PHYSICOCHEMICAL STUDIES ON XYLANASES FROM *CHAINIA* Sp. (NCL-82-5-1)

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BY

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DEDICATED TO MY PARENTS

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DECLARATION

Certified that the work incorporated in the thesis entitled **"PHYSICOCHEMICAL STUDIES ON THE XYLANASES FROM *CHAINIA* Sp. (NCL-82-5-1)"** submitted by **Mr. Subray S. Hegde** was carried out under my supervision. Such material as has been obtained from other sources has been duly acknowledged in the thesis.

Dr. M. I. Khan

Research Guide

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ABSTRACT

Xylanases (β-1,4 xylan xylanohydrolase E.C. 3.2.1.8.) catalyze the random hydrolysis of backbone β-1,4 xylosidic bonds in xylans - the major components of plant hemicelluloses found in the cell wall of all terrestrial plants. Cellulase-free xylanases have drawn increasing attention in recent years because of their application in paper and pulp industries where, they have been shown to be effective in reducing the requirement for toxic chemicals such as, chlorine, chlorine dioxide and the formation of environmentally hazardous chemicals (*e.g.* chloro-organic materials) as a consequence of chemical bleaching. A comprehensive understanding of substrate specificity, mode and mechanism of action is an essential prerequisite for their effective application in industrial processes. Despite the biotechnological promise of xylanases, little is known about the molecular enzymology of these enzymes. The biotechnological potential of xylanases and their functional relationship to lysozyme and cellulases make them an ideal candidate for structural and structure-function relationship studies. An actinomycete *Chainia* sp. (NCL-82-5-1) produces an extracellular cellulase-free endoxylanase. Hence the present investigation was carried out to purify and characterize cellulase-free xylanase from *Chainia* sp. and study its structure-function relationships *viz.* active site nature, subsite architecture, thermodynamic parameters of substrate binding and thermostability.

Chapter 1 : General Introduction

This part comprises of literature survey with reference to microbial xylanases, their occurrence, production, properties and industrial application.

Chapter 2 : Purification and characterization of extracellular endoxylanase(s) from *Chainia* sp.

An extracellular cellulase-free endo β-1,4 xylanase from *Chainia* sp. has been purified to homogeneity by ammonium sulfate precipitation, successive chromatography on CM-Sephadex C-50 and Sephadex G-50 followed by FPLC (Mono S). The molecular mass of the enzyme indicates a single species with a mass of 21,000 and 21,485, as determined by SDS/PAGE and Laser desorption mass spectroscopy, respectively. The purified xylanase is non-glycosylated protein with a pI of 9.4-9.5 and belongs to low Mr/basic, family G/11 glycosyl hydrolases. The enzyme is low in basic, aromatic and sulfur containing amino

acids and the partial N-terminal sequence exhibits a remarkable homology with other actinomycete xylanases. The pH and temperature optima for the enzyme are 6.0 and 50°C, respectively. The xylanase is stable over a wide range of pH (3-10, 120h) and is stable at 50°C for 24h in pH 6.0. The kinetic parameters showed that the enzyme has a higher affinity for the oat spelt arabinoxylan as compared to birch wood (acetylated) xylan. The mode of action on xylo-oligosaccharides and xylan suggests that *Chainia* xylanase is a non-debranching endoxylanase and a minimum of four xylose units (xylotetraose) are required for the initiation of hydrolytic action.

Chapter 3 : Active site characterization of *Chainia* xylanase

The chemical modification studies on purified xylanase revealed the involvement of a cysteine and carboxyl group in the catalytic activity of the enzyme. Based on isolation and partial N-terminal sequencing of the modified carboxylate containing peptide and sequence homology comparison with other family G/11 xylanases, it was proposed that the carboxyl group acts as acid/base catalyst and the cysteine may function as the catalytic nucleophile. Modification of the enzyme either by p-nitrophenylglyoxal or 2,3, butanedione resulted in significant loss of enzyme activity. Fluorescence quenching studies on native and modified enzyme samples using xylooctaose (X_8) revealed that arginine may have a role in substrate binding. On the contrary, the N-bromosuccinimide mediated inactivation of the enzyme was due to peptide cleavage and fluorescence quenching studies suggested that all the three tryptophans are located in a similar, moderately hydrophobic, microenvironment.

Chapter 4 : Equilibrium and thermodynamic parameters of xylo-oligosaccharide binding and thermostability of *Chainia* xylanase

Binding of xylo-oligosaccharides to the enzyme resulted in a decrease in fluorescence intensity of the enzyme. The percentage quenching of the xylanase fluorescence increased from 5% for xylobiose (X_2) to 12% for xylopentaose (X_5). Oligosaccharides of higher chain length gave a uniform 15% quenching. Equilibrium and thermodynamic parameters of substrate / inhibitor binding were determined by following the fluorescence changes and by using titration calorimetry. The standard free energy changes ($-\Delta G°$) at 20°C were 17.84, 19.45, 20.21 and 20.50 kJ mol^{-1} for X_2, X_3, X_4 and X_5, respectively. The Gibb's free energy changes as determined from titration calorimetry were 13.10 kJ mol^{-1} for

X_3 at 10°C, 16.50 kJ $mol^{-1}$for X_4 at 10°C and 17.30 kJ mol^{-1} for X_5 at 7°C. Contribution from the enthalpy towards the free energy decreased with increasing chain length from X_2 to X_4 whereas, an increase in entropy was observed. The change in enthalpy and entropy of binding were compensatory and entropically driven binding process suggested that hydrophobic interactions play a predominant role in binding. The difference between the ΔH and ΔS for X_4 and X_5 were marginal as compared to X_2 and X_3 indicating that the enzyme has 4 subsites. The xylanase showed a single irreversible transition peak with the T_m dependent on pH, enzyme concentration and scan rate.

Chapter 5 : General discussion and conclusion

This chapter discusses the salient features of the present investigation with respect to structure-function relationship of the enzyme.

List of publications

1. Purification and characterization of an extracellular endoxylanase from an actinomycete *Chainia* sp. Hegde S. S., Kumar A. R., Suresh C. G., Kotwal S. M., Ganesh K. N. and Khan M. I. (1997) J. Biochem. Mol. Biol. Biophys. (in Press)

2. Endoxylanase from *Chainia* sp.: Involvement of a cysteine and carboxylate in catalysis and arginine in substrate binding. Hegde S. S., Kumar A. R., Ganesh K. N. and Khan M. I. (communicated).

