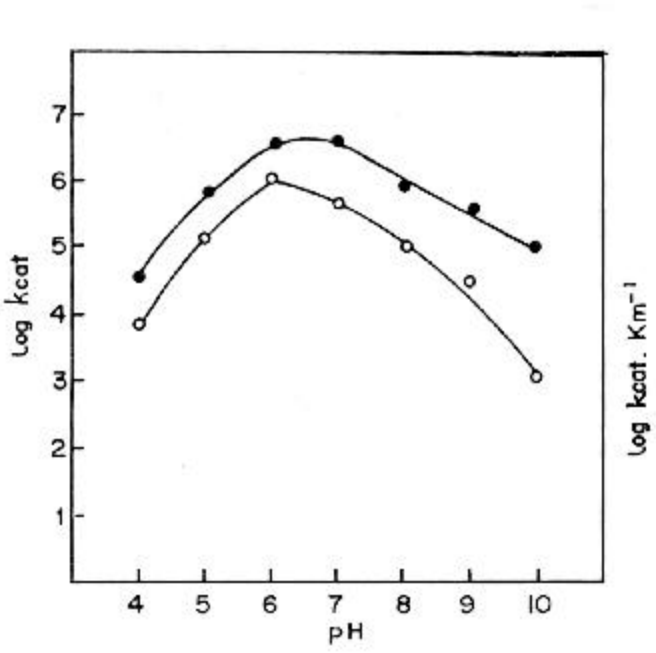


Fig.3 **Dependence of kinetic parameters on pH**

Xylanase (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 4-10. K_m and k_{cat} values were derived from Lineweaver-Burk plots. The effect of pH on log k_{cat} (•—•) and log $k_{cat}.K_m^{-1}$ (**o—o**) are shown.

The log k_{cat} versus pH plot gave p*K*e values of 5.5 and 7.0 for the free enzyme. Values of 5.6 and 6.7 were obtained from the log $k_{cat}.K_m^{-1}$ versus pH plot for the enzyme-substrate complex. The values obtained from the acidic limb of the curves are consistent with the participation of a carboxylate in the Xyl II 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 His residue since imidazolium groups in proteins have pK_a values usually in the range of 5.6 –7.0 (Cohn and Edsall, 1943; Greenstein and Winitz, 1961). Alternatively, a carboxyl group located in a non-polar environment may exhibit abnormally high p*k*a values of between 6 and 7, as in the case of hen egg white lysozyme (Imoto, *et al*., 1972; Inoue *et al.*,1992). Slopes obtained from the log $k_{cat}\ K_m^{-1}$ versus pH and log k_{cat} versus pH plots were +0.83, -0.71, and +0.57, -0.33 respectively. The slopes of the acidic and basic limb of the curves in Figure 3 are less than unity, indicating that the simple model of the pH dependence of enzyme action does not describe adequately the Xyl II 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 II system. (Wakim *et al*., 1969). The nature of the essential amino acid residues for the function of Xyl II activity was resolved through group specific chemical modifiers.

Effect of various chemical modifiers on Xyl II

The importance of particular functional groups for the activity of Xyl II was investigated by the use of various chemical modifiers with restricted amino acid specificity. DEPC, NAI, phenyl glyoxal, PMSF are not inhibitory to Xyl II indicating that His, Tyr, Arg, and Ser are not involved in catalysis of the enzyme (Table 2). Modification with NBS, HNBB, and WRK led to the inactivation of the enzyme suggesting the involvement of Trp and carboxyl residues in the active site of Xyl II.

Table 2 Effect of chemical modifiers on the activity of Xyl II from AT *Bacillus* sp.

Reagent	Inhibitor concentration (mM)	Residual activity (%)	Incubation Buffer
Diethyl pyrocarbonate	10	100	Potassium phosphate, pH 6.0
Trinitrobenzoic acid	1	100	Sodium bicarbonate, pH 8.5
N-acetyl imidazole	10	100	Potassium phosphate, pH 7.0
Phenylglyoxal	10	100	Tris HCl, pH 8.0
Phenyl methyl sulfonyl fluoride	10	100	Potassium phosphate, pH 7.0
N-ethylmaleimide	10	100	Sodium acetate, pH 6.0
p-hydroxymercury benzoate	10	100	Sodium acetate, pH 6.0
N-Bromosuccinimide	1	0	Sodium acetate, pH 6.0
2-hydroxy-5-nitrobenzyl bromide	10	20	Sodium acetate, pH 4.0
Woodward's reagent K	10	20	Sodium acetate, pH 6.0

Enzyme (5 mg) was incubated with various reagents at room temperature and after terminating the reaction, residual activities were measured under standard assay conditions.

Role of carboxyl group in the catalytic mechanism

Kinetics of inactivation of Xyl II by WRK

The participation of carboxyl groups in the mechanistic pathway was investigated using WRK at pH 6.0. After 20 min incubation of Xyl II (6.25 x 10^{-7} M) with 50 mM WRK (pH 6.0, 20 °C) residual activity against xylan is only 40 % (Fig. 4). Analysis of a log (K_{app}) versus log [WRK] by the method of Levy *et al*. (1963) yields a reaction order with respect to WRK of 1.0 (Fig. 4, inset) and indicates that at least one mole of carbodiimide binds an equivalent mole of xylanase for inactivation.

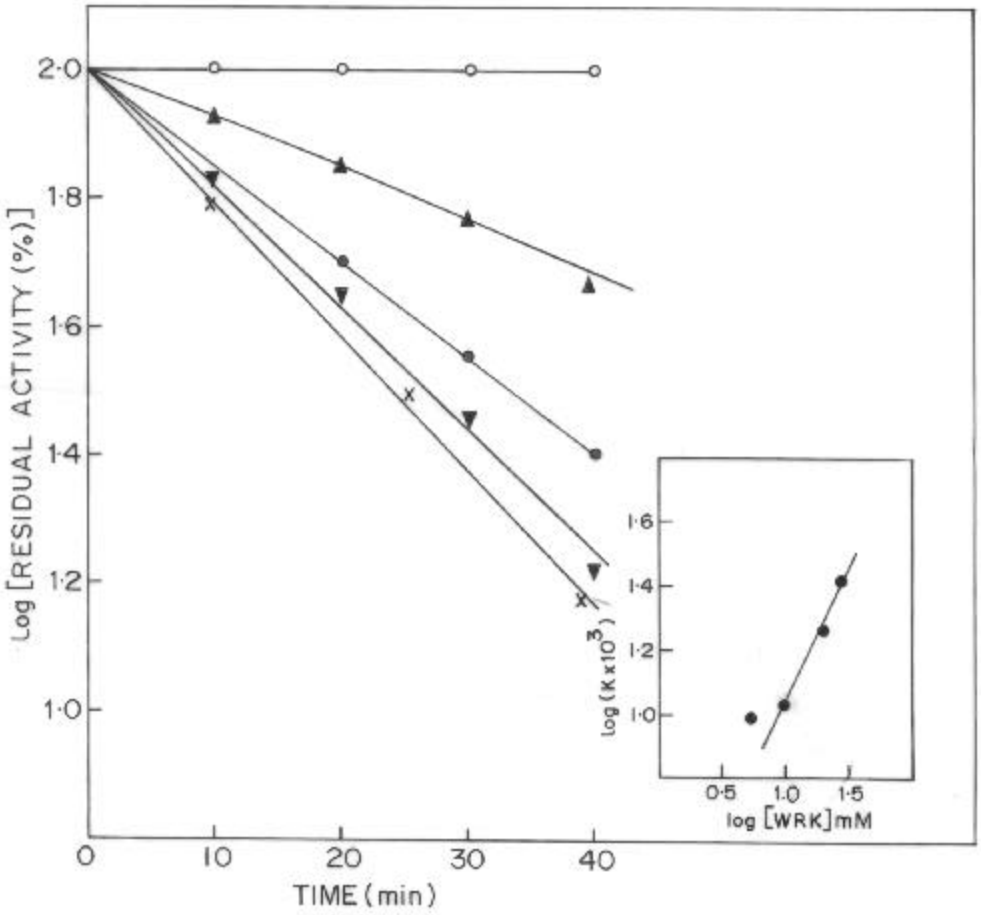


Fig.4 **Inactivation of Xyl II by WRK**. Plot of residual activity *versus* time for the inactivation of Xyl II by WRK. Xyl II (10 µg) was incubated with WRK 0 mM (-o-), 5 mM (- -), 10 mM (-· -), 20 mM (- -), and 30mM **(-x-)** at 25 °C. **Inset**: Apparent order of reaction with respect to reagent concentration.

A plot of the pseudo-first order rate constants against the modifier concentration appears to be hyperbolic (Fig. 5). The reaction probably proceeds with the formation of an intermediary reversible enzyme modifier complex (saturation with respect to WRK). The modification thus seems active site directed. The linearised $1/K_{app}$ versus [1/I] plot yields an apparent binding constant $K_{i\ app}$ is 1.56 x 10^{-3} and a first order rate constant at saturation of 0.076 min^{-1} at pH 6.0.

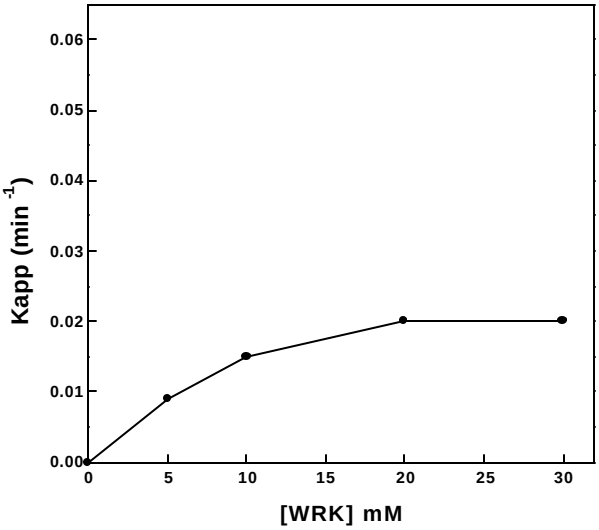


Fig. 5 Plots of the K_{app} value derived from slopes in *Figure 4*, versus WRK concentrations

The catalytic rate constant of modified enzyme was found to be 78x10^6 min^{-1}, which were two orders lower than the native xylanase (4.21 x 10^6 min^{-1}). The K_m was unaltered indicating that the catalytic hydrolysis and not the substrate binding is affected by chemical modification. Xylan (0.5 %) was able to provide partial protection against inactivation probably due to the bulky nature of the ligand, whereas xylooligosacharides (0.5-1 %) were able to provide 90 % protection against inactivation. The modification proceeds most rapidly at pH 6.0 and the bell shaped pH inactivation curve indicates that two groups with apparent pk_a values of 5.7 and 6.3 are implicated (Fig. 6).

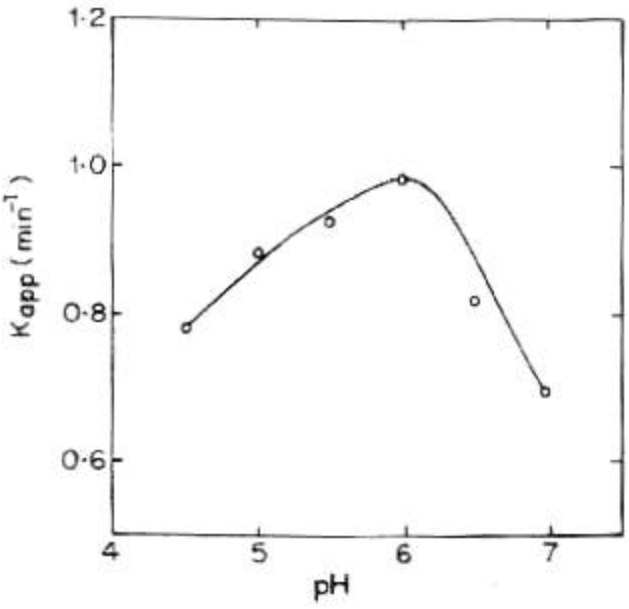


Fig. 6 **Influence of pH on the pseudo-first-order reaction constants for the inactivation of Xyl II by WRK**

Xylanase (10 µg) was modified by WRK (20 mM) in 50 mM buffers of different pH (pH 4-5, citrate-phosphate; pH 6-7 potassium phosphate). The pseudo-first-order reaction constants were obtained from the slopes of the plots of logarithm of residual activity against time.

The residues could either be histidyl groups with an abnormally low pk_a or carboxyl with a high pk_a value. Chemical modification of Xyl II with diethyl pyrocarbonate did not result in any loss of activity suggesting that the histidine residues are neither mechanistically essential nor involved in the maintenance of a conformation necessary for an active enzyme. Hence, the pH dependence of inactivation process provided support for the identification of the ionizable groups as carboxylates. Carboxyl group modification by carbodiimides requires a protonated carboxyl group (Hoare and Koshland, 1963) thus the observed increase in the apparent rate of inactivation between pH 4-6 indicates ionization of the essential carboxyls in agreement with the pH-activity data. The possibility of interference of sulfhydryl groups can be excluded as Xyl II did not possess any cysteinyl residues.

Kinetics of inactivation of Xyl II by Gdn. HCl

The xylanase activity was decreased 30 % by 0.5 M Gdn HCl (Fig. 7). The Trp fluorescence was unaffected by 0.5 M Gdn HCl and higher concentrations of Gdn HCl were required to physically disrupt protein structure (Fig. 7). These results suggest a specific inhibition of the enzyme. The Lineweaver-Burk plot indicated the inhibition of Xyl II by 0.25 M Gdn HCl to be purely competitive. The K_m values were 3.5 mg/ml and 14 mg/ml in the absence and presence of inhibitor, respectively. From these data, K_i was calculated to be 40 mM. Since maximum inhibition occurred between pH 6.0-7.0, which is similar to the pH range for maximum activity, it would appear that the catalytic groups are involved in binding to Gdn HCl. It has been proposed that the positively charged guanidinium group of Gdn HCl interacts electrostatically with the active site residue to form the enzyme inhibitor complex, thus causing inhibition.

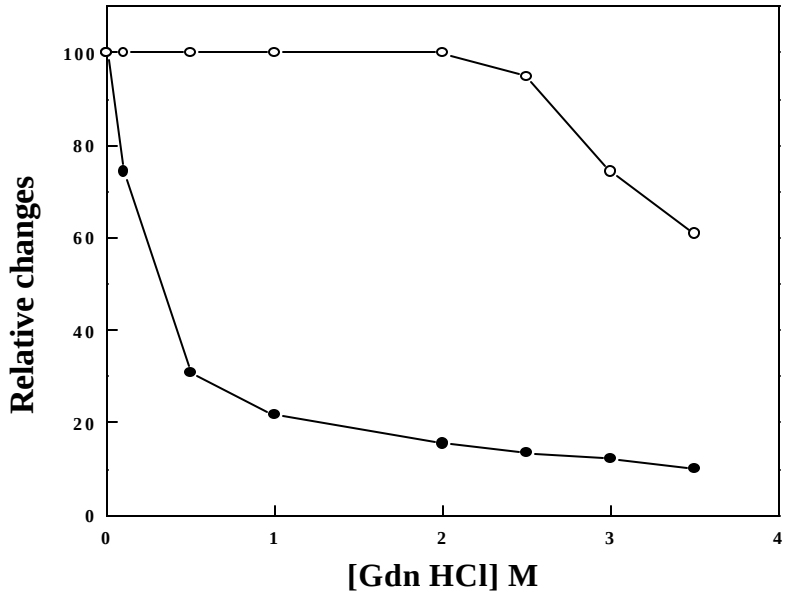


Fig.7 Effects of guanidine on the activity and the unfolding of Xyl II.

Enzyme activities in Gdn HCl of different concentrations were measured after incubation with the denaturant (•) and unfolding of the enzyme was followed by fluorescence emission at 340 nm (**o**).

One of the most commonly used procedures for the modification of protein carboxyl groups has been that of coupling with nucleophiles mediated by water-soluble carbodi-imide (Glazer *et al.*, 1975). Treatment of Xyl II with WRK inactivated the enzyme rapidly with the modification of one carboxyl residue. Xylooligosaccharides afforded significant protection against inactivation. A variety of strategies including affinity labeling and chemical modification (Legler, 1990; Clarke *et al*., 1963) have served to support the current model of carbohydrase action. Experimental evidence from stereochemical studies suggests that the xylanases perform catalysis through a double displacement mechanism similar to hen egg white lysozyme. Such mechanism requires the presence of a nucleophilic residue, which will assist the catalytic acid/base residue during transfer of protons. This acidic residue in order to play its role would have to be in an ionized state under the catalytic conditions. The acid base catalyst is conserved in xylanases of both families and surrounded by a highly homologous region specific to each family. The observed high pK_a value of the amino acid residue that participates as the acid catalyst in the carbohydrases may be due to the low accessibility of the solvent to the active site cleft, electrostatic interactions with the charged amino acids (Asp52) and location at the negative charge containing site of an α-helix microdipole as shown in case of lysozyme (Spassov *et al*., 1990). The stability of an enzyme is an intrinsic property of the protein based on the presence of hydrophobic residues and the net surface charge. However, our results suggest that the functional activity depends on the microenvironment and the spatial biased distribution of charged residues on the protein. The pK_a of the ionizable side chain will depend on the microenvironment of the amino acids and as a consequence, various xylanases show different optimal pHs. In *Trichoderma reesei* xylanases (Törrönen & Rouvinen, 1995), it has been shown that the acidic xylanase is active in the pH range of 3-6, whereas the alkaline xylanase is active in a higher pH range from 4.0-8.0. Krengel and Dijkstra (1996) have proposed that the higher pH optimum of xylanases is influenced by an Asn residue, which is critical in determining the pH dependence of xylanase activity. Xylanase from *Bacillus pumilus* has revealed Glu93 and Glu182 to be the most suitable residues responsible for the essential activity of the enzyme (Ko *et al*., 1992). The conserved residues Glu87 and Glu184 have been shown to be present in the active site of xylanase A from *S. commune* (Oku *et al*., 1993). Mutagenesis and analysis of 3D structure

of xylanase A from *S. lividans* revealed Asn127 to be the important residue in maintaining the ionization states of two catalytic residues and in the stabilization of catalytic intermediates (Roberge *et al*., 1997). In case of *B. circulans* the two residues Glu78 and Glu172 have been found to be involved in catalysis (Wakarchuk *et al*., 1994). The mutational analysis of Xylanase J from alkalophilic *Bacillus* sp. strain 41M-1 indicated that Glu93 and Glu183 play an important role in the catalytic activity (Nakamura *et al*., 1994). Sequence homology studies of Xyl II revealed two conserved residues Asp94 and Glu117. Asp94 was analogous to Asp 106 of *B. circulans*, which played an important role in catalysis. Glu 117 may function as nucleophile acid-base catalyst (Kulkarni *et al*., 1999). We propose that the catalytically essential carboxyl residue in Xyl II may be Asp94.

Role of Trp residues at the active site of Xyl II

Inactivation kinetics of NBS modified enzyme

The inactivation of Xyl II by NBS measured in terms of xylanase activity was found to be dependent on both time and inhibitor concentration. The plot of log of residual activity versus time at all concentrations of the reagent was always linear indicating that the inactivation followed first order kinetics (Fig. 8). Applying the analysis described by Levy *et al*. (1963) the double logarithmic plots of the observed pseudo first order rate constants against reagent concentration also appeared to be linear. Complete inactivation of Xyl II activity was due to the oxidation of one Trp residue, as determined from the slopes of the plots (Fig. 8, inset).

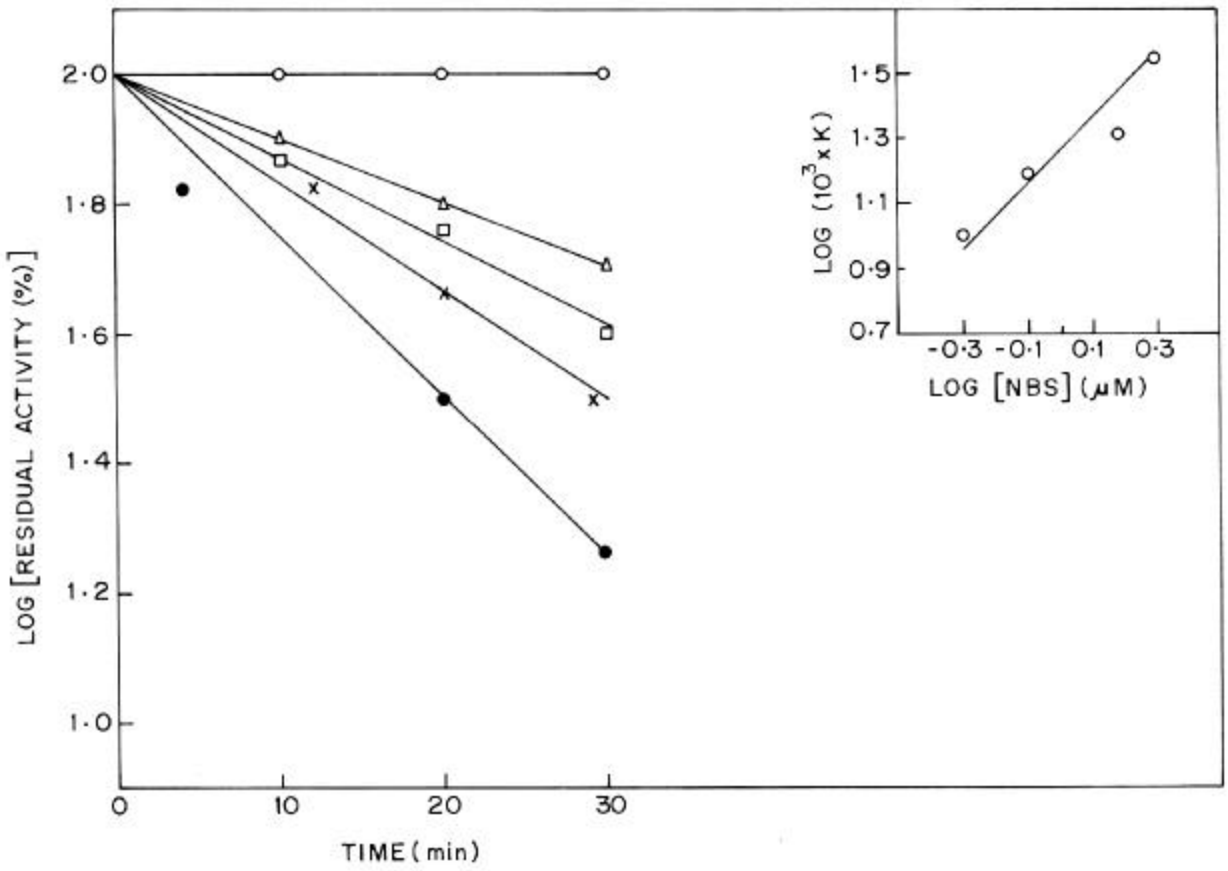


Fig. 8 **Inactivation of Xyl II by NBS**

Xyl II was incubated with NBS 0 μM (o), 0.5 μM ( ), 1.0 μM ( ), 1.5 μM (x), and 2.0 μM (•) at 25 °C. **Inset**: Apparent order of reaction with respect to NBS concentration.

Titration of Xyl II by NBS

The effect of NBS on the xylanase activity showed that after each addition of NBS there was a progressive decrease in absorption at 280 nm with concomittant loss in activity (Fig. 9). The reaction of proteins with NBS is accompanied by a decrease in the absorbance at 280 nm. This is attributed to the NBS induced oxidation of indole chromophore of Trp which absorbs at 280 nm to oxyindole a much weaker chromophore at this wavelength (Eyzaguirre, 1986). The residual enzymatic activity plotted against the number of Trp residues modified is shown in Figure 10. The extrapolation with the Y-intercept passing through 0 % inactivation confirmed the presence of one Trp residue at the catalytic site. The second order rate constant was determined to be 9700 M^{-1} min^{-1}. The values of V_{max} for the native and modified enzyme were 0.742 µmoles min^{-1} mg^{-1} and 0.1886 µmoles/ min^{-1} mg^{-1}, respectively. The K_m values for the modified enzyme (5 mg/ml) increased as compared to the native enzyme (3.5 mg/ml) indicating the decreased affinity of the enzyme to the substrate, which apparently affected the efficiency of the modified enzyme. The inhibition of Xyl II was solely due to Trp residues was confirmed by the modification of Xyl II with HNBB which is a more specific reagent for tryptophan (Fig. 11) and the second order rate constant was determined to be 33 M^{-1} min^{-1}.

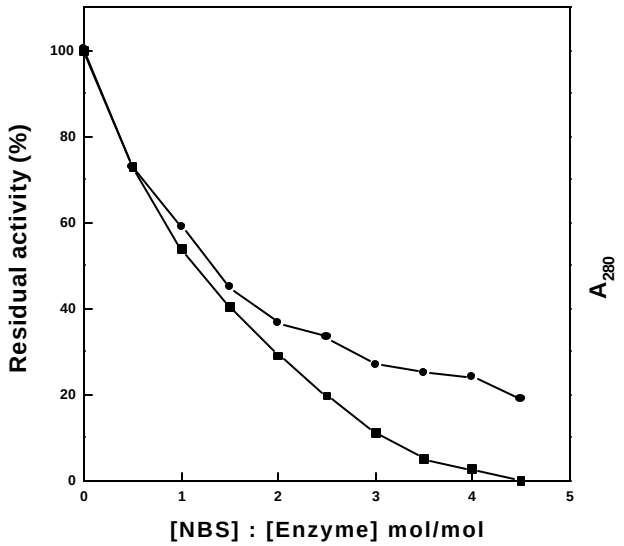


Fig. 9 **Titration of Xyl II by NBS.** Residual activity of Xyl II ( ) and A_{280} nm (•) on titration with NBS

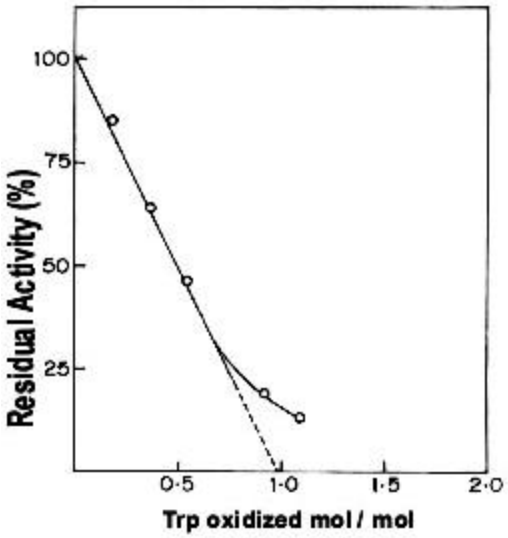


Fig. 10 **Plot of residual activity against number of tryptophan residues modified as deduced by spectroscopic studies.**

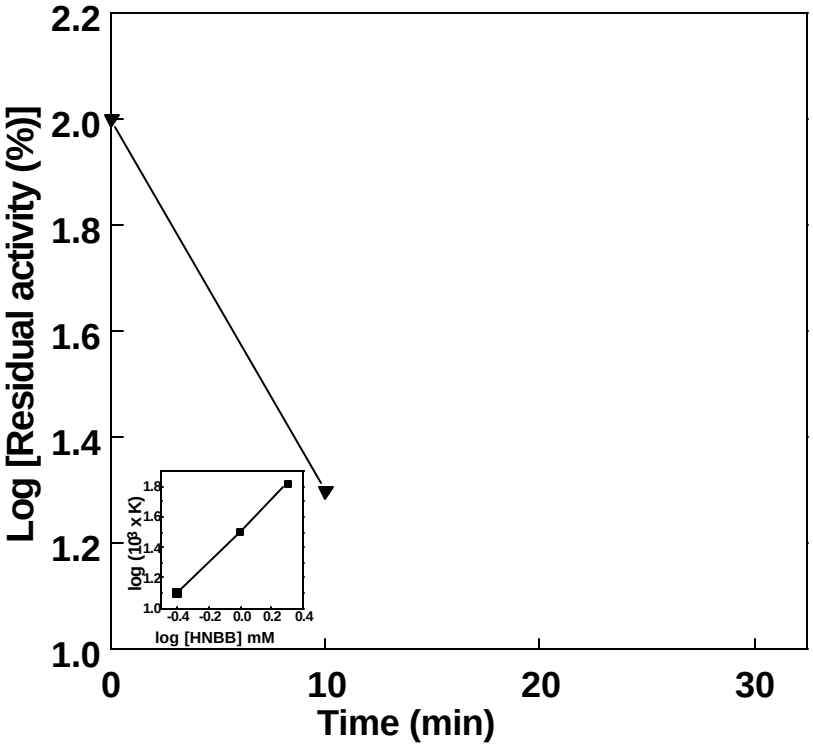


Fig. 11 **Kinetics of inactivation of Xyl II by HNBB**

Plot of residual activity *versus* time for the inactivation of Xyl II by HNBB 0 mM ( ), 0.5 mM (o), 1.0 mM ( ), and 2.5 mM ( ). **Inset** Double logarithmic plot of pseudo-first order rate constant *versus* [HNBB]

Tryptophanyl fluorescence analysis of native and NBS modified Xyl II

On excitation (295 nm) the native and NBS modified Xyl II fluoresce with an emission maximum at 339 nm, typical of proteins containing Trp residues in an hydrophobic environment. (Fig. 12A). The fluorescence spectum of native enzyme showed a rapid decrease on addition of NBS upto a molar ratio of ~5. A negligible change on further addition was observed suggesting that in a molecule of Xyl II the reactivities of the Trp residues were different (Fig. 12B).

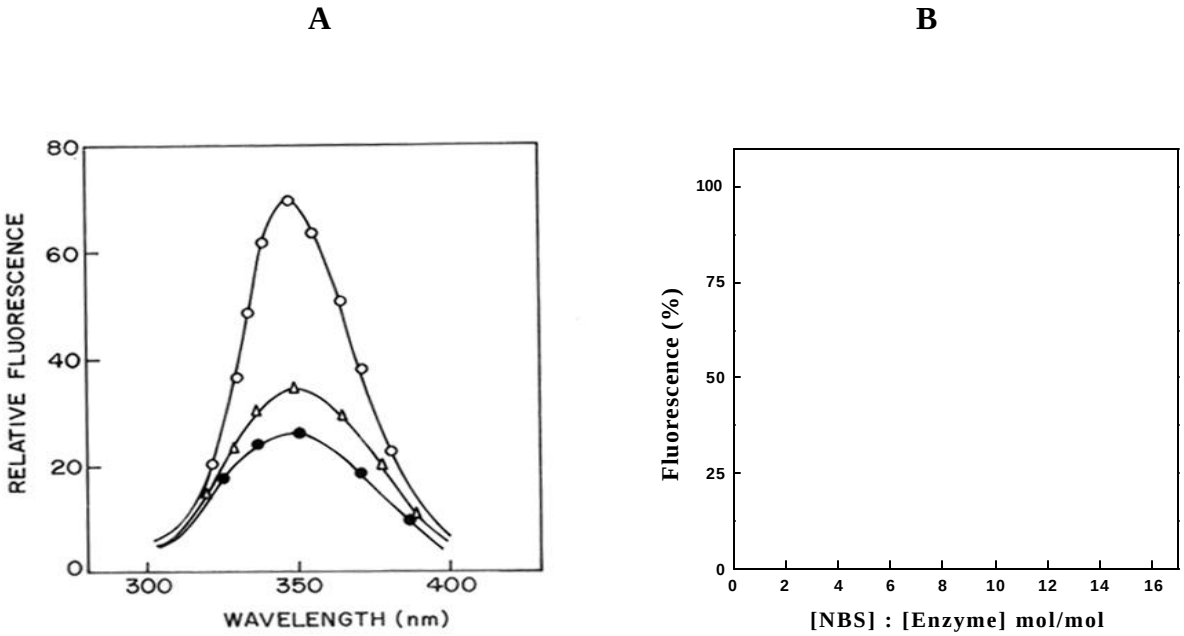


Fig.12 Effect of NBS on the tryptophanyl fluorescence of Xyl II

A. Fluorescence spectra of 25 µg Xyl II (o) treated with 20 µM NBS in presence of 0.5 % xylan (Δ) and in presence of NBS alone (•).

B. Xyl II (1.87×10^{-6} M) in 0.05 M sodium phosphate buffer, pH 6.0 was titrated with aliquots of NBS (1×10^{-3} M).

Substrate protection

Xylan (1 mg) was able to provide 100 % protection against inactivation of the enzyme by NBS (Table 3). This was further confirmed from the fluorometric experiments where substrate (0.5 %) quenched the fluorescence to 50 %. However, on addition of NBS a minor change in quenching of fluorescence was observed (Fig.12A).

Table 3 Protection of xylanase from inactivation

Addition	Residual activity (%)
None	100
NBS	0
Xylan (0.2 mg)+ NBS	50
Xylan (0.5 mg)+ NBS	80
Xylan (1.0 mg)+ NBS	100

NBS concentration was 5 mM

Assessment of the microenvironment of Trp residues of XylI

The fluorescence characteristics of Trp residues depend strongly on the microenvironment and thus provide a sensitive probe of the conformational state of the protein. Fluorescence quenching studies have been widely used for studying the degree of exposure and electronic environment of aromatic amino acid residues. Acrylamide is an efficient neutral quencher of tryptophan fluorescence and can distinguish between buried and exposed side chains (Lehrer, 1971) while KI and CsCl are charged, highly hydrated molecules and their quenching ability is limited to surface exposed Trp and also depends upon the neighbouring charged groups (Lehrer, 1971; Eftink and Ghiron, 1981). The Stern-Volmer plots of native and Gdn HCl treated Xyl II with various quenchers are shown in (Fig. 13, 14A, 14B). The quenching of Xyl II with increased concentration of acrylamide resulted in a Stern-Volmer plot with an upward curvature, whereas those for CsCl and KI were linear. The static parameter V was calculated to be 1.0 M^{-1} for acrylamide. The $K_{(Q)(eff)}$ and f_a values were extrapolated from a replot of quenching data according to modified Stern-Volmer equation (Fig. 15).

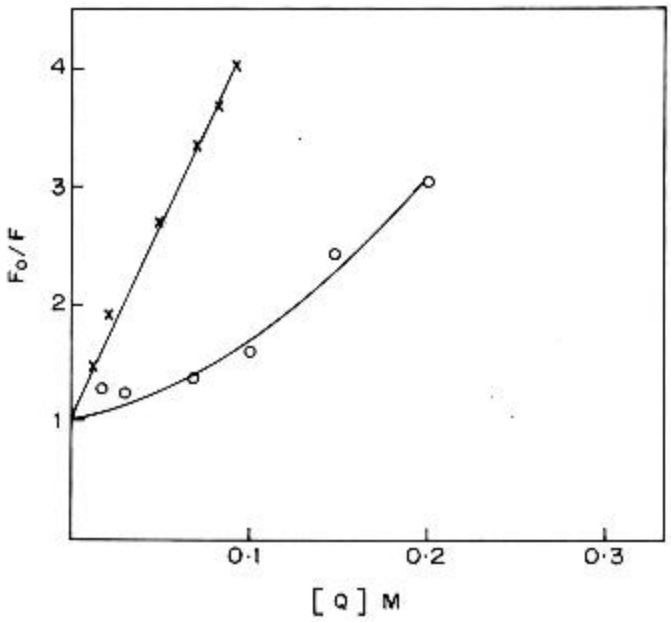


Fig.13 **Acrylamide of quenching of Xyl II**

Stern-Volmer plots of fluorescence quenching of Xyl II (o) and 6 M Gdn HCl treated Xyl II (x)

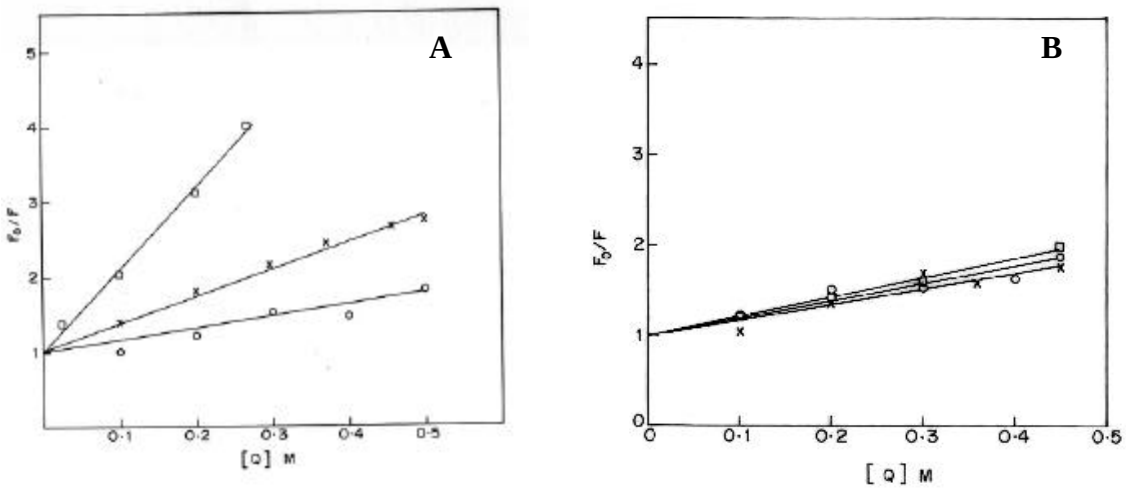


Fig.14 **Assessment of the microenvironment of Xyl II:**

Stern Volmer plots of quenching of free tryptophan ( ), Xyl II (o) and of enzyme treated with 6 M Gdn HCl (x).