3. Evaluation of equilibrium and thermodynamic parameters of binding of xylo-oligosaccharide inhibitors/substrates to the endoxylanase from *Chainia* sp. Hegde S. S., Kumar A. R., Ganesh K. N. and Khan M. I. (communicated).

List of abbreviations

β-ME	β-mercaptoethanol (2-mercaptoethanol)
CAPS	3-(cyclohexylamino-1-propanesulfonic acid)
CBD	cellulose binding domain
ConA	concanavalin A
CM-Sephadex	carboxymethyl Sephadex
DAM	2,4, dinitroanilinomaleimide
DEP	diethylpyrocarbonate
DP	degree of polymerization
DSC	differential scanning calorimetery
DTT	dithiothreitol
DNSA	3,5 dinitrosalicylic acid
DTNB	2,2'-dithiobis nitrobenzoic acid
EAC	1-ethyl-3-(3-dimethylaminopropyl) azonia carbodiimide
EDC	1-ethyl-3-(3-dimethylaminopropyl) carbodiimide
HEPES	(N-[2-Hydroxyethyl]piperazine-N'-[2-ethanesulfonic acid])
HEWL	hen egg white lysozyme
HNBB	2-hydroxy-5-nitrobenzylbromide
IEF	isoelectricfocusing
ITC	isothermal calorimetry
MES	(2[N-Morpholino]ethanesulfonic acid)
NAI	N-acetylimidazole
NAG	N-acetylglucosamine
NAM	N-acetylmuramic acid
NEM	N-ethylmaleimide
NBS	N-bromosuccinimide
NTEE	3-nitro-L-tyrosine ethylester hydrochloride
PAGE	polyacrylamide gel electrophoresis
PHMB	*para* hydroxymercurybenzoate
PCMB	*para* chloromercurybenzoate
PMSF	phenylmethylsulfonylfluoride
pNPG	*para* nitrophenylglyoxal
PVDF	polyvinylidenedifluoride
RBB-xylan	remazol brilliant blue-xylan
SDS	sodiumdodesyl sulfate
TCA	trichloroacetic acid
TFA	trifluoroacetic acid
TNM	tetranitromethane
X, X_2, X_3------X_n	xylo-oligosaccharides where 1, 2, 3, ---- n designates the number of xylose units

CHAPTER 1

General Introduction

Xylans : Occurrence, structure and functions.

Xylans - the major components of plant hemicellulose, are amorphous heteropolysaccharides found in the cell walls of all plants [1], tightly associated with other polymeric cell wall materials. After cellulose, xylans are one of the most abundant biopolymers, synthesized in the biosphere [2,3]. They usually are the constituents of secondary walls of tissues (major component of woody tissue) [4] but are also present in the primary walls of growing cells as well as in seeds and bulbs of certain plant species [5,6]. The chemical structure and cytological localization of xylans vary based on their botanical origin [2,4-8]. Xylans from terrestrial plants are generally characterized by β-1,4 linked D-xylopyranosyl main chain, which carries a variable number of neutral monosaccharides or uronic acid or short oligosaccharide chain. A few homoxylans have also been reported [6,9-16]. Marine algae also synthesize β-1,3 xylan [17] and in some species of *Chlorophyceae* and *Rhodophyceae* where cellulose is absent, xylans are in highly crystalline fibrillar form [2].

Based on the substituents found on the backbone, xylans are divided into different categories *viz.*, linear homoxylan, arabinoxylan, glucuronoxylan, glucuronoarabinoxylan, galactoglucuronoarabinoxylan etc. and in addition several polymers with unusual characters (like neutral arabinoxylan with xylosyl residues, on an average doubly substituted with arabinofuranosyl residues at O-3 and two (1→3) linked L-arabinofuranosyl residues at O-2) have also been reported [18-20]. The complexity of xylan increases with the number of substituents attached to the main chain and the schematic features of all these xylans are given in Figure I.

In hard wood xylans, 10% of xylose residues carry a side chain of 4-O-methyl glucuronic acid attached to C2 of the xylose and 70% of the xylose residues contain O-acetyl groups either at C2 or C3 positions [21,22]. Grass xylans are highly branched and contain large amounts of L-arabinofuranose but are low in uronic acid [22]. Graminaceous plants contain O-acetyl groups and also contain p-coumaric acid and ferulic acid [23].

Figure 1

A) A hypothetical xylan fragment showing the sites of action of enzymes involved in its hydrolysis

[1] Endo β-1,4 xylanase (E.C. 3.2.1.8)

[2] α-L-arabinofuranosidase (E. C. 3. 2. 1. 55)

[3] Xylobiase or β-xylosidase (E.C. 3.2.1.37)

[4] Acetyl xylan esterase (E.C. 3.1.1.6)

[5] α-(4-O-methyl)-D-glucuronidase (E.C. 3.2.1.?)

[6] Feruloyl (p-Coumaryl) esterase (E.C. 3.1.1.?)

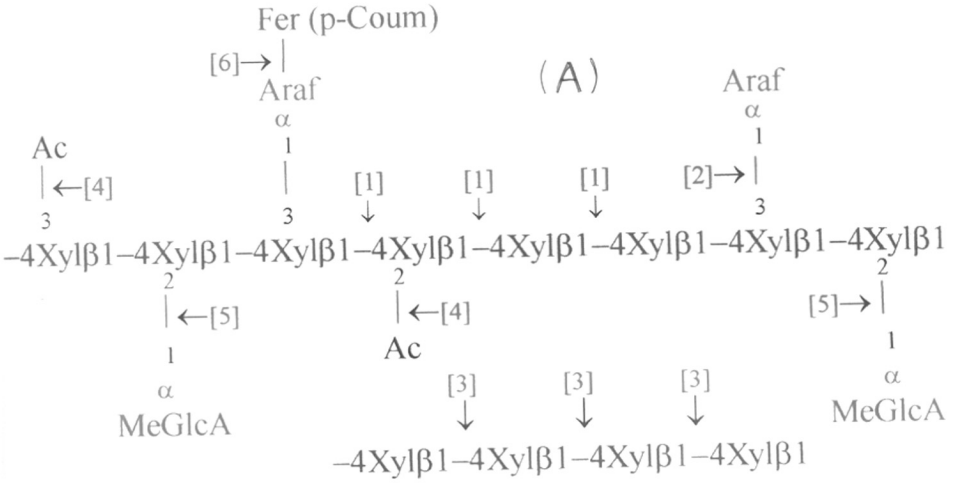
Abbreviations used : Ac - acetyl group, Araf - arabinofuranose, MeGlcA – 4-O-methyl-D-glucuronic acid, Xyl – D-xylose, Fer – ferulic acid and p-Coum – p-coumaric acid. Modified from Reference [58]