A: Quenching with I^-

B: Quenching with Cs^+

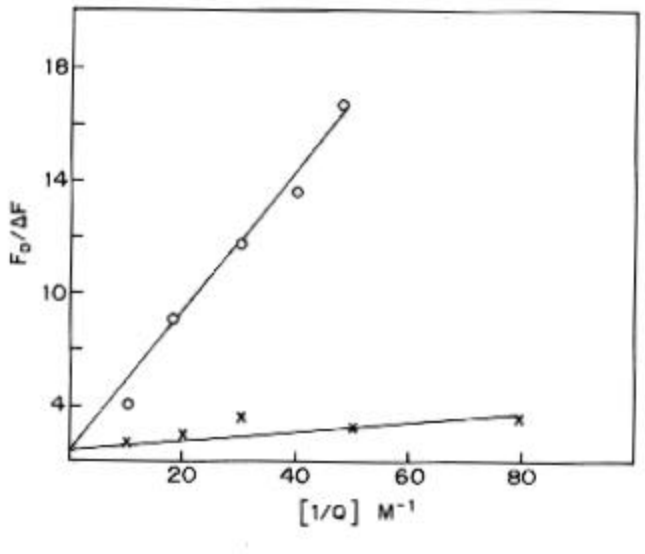


Fig.15 Modified Stern-Volmer plot of Xyl II due to acrylamide quenching
Native (o) and in the presence of Gdn HCl (x)

The K_{sv} and f_{eff} values are given in Table 4. The fluorescence emission of Xyl II in 6 M Gdn HCl shifted to 353 nm. The K_{sv} values for acrylamide and KI were increased 10 and 2 fold respectively in presence of 6 M Gdn HCl indicating exposure of the fluorophores to solvent whereas in case of CsCl there was only a slight increase (Table 4).

Table 4 Quenching Parameters

	Acrylamide			**K_{sv}**	
	K_{sv}	**f_a**	**K_a**	**I^-**	**Cs^+**
Native Xyl II	5.2	1	3.33	1.5	2.0
Xyl II in 6 M Gdn HCl	40.0	1	37.50	2.8	2.0
Free tryptophan	_	_	_	12.5	2.5

The K_{sv} value for acrylamide was much higher than that of KI and CsCl since it can penetrate into the interior and is an efficient quencher. The ratio of the Stern-Volmer constant of Trp to that of a protein using the same quencher is the steric factor. In the expression (Pownall and Smith, 1974)

$$R = [K_{sv}(\text{protein}) / K_{sv}(\text{Trp})] \times 100\ \%$$

the steric factor R represents the percent accessibility of the Trp residues of Xyl II. Since iodide and cesium are oppositely charged,

$$R = R(I^-) + R(Cs^+)/2$$

is used as a measure of fluorophore accessibility. The stearic values R for Xyl lI are 10 with I^- ion and 80 with Cs^+ ion (Fig 8 A&B). The average steric value is 48, which indicates that 48 % of the Trp residues in Xyl II are exposed to solvent and quencher.

NBS oxidation of Xyl II from AT *Bacillus* imparted a complete loss of the enzymic activity suggesting an essential role of one Trp residue in the mechanism of action of this enzyme. NBS is also highly selective with sulfhydryl and tyrosine residues. Absence of inactivation of Xyl II by Cys specific and Tyr specific reagents suggests that the inactivation by NBS is solely due to reaction with Trp residues. The kinetic analysis revealed the participation of one Trp residue at the active site of the enzyme. Substrate protection studies by kinetic and fluorometric analysis indicated the involvement of Trp at the substrate-binding site. This was further confirmed using fluorometric analysis showing 35 % protection against loss in fluorescence indicating that not all the Trp residues are involved in the substrate-binding site. It has been suggested that interaction between substrate and tryptophan stabilizes the transition state during enzymatic hydrolysis of glycosydic bond (Sierks *et al*., 1990). The stacking interaction of tryptophan with the face of a xylose ring is similar to a predicted stacking interaction of Trp 457 with a glucose residue at the subsite D in the CelD active site. The stacking interaction is supposed to be a common binding interaction in glycosidases (Juy *et al*., 1992). In general when the protein unfolds, it exposes buried Trp to the aqueous solvent, this results in the shift of fluorescence emission maximum toward longer wavelength. Upon unfolding in 6 M Gdn HCl the fluorescence emission maxima of Xyl II shifts to 353 nm characteristic of tryptophans fully exposed to water. Further insight into the tertiary stucture of Xyl II may be obtained from fluorescence quenching experiments. Acrylamide is a useful neutral quenching probe for topological studies with proteins. It can penetrate a protein matrix and its ability to collisionally quench the emitting groups depends only on the ease with which it is able to diffuse to meet the excited groups (Eftink & Ghiron, 1976). KI and CsCl have been suggested as companion probes since these bear opposite charges and their relative quenching efficiencies should be related not only to the accessibility of the protein

fluorophore but also to the net charge in the vicinity of the fluorophore. In presence of acrylamide, Xyl II showed an upward curving Stern-Volmer plot, indicating that the Trp residues may be present in an homogenous environment and that the side chains of all Trp residues have similar collisional constants (Eftink & Ghiron, 1976). The acrylamide quenching of Trp fluorescence proceeds via both a static and dynamic process. The magnitude of V is related to the probability of finding the quencher molecule close to the fluorophore at the moment of excitation to quench it immediately or statically (Eftink & Ghiron, 1976). When V is less than 2.0 M^{-1} as is in this case, the steady-state concentration of the quencher in the 'active' volume surrounding the indole ring is very low. This can be explained by a restricted accessibility of the quencher to the side chains of the Trp residues. This means that static quenching can occur even when the quencher and fluorophore are not in physical contact at the moment of excitation. A linear Stern-Volmer plot with KI and CsCl indicates that no static quenching was observed, as is usually the case. The larger steric value obtained with Cs^+ ion compared to KI^- as quencher suggests that the Trp(s) are located in a relatively negatively charged environment which may be due to the Trp(s) being present in the close vicinity of a carboxylic acid residue. Our experiments have indicated the presence of essential carboxylic groups at the active site of Xyl II with high pka value (*described earlier in this chapter*), which may be responsible for the activity of the enzyme at alkaline pH. Analysis of the nucleotide sequence of Xyl II based on the multiple alignment has shown Trp61 to be highly conserved (Kulkarni *et al*., 1999) and may play a crucial role in the interaction with the substrate. In lysozyme, belonging to the family of glycanases the hydrophobicity of Trp108 is reported to play an important role in maintaining stability and high pka value of Glu35 (Inoue *et al*., 1992). NMR assignments of xylanases from *B. circulans* indicate a potential amide aromatic hydrogen bond to the indole ring of Trp71. This interaction conserved in all low molecular weight xylanases of known structure play an important role in establishing the active site conformation of these enzymes (Plesniak *et al*., 1996).

CHAPTER 4

FOLDING/UNFOLDING STUDIES OF XYL II

SECTION I

A PARTLY FOLDED STATE OF XYL II AT LOW pH

SUMMARY

The conformation and stability of Xyl II at acidic pH was investigated by equilibrium unfolding methods. Using intrinsic fluorescence and CD spectroscopic studies, we have established that Xyl II at pH 1.8 (A-state) retains the helical secondary structure of the native protein at pH 7.0, while the tertiary interactions are much weaker. At variance, from the native species (N-state), Xyl II in the A-state binds 1-anilino-8-sulfonic acid (ANS), a property considered most typical of a protein in the molten globule state. Lower concentration of Gdn HCl are required to unfold the A state. For denaturation by Gdn HCl, the midpoint of the co-operative unfolding transition measured by fluorescence for the N-state is 3.5 M, which is higher than the value (2.2 M) observed for the A-state at pH 1.8. Thus, our results suggest that an equilibrium unfolding intermediate may be present on the unfolding pathway of Xyl II at acid pH.

INTRODUCTION

It is well known that protein folding goes through some kinetic intermediates, which can accumulate in the folding process. It has become increasingly evident that a polypeptide chain can adopt conformations different from the functional, native conformation of the protein. In order to understand the principles governing protein folding it is important to study these partly folded conformations (Baldwin, 1991; Dill and Shortle, 1991; Dobson, 1992). The development of wide range of sensitive techniques have led to the identification and characterisation of stable intermediates in several proteins (Kuwajima, 1989). The classic examples of such conformations are the molten globule states adopted by several proteins at extremes of pH (typically acid pH) and ionic strength (Goto *et al*., 1990). It has been widely accepted that the molten globule state is a general intermediate populated during the early stage of protein folding (Kuwajima, 1989; Matthews, 1993; Baldwin, 1995). Molten globule is as compact as the native conformation with pronounced secondary structure but has no rigid tertiary structure with exposed hydrophobic surface that binds a hydrophobic dye or induces extensive aggregation (Ptitsyn, 1987). Though many proteins in their molten globule and partially folded states have been characterised, it has not yet been possible to generalise the concept of the molten globule as the universal equilibrium protein folding intermediate(s). Interest in the molten globules has intesified because the properties of the molten globule support the argument that it may be one of the first conformations embraced by the polypeptide chain in folding from the unfolded state (Kim and Baldwin, 1990). It has been suggested that the molten globule could be the state of non-native proteins in the living organisms, and they might be involved in physiologically important processes other than translocation such as interaction with chaperones and release of ligands.

In this work, we have shown the presence of a partially structured intermediate at low pH that binds to the hydrophobic dye 1-anilinonaphthalene-8-sulfonic acid and resembles a 'molten globule'-like state in the equilibrium-unfolding pathway of xylanase.

MATERIALS AND METHODS.

Materials

ANS was purchased from Sigma Chemical Co. USA. Gdn HCl was obtained from Fluka. All other reagents were of analytical grade and solutions were prepared for spectroscopic measurements were in de-ionized water. Samples for spectroscopic measurements were centrifuged, filtered through 0.45 μm filters and the exact pH and protein concentrations were determined before measurements.

Spectroscopic measurements

CD spectroscopy studies were carried out on a Jasco Model J500A spectropolarimeter. Secondary structure of Xyl II (200 μg) was monitored in the wavelength range 200-250 nm using a cell of pathlength 0.1 cm. The tertiary structure of the enzyme was (1 mg/ml) was monitored in the wavelength range 250-320 nm using a cell of pathlength of 0.5 cm. Each spectrum was recorded as an average of 4 scans. Fluorescence spectroscopy studies were measured using a Perkin-Elmer Luminiscence spectrofluorimeter LS 50B. Intrinsic tryptophan fluorescence spectra were recorded by exciting the samples at 295 nm with excitation and emission slit widths set at 5 nm. All measurements were done at 25 °C.

Acid denaturation of xylanase: Acid denaturation of Xyl II was carried out by incubating protein solution in an appropriate buffer for 3-24 h at 25°C over a wide pH range.

Guanidine hydrochloride unfolding Protein samples were incubated at different denaturant concentrations at a given pH for approximately 24 h to attain equilibrium.

ANS binding assay

A stock solution of 10 mM ANS was prepared and the concentration was checked using an extinction coefficient of 5000 M^{-1} cm^{-1} at 350 nm (Khurana and Udgaonkar, 1994).

RESULTS AND DISCUSSION

Effect of low pH on the intrinsic fluorescence and circular dichroism spectra of Xyl II

Circular Dichroism: (CD) spectra of the native (N), acid (A), and Gdn HCl denatured (U) states of enzyme were recorded in the peptide region (far-UV region Fig. 1a) as well as the aromatic region (near-UV region Fig. 1b). The far-UV CD spectra of the N and A states show that both forms have considerable secondary structure. The mean residue ellipticities at 220 nm are –3631 deg cm^2 d mol^{-1} for the A state, -5557 deg cm^2 $dmol^{-1}$ for the N state, and -1508 deg cm^2 $dmol^{-1}$ for the U state. The CD signal in the near-UV region at pH 1.8 is significantly lower (~60%) than that measured at pH 7, suggesting that the addition of acid induces a looser and more flexible environment nearby aromatic residues (Strickland, 1974). Nevertheless, the overall features of the near-UV CD spectrum and in particular, the presence of a fine structure in the 258-270 nm region, associated with phenylalanine residues suggest that tertiary interactions do exist in the protein in its A-state. On the other hand, in the presence of the strong protein denaturant 6 M Gdn HCl, XyL II loses its secondary and tertiary structure, as given by far- and near-UV CD spectra.

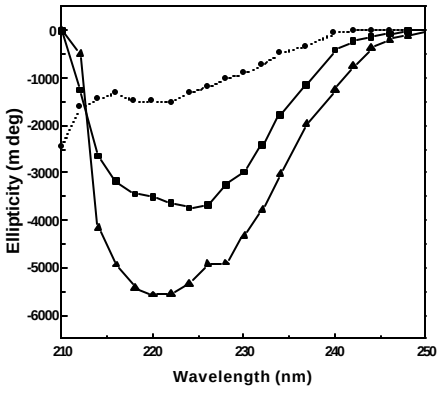


Fig. 1. A. Far-UV CD spectra of Xyl II

The samples were incubated for 24 h at 25 C before the measurements. The CD spectra of Xyl II at pH 7.0 (▲), pH 1.8 (■) and 6 M Gdn HCl denatured (●).

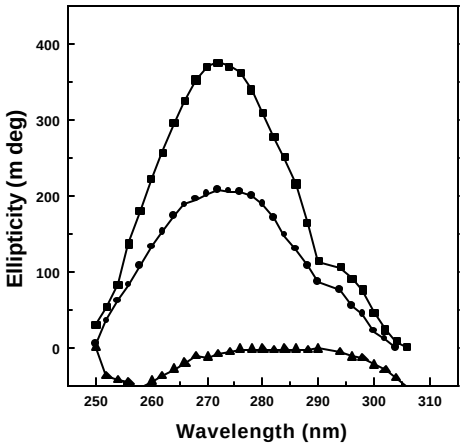


Fig. 1. B. Near-UV CD spectra of Xyl II.

The samples were incubated for 24 h at 25 C before the measurements. The CD spectra of Xyl II at pH 7.0 (■), pH 1.8 (●) and 6 M Gdn HCl denatured (▲).

Fluorescence emission*:* The fluorescence spectrum of Xyl II taken at pH 7 shows a maximum at 339 nm. The spectrum of the protein at pH 1.8 shows a reduced Trp fluorescence intensity, a blue shift of λ_{max} to ~336 nm (Fig.2), this reveals that the reduction of the Trp emission observed for Xyl II at pH 1.8 is primarily caused by an acid quenching of fluorescence, while the shift of λ_{max} to shorter wavelengths may arise from changes in the polarity of the microenvironment near the Trp residues. At low pH the carboxylate groups become uncharged, causing a less polar environment near the Trp residues and thus a blue shift of the Trp fluorescence (Lacowicz, 1986).

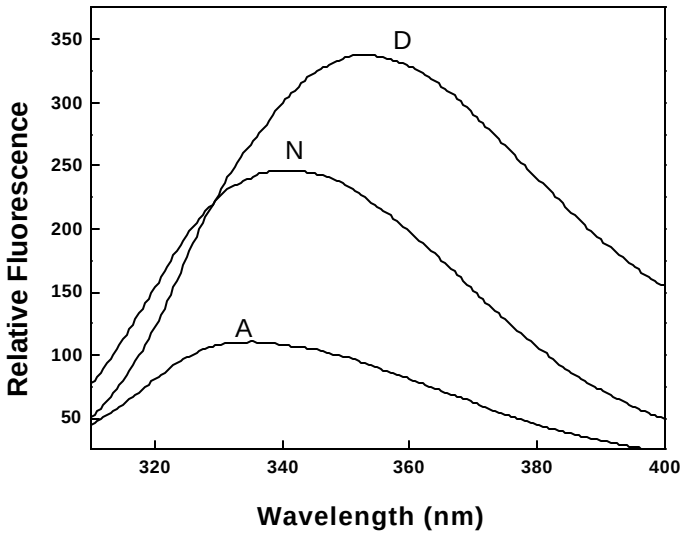


Fig. 2 **Fluorescence emission spectra of Xyl II.**

Spectra were taken, exciting the native enzyme (5 μM) at 295 nm and 25 °C in 50 mM phosphate buffer, pH 7.0 (N), 50 mM HCl KCl, pH 1.8 (A), or 6 M Gdn HCl in phosphate buffer pH 7.0 (D).

ANS binding studies

The hydrophobic dye ANS is widely used tool to identify and characterize partly folded states of proteins, including molten globule states (Ptitsyn, 1992). ANS binds to the hydrophobic regions of proteins with a dramatic increase of the fluorescence intensity and a blue shift of λ_{max} reflecting the burial of the ANS molecule in a more hydrophobic environment. The fluorescence intensity of ANS in presence of Xyl II at pH 1.8 is much higher than that observed at pH 7 or with fully unfolded protein in the presence of 6 M Gdn HCl (Fig. 3A). However, the ANS fluorescence does not change sigmoidally, but mostly in the region pH 3 to 1 (Fig. 3B). Moreover, at acidic pH, the λ_{max} of the fluorescence emission is shifted from 520 nm to 490 nm.

These fluorescence data indicate that ANS binds to Xyl II in its A-state only, suggesting that hydrophobic region becomes exposed to solvent at low pH.

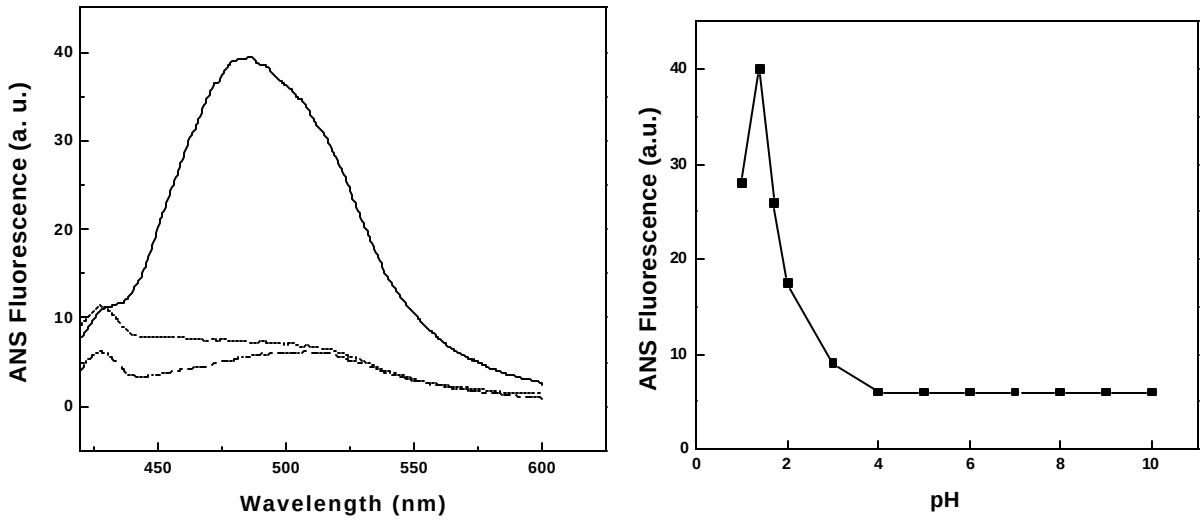


Fig. 3 ` ANS binding

ANS emission spectra were recorded with excitation at 375 nm. Protein concentrations were 5 µM.

A. pH 7.0, (—); pH 1.8, (- -); and 6 Gdn HCl (…..).

B. Effect of pH on the binding of ANS to Xyl II.

Acrylamide quenching: The sensitivity of the Trp residues to quenching by acrylamide is also similar in the native and A-states. Table 1 shows the Stern-Volmer constants fitted to the early linear parts of the curves. Although these constants, in the absence of the microscopic rate constants of quenching, cannot be rigorously interpreted as representing the degree of solvent exposure of the fluorophore (Eftink and Ghiron, 1981), the similar sensitivities of the native and A-states to quencher are consistent with the folded structure in the A-state.

Table.1 Stern-Volmer constants

State	K_{sv}

N-state	5.2
A-state	4.0
D-state	40.0

Equilibrium unfolding of the N and A forms of Xyl II

Gdn HCl: induced changes in the tertiary structure content of Xyl II under neutral conditions, are co-operative and the transition curves are sigmoidal. (Fig. 4). All the structural changes take place between 3 and 4 M Gdn HCl with a transition midpoint of 3.5±0.1 M Gdn HCl. Wavelength maximum shifts by 13 nm upon unfolding. At pH 1.8 Gdn HCl induced unfolding of the enzyme was co-operative, as at neutral pH, but with a lower transition midpoint of 2.2±0.1 M

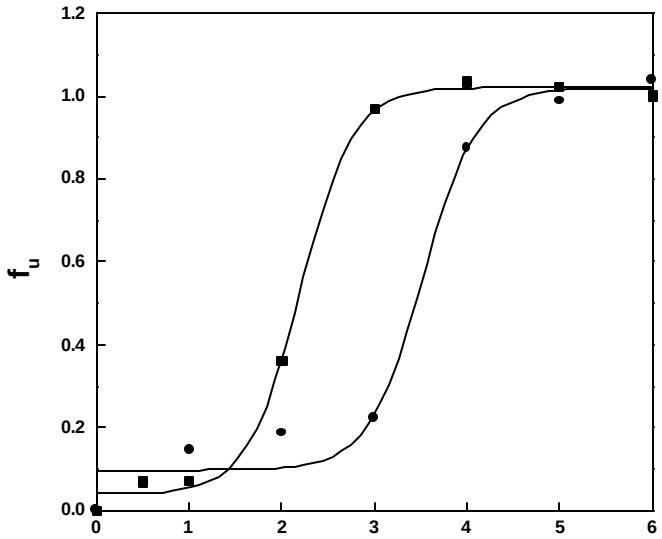


Fig. 4 **Equilibrium Gdn HCl denaturation of Xyl II**

The unfolding of Xyl II was monitored by by changes in the wavelength of maximum emission at pH 7.0 (●), and pH 1.8 (■).

The results of this study indicate that Xyl II from family 11 at low pH retains the secondary structure elements of the native species at neutral pH, while the acid induced state state exhibited partial unfolding of the protein leads to a weakening of tertiary interactions and an

enhanced solvent exposure of hydrophobic groups. However, the reduced (~60%) but still significant near -UV CD signal of Xyl II in its A-state does not imply global disorder of the protein molecule, but may reflect local perturbations of the tertiary structure. Although the present data alone does not allow a detailed picture of the structural features of the A-state, they provide useful information on the N to A conformational transition. In particular, the pH dependence of both fluorescence and ANS binding indicates that the partial unfolding of Xyl II occurs at acid pH thus indicating that the conformational change is mediated by the titration of Glu and or Asp residue(s). It can be proposed that upon acid titration, intramolecular charge repulsion is the driving force for partial unfolding of the protein molecule, thus inducing looser side-chain packing and the exposure to solvent of hydrophobic groups. In recent years, several proteins were shown to acquire partly folded state at acidic pH, commonly called the A-state. It was found that the A-state of proteins is characterized by significant structural variability, so that only general features of the state can be given (Fink, 1994; Fink *et al.*, 1995). The A-state of Xyl II appears to maintain not only a native-like structure but also some packing of side chain groups in the tertiary structure as given by CD measurements.

Various molten globule states are reported, however it has been revealed that the degree of structural organization in the molten globule like state is considerably dependent on the proteins. Recently reports on premolten globule (Ptitysn, 1995), molten-coil (Redfield *et al*, 1994) and highly ordered molten globule states have been documented (Redfield *et al*, 1994; Ferrer *et al*, 1995; Morozova *et al*, 1995). We conclude that the A-state of Xyl II is similar to a putataive molten globule state. Not much information is available about folding/unfolding of xylanases of family 11. Hence, such studies about individual variations would certainly help to generalise the folding behavior of xylanases. The present investigation also paves the way for further characterization of intermediate state in the enzyme. The formation of this intermediate is a reversible reaction, upon titration to neutral pH it refolds into the native enzyme. The possible significance of the molten globule state of proteins has been recognized recently (Bychkova, 1995). It is becoming clear that in order to regulate protein function and turnover in a living cell, molten globule states of proteins can be generated by exposing them to "unusual" denaturing conditions (Ptitsyn, 1995). In fact, the native-like overall fold of a molten globule, together with

its enhanced flexibility, can dictate a number of ideal properties for a functional protein in a living cell and could facilitate the insertion into and translocation across membranes, chaperone-mediated protein folding *in vivo*, cell fusion and protein -receptor interactions (Ptitsyn, 1995).

SECTION II

a -CRYSTALLIN MEDIATED REFOLDING OF XYL II

SUMMARY

Chaperones are catalysts in the sense that they transiently interact with their substrate proteins but are not present in the final folded product, and in that, they increase the yield of folded protein. Crystallins are group of structural proteins, which share both sequence and structural homology with small heat shock proteins. For the functional *in vitro* analysis of α-crystallin, Xyl II from alkalophilic thermophilic *Bacillus* was used as a model system. We have attempted to study the refolding of Gdn HCl denatured Xyl II in the presence of the molecular chaperone, α-crystallin. Our results based on the intrinsic tryptophan fluorescence and the hydrophobic flurophore 8-anilino-1-naphthalene sulfonate binding studies, suggest that α-crystallin forms a complex with a putative molten globule like intermediate in the refolding pathway of Xyl II. The complex exhibited no functional activity. Addition of ATP to the α-crystallin· Xyl II complex initiates the renaturation of Xyl II and 30-35 % activity was recovered. ATP hydrolysis was not a prerequisite for Xyl II release, since the nonhydrolyzable analogue 5'-adenylyl imidodiphosphate (AMP-PNP) was capable of reconstitution of active Xyl II. Although metal ions Ca^{2+} or Mg^{2+} were not required for the *in vitro* refolding of Xyl II, the renaturation yields were enhanced in their prescence. The ability of refolding was quite specific for α-crystallin since bovine serum albumin and PEG had no effect on the refolding of Xyl II.

INTRODUCTION

It is well established that the amino acid sequence of a polypeptide chain contains the information that determines the three-dimensional structure of a functional protein (Anfinsen, 1973) however the folding of many proteins in vivo requires the assistance of a preexisting machinery of molecular chaperone proteins (Creighton, 1990; Ellis, 1987). Chaperones are catalysts in the sense that they transiently interact with their substrate proteins but are not present in the final folded product, and in that, they increase the yield of folded protein. Despite the fact that in vitro folding may not exactly mimic intracellular environment in the cell, it is minimally a good model for *in vivo* protein folding (Fink, 1999). In the past decade vide interest has been generated in crystallins, a group of structural proteins initially thought to be lens-specific, have also been identified in non-lenticular tissues (Klemenz *et al.,* 1991; Iwaki, *et al*., 1989). α-Crystallin shares both sequence and structural homology with small heat shock proteins (sHSPs) and behaves in many ways like them (Klemenz *et al.,* 1991; Horowitz, 1993; de Jong *et al*., 1993; Ingolia and Craig, 1982). The chaperone like activity of α-crystallin may be important in the formation and maintenance of eye lens transparency and that age related deterioration of the chaperone function could contribute to the development of cataracts (Horowitz, 1993). The expression of α-crystallin has been shown to be induced by thermal (Klemenz *et al.,* 1991) or hypertonic stress (Kelley *et al.,* 1993). α-crystallin has been reported to be functionally equivalent to the small heat shock proteins murine Hsp25 and human Hsp27 in the *in vitro* refolding of α-glucosidase and citrate synthase respectively (Jakob *et al.,*1993). It was however, unable to refold rhodanase denatured in 6 M Gdn HCl (Das and Surewicz, 1995). Recently, α-crystallin has also been reported to bind the temperature-induced molten globule state of proteins (Rajaraman, *et* al., 1996) and prevent photoaggregation of γ-crystallin by providing hydrophobic surfaces (Raman and Rao, 1994).

The participation of α-crystallin and other sHSPs in the folding of proteins have been investigated recently. Several studies have supported a structural and functional relationship between ATP and sHSPs. Equilibrium binding studies, intrinsic tryptophan fluorescence, and ^{31}P nuclear magnetic resonance spectroscopy demonstrated an

interaction between ATP and bovine α-crystallin (Reddy *et al.,* 1992). ATP is an abundant phosphorous metabolite present in high concentrations in lens cells from many species (Pirie, 1962). High concentrations of ATP are present in the skeletal muscle (Burt, et *al.,* 1976) in which high levels of α-crystallin are coexpressed (Bennardini *et* al., 1992). However, the role of ATP in the mechanism of folding of proteins by α-crystallin remains to be elucidated. Despite the growing interest in the chaperone action of α-crystallin, little is known about its mechanism of chaperoning. For this functional *in vitro* analysis of α-crystallin, Xyl II from alkalophilic thermophilic *Bacillus w*as used as the model system. In the current study, experimental evidence serves to implicate that α-crystallin forms a complex with a putative molten globule like intermediate in the refolding pathway of Xyl II. ATP dependent release of Xyl II, and enhancement of the reactivation yield has been investigated. We particularly focussed our attention on the effect of metal ions in the α-crystallin mediated refolding of Xyl II and have shown for the first time the enhancement of the refolding yield of the enzyme in their presence.

MATERIALS AND METHODS

MATERIALS

ATP, ADP, AMP-PNP, and α-crystallin were purchased from Sigma. All chemicals used were of analytical grade.

METHODS

Denaturation/renaturation studies of xylanase:

All denaturation and renaturation experiments were carried out in 50 mM potassium phosphate buffer, pH 7.2. Xyl II (100 μM) was denatured for 16 h in 6 M Gdn HCl. Renaturation was initiated by diluting 10 μl of the sample into a final volume of 1 ml of potassium phosphate buffer pH 7.2. 100 μl aliquots were withdrawn at various time intervals of refolding and assayed for Xyl II activity. Renaturation in presence of α-crystallin was carried out at 37 °C. After 1 h ATP (0.3 mM) was added to the refolding solution and the activity was assayed at incubated further. The final concentration of Xyl II and α-crystallin were 1 μM and 0.2 mg/ml respectively. Renaturation of Xyl II in presence of ATP analogues like AMP, ATP and AMP-PNP were also carried out.

Temperature dependent refolding of Xyl II- a -crystallin complex

Renaturation of Xyl II was initiated at 37 °C in the presence of α-crystallin. After 1 h, ATP (0.3 mM) was added to the complex and further incubated at different temperatures. Aliquots were removed at various time intervals and the enzyme activity was assayed.

Fluorescence of the free and a -crystallin bound complex.

Fluorescence spectra were recorded with a Perkin-Elmer LS 50B spectrofluorometer. Corrections due to inner filter effect were made.

RESULTS AND DISCUSSION

In vitro refolding of Xyl II

In the initial experiments, measurements of circular dichroism indicated that Xyl II is essentially devoid of secondary structure when incubated at 25 °C in either 6 M guanidine hydrochloride as shown in *Section 1 of Chapter* 3. This unfolded material was used as the substrate for refolding experiments. The refolding was initiated by diluting the unfolded enzyme (100 fold) in presence of α-crystallin. The refolded enzyme did not exhibit activity in the presence of α-crystallin. Significantly, under the conditions described, the enzymatic activity of native Xyl II was not influenced by the presence of even the largest concentration (0.8 mg/ml) of α-crystallin tested.

Intrinsic tryptophan fluorescence spectroscopy (ITF)

The intrinsic fluorescence of α-crystallin bound Xyl II as a measure of tertiary structure was examined. Generally, increased exposure of Trp residues to solvent on unfolding results in a red shift of the Trp fluorescence (λ_{ex} 295 nm). The wavelength of maximum emission of α-crystallin bound Xyl II was at 343 nm, indicating that the Trp residues were still in a non -native, but in a more hydrophobic environment than in the fully unfolded state (Fig. 1).

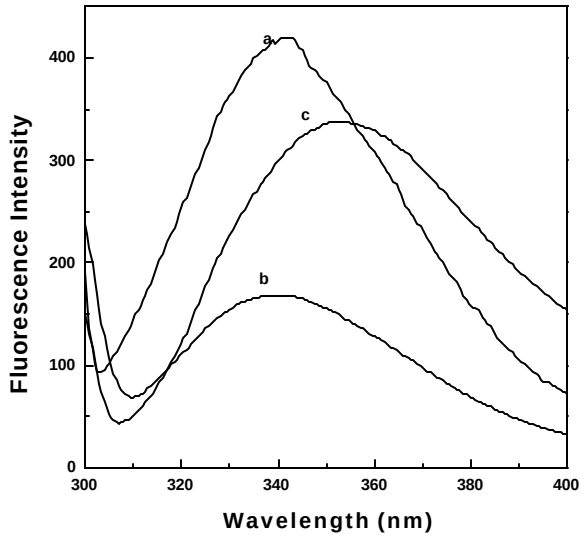


Fig. 1 **Tryptophanyl fluorscence of a -crystallin bound Xyl II (a); native (b); denatured (d).**

ANS binding

Intermediate emission of Trp fluorescence can be observed with certain collapsed folding states containing a fluctuating hydrophobic core that adsorbs the fluorescent compound 1-anilino-naphthalene-8-sulphonate (ANS). In contrast to the native and fully unfolded states, Xyl II bound by α-crystallin showed significant ANS fluorescence, which was blue shifted to 490 nm, indicating that ANS was in a hydrophobic environment (Fig. 2).

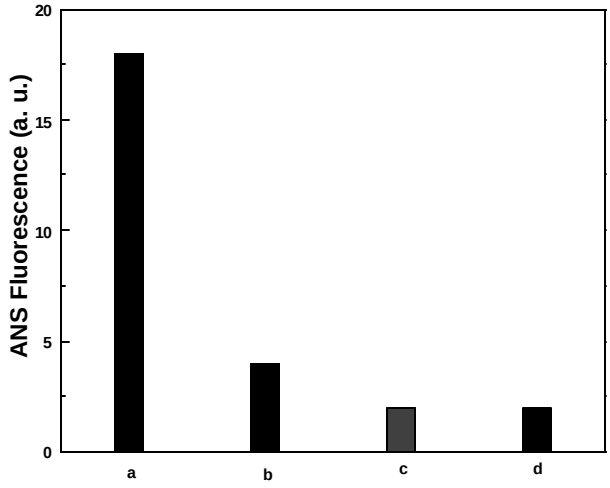


Fig. 2 **ANS fluorescence of the free and ANS bound Xyl II**
a; α-crystallin· Xyl II complex; b, native Xyl II; c, Gdn HCl denatured Xyl II; d, refolded enzyme.

Addition of ATP to the a -crystallin· Xyl II complex initiates renaturation

The addition of ATP to the refolded enzyme in presence α-crystallin· Xyl II complex initiates the renaturation of Xyl II (Fig. 3). The refolded enzyme was kept in a reactivable form, since after the addition of ATP, recovery of enzyme activity (30-32 %) was obtained. The α-crystallin mediated renaturation of Xyl II was examined as a function of the chaperone concentration.