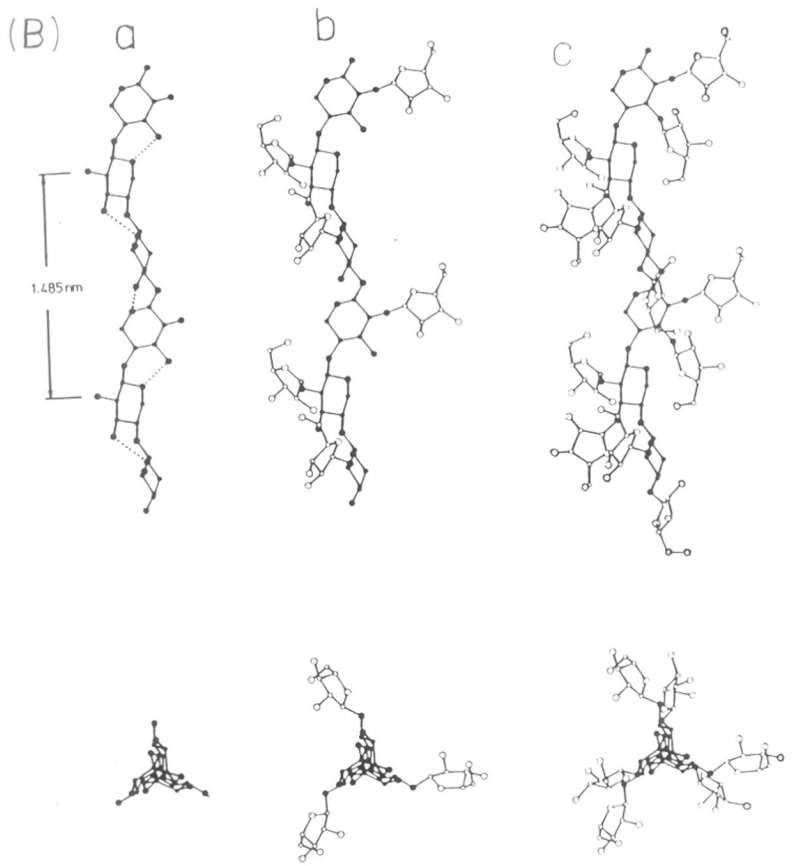
B) Projections of unsubstituted and L-arabinose substituted xylan

Top : perpendicular projections a) xylan backbone, b) substituted with one arabinose side group and c) two arabinose side groups.

Bottom : parallel projection a) xylan backbone, b) substituted with one arabinose side group and c) two arabinose side groups.

Atkins, E. D. T. in Xylanas and xylanases (Reference [36])


Fer (p-Coum)
[6]→
Araf
α
1
3
(A)
Araf
α
1
3
Ac
←[4]
3
[1]
[1]
[1]
[2]→
–4Xylβ1–4Xylβ1–4Xylβ1–4Xylβ1–4Xylβ1–4Xylβ1–4Xylβ1–4Xylβ1
2
←[5]
1
α
MeGlcA
2
←[4]
Ac
[3]
[3]
[3]
–4Xylβ1–4Xylβ1–4Xylβ1–4Xylβ1
2
[5]→
1
α
MeGlcA


(B)
a
b
c
1.485nm

In soft wood xylan, 70% of the xylose units carry terminal 4-O-methyl glucuronic acid attached to C2 and α-L-arabinofuranose directly attached to C3 [24].

Xylans are attached to other xylan chains or other polymeric cell wall constituents through covalent as well as non-covalent interactions [2]. These covalent linkages include glycosidic, ester and ether bonds of either main chain xylopyranose or side chain substituents like glucuronic acid, 4-O-methyl glucuronic acid with other polysaccharides and non-carbohydrate moieties like p-coumaric acid, Cα and Cγ lignin, ferulic acid etc. [25-33]. Xylans are believed to form hydrogen bonding with cellulose microfibrils with a strength inversly proportional to the degree of substitution [34].

X-ray diffraction analysis of xylan [35] and xylan hydrate [36] revealed that β-1,4 linked xylan chain forms twisted ribbon like strands of three fold symmetry with a 1.49 nm repeat of the three fold helix giving a value of 0.5 nm for the axial advance of each xylose unit, which compares very well with the values of 0.5-0.52 nm for cellulose, mannan, chitin and polymannuronic acid [37]. On the other hand, β-1,3 xylans form a complex, robust-rope like triple helix structure [37,38].

Although the chemical structure of xylans has been known for long, very little or no information exists on the minor structural elements. Little is known about the true primary structure of xylan due to lack of knowledge about the sequence and distribution pattern of side-chain substituents which are determinental to the solubility, physical conformation, interaction with other cell wall constituents and hence for the mode and extent of enzymatic cleavage.

As a constituent of secondary wall tissue, xylans have structural functions and appear to be the major interface between lignin and other carbohydrates [2,3]. Xylans are also present in primary cell walls of growing cells and have been considered to play a crucial role in growth and differentiation [39,40] and they are present as storage polymers in seed [41].

Xylanolytic system

Xylanolytic enzymes are widely distributed in nature and are produced by a variety of organisms such as bacteria (of marine, terrestrial and rumen environment), fungi (saprophytic as well as phytopathogenic and mycorrhize), protozoa, marine algae, insects, snails, crustaceans and germinating seeds of terrestrial plants [41-44]. Majority of the xylanases is secreted extracellularly but a few reports on intracellular xylanases exist [42,45-47]. All xylosidic bonds in lignocellulose are not equivalent or equally accessible to xylanolytic enzymes [3]. The microorganisms have developed a system of enzymes with a specialized function, which act synergistically [48-57] for the efficient break down and utilization of complex xylans [3,58]. The following are the enzymes of xylanolytic system.

Endo β-1,4 xylanase (1,4-β-D-xylan xylanohydrolase, E.C. 3.2.1.8) - depolymerises the β-1,4-D-xylan main chains randomly producing xylo-oligosaccharides of variable chain length.