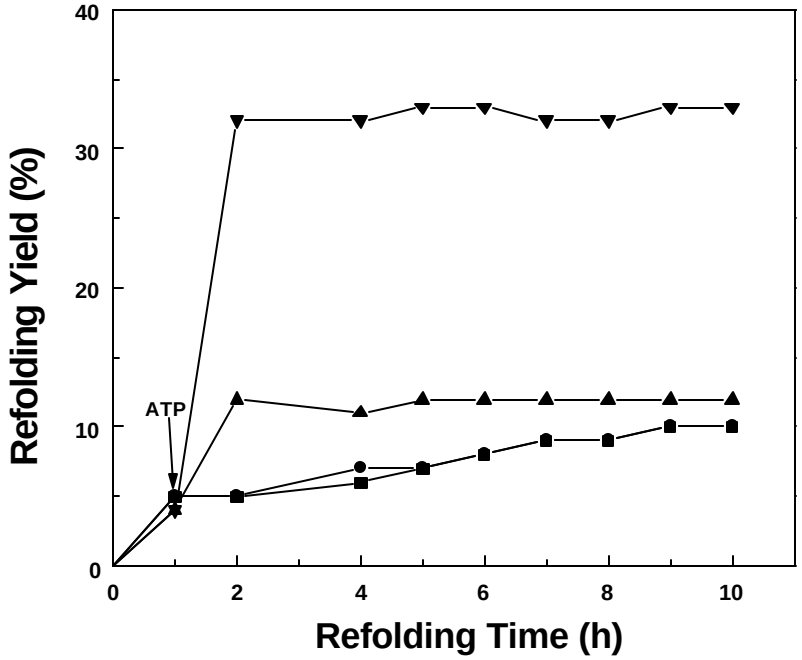


Fig. 3 **The refolding of Xyl II in the presence of various factors which facilitate folding, as monitored by enzymatic activity.**
The molar concentration of Xyl II was 1 μM. ( ), refolding in the presence of buffer alone; (■), refolding in the presence of 1 mg/ml of BSA or (●),PEG; ( ) refolding in the presence of α-crystallin (0.2 mg/ml), and at the time indicated by an arrow, ATP was added to the refolding mixture to a concentration of 0.3 mM.

The α-crystallin mediated renaturation of Xyl II was examined as a function of the chaperone concentration. As shown in Figure 4, 15 % of the original Xyl II activity was recovered at the lowest concentration of α-crystallin (0.05 mg/ml). The extent of renaturation increased in a concentration -dependent manner, and a maximum of 30-35 % of the original activity was recovered at α-crystallin concentration of 0.2-0.5 mg/ml. The increase in the α-crystallin concentration would increase the collisional frequency so as to favour the formation of the complex. The ability of refolding was quite specific for α-crystallin since, bovine serum albumin or PEG had no effect on the refolding of Xyl II.

When unfolded Xyl II was allowed to renature in the absence of α-crystallin for 2 h and if α-crystallin and ATP were added at this time, there was no effect on rate or

extent of the observed renaturation. This indicates that α-crystallin cannot rescue misfolded Xyl II aggregates once they are formed.

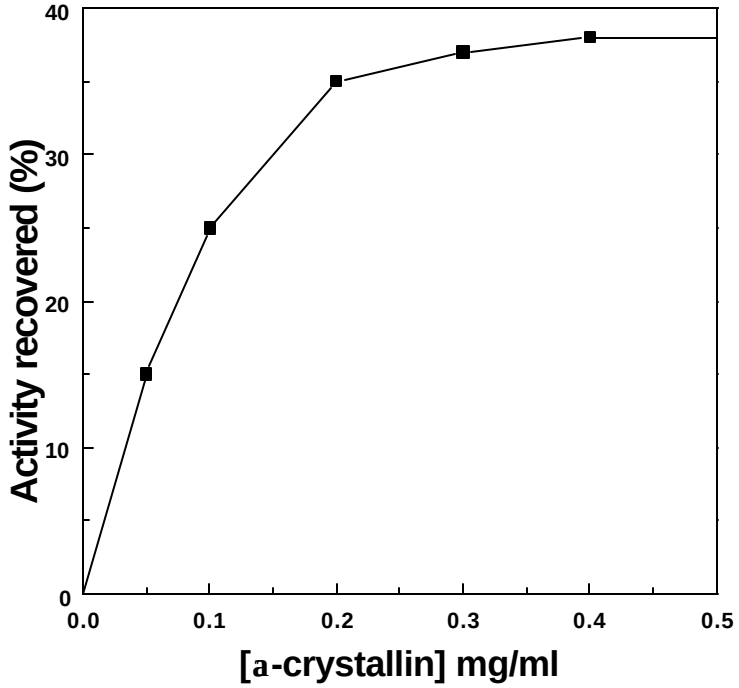


Fig. 4 Reactivation of Xyl II at varying concentrations of a -crystallin

Xyl II was renatured as described in the (legend to Fig. 3) in the presence of varying concentrations of α-crystallin. After 1 h, ATP (0.3 mM) was added to each sample and incubated further. Aliquots were withdrawn after 4 h and assayed for Xyl II activity.

Temperature dependence of the release of Xyl II from the complex on addition of ATP

The temperature shift experiments of α-crystallin assisted refolding of Xyl II is shown (Fig. 5). In all cases, the complex had been previously formed by diluting unfolded Xyl II into a buffer containing α-crystallin at 37 °C. The addition of ATP to the complex enabled the recovery of activity in the range 28 to 45 °C whereas, there was no activity when the complex was shifted to 10 °C. Thus the temperature dependence of release of the active Xyl II may be due to the conformational changes occurring at these

temperatures. Equilibrium binding studies of ATP/ α-crystallin conducted at 37, 22 and 4 °C revealed temperature dependence of the interaction of ATP and α-crystallin. Binding occurred at 37 °C but was not significant at 22 °C and was absent at 4 °C (Palmisano *et al.*, 1995). Thus the differential binding of ATP to α-crystallin is due to the observed modifications of α-crystallin between 25 -45 °C and may result in the temperature dependent release of the active Xyl II.

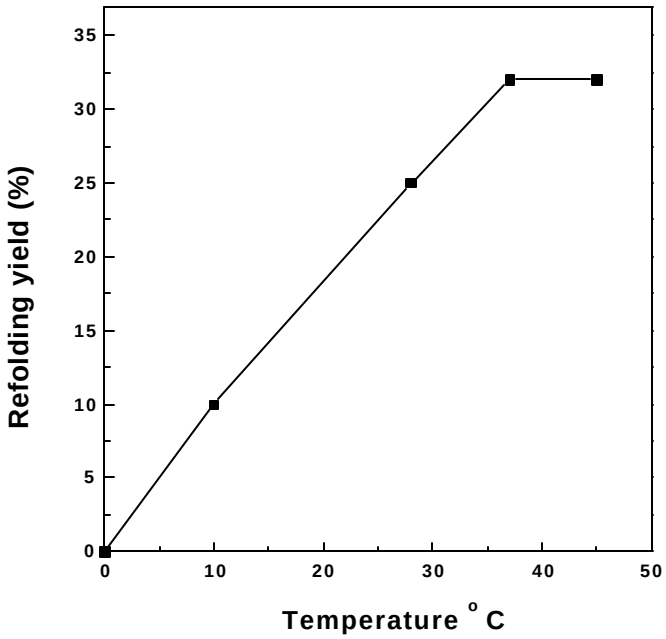


Fig. 5 **Temperature dependent refolding of Xyl II**

The α-crystallin Xyl II complex was formed at 37 °C. After 1 h ATP was added to the refolding mixture and further incubated at the indicated temperatures.

The effects of adenine nucleotides upon the α-crystallin mediated reconstitution of active Xyl II were investigated. As shown in Figure 6 addition of addition of AMP-PNP, an ATP analog with a non-hydrolyzable β-γ bond, resulted in a maximum of 20 % of Xyl II activity in 5 h. Addition of ADP and AMP did not have any effect on refolding.

The ability of refolding was quite specific for α-crystallin since bovine serum albumin and PEG virtually had no effect on the refolding (Fig. 1). BSA and PEG facilitates reactivation of several proteins in an unspecific way (Jaenicke and Rudolph, 1989).

However, BSA, as well as PEG, does not exert a significant effect on the reconstitution of Xyl II

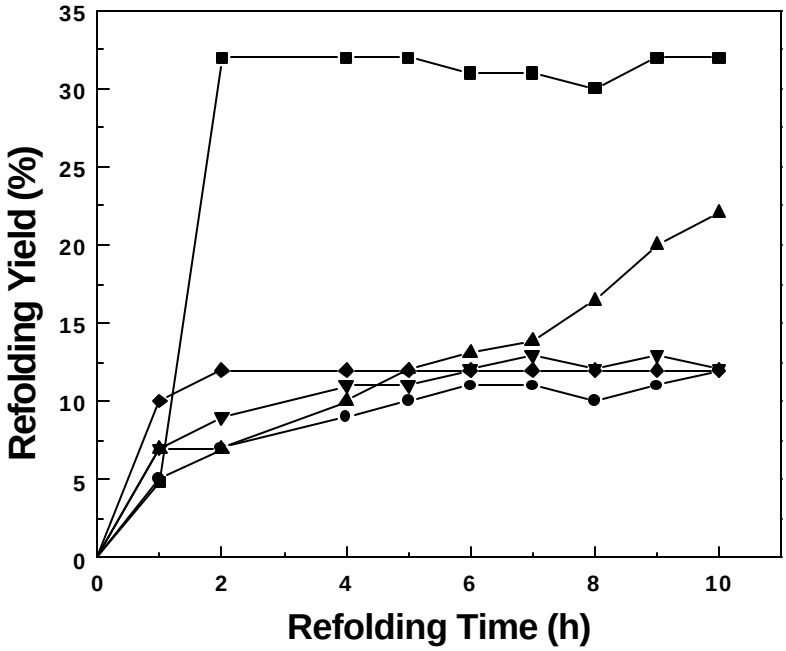


Fig. 6 **Effect of adenine nucleotides on the a -crystallin mediated reactivation of Xyl II**
Reactivation of Xyl II (1 μM) in the presence of (♦), no nucleotide; ( ), AMP; (●), ADP; ( ) AMP-PNP; and ( )ATP.

Effect of metal on the renaturation profile

The effect of Ca^{2+}/Mg^{2+} on the α-crystallin assisted Xyl II renaturation were performed in parallel with those of where only α-crystallin and ATP were used. When Ca^{2+} and Mg^{2+} were initially present with the pre-formed complex, the ATP induced release of Xyl II was characterized by substantial increase in the renaturation rates as compared with the values observed with α-crystallin alone. It was also observed that changes in the order of addition of unfolded Xyl II and Ca^{2+}/Mg^{2+} to α-crystallin resulted in significant differences in the renaturation yield (Fig. 7). When Ca was added to the α-crystallin·Xyl II complex formed prior to the addition of ATP, the yield of the renatured enzyme was 40 % as compared to the native enzyme. However, when α-crystallin was

preincubated with Ca^{2+}/Mg^{2+} prior to the addition of Xyl II andATP, the extent of regain in activity was found to decrease (Fig. 7). This may due to some conformational changes brought about by Ca^{2+} or Mg^{2+}. It is documented that the presence of calcium ions in lens water (Spector *et al*., 1974; Rink *et al*., 1977) may play a significant role in the stabilization of α-crystallin *in vivo*. Inspite of the ionic radii of Ca^{2+} and Mg^{2+} being different, they seem to catalyze the refolding. Xylan shows a marginal increase increase in reactivation which may be due to the substrate induced protection or stabilization of the refolded enzyme.

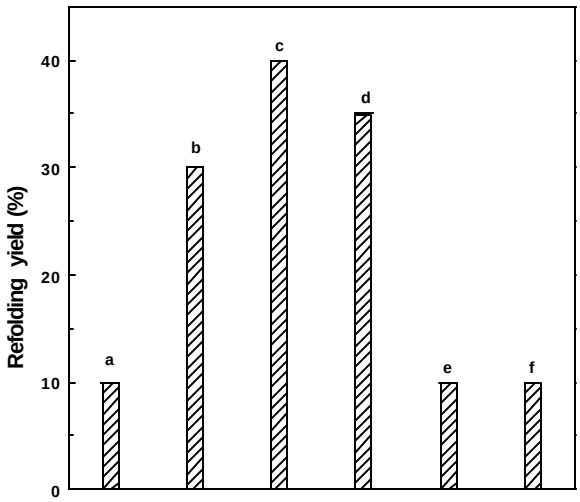


Fig. 7 **Effect of metal ions on the a -crystallin mediated reactivation of Xyl II**

Renaturation of Xyl II was initiated by diluting 10 µl of the sample into 1 ml final volume of potassium phosphate buffer pH 7.2 with α-crystallin (0.2 mg/ml). a, refolding in bufer alone; b, refolding in presence of α-crystallin and ATP; Ca^{2+}; and d, Mg^{2+}, at a concentration of 5 mM were added to the incubating mixture, e and f, Ca^{2+} and Mg^{2+} were preincubated with α-crystallin prior to the addition of Xyl II.

Our results demonstrate that α-crystallin behaved as a molecular chaperone by actively participating in the refolding and reactivation of Xyl II. The presence of ATP is necessary for the recovery of the active enzyme or for the dissociation of Xyl II from α-crystallin. The conditions for the unfolding of native Xyl II were sought in belief that the unfolded enzyme or its folding intermediates would serve as a substrate for the α-crystallin mediated reconstitution of active Xyl II. We have shown that under ATP depleted conditions Xyl II is bound tightly to α-crystallin. The presence of ATP is necessary for the recovery of the active enzyme or for the dissociation of Xyl II from α-crystallin. Conformational changes have been proposed to play a major role in the binding of folding intermediates and in the discharge of polypeptides from molecular chaperones. One of the signals for inducing such structural changes is the hydrolysis of ATP as reported in case of the chaperone GroEL (Libarek *et al*., 1991) and DnaK (Mendoza *et al*., 1991). However, in some cases the chaperones GroEl (Goboubinoff *et al*., 1989) and BiP (Kassenbrock and Kelly, 1989) do not require ATP hydrolysis. The mere binding of the adenine nucleotide to the chaperone induces a typological change in the chaperone but weakens its interaction with the bound protein. Studies performed on the refolding of lactate dehydrogenase (Badcoe *et al*., 1991) and dihydrofolate reductase (Viitanen, 1990) in the presence of Gro EL, it was found that the hydrolysis of ATP is not absolutely required for the dissociation of these enzymes from Gro EL, as both these enzymes are released from Gro EL in the presence of a non-hydrolyzable ATP analogue, AMP-PNP. When similar experiments were performed on Xyl II, it was found that Xyl II dissociated from α-crystallin in the presence of ATP and to some extent in the presence of AMP-PNP. ADP and AMP did not facilitate the release of the enzyme. These results might indicate that, in some cases, simple binding ATP or an equivalent structure is sufficient to disrupt the complex between α-crystallin and Xyl II. Quenching of intrinsic fluorescence in the presence of ATP has been reported for both α-crystallin and for αB-crystallin suggesting ATP-induced conformational changes (Viitanen, 1990; Muchowski, 1999). The refolding of citrate synthase by α-crystallin was enhanced 2 -fold in the presence of ATP (Viitanen, 1990). The specificity of α-crystallin was confirmed by the fact that neither addition of BSA nor PEG to the refolding mixture resulted in a similar

improvement. Refolding experiments in the presence of various concentrations of α-crystallin revealed that the improvement in refolding yield was a saturable phenomenon with respect to the concentration of α-crystallin.

The temperature shift experiments indicated that when the α-crystallin Xyl II complex was formed at 37 °C and then further incubated at 45 °C functional activity could be obtained with the subsequent addition of ATP. The complex appears to be stable at temperatures even as high as 45 °C. This suggests that the multiple contacts between the two proteins are necessary to prevent intra-or inter molecular interactions that would otherwise lead to irreversibly inactive enzyme. The result is the formation of a very stable binary complex that keeps the enzyme in a reactivable state. Delay experiments addition of α-crystallin revealed the inability of α-crystallin and ATP to rescue misfolded Xyl II aggregates once they have formed.

The structural characteristics of Xyl II bound to α-crystallin was determined using a fluorescent probe, ANS. We could detect an intermediate state with enhanced ANS fluorecence at 490 nm. This suggests that α-crystallin probably binds to a putative molten globule state. ther studies have shown that α-crystallin binds to molten globule states of carbonic anhydrase (Rajaraman *et al.*,1996) and xylose reductase (Rawat and Rao, 1998). Our results do not completely rule out the possibility that Xyl II is undergoing refolding while bound to the surface of α-crystallin, when we consider, the protein–protein interaction involved in the binding, it seems more plausible that the folding of Xyl II is initiated during the bound state and the functional activity is obtained during the free state of equilibrium.

Das and Surewicz (1995) reported that the specificity of α-crystallin appears to be limited to specific conformational intermediates that occur on the denaturation pathway, with no affinity for the intermediates formed on the refolding pathway, however it has been shown in the case of lysozyme that α-crystallin inhibits the aggregation and as well as the oxidative renaturation of lysozyme in the refolding pathway (Raman *et al.*, 1997). Our results showed that although metal ions (Ca^{2+} and Mg^{2+}) were not required to ensure the proper release and association of Xyl II in vitro, their presence affects the observed renaturation rates. Ca^{2+}/Mg^{2+} must somehow influence the conformation of α- crystallin

to increase of refolding since Ca/Mg independently does not affect the extent and rate of Xyl II renaturation. Recently an investigation on the role of calcium in the protection of thermal unfolding of mesophilic xylanase from *Pseudomonas flouorescence* has been documented. Occupancy of the calcium binding domain with its ligand protected the enzyme from inactivation, however the addition of calcium or EDTA did not influence the catalytic activity of the xylanase (Spurway, 1997). Although, calcium ions often play an important role in conferring structural stability on cellulases (Keitel *et al*., 1993; Chavaux *et al.*,1995). To our knowledge, this is the first report on α- crystallin mediated refolding of xylanase from an extremophilic source. The *in vitro* conditions, which are used in the present studies for the α-crystallin mediated system, may not represent the complex in vivo cellular conditions, probably essential for extremophiles. To modulate refolding of induced enzyme systems such as xylanase may require other chaperone and non-chaperone proteins. The translocation of a partially unfolded structure could be greatly facilitated compared to the native state. The folding of the protein might occur on the external side of the cytoplasmic membrane since the metal binding properties of the cell wall possibly provide high metal ion environment. (Petit -Glatron , 1993). It is tempting to speculate that extremophilic organisms might be synthesizing chaperone like molecules hitherto unknown, which are crucial for the refolding of enzyme at elevated temperature and pH imparting stability to the protein during the folding in *vivo*.

SECTION III

ARTIFICIAL CHAPERONE MEDIATED REFOLDING OF XYL II

SUMMARY

To gain insight into the molecular aspects of unfolding/refolding of enzymes from extremophilic organisms, we have used Xyl II from an alkalophilic thermophilic *Bacillus* as the model system. Kinetics of denaturation/renaturation were monitored using intrinsic fluorescence studies. The shift in fluorescence maximum was used to detect the folding intermediate(s). The structural changes during the process showed nonlinear curves. The refolding occurs in at least two phases. The protein fluorescence measurement suggested a state of the protein present in 0.08 M Gdn HCl a species with an emission maximum of 345 nm and was interpreted as a partially folded intermediate state. Far-UV CD spectra revealed that the putative intermediate had a similar content of secondary structure as the native enzyme. Studies with the fluorescent apolar probe 1,8 ANS were consistent with the presence of increased hydrophobic surfaces as compared with the native or fully unfolded protein. The refolding of xylanase (Xyl II) was attempted by a relatively new strategy using an artificial chaperone assisted two-step mechanism. The unfolded xylanase was found to bind to the detergent transiently and the subsequent addition of cyclodextrin helped to strip the detergent and assisted in folding. Our findings suggested that the detergent stabilized an early intermediate in the folding pathway seemingly equivalent to the folding state as molten globule. The reactivation of Xyl II was affected by ionic as well as non-ionic detergents. However, the cationic detergent CTAB provided a maximum reactivation (3-fold) of the enzyme. The delayed detergent addition experiments revealed that the detergent was effective by suppressing the initial aggregate formation but not in dissolving aggregates. The relevance of our findings to the role of artificial chaperones *in vivo* is discussed.

INTRODUCTION

The mechanism by which protein molecules fold to assume their active conformation has been a subject of intense study in the recent years. Refolding denatured proteins *in vitro* has been an important issue both at the fundamental as well as at the biotechnological level. Newly synthesized proteins expressed from cloned genes frequently accumulate as inactive aggregates with only a small portion actually reaching native and active conformation. The folding pathway of proteins have not been accurately determined, however it has been established that folding usually occurs through a succession of a definite number of intermediate conformational states (Cleland and Wang, 1990; Brems, 1988). Denaturation and renaturation are thermodynamic processes, involving a change in free energy and large changes in conformation between the denatured and the native states (Ruddon and Bedows, 1997). Misfolding and aggregation poses a serious problem in the industrial process of recombinant proteins. Aggregation may be due to the association of hydrophobic surfaces that are exposed during the refolding process (Horowitz and Criscimagna, 1986). Understanding of the competition between folding and aggregation in *'in vitro'* protein folding will allow the development of efficient refolding processes. Recent discoveries have shown that the problems of aggregation and association may be circumvented *in vivo* by 'molecular chaperones'. The chaperones interact with the non-native protein intermediates, prevent non-specific aggregation, and catalyze correct protein folding. Despite these functional similarities, the structures and modes of action of these molecules are different. GroEL and GroES proteins from *E. coli* are among the most detailed characterized chaperones (Fink, 1999). α-crystallin acts as a molecular chaperone and shows extensive structural similarity with heat shock proteins which act like molecular chaperones *in vitro* (Jakob, 1993). The α-crystallin mediated renaturation of xylose reductase at 37° C may be functionally similar to GroE chaperone system, where it has been demonstrated that he stabilized partially folded state of xylose reductase forms a complex with the α-crystallin and the addition of ATP releases the native enzyme (Rawat and Rao, 1998). Elucidating the mechanistic details underlying the efficient refolding of proteins by chaperones now appears to be an important consideration for defining how proteins fold *in vivo.* A strategy to prevent aggregation by interfering with inter molecular hydrophobic interactions is to use additives, small molecules that are

relatively inexpensive and easy to remove once folding goes to completion. Surfactants and detergents have proven to be very efficient folding aids and have been shown to work with a few proteins (Tandon and Horowitz, 1986, 1988; Horowitz, 1993; Zardenta and Horowitz, 1992, 1994). The development of inexpensive folding catalysts, which act like chaperonins, will help in the optimization of refolding processes.

We have used Xyl II from alkalophilic thermophilic *Bacillus* as a model system to understand the mechanism of folding of enzymes from extremophilic organisms. Our results reveal for the first time the refolding of Xyl II by a new strategy using an artificial chaperone assisted two-step mechanism. We show that during refolding, Xyl II binds to detergent transiently and cyclodextrin helps to strip the detergent and assist in correct folding. It was found that the detergent stabilizes an early intermediate in the folding pathway seemingly equivalent to the folding state as molten globule. Since the procedure of protein refolding via sequential use of a detergent and a cyclodextrin is inspired by the two step mechanism of GroE chaperone system (Hartl, 1996), hence it was named as artificial chaperone assisted refolding (Rozema and Gellman, 1995).

MATERIALS AND METHODS

MATERIALS

Methyl-β-cyclodextrin, TTAB (tetradecyl ammonium bromide), CTAB, (cetyltrimethylammonium bromide) were purchased from Sigma. Gdn HCl (Guanidium chloride) was purchased from Fluka. CTAHS (cetyltrimethyl ammonium hydrogen sulfate) was purchased from Aldrich Co. USA. All other chemicals used were of analytical grade.

Denaturation/renaturation studies of xylanase

All denaturation/renaturation experiments were carried out in 50 mM potassium phosphate buffer, pH 7.2. Xyl II (0.3 mM) was denatured for 16 h in 6 M Gdn HCl. The Xyl II solution was then diluted by a factor of 60 with potassium phosphate buffer pH 7.2 containing detergent to give a concentration of 5 μM Xyl II, 5 mM detergent. After 15 min methyl β-cyclodextrin stock solution was added to bring the final concentration to 4.1 mM detergent and 16 mM methyl-β-cyclodextrin. After sitting for 20 h, the solutions were assayed for enzymatic activity.

Kinetics of unfolding and refolding

The kinetics of the structural changes during denaturation/renaturation were followed at 25 °C using fluorescence spectroscopy. For denaturation studies, the enzyme (5 μM) was dissolved in buffer containing various concentrations of Gdn HCl, and the fluorescence spectra of the protein were recorded versus time. The samples were excited at 295 nm. The native enzyme has a fluorescence emission maximum at 339 nm, while the denatured enzyme has an emission maximum at 353 nm. The ratio, R, of the fluorescence intensities at 339 nm to 353 nm (339/353) was used to follow the shift in the fluorescence spectrum. Fluorescence spectra were recorded with a Perkin-Elmer LS 50B spectrofluorometer. The percent native structure was calculated according to the following relationship:

$$\% \text{ native structure} = 100 \times [(P_t - P_0)/(P_N - P_0)]$$

where, P_N is the ratio of intensities of native protein, P_0 is the intensity ratio after dilution in Gdn HCl and overnight incubation, and P_t is the intensity ratio at various time points after dilution in the indicated Gdn HCl concentrations.

The percent unfolding was calculated using the following relationship;

$$\% \text{ unfolded} = 100 \times (P_0 - P_t) / (P_0 - P_D)$$

where, P_0 and P_t are the intensity ratios after 24 h and at selected time t after dilution in Gdn HCl and P_D is the intensity of the denatured enzyme.

Circular dichroism and Fluorescence studies

Fluorescence and CD spectra were recorded within 2 min after the initiation of refolding and were completed within 5 min of initiation of refolding. These early recordings represent spectra of an intermediate. Circular dichroism (CD) spectra were recorded on a Jasco 715 model spectropolarimeter. Changes in the secondary structure of Xyl II were monitored in the far UV region (205-250 nm) using 0.1 mm path length. The enzyme concentrations in these experiments were 5 μM. For all measurements a reference sample containing buffer, detergent was subtracted from the CD signal. The secondary structure was analyzed using the program K2d (Andrade *et al*., 1993; Merelo *et al*., 1994). Additional experimental details have been described in the text and figure legends.

RESULTS AND DISCUSSION

Concentration dependence of the renaturation yield

The influence of protein concentration on refolding of Xyl II denatured in 6 M Gdn HCl is shown in Figure 1. The refolding was examined by diluting the denatured enzyme (concentration ranging between 0.03 mM and 0.625 mM) rapidly in the renaturation buffer. After an overnight incubation at 25 °C, the different dilutions containing 5 μM as final concentration of protein were assayed for xylanase activity. The results of this experiment, established that under identical refolding conditions, when the initial denatured protein was increased the yield of the renatured enzyme decreased.

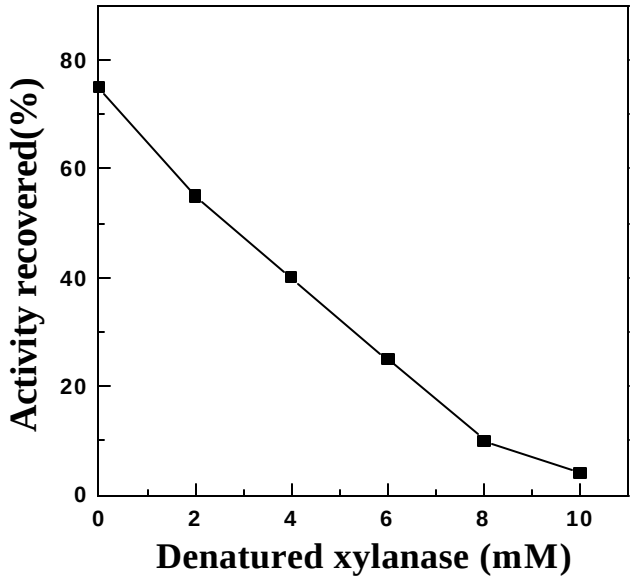


Fig. 1 **Yield of enzymatic activity as a function of the initial concentration of denatured xylanase.**
Denatured Xyl II at concentrations ranging from (0.03-0.625 mM) were prepared and diluted into renaturation buffer to a final xylanase concentration of 5 μM. Gdn HCl was added in order to maintain the same final Gdn HCl in all samples. After overnight incubation at room temperature the enzymatic activity was assayed; it is expressed as the yield (in percentage) relative to the initial activity.

Kinetics of unfolding and refolding

The time dependence of the shift in fluorescence wavelength maximum was measured at various concentrations of Gdn HCl. There was no observable shift in the fluorescence spectrum of the native control, which contained 0.08 M, a concentration accounting for the Gdn HCl carried over into the refolding buffer. In Gdn HCl at 3 M or above, the fluorescence response was very fast and was over within the mixing time of the experiment. The structural changes during denaturation are shown in Fig.2a. The denaturation process was found to be biphasic. The first phase was very fast, and the second phase was relatively slow.

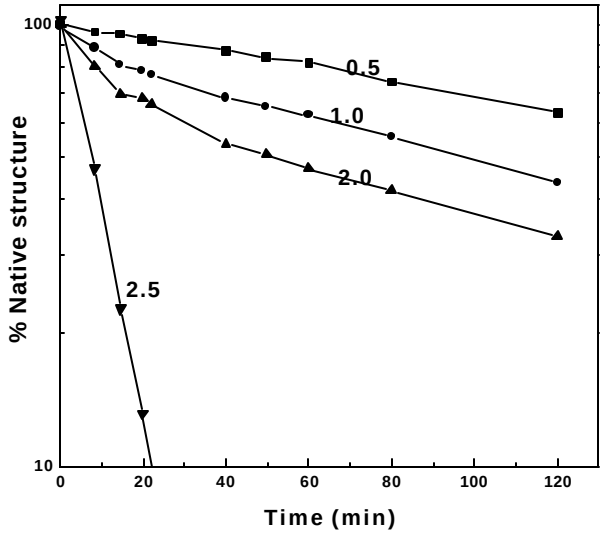


Fig 2a **A semilogarithmic plot showing denaturation kinetics of xylanase in various concentrations of Gdn HCl.**
The enzyme (5 µM) was allowed to unfold in various concentrations of Gdn HCl, and the percent native structure was calculated as described in the 'Materials and methods'.

Refolding kinetics was studied by diluting the random-coiled protein in 6 M Gdn HCl to its native state in 0.08 M Gdn HCl. Figure 2b shows a semi logarithmic plot for the renaturation. The conformational change that results in the burying of aromatic acid residues from the solvent during refolding was monitored by fluorescence measurements. The results show that about half of the total change takes place before the first experimental point was obtained (~1 min). Thus, the structure is regained in two phases.

The percentage of inactive enzyme, capable of reactivation was calculated using the expression

$$\% \text{ inactive} = 100 \times (A_0 - A_t)/ A_0$$

where, A_t and A_0 are the activities of renatured enzyme at selected time t, and after 24 h respectively after dilution of the denatured enzyme in the refolding buffer.

The regain in activity was of first order with a half time of 2.5 h. Thus, during refolding, the enzyme goes through an intermediate stage where some of the structure is regained but not the activity.

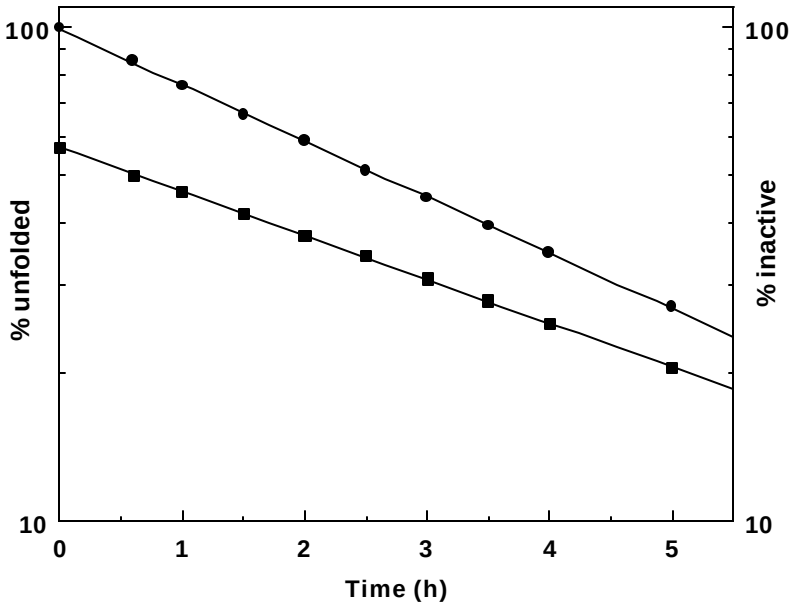


Fig 2b **A semi logarithmic plot of renaturation kinetics of Xyl II.**
The enzyme was denatured in 6 M Gdn HCl at 0.3 mM. After 24 h of denaturation, the enzyme was allowed to refold at 5 μM in renaturation buffer. The changes in activity (•) and the fluorescence ratio ( ) were monitored on the same sample at 25 °C. The % inactive and % unfolded enzyme were calculated as described earlier.

Trapping of a possible intermediate

The reversibility of unfolding was studied using various parameters such as exposure of Trp residues, loss of secondary structure and the exposure of hydrophobic surfaces. The fluorescence changes markedly when the protein is in the unfolded state (referred to as X-u) with a shift in the λ_{max} from 339 to 353 nm. The red shift of fluorescence generally reflects transfer of Trp residues to a more polar environment. When refolding of the enzyme from the Gdn HCl-denatured state was initiated, there was a rapid return of the fluorescence wavelength maximum to 345 nm (Fig. 3), which occurred within the time required to make the first measurement. The blue shift of the maximum of fluorescence emission from the X-u state suggested that some of the Trp residues have recovered the environment they have in the native protein state. Then there was a slower return of the fluorescence maximum to 339 nm, with a half time of approximately 2 h. During this period, there was no corresponding regain of enzyme activity

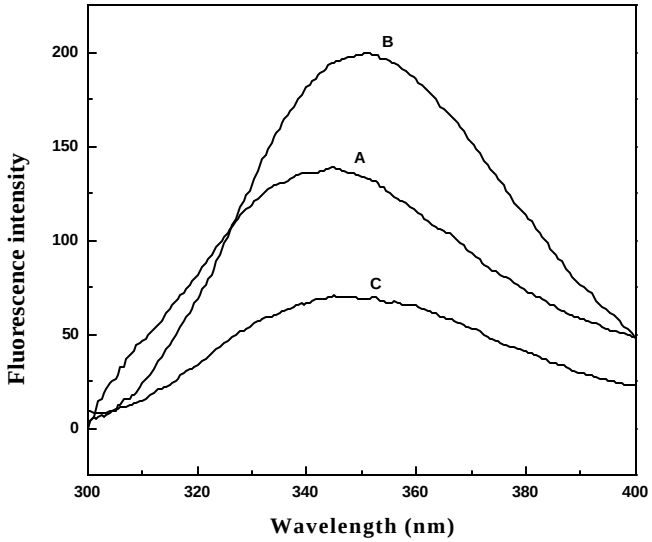


Fig. 3 **Fluorescence spectra of xylanase species**.

The spectra are as follows: **A**, native enzyme; **B**, enzyme in 6 M Gdn HCl; **C**, enzyme diluted from 6 M to 0.1 M Gdn HCl, representing the intermediate state. This spectrum was taken within 2 min of dilution.

Circular dichroism (CD) measurements were carried out to test whether this partial refolding was associated with the recovery of a part of the protein secondary structures. The CD spectra of the native Xyl II and the folding intermediate were recorded under the same conditions (0.05 M) potassium phosphate HCl, pH 7, 50 mM Gdn HCl at 25° C (Fig.4). Because of the presence of 50 mM Gdn HCl, the spectra were monitored only in the range 250-205 nm. The estimates of the secondary structure content using the program K2d (Andrade *et al*., 1993; Merelo *et al*., 1994) showed that the alpha and beta helical content of the native enzyme were 7 and 45 % and that of the partially folded structure were 5 and 47 % respectively, indicating that the intermediate was partially folded.