Exo β-1,3 xylanase (1,3-β-D-xylan xylanohydrolase, E.C. 3.2.1.72) - produces xylose from β-1,3 xylans and xylo-oligosaccharides in exo manner.

Exo β-1,4 xylanase (1,4-β-D-xylan xylanohydrolase) - produces xylose from xylan and xylo-oligosaccharides in an endwise attack from the non-reducing end with the inversion of anomeric configuration.

Xylobiase or β-xylosidase (1,4-β-D-xylan xylanohydrolase, E.C. 3.2.1.37) - acts on xylobiose and xylo-oligosaccharides from the non-reducing end to form xylose with the retention of anomeric configuration.

α-L-arabinofuranosidase (E.C. 3.2.1.55) - cleaves side chain α-1,3 arabinofuranose from the xylan main chain.

α-(4-O-methyl)-D-glucuronidase (E.C. 3.2.1.?) - removes D-glucuronosyl or 4-O-methyl glucuronosyl residues from xylan.

Acetyl xylan esterase (E.C. 3.1.1.6) - liberates acetyl groups from the xylan backbone.

Feruloyl esterase and *p-coumaryl esterase* (E.C. 3.1.1.?) - removes ferulic acid and p-coumaric acid respectively, from xylan.

Endo β-1,4 xylanases; the crucial enzymes of xylanolytic system, vary to a great extent in substrate specificity and substrate side chain positional specificity. They also vary in the product formation and hence are grouped into four different categories [43,44,59].

A) Non-arabinose liberating endoxylanases: These produce only xylobiose and xylose as the major end products and cannot act on L-arabinosyl initiated branch points at β-(1→4) linkages. These enzymes can break down xylo-oligosaccharides as small as xylobiose.

B) Non-arabinose liberating endoxylanases: These produce mainly xylo-oligosaccharides larger than xylobiose and have no action on xylotriose and xylobiose. These enzymes cannot cleave α-(1→2) and α-(1→3) branch points.

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

D) Arabinose liberating endoxylanases: These can hydrolyze the branch points and produce intermediate size xylo-oligosaccharides and arabinose.

Physiological role of xylanases

In majority of the cases, xylanases are secreted extracellularly and act on the hemicellulosic materials present in the growth medium, liberating xylose - an assimilable end product and thus allow the organism to grow on xylan. Xylanases play a key role in invasiveness of plant pathogens by hydrolysis of plant cell wall xylan [60-63]. Xylanases present in the plant cell wall are believed to play an important physiological role [58] and those present in the germinating seeds convert reserve food into assimilable product [64]. They also seem to be involved in fruit softening [65,66] and certain xylanases act as elicitors of plant defense mechanism [66-68]. It has recently been reported that challenge of *Nicotiana tabacum* cv Xanthi with ethylene inducing xylanase from *Trichoderma viride*

causes rapid induction of plant defense responses leading to hypersensitive necrosis but the phenomena is cultivar specific. The binding of xylanase is localized to the plasma membrane. Chemical cross-linking of xylanase to microsomal membranes from responding plants revealed a 66 kDa protein complex, which has been proposed as the receptor that mediates the hypersensitive response [69].

Xylanase production

Xylanolytic enzymes are inducible by fragments of polysaccharides, but a few examples of constitutive xylanase synthesis have also been reported [70-77]. Most of these microbes produce low levels of constitutive xylanase activity which liberate smaller fragments from xylan and the low molecular weight fragments thus formed enter the cell and trigger the xylanase biosynthesis [71,78]. In non-cellulolytic microbes, mainly yeasts, the xylanolytic enzyme production is controlled by induction and catabolic repression [58,79]. Xylan has been shown to be the best inducer of xylanase biosynthesis in many cases [80-85] and a few reports of cellulose as an inducer of xylanases have also been reported [3,86,87]. Among the low molecular weight derivatives, xylobiose, xylose, xylo-oligosaccharides and their positional isomers, non metabolisable analogues of xylo-oligosaccharides, various aryl, alkyl and methyl β-xylosides are good inducers of xylanase [58,79,88-98]. Cheaper hemicellulose rich agricultural residues like wheat bran, rice bran, corncob, corn stalk, rice straw and bagasse have also been found to be better inducers in certain cases [99-107]. A recent study on production of xylanase from an alkaophilic *Bacillus* sp. [108] has shown that the addition of DL-norvaline, glycine and casamino acids to the fermentation medium resulted in 2-5 fold increase in xylanase secretion suggesting that the inhibition of proteolytic activity as a possible reason for enhanced xylanase activity.

The xylanase induction is a complex phenomenon and since there is no uniform mechanism for regulating the xylanolytic enzyme formation among the microbes [58], the response to an individual inducer is species specific. Occurrence of both inducible and constitutive xylanase activities has also been reported in the

culture filtrate of single species [108-110]. Low molecular weight inducers like positional isomers and structural analogues need transferases for their translocation into the cell and hence the presence and level of these enzymes may also affect xylanase synthesis.

Xylanase multiplicity and xylanosomes

Many of the xylanolytic microbes show multiple protein bands associated with xylanase activity [3,55,99,111-115]. This phenomenon arises due to several reasons [3,55,78,115] such as;

1). *Differential post-translational or post-secretional modification*: *viz.* glycosylation or proteolysis or both. Many of the xylanases are glycosylated [116-121] and some are translated as precursors with signal peptides [119, 122-124]. The gene xysA from *Streptomyces halstedii* JM8 encodes for a 461 amino acid xylanase (xys 1L) which is proteolytically processed to produce 362 amino acid protein (xys 1S) that retains the same xylanolytic activity [125]. The domain of 99 amino acid produced as a result of proteolytic cleavage shows similarity to bacterial cellulose binding domains. By using anti-xys 1L serum, xylanases similar in size to xys 1L and xys 1S have been detected in many *Streptomyces* species, suggesting the ubiquity of these types of processing mechanism [125].

2). *Presence of distinct gene product*: It has been shown that some of the xylanolytic microbes possess multiple xylanase genes [111,113,117,126] and immunological distinction among xylanases from the same strain has also been reported [127-130].

3). *Differential substrate specificity of xylanases and substrate cross-specificity of glycanases*: Xylanases show a wide range of specificity towards xylan. The microspecificity varies to a great extent with the nature of xylan such as, soluble, insoluble, side chain substituents etc. Substrate cross-specificity has been reported in numerous lignocellulolytic enzymes [117,118,128,131-137]. Some of them show wide specificity while some others substantially high secondary activity [3].