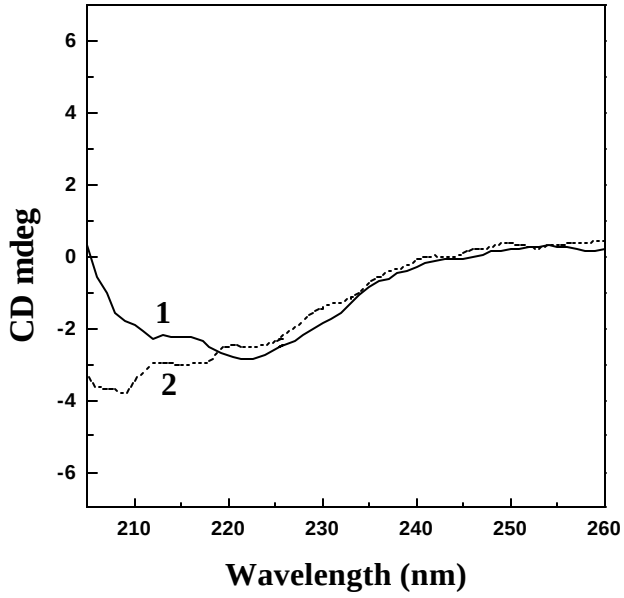


Fig 4 **Far-ultraviolet circular dichroism spectra**
Native **(1)** and intermediate state **(2).**

We attempted to determine the structural characteristics of the refolding intermediate of Xyl II by using a fluorescent probe, 1-anilino-8-napthalenesulfonate (ANS). ANS is not fluorescent in aqueous solutions ($\lambda_{max,\ em}$ 525); however, on addition of proteins, containing hydrophobic pockets its emission maximum shifts to shorter wavelengths, and emission intensity is enhanced. Figure 5 shows the fluorescence of ANS bound intermediate during the Gdn HCl induced refolding of Xyl II. The $\lambda_{max,\ em}$ of ANS

shifts to 490 nm in presence of the refolding enzyme with an increase in the fluorescence intensity, these results indicate that Xyl II possess a hydrophobic region which is buried in the native state but is exposed during the refolding reaction. The fluorescence of native as well as denatured Xyl II shows no binding to ANS. The intermediate which appears to contain considerable secondary structure but altered tertiary structure is probably similar to the "molten globule" states proposed as an accessible conformation for several proteins (Pain, 1987; Dolgikh, 1982).

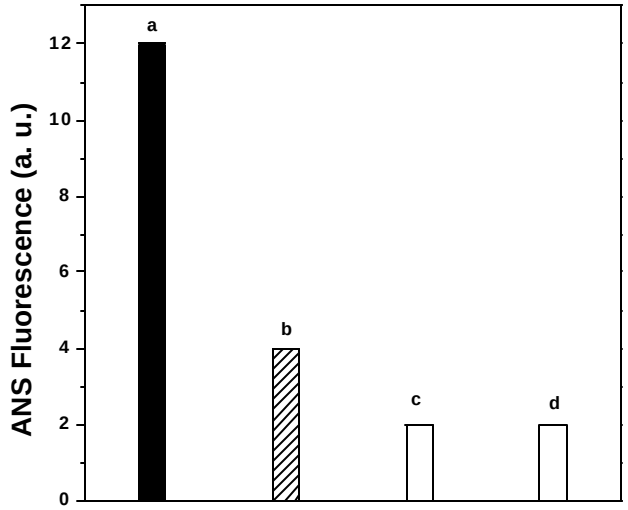


Fig 5 **Exposure of hydrophobic surfaces during refolding measured by ANS binding.**

The fluorescence of ANS bound to the refolding xylanase was performed by adding ANS (40 μm) 3 min after the initiation of refolding into the buffer. The excitation wavelength was set at 365 nm. The excitation and emission band passes were fixed at 5 nm. **a**, refolding intermediate; **b**, native Xyl II **c**, 6M Gdn HCl treated enzyme; **d**, refolded enzyme.

Folding via artificial chaperone protocol

Further, experiments were carried out to study the renaturation of Xyl II by the sequential addition of a detergent and a stripping agent. When 0.3 mM Xyl II was denatured with 6 M Gdn HCl or 8 M urea for 16 h and then diluted 60-80 fold in renaturation buffer, only 20 % and 38 % of the native Xyl II activity were recovered

respectively. The chemically denatured Xyl II on simple dilution yields very little activity and showed an increase in the turbidity due to the aggregation of protein as indicated by the light scattering experiments monitored by fluorescence at 350 nm. However refolding of Xyl II in the presence of various detergents, followed by the addition of methyl- β-cyclodextrin yielded an increase in the activity. β-cyclodextrin are cyclic oligosaccharides which exhibit amphiphilic properties by solubilizing lipophilic compounds by accommodating them in their inner hydrophobic cavities. Since the naturally occurring cyclodextrins have limited water solubility, we have used methyl-β-cyclodextrin, which are more soluble.

The effect of different types of ionic as well as non-ionic detergents on the reactivation of xylanase is shown in Table 1. The cationic detergent CTAB provides a maximum reactivation (3-fold) of the enzyme. Besides, TTAB the shorter analogue of CTAB, the non-ionic detergents Triton X-100, and Tween-80 were also effective to some extent. These results suggest that reactivation of Gdn HCl denatured Xyl II can be affected by both ionic as well as non-ionic detergents, but not all of the detergents are equally effective. Anionic detergents were incompatible with Gdn HCl denatured xylanase because of their tendency to precipitate, hence we used urea denaturation to test the effect of SDS. The yield of the urea denatured Xyl II refolding yielded 1.5 fold increase in the activity.

The CD spectra of Xyl II -detergent complex referred to as X-d was examined in order to determine whether there was any residual conformational order in the detergent-complexed protein. CD measurements were carried out using CTAHS (cetyltrimethylammonium hydrogen sulfate) because the strong absorption of the bromide ion of CTAB may interfere with the CD. The far-UV CD data for native Xyl II and for the complexes formed by diluting Gdn HCl denatured protein in the presence of the detergent is shown in Figure 6. The far-UV ellipticity is an indicator of secondary structure. The Xyl II complex with CTAHS appears to contain considerable secondary structure. The tertiary structure is determined by the intrinsic fluorescence studies.

Table 1 Xylanase refolding

Additives	Recovery of activity (%)
Controls	
Native Xyl II	100
+CTAB	85
+TTAB	80
+Methyl-b -CD	100
+Triton X-100	86
+Tween -80	93
Gdn HCl denaturation	
None	20
+CTAB	10
+CTAB+Methyl-b -CD	55
+TTAB	10
+TTAB+Methyl-b -CD Tween -80	45
+Triton X-100	15
+Triton X-100+Methyl-b -CD	38
+Tween-80	10
+Tween-80+Methyl -b -CD	27
Urea Denaturation	
None	38
+SDS	20
+SDS+Methyl-b -CD	60

Xylanase, 0.3 mM, was denatured in 6 M Gdn HCl for ³ 16 h in 50 mM potassium phosphate buffer pH 7.2. This solution was then diluted to 5 mM xylanase, with 50 mM potassium phosphate buffer, pH 7.2, 100 mM Gdn HCl and 5 mM detergent when indicated. After 15 min, these aliquots were diluted with aqueous methyl-b-cyclodextrin or water to give 4.2 mM Xyl II, 83 mM Gdn HCl, 0.05 mM potassium phosphate buffer, pH 7.2 and when indicated 4.1 mM detergent and 16 mM methyl-b-cyclodextrin. These solutions were assayed after sitting for 20 h. For control experiments, the reaction solutions were assembled in the same way, but no Gdn HCl was added.

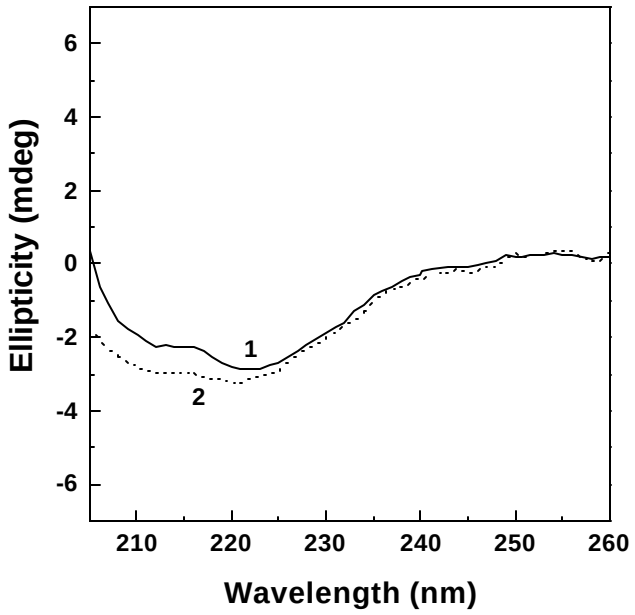


Fig. 6 **Far-UV circular dichroism spectra of Xyl II-detergent complex (1),** Native Xyl II and **(2),** the complex of Xyl II-CTAHS complex.

The fluorescence data for the native and X_I-d complex is shown in Figure 7 (excitation at 295 nm). The detergent differs substantially from the native Xyl II, indicating that the native tertiary structure is not retained in the X_I-d complexed state. The CTAB complex (which is identical with CTAHS complex) displays considerably enhanced fluorescence intensity relative to the native enzyme and is red shifted to the native enzyme. The resulting X_I-d complex does not exhibit significant activity (Table 1). This shows that CTAB molecules bind to the intermediate and inhibits its refolding. The fluorescence emission spectra recorded after renaturation exhibits a maximum of 340 nm (Fig. 7), which is close to the native.

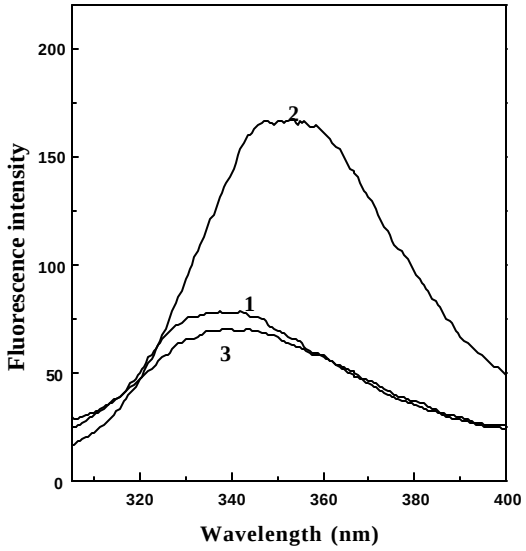


Fig 7 **Intrinsic fluorescence spectra**

Native Xyl II, **(1)**; Xyl II-CTAB complex formed by capture from Gdn denatured state, **(2)**; and spectrum of the protein renatured with 5 mM detergent and 16 mM methyl-β-cyclodextrin **(3)**. Each spectrum was obtained with a 3x3 mm cell. In each case the final protein concentration was 5μM. All spectra have been corrected for their respective blanks.

Dependence of refolding yield on the cyclodextrin-detergent ratio

The variation in the recovery of Xyl II activity from the Gdn HCl generated CTAB complex as a function of number of equivalents of methyl-β-cyclodextrin (relative to detergent) employed in the stripping step. Two different initial detergent concentrations were tested, the curves were found similar indicating that the methyl-β-cyclodextrin functions by interacting with the detergent, rather than with the protein. Addition of 0.5 or 1 equivalent of methyl-β-cyclodextrin causes 0-20% reactivation, but addition of 2 equivalent provided maximum reactivation (55%) as shown in Figure 8.

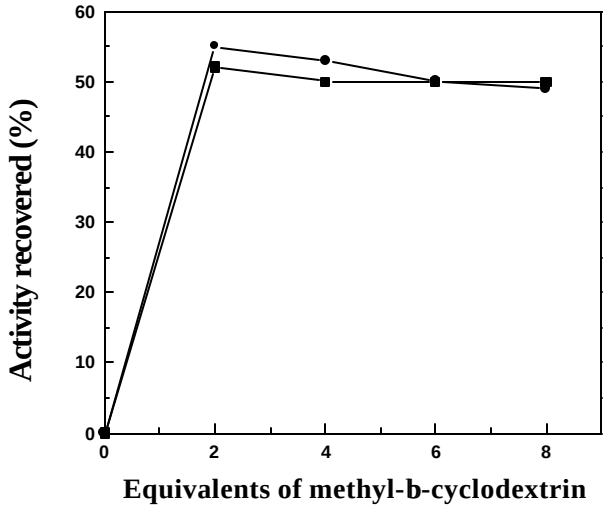


Fig. 8 **Yield of refolded Xyl II as a function of the number of equivalents of methyl-b -cyclodextrin relative to the detergent CTAB**.

Solutions contained 5 μM Xyl II, 50 mM potassium phosphate, pH 7.2. 5 mM, (●); 8 mM, (■); CTAB. After methyl-β-cyclodextrin addition, solutions were allowed to stand overnight before the assay. The lines are arbitrary.

Dependence of renaturation process on the time interval between initiation of refolding and addition of detergent

The effects of the time interval between initiation of refolding of xylanase and the addition of detergents are shown (Fig. 9). In a series of experiments CTAB was present from zero time in the refolding solvent, in the second CTAB was added 30 s after zero time and in the third the detergent was added after 1 min. When CTAB is present at the start of refolding, the yield is greater than the control. When the addition is delayed to 30 s or 1 min, the activity is superimposed on the control. Thus the "delayed detergent addition" experiments fit the idea that the detergents could act only on the early forming intermediates and do not facilitate dissolution of aggregates.

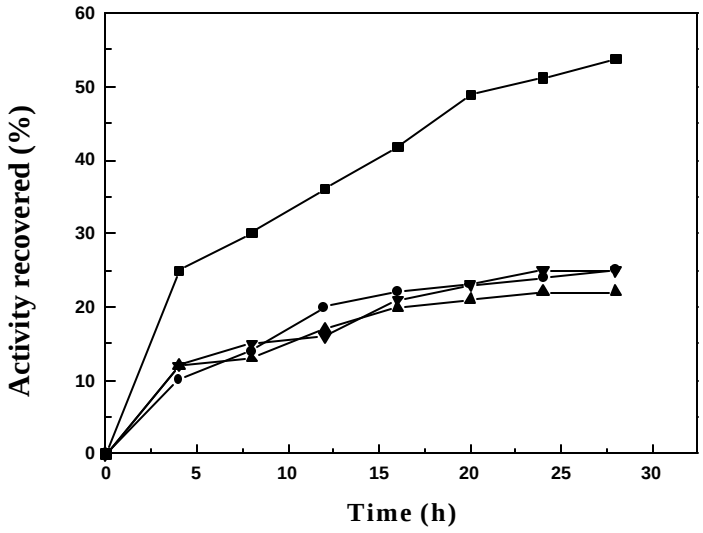


Fig. 9 Xyl II refolding with 5mM CTAB added at zero time, (■); after 30 s, (●); after 60 s ( ); and in the absence of detergent, ( ). The final protein concentration after dilution was 5 μM in each case.

Influence of protein concentration and pH on the reactivation efficiency using artificial chaperone method

The effect of various protein concentrations on the reactivation efficiency of Xyl II upon stripping of the protein-bound detergent by β-methyl cyclodextrin at various concentrations are assessed (Fig. 10). The maximum recovery of Xyl II (50 %) was obtained at the enzyme concentration of 4-6 μM. At higher protein concentrations the yield of enzyme activity by the artificial assisted refolding decreases to 20 % as compared to 4 % by the unassisted folding. Thus, the recovery of active enzyme decreased at higher concentrations due to increased protein aggregation. The maximum yield of reactivation was observed over a pH range 6-7.5 and was found to decrease above pH 7.5.

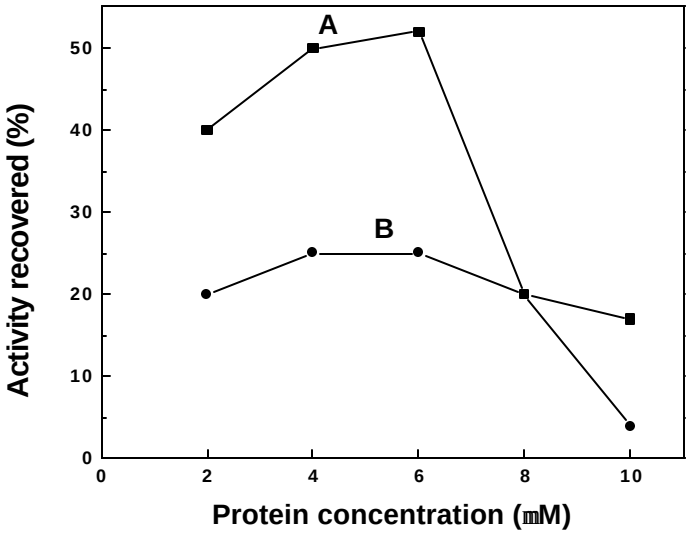


Fig. 10 **Effect of protein concentration on refolding kinetics of Xyl II**.

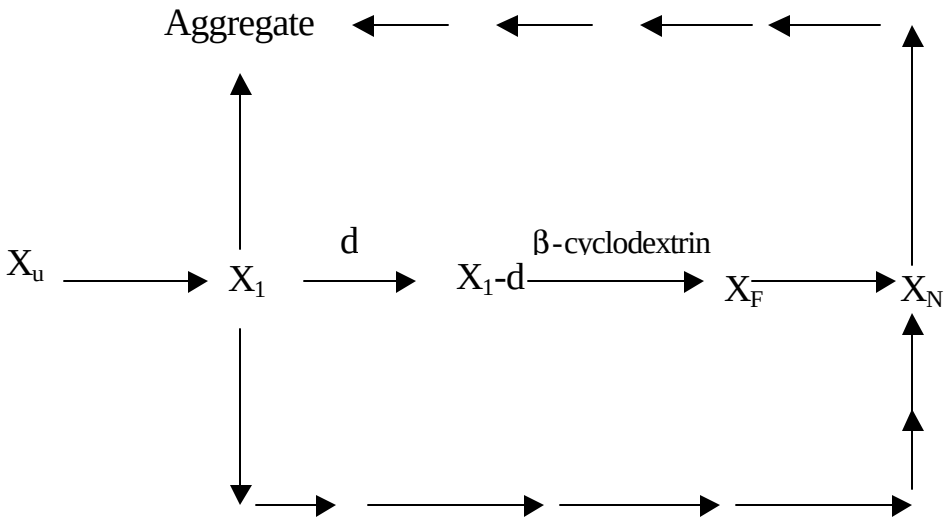
Native protein (0.5 mM) was denatured in 6 M Gdn HCl overnight. The denatured enzyme was diluted to 2-10 μM protein. Curve **A**, represents enzyme recovery obtained in the presence of 5 mM CTAB and 16 mM cyclodextrin while curve **B**, represents recovery in their absence.

Many natural or recombinant proteins do not fold properly and various techniques have been developed in recent years to refold them from their inactive state to the fully functional form. As the protein proceeds along the folding pathway towards the native state, it may be diverted off the proper pathway into side reactions, any of which act to reduce the yield of active protein. The off–pathway reactions during refolding, in particular aggregation, can be prevented by molecular chaperones and the problems of aggregation and association can be circumvented by the molecular chaperone mediated refolding.