4). Multiplicity may also arise from the protein aggregation and/or degradation of other xylanases in the culture filtrate [3]. In addition to endoxylanases, the enzyme multiplicity has also been observed in other xylanolytic enzymes *viz.* β-xylosidases [138,139], α-L-arabinofuranosidase [140-142], acetyl xylan esterases [143,144] and ferulic acid esterases [145]. Multiple components of xylanolytic system are known to act cooperatively in xylan degradation such as reducing the degree of polymerization, removal of side chain substituents, solublization of xylan, generation of lower oligosaccharides and xylose.

Discrete multifunctional, multienzyme complexes, which play an important role in the degradation of cellulose and hemicellulose, found, either cell-associated or extracellularly, of several cellulolytic microbes are called cellulosomes [146-148]. The presence of a structure analogous to cellulosome, the xylanosome, has been reported [112]. The extracellular complex B from *Butyrivibrio fibrisolvens* exists as multisubunit protein aggregate of molecular weight 669 kDa and is composed of 11 protein bands with xylanase activity and 3 bands with endoglucanase activity. The cellulase-xylanase multicomplex from *Clostridium papyrosolvens* composed of at least 7 protein bands has been reported [149]. The presence of xylanosome was also reported in anaerobic bacterium *Thermoanaerobacterium saccharolyticum* [150]. The molecular weight of these complexes range from 500 - 660 kDa. A single non-catalytic glycoprotein of molecular weight 125 kDa is present in all the complexes, which may have the function in substrate binding and scaffolding [55]. The xynC from *Clostridium thermocellum* codes for xylanase C containing 619 amino acids and the enzyme contains a typical N-terminal signal peptide, thermostabilising domain, catalytic domain, and a dockerin domain responsible for cellulosome assembly [151]. Immunological and sequence analysis suggested that full-length xylanase C is one of the major components of *C. thermocellum* cellulosome [151].

Xylanase purification

Xylanases from microbial origin are in general extracellular enzymes and conventional procedures have been used for their purification. Majority of the procedures involve concentration of culture filtrate either with salt or solvent precipitation or membrane filtration followed by a combination of chromatographic procedures like ion exchange, gel filtration [152], hydrophobic interaction [128,153,154], immunoaffinity [155] or affinity adsorbent techniques like chromatography on cross linked xylan [156] or conA Sepharose chromatography (in case of glycosylated xylanases) [157]. In addition, more sensitive tools like IEF [126,158], preparative PAGE [159], HPLC [160-162] and FPLC [163,164] have also been used. Some of the xylanases have been purified using gel filtration matrices like Biogel-P-6, Sephadex G 25 based on the adsorptive interaction of these apparent low molecular weight xylanases with the gel matrices [152] and a few low molecular enzymes have been purified by ultra filtration [68,136].

Biochemical and physicochemical properties of xylanases

Microbial xylanases display a wide range of variability in their biochemical and physico-chemical properties. The pH optima of xylanases from neutrophiles are generally in the range of pH 3.5-7.0. However, a few xylanases from acidophilic and alkalophilic organisms have been shown to be optimally active at pH 2 and 9, respectively [129,132,165-180]. Most of the fungal xylanases are stable over a wide range of pH, i.e. from pH, 3-10. The pH stability of bacterial and actinomycete xylanases is comparable to the fungal enzymes with a few exceptions [55,82,181]. Majority of the xylanases has the temperature optima in the range of 40-60°C. Several *Bacillus* sp. and thermophilic actinomycetes produce stable xylanases with an optimum temperature in the range of 60-80°C [108129,151, 172,174,182-185]. Fungal xylanases have comparatively lower temperature optima in the range of 40-50°C; nevertheless a few xylanases with high temperature optima (80-105°C) are known [186,187]. Xylanases from

various species belonging to the genus *Thermotoga* are optimally active at temperatures between 80-105°C [185,188,189]. The enzymes from thermophilic organisms generally show higher thermal stability.

Microbial xylanases are monomeric proteins with a molecular weight in the range of 20-50 kDa. However, xylanase from *Penicillium herquei* [190] showed two protein bands on SDS/PAGE under reducing conditions, corresponding to 8 and 11 kDa indicating it to be a hetero dimer. The xylanses from *Thermotoga thermarum, T. maritima, Melanocarpus albomyces, Aeromonas sp. Y-20, Humicola grisea var. thermoidea, Thermoanaerobacterium sp.*, and *Cellulomonas fimi* showed very high molecular weight of 150, 120, 104, 145, 95, 180 and 150 kDa, respectively [188,189,191-195]. A few xylanases have been reported to have unusually low molecular weight [44,62,68,109,148,163,134,196-200]. However, majority of the above reports is based on the molecular weight determination by gel filtration where anomalous behavior of the xylanases is not uncommon. Retardation of protein, on gel filtration, has been reported for many xylanases either due to interaction of the enzyme with the gel filtration matrix or due to molecular shape. The bacterial xylanases are distinctly classified into two categories. The low molecular weight enzymes in the range of 16-22 kDa and the high molecular weight ones in the range of 43-50 kDa. The molecular weight of fungal xylanases is generally in the range of 16-50 kDa whereas actinomycete and *Streptomyces* display the molecular weight in the range of 20-55 kDa.

The pI values of the xylanases range from pH 3.75 - 10.3. There appears to be a strong relationship between the molecular weight and pI of microbial xylanases and based on this, xylanases are classified into two groups *viz.* low molecular weight basic (molecular weight < 30 kDa) and high molecular weight acidic (molecular weight > 30 kDa) enzymes [3]. Almost 70% of the purified fungal, bacterial and actinomycete basic xylanases have molecular weight less than 30 kDa and nearly the same number of acidic xylanases show molecular weight above 30 kDa [55]. However, there are several exceptions to this general pattern and both low molecular weight acidic [166,201-205] as well as high molecular

weight basic [206-211] xylanases have been reported. Some of the xylanases contain carbohydrates either covalently attached or as a dissociable complex. Carbohydrate moieties are believed to play an important role in stabilizing the enzyme structure, activity and thermostability [59] and may be playing some role in the multiplicity of xylanases [3]. The carbohydrate content of xylanases varies from 1-40% [130,133,186,200,212-214]. The amino acid composition of several xylanases has been reported and in general they contain high amounts of acidic as well as neutral amino acids while sulfur containing amino acids are low or absent [117,133,134,215].

The xylanases show a wide range of affinity towards the substrate xylan. Michaelis-Menten constant (K_m) of different xylanases varies with the source and nature of the xylan and has been reported in the range of 0.27 - 14 mg/ml [3]. Among divalent and trivalent metal ions, Hg^{+2}, Fe^{+3}, Fe^{+2} and Pb^{+2} are the potent inhibitors of xylanases. Other metal ions like Mn^{+2}, Cu^{+2}, Mo^{+2}, As^{+2}, Zn^{+2}, Cd^{+2}, Ca^{+2}, Ni^{+2}, Al^{+3} etc. also inhibit the activity of certain xylanases [55].

Substrate specificity

An endoxylanase may be classified as being specific or non-specific glucanase depending on whether its action is restricted to β-1,4 linked xylopolymers or whether it can also hydrolyze other lignocellulosic materials such as: CM-cellulose, avecil, starch, and synthetic substrates (like PNP-xylosides). Substrate cross-specificity has been reported in numerous xylanases (lignocellulolytic enzymes)[117,128,131-137,187,215-221]. However, determination of K_{cat}/K_m values will usually suffice to ascertain whether the enzyme is primarily a xylanase or xylanase activity is secondary [222]. Endoxylanases catalyze the hydrolysis of xylan and xylo-oligosaccharides of degree of polymerization (DP) 3 or more but usually have no action against synthetic substrates [222] or cellulosic material. The affinity or catalytic efficiency of xylanases towards xylo-oligosaccharides increases with increasing DP. In early studies, xylanases were classified as debranching or non-debranching depending on

whether or not they catalyze the removal of side chain arabinose in addition to main chain linkages [42-44,223]. Several reports exist on the same strain producing both types of enzymes [3,170,224-226] and some of these enzymes liberate feruloyl-arabinoside. In many cases, better efficiency was observed in xylan hydrolysis when side chain substituents were removed by relevant ancillary enzymes prior to hydrolysis by main chain delinking enzymes [215,227]. In contrast, a few enzymes have been reported to show high affinity for the main chain linkages near the branch points [65,133,216,228]. The presence of side chain substituents can be a requirement or hindrance to the activity of an individual xylanase.

Endoglucanases appear to be quite specific about anomeric configuration [229] and very few enzymes act on both β-1,4 and β-1,3 linkages [230]. Trans-glycosylation by endoglucanases is believed to form the products with the same linkage as the substrate [229]. However, an endo β-1,4 xylanase from *Cryptococus albidus* formed β-1,3 linkages during transxylosylation reaction [231]. Several organisms produce both β-1,3 as well as β-1,4 xylanases and in majority of the cases these enzymes are specific for the corresponding linkage type [180]. The mixed β-1,3, 1,4 xylan (produced by *Rhodymenia palmatum*) is hydrolyzed by some xylanases [197, 232-235] with the formation of xylose, 1,4-linked X_2, X_3 and 1,3, 1,4 linked mixed X_4 or higher oligosaccharides [180]. Whether β-1,3 linkages are cleaved by the enzyme or not are in question and a few authors have reported that only β-1,4 linkages are cleaved by such enzymes [233,234].

Domain structure and xylanase families

Microbial cellulases and xylanases comprise of various combinations of discrete functional elements such as catalytic domain, cellulose binding domain (CBD), the linkers connecting such domains and repetitive amino acid sequences [235, 236]. In addition to the above-mentioned domains, cellulosome forming and other homologous domains with unknown functions have also been described.

In the early classification studies, 1,4 - glycosyl hydrolases were grouped into 8 families; A-H, based on conserved amino acid sequence in catalytic domain and hydrophobic cluster analysis [235,237]. The xylanases were placed in family F and family G [235]. Later, Henrissat and Bairoch [238] classified 1,4 - glycosyl hydrolases into 11 families; 1 - 11, where xylanases lie in family 10 and 11 (family F and G respectively, according to earlier classification). The total number of glycosyl hydrolase families is 45. Family 10 (F) xylanases have relatively high molecular weights and show broader substrate specificity. Family 10 also includes cellulases while family 11 has only xylanases. Family 10 enzymes contain a cellulose-binding domain, which is connected to catalytic domain via linker sequences whereas family 11 xylanases are single domain proteins. Family 11 xylanases display narrow substrate specificity and are of relatively low molecular weights. The members of individual families show common fold, active site architecture and catalytic mechanism.

Subsite mapping and mechanism of action

Subsite mapping is one of the important means of investigating the active sites of xylanases in terms of subsite heterogeneity such as number of subsites involved in substrate binding, their size, individual contribution to overall affinity for the substrate and spatial relationship to the catalytic groups which in turn dictate the specificity, action pattern and kinetic parameters of the enzyme acting on a homopolymer [239,240]. Subsite mapping is usually carried out by monitoring the reaction between the enzyme under investigation and xylo-oligosaccharides, either radio labeled at the reducing end (H^3) or reduced to corresponding alcohol (xylitol, xylobiitol etc.) at the reducing end with DP values ranging from 2 - 8. Separation and quantification of the products either by TLC or HPLC and determination of DP of the product containing radio label/xylitol gives information about bond cleavage frequencies, multiple attack, condensation and transxylosylation. From the values of K_m, V_{max} and K_{int} (the dissociation constant of the ES complex when all subsites are occupied by D-xylosyl residues [241]), the

number of subsites, location of catalytic site, and the values of all the binding energies can be calculated [180]. From the above studies it has been shown that an endoxylanase from *Aspergillus niger* contains 5 subsites with the catalytic site located between the 3rd and 4th subsites from the non-reducing end [241] whereas xylanase from *A. niger* strain 14 was found to have 7 subsites [242] and *C. albidus* xylanase has 4 subsites with the catalytic groups in the middle [243]. The size of subsites was shown to be 5 xylose units for 3 xylanases from mesophilic fungal strain Y-94 [244] while endoxylanase from *Schizophyllum commune* has 7 subsites with the catalytic residues between 4th and 5th subsites from the non-reducing end [239].