Despite the biotechnological importance of xylanases very few systematic studies on the unfolding/refolding of these enzymes are available. The present investigation was carried out to gain insight about the utility, and mechanism of the artificial chaperone technique when applied to Xyl II. The proteins that have been successfully renatured by the approach of artificial chaperone technique include carbonic anhydrase B from human erythrocytes, hen egg white lysozyme, pig heart citrate synthase and MM creatine kinase from rabbit muscle (Rozema and Gellman, 1996a, 1996b, 1998). The present studies using

the structure-perturbing agent Gdn HCl revealed that Xyl II refolding occurs through an intermediate state in which the structure of the enzyme is altered enough to cause partial exposure of Trp residues with minimum loss of secondary structure. Our results on the binding of the fluorescent probe 1,8 ANS to the protein revealed that Xyl II possesses a hydrophobic region, which is buried in the native state but is exposed during the refolding reaction. Thus, we suggest that on the folding pathway for Xyl II there may exist an intermediate with exposed hydrophobic patches. The *in vitro* folding reaction of Xyl II probably involves a kinetic competition between correct folding and irreversible aggregation of folding intermediates. In artificial chaperone mediated refolding detergents may in part operate by interacting with exposed hydrophobic regions that appear on the surface of folding intermediates. The role of the detergent in the refolding reaction is a passive one, which consists of segregation of aggregating folding intermediates. Detergents are able to protect the hydrophobic regions on the unfolded and partially folded molecules, which would interact with each other to form aggregates. The results in Table 1 indicated that the refolding yield of Xyl II was specifically improved by the presence of a detergent and methyl-β-cyclodextrin in the refolding mixture. CTAB probably binds to the intermediate, preventing the aggregation but the resulting solution does not exhibit significant activity. The folding of the Xyl II is arrested in the X_I-d complex and is resumed after the stripping step. The presence of the stripping agent is necessary for efficient recovery of the protein. This shows that CTAB molecules bind to the intermediate of Xyl II, which has substantial amount of secondary structure with, altered tertiary structure resembling a molten globule state (Dolgikh *et al*., 1982). It appears that some specificity is involved in the interaction of detergent and substrate proteins. The chaperonin GroEL is known to bind to flexible molten-globule state of rhodanase and chicken dihydrofolate reductase (Martin *et al*., 1991) but does not interact strongly with α-lactalbumin either in its compact or in its reduced state which assumes extended conformation (Okazaki *et al*, 1994). Bam *et al*. (1996) have shown the stabilization of an intermediate of recombinant growth hormone with a non-ionic surfactant Tween. The ionic as well as the non-ionic detergents are able to capture the non-native protein upon dilution from concentrated Gdn HCl solution.

In our studies, 55% renaturation yield of Xyl II was obtained in the presence of a detergent and methyl-β-cyclodextrin. This represented a nearly 3-fold improvement in the renaturation yield obtained in the presence of the 'folding helpers'. The concentration of the solubilizing agent during refolding was such that it did not destabilize the native structure. Partitioning between folded and captured forms must be kinetically controlled, however, because detergents employed in these studies do not spontaneously disrupt native xylanase. These set of results on the artificial chaperone mediated reconstitution of a xylanase from an extremophilic source are likely to be of significance in understanding the mechanism of folding of an biotechnologically important enzyme.



Scheme 1 A model deciphering the mechanism of artificial chaperone mediated refolding of xylanase

In the scheme 1, refolding from Gdn HCl denatured state (X-u) involves the formation of an intermediate (X_I) that is not enzymatically active and can partition into inactive aggregates and the active product X_N. The effect of detergent would be then to stabilize the intermediate X_I by forming a complex (X_I-d). Addition of methyl -β-cyclodextrin would assist in the stripping process to form a partially folded state X_F which may be partially folded, which then folds to the native state X_N. The results in Figure 8, show that detergent acts by interacting with the early intermediates during folding. It can be speculated that these folding intermediates are different from the species X_F, the initial state of protein released from the detergent. Hence, it can be assumed that in the artificial

chaperone method the substrates are released in distinct conformational states. The identical reactivation yields generated by heating or by dilution of Gdn HCl denatured carbonic anhydrase B supports the view that the intermediates produced in these two routes are similar. In contrast, the activity of the heat denatured Xyl II system was not recovered by the approach of artificial chaperone assisted folding. This indicates that the mechanism of refolding by this method is may be different from that of Gdn HCl denaturation.

To our knowledge, no other report on the applicability of this method to xylanase from an extremophilic source is available. The artificial chaperone method have shown higher refolding yields when extended to two other monomeric proteins, lysozyme and carbonic anhydrase. The *in vitro* conditions, which are used in the present studies for the artificial chaperone mediated systems may not represent the complex *in vivo* cellular conditions, probably essential for extremophiles. To modulate refolding of induced enzyme systems such as xylanase may require other chaperone and non-chaperone proteins. It is tempting to speculate that extremophilic organisms might be synthesizing chaperone like molecules hitherto unknown which are crucial for the refolding of enzyme at elevated temperature and pH imparting stability to the protein during the folding in *vivo*.

CHAPTER 5

INCREASE IN STABILITY OF XYLANASE FROM AN ALKALOPHILIC THERMOPHILIC BACILLUS (NCIM 59)

SUMMARY

The cellulase-free xylanase from the alkalophilic thermophilic *Bacillus* was stable at pH 7.0 to 10.0 at 50 °C for 3 days. At 60 °C, the enzyme showed a decrease in stability with a half-life of 3 h. The effect of various additives on the stability of the enzyme at 60 °C was studied. Polyhydric alcohols such as glycerol, sorbitol, mannitol and sugars like glucose, sucrose and xylose did not have any effect on the stability of the enzyme. Similarly, salts such as KCl, $CoCl_2$, $CaCl_2$, and NaCl did not enhance the stability of the enzyme. However, glycine (0.5 M) increased the enzyme half-life by 6-fold in the pH range 7.0-9.0 and at 60 °C and 70 °C. Xylan could offer thermoprotection against inactivation of the xylanase at pH 7.0 and 8.0 at 60 °C and only a marginal increase at pH 9.0 at 70 °C was observed.

INTRODUCTION

Thermostability is an intrinsic property of the protein determined by its primary structure. Studies on enzyme stability have two main purposes: to understand the mechanism of denaturation and stabilization of proteins from a fundamental viewpoint, and to improve the stability of an enzyme for its industrial applications. The enzymatic reactions in industrial processes often need to be performed at high temperatures and reaction temperature is probably the most important physical factor, which can be optimized. Carrying out conversions at higher temperature markedly reduces microbial infection of the material being processed (Brock, 1986). Higher temperatures also increase the solubility of polymeric substrates such as carbohydrates, thereby increasing their mechanical handling characteristics and rendering them more amenable to enzymic attack. Hence, for these reasons, thermostable enzymes have been the aim of numerous studies for developing rational strategies for thermostability enhancement (Klibanov, 1983)

An industrial disadvantage of the commercially used biocatalysts and enzyme complexes is their relatively low stability. Strategies to identify thermally stable enzymes is to exploit natural sources such as thermophilic organisms or stabilize already known, existing enzymes. Thermophiles are known to produce enzymes with higher thermostability than those from their mesophilic counterparts (Nicolson *et al*., 1988). External environmental factors including cations, substrates, co-enzymes, modulators, polyols and proteins often increase enzyme stability (Ward and Young, 1988). Reduction of the water activity in the medium has sometimes been proposed as a method of increasing the stability of the enzyme (Han-Hagerdal, 1986). The stability of the enzymes can also be increased by chemical modification and cross linking, immobilization, treatment with additives, polyols or osmolytes (Gupta, 1991) or by protein engineering (Wetzel *et al*., 1988). Addition of low molecular weight additives like sugars and polyols to aqueous enzyme solutions has been demonstrated to strengthen hydrophobic interactions among non-polar amino acid residues, thereby making them more resistant to unfolding and thermal denaturation (Klibanov, 1983). Protein engineering requires sophisticated techniques and the knowledge of the gene sequence of the protein.

The xylanase from alkalophilic thermophilic *Bacillus* is stable at 50 °C, which is the cultivation temperature of the organism. However, at higher temperature and alkaline

pH the enzyme loses activity. Enhancement of the half-life of the enzyme at elevated temperature would be desirable for biotechnological applications. The present chapter deals with the effect of addition of substrate and various additives on the stability of the xylanase.

MATERIALS AND METHODS

MATERIALS

Glycerol, sorbitol, mannitol, glucose, sucrose, glycine, and β-alanine were purchased from Qualigens. Xylose was purchased from Sigma. All other chemicals were of analytical grade.

METHODS

Enzyme production

Xylanase from AT *Bacillus* was produced in a 50 ml medium containing wheat bran (10 %) and yeast extract (0.5 %) with 1 % Na_2CO_3. The centrifuged culture broth was precipitated with 3 volumes of chilled ethanol. The precipitate was dissolved in 10 ml of 50 mM potassium phosphate buffer, pH 7.0 and was used for further experiments.

Thermal and pH stability

The stability of the enzyme was measured by incubating 10 U in 0.1 ml for different time intervals in presence of 0.05 M phosphate buffer (pH 7.0), Tris HCl buffer (pH 8.0) and glycine-NaOH buffer (pH 9.0-10.0) at various temperatures. The effect of various compounds on the stability was determined by incubating the enzyme at different temperatures in presence of the additives. At the end of incubation the enzyme was cooled for 5 min and the residual activity was determined. The enzyme at 4 °C in the absence of additive was assumed to have 100 % activity.

RESULTS AND DISCUSSION

Stability of xylanase from alkalophilic thermophilic *Bacillus* at 60 °C

The xylanase from the alkalophilic thermophilic (AT) *Bacillus* was stable for 3 days at 50 °C. However, at higher temperature the enzyme showed decrease in the half-life. At pH 7.0 and 8.0 at 60°C the enzyme had half-lives of 3 and 2 h, respectively. However, at pH 9.0 and 10.0 the native enzyme lost 50 % activity in 15 and 5 min, respectively (Fig. 1).

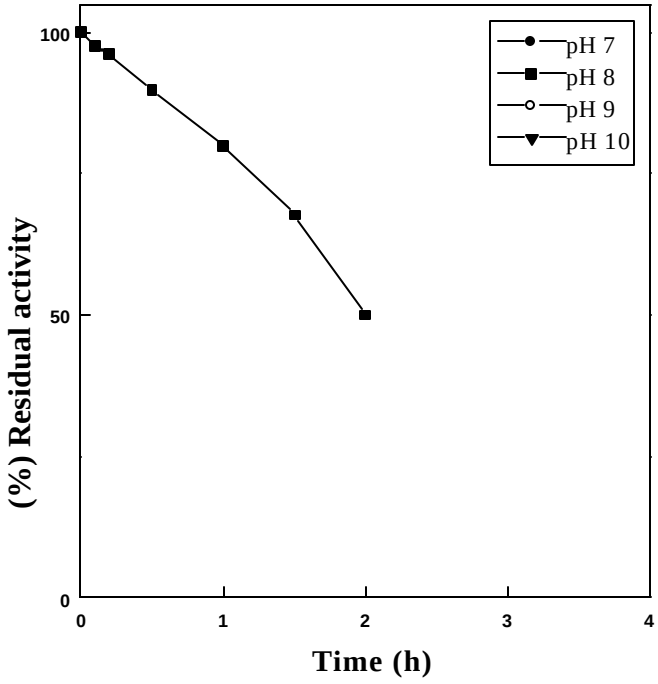


Fig. 1 The stability of xylanase from AT *Bacillus* at 60 °C.

Effect of various additives on the stability of xylanase

The protective effect of different additives on the stability of the enzyme at 60 °C was studied. Polyhydric alcohols such as glycerol, sorbitol, mannitol and sugars like glucose, sucrose and xylose did not affect the stability of the enzyme. Similarly, various salts such as $CoCl_2$, $CaCl_2$, KCl and NaCl did not have any effect on the stability of the enzyme (Table 1). However, neutral amino acids like glycine and β-alanine increased the

stability of the enzyme significantly at 60 °C. The effects of the amino acids on xylanase stability were investigated in detail.

Table 1 **Effect of different compounds on the stability of xylanase from alkalophilic thermophilic *Bacillus*.**

Additives	Concentration	Residual activity (%)
None	10 mM	50
NaCl	10 mM	50
KCl	10 mM	38
$CaCl_2$	10 mM	50
Sorbitol	1 %	45
	10 %	52
Glycerol	10 %	50
Sucrose	1 %	50
	10 %	50
Glucose	1%	50
	10 %	51
Polyethylene glycol	10 %	30
Xylose	10 %	40
Inositol	10 mM	50
Glycine	1M	100
b -alanine	1M	100
Phytic acid	5 %	1

(10 U) of enzyme was incubated with the different compounds at the concentrations mentioned above at 60°C in 0.05 M phosphate buffer, pH 7.0 for 3 h.

The addition of glycine (0.5 M) to the enzyme at pH 7.0 and 8.0 at 60 °C showed four to six fold increase in the half-life. At pH 9.0 and 10.0 in the presence of glycine the stability increased with half-lives of 10.0 and 4 h respectively (Fig. 2). However, the enzyme retained 80 % activity for 4 h at pH 10.0, when glycine (1 M) was added. The xylanase at 70 °C and pH 7.0 showed a half-life of less than 5 min. In the presence of glycine (1 M), 50 % activity was retained for 30 min. At pH 8.0 and 9.0 at 70 °C, the enzyme showed complete loss in activity in 5 min but 50 % activity was retained for 15 min in the presence of 1 M glycine.

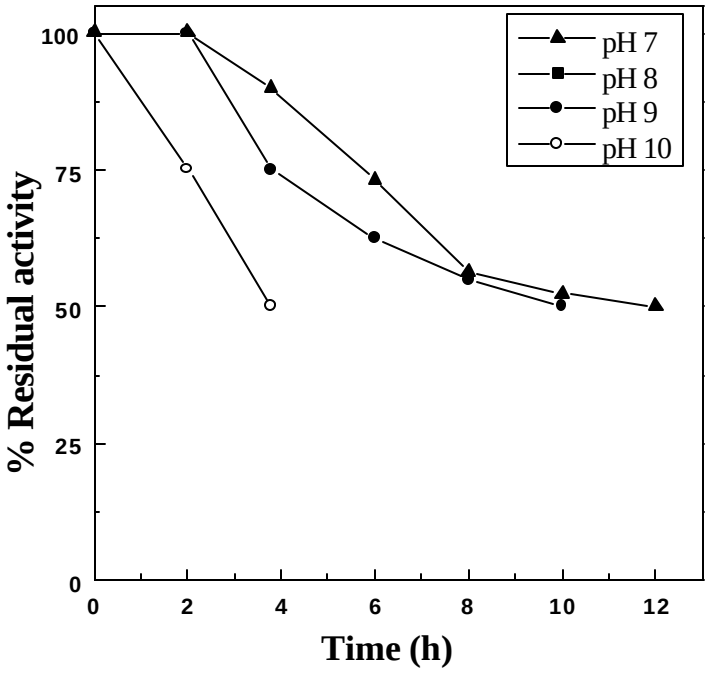


Fig. 2 **The stability of xylanase from AT *Bacillus* at 60 °C in presence of 0.5 M glycine.**

Substrate protection:

The enzyme at 60 °C showed half-lives of 4 and 3 h at pH 7.0 and 8.0 respectively in presence of xylan (3 %). Increasing the pH of incubation to 9.0 and 10.0 in the presence of the substrate, the half-lives obtained were 30 and 15 min respectively (Fig. 3). At 70 °C and at pH 7.0, the enzyme showed 50 % activity for 20 min in presence of xylan.

However, at alkaline pH (8.0 and 9.0) a half-life of 5 min was obtained. The addition of glycine in presence of xylan increased the half-life to 15 min.

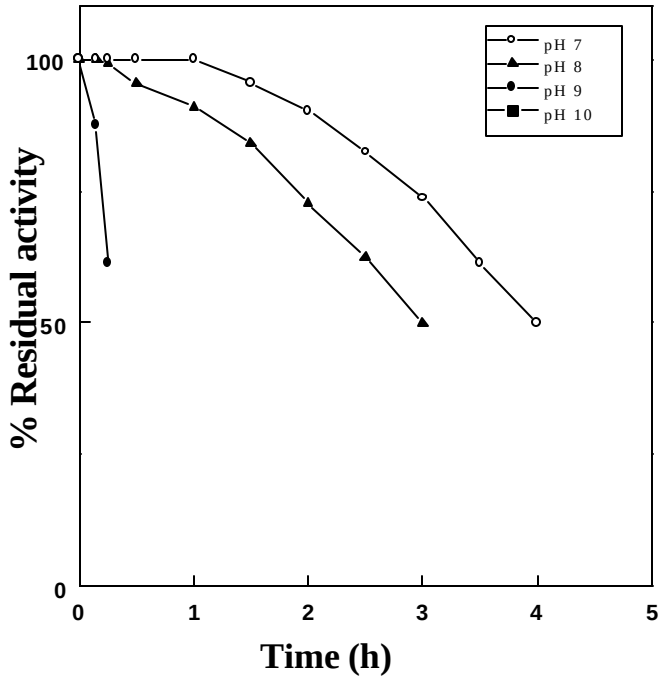


Fig. 3 The stability of xylanase from AT *Bacillus* at 60 °C in presence of xylan (3%)

The oxidation of indole group of tryptophan has been implicated during thermal inactivation of proteins (Gupta, 1991). It has been shown that substrates by binding to the active site may increase enzyme stability (Sudi, 1970; Citri, 1973). Earlier we have shown the protection of xylanase by substrate against inactivation by N-bromosuccinimide, which is a tryptophan specific reagent (*Chapter 3*). In practical applications, for example in biobleaching process considerable amounts of xylan are present which might be sufficient to protect the enzyme against thermal inactivation. It has also been reported that the substances that raise the surface tension of water also stabilize the protein structure and induce preferential hydration of water molecules. Glycine a neutral amino acid has been shown to increase significantly the surface tension of water (Arakawa and Timasheff, 1983). The increase in thermal stability of xylanase in presence of glycine is significant and may be comparable with some of the recombinants designed using protein engineering.

However, the increase in thermostability in the presence of glycine may not be universal. It clearly appears that the thermostabilization efficiency of the additives is linked to the intrinsic property of the enzyme. Thus, the results reported here show that this approach of enzyme stabilization by modification of the microenvironment may be very efficient.

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