From subsite mapping of *C. thermocellum* xylanase followed by determination of primary structure of the products (by methylation analysis and NMR spectroscopy) of long term hydrolysis of larch wood 4-O-methyl glucuronoxylan, Debeire *et al.* [109] concluded that the enzyme is composed of 5 subsites A to E, binding 5 xylosyl residues. Based on the structure of $(Xyl)_nGlcA$ it was also concluded that the catalytic site was located between the sites B and C and subsites D and E were able to bind substituted xylosyl residues whereas, the others could not. Recent studies on *Pseudomonas fluorescens* subspecies *cellulosa* xylanase [245] showed that the enzyme contains a sixth xylose binding site (subsite F) comprising of conserved residues (Glu 43, Asn 44 and Lys 47) which prevents small oligosaccharides from forming nonproductive enzyme-substrate complexes.

All investigated glycanases and glycosidases catalyze the hydrolysis of glycosidic bonds stereoselectively, such that the anomeric configuration of the substrate is either retained or inverted [246]. Retention of anomeric configuration occurs via double displacement reaction while inversion via single displacement [236,246,247]. Recent crystallographic studies have suggested that the active site in exoenzymes is situated in a tunnel and in endoenzymes it is located in a cleft [248,249]. Endoxylanase from *Bacillus pumilus* [250], *Bacillus subtilis* [124], *C. thermocellum* [251], *S. commune* [251], β-xylosidase from *Penicillium wortmanii*

[252], a cellobiohydrolase with high xylanase activity from *C. fimi* [220,253] and two arabinofuranosidases from *Monilinia fructigena* [254] were found to be configuration retaining enzymes. The β-xylosidase from *B. pumilus* [255,256] and an endoxylanase from *A. niger* [215] invert the configuration of their substrates.

Chemical modification and site-directed mutagenesis

The effect of chemical modifiers on enzyme activity, kinetic measurements of inactivation process, determination of number of amino acid residues modified and site directed mutagenesis of specific amino acid residues can lead to important information on active site and structure-function relationship of enzymes. Based on these studies, involvement of Trp, Tyr, Cys, His, and carboxylate (Asp / Glu) has been reported for different xylanases.

Reports on the involvement of Trp in catalytic function of xylanases and β-xylosidases are all based on inactivation of enzyme by NBS [55,114,180] and Trp was shown to be essential for enzyme activity of a few xylanases [181,257,258]. But quantitative determination of the number of Trp residues modified and substrate protection studies has been done only in a few cases. Three Trp residues have been reported to be present at or near the active site of an Alkalophilic thermophilic *Bacillus sp.* [259]. However, Trp was not involved in catalysis of xylanases from *S. commune* [260] and *Streptomyces* sp. [261,262].

Participation of single Tyr residue in substrate binding has been reported for a xylanase from *S. commune* [260,263]. Differential modification of the enzyme with tetranitromethane (TNM), in absence and presence of xylo-oligosaccharides, proteolytic digestion and amino acid sequencing of labeled peptide and alignment of the sequence with parent sequence led to the conclusion that a highly conserved Tyr 97 is involved in substrate binding [260]. Involvement of two Tyr residues in substrate binding has been shown by site directed mutagenesis in a xylanase from *Bacillus circulans* [264].

Histidine was shown to either participate in catalytic function or conserved in the active site of a few xylanases [181,265,266] whereas modification of His

residues did not alter the enzyme activity in some other xylanases [180,263,267]. Wakarchuk *et al.* [265] have recently identified a conserved His residue having very low pK_a by NMR assignment, pH titration and proton exchange studies in *B. circulans* xylanase. A recent report of site-directed mutagenesis and structural studies on xylanase A from *Streptomyces lividans* revealed the involvement of two conserved His residues in hydrogen bonding network in the vicinity of catalytic residues and in maintaining the enzyme stability [268]. Xylanase A contains three His residues in the active site, of which two are almost conserved in family F/10 glycanases. Site-directed mutagenesis of H 207 E/K/R and H 81 R/S/Y resulted in drastic decrease in specific activity. The pK_a values of catalytic residues are decreased in all the mutant enzymes indicating the importance of these two residues in hydrogen bonding network responsible for maintaining the proper ionization states of catalytic residues. Furthermore, guanidine hydrochloride induced unfolding studies on native and mutant enzymes revealed the role of these His residues in maintaining the structural integrity of the enzyme [268].

Participation of carboxyl group(s) in catalytic function has been reported in variety of glycoside hydrolases and the carboxylate seems to be the integral part of catalytic site of glycoside hydrolases where acid-base catalysis is involved. All xylanases to date, on which chemical modification and site-directed mutagenesis studies have been carried out, revealed the participation of either one or two carboxyl groups in catalysis with the exception of *T. saccharolyticum* [269] where three essential acidic amino acid residues were identified. Water-soluble carbodiimide, 1-ethyl-3-(3-dimethylaminopropyl) azonia carbodiimide (EAC), inactivated an endoxylanase from *S. commune* with the resultant modification of one carboxyl group that could be protected against inactivation by substrate [263]. Later it was demonstrated by labeling of the enzyme with [^{14}C] EAC followed by proteolytic digestion, amino acid sequencing of labeled peptide, sequence homology, hydrophobic cluster analysis and secondary structure prediction that Glu87 was the residue modified by EAC which acts as the nucleophile in acid/base catalysis [270]. Based on the inactivation of xylanase from *Streptomyces* sp. by

EDC and triethyloxonium fluoroborate, it was reported that one or more carboxyl groups are involved in the catalytic function of the enzyme [262]. Use of specific active site carboxyl modifying reagent *viz.* tritiated 2', 4'-dinitrophenyl 2-deoxy-2-fluoro-β-D-glucopyranoside, isolation of the radio labeled peptide and sequencing allowed the identification of Glu 233 as the nucleophile in *C. fimi* exoglucanase (Cex) [271-273]. Glu 127 of Cex was also shown to be involved in catalysis by site-directed mutagenesis [272]. Newly synthesized inhibitor, 2', 4'-dinitrophenyl 2-deoxy-2-fluoro-β-D-xylobioside, was used to trap the covalent intermediate during the catalysis of *B. subtilis* xylanase [274]. Finally, by the use of Electrospray Mass Spectrometry, Glu78 was identified as the nucleophile. Among the family 11 xylanases, Glu 78 and Glu 172 of *B. circulans* xylanase and Glu 93 and Glu 182 of *B. pumilus* xylanase were shown to be the catalytic residues by site-directed mutagenesis [264, 275]. Two glutamates (Glu128 and Glu236) of *S. lividans* xylanase A [276] and putative nucleophile of *P. fluorescens* (Glu 246) [277] were shown to be catalytic residues in Family 10 xylanases. Chemical labeling and/or site-directed mutagenesis of carboxylate that acts as nucleophile as well as the acid/base catalyst and the sequence alignment revealed that both these residues are highly conserved in xylanases of both families and are surrounded by a highly homologous region specific to each family [278].

Quite a number of microbial xylanases and β-xylosidases are sensitive to thiol reagents like PCMB, PHMB, DTNB, NEM, DTT, iodoacetamide, iodoacetic acid, β-ME etc. [55,180]. Several reports on thiol inactivation also reported the reversal of such inactivation [180]. However, Hg^{+2} has been found to inactivate some enzymes devoid of Cys residues [117,133,134,180]. Strong protection against inactivation of xylanases and xylosidases by thiol reagents was obtained by substrate, substrate analogues and competitive inhibitors in certain cases [180,279]. Use of active-site thiol labeling reagent DAM and subsequent sequencing of the labeled peptide has shown that a xylanase from thermotolerant *Streptomyces* sp. contains a Cys residue in its active site [181, 280]. Studies on

Thermomyces xylanase have also indicated the presence of sulfhydryl group in the active site [281,282].

Recent crystallographic and site-directed mutagenesis studies on xylanases revealed the presence of Asn residues in the active site and their role in maintaining the ionization states of catalytic residues. Site-directed mutagenesis of Asn 127 (N 127 D) of *S. lividans* xylanase A resulted in 70 fold decrease in specificity constant (k_{cat}/K_m) and also in the ionization states of catalytic residues (as shown by modified pH profile) suggesting the role of conserved Asn 127 in stabilization of the catalytic intermediate and maintaining the ionization states of catalytic residues [283]. Xylanase A from *P. fluorescens* contains three conserved Asn residues on the surface of the active site cleft, which have been shown to have a role in catalytic activity of the enzyme [245]. Asn 44 along with Glu 43 and Lys 47 is situated in the subsite F that prevents the binding of small oligosaccharides to form nonproductive enzyme–substrate complex. Asn 126 is located adjacent to acid/base catalyst and it is thought that its interaction with the substrate is important in positioning the glycosidic oxygen in close proximity to Glu 127. Asn 182 is situated at subsite C, proximal to subsite B and influences the activity of the enzyme against the oligosaccharides larger than X_3 [245].

X-ray crystallography and three dimensional structure

The three dimensional structure of xylanases from both the families (F/10 and G/11) have been determined. Four structures of xylanases of family 10 and six of family 11 have so far been reported. Family 10 xylanases whose structure has been reported are *S. lividans* Xyn A [284], *C. fimi* Cex [285], *C. thermocellum* Xyn Z [286] and *P. fluorescens* Xyn A [277,287]. Xylanases from family 10, fold into a common $(\alpha/\beta)_8$ barrel motif and show a general "salad bowl" shape [278] (Fig 2). The active site crevice is invariably found at C-terminal. An enzyme-substrate complex of Xyn A from *P. fluorescens* [277] has been crystallized with the substrate located in the crevice.

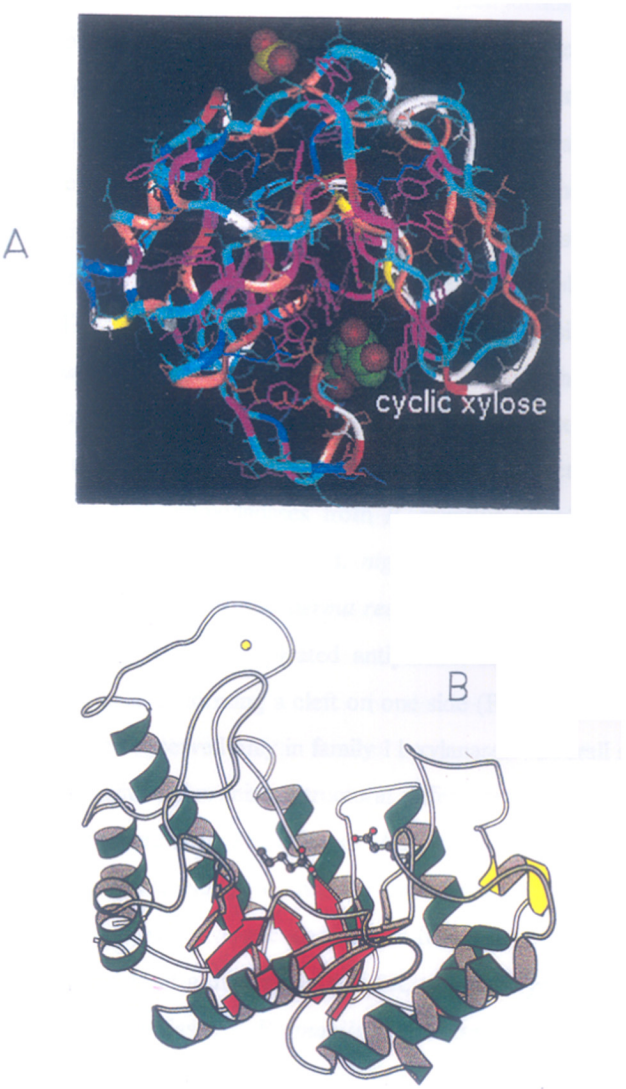


Fig 2. Three-dimensional structure of xylanases : **A)** *Bacillus circulans* xylanase (Reference 264 and http://www-imb.jena.de/image_PROTEINS/proteins/1.bcx/bcx_midas2s.gif) **B)** *Pseudomonas fluorescens* xylanase : viewed perpendicular to the barrel axis showing catalytic acid/base Glu 127 and nucleophile Glu 246. Calcium atom is shown as the yellow dot, which stabilizes the long loop. (From Reference 287)

Numerous amino acid residues, which had already been identified or suggested as part of the active site, are located in the cleft, which is well exposed to solvents. The active site residues are held in place by extensive hydrogen bonding net work and β-bulge type distortions that serve to orient important active site residues. However, there are some minor structural differences in some regions. A long loop observed in *P. fluorescens* XynA [277,287] between β strands 7 and 8 has been identified as the binding site for calcium ion that plays a role in stabilization of the enzyme. The corresponding loop in other xylanases is significantly shorter. Xyn A of *P. fluorescens* contains an additional helix between the residues 149-154 while *C. thermocellum* Xyn Z has an additional N-terminal helix.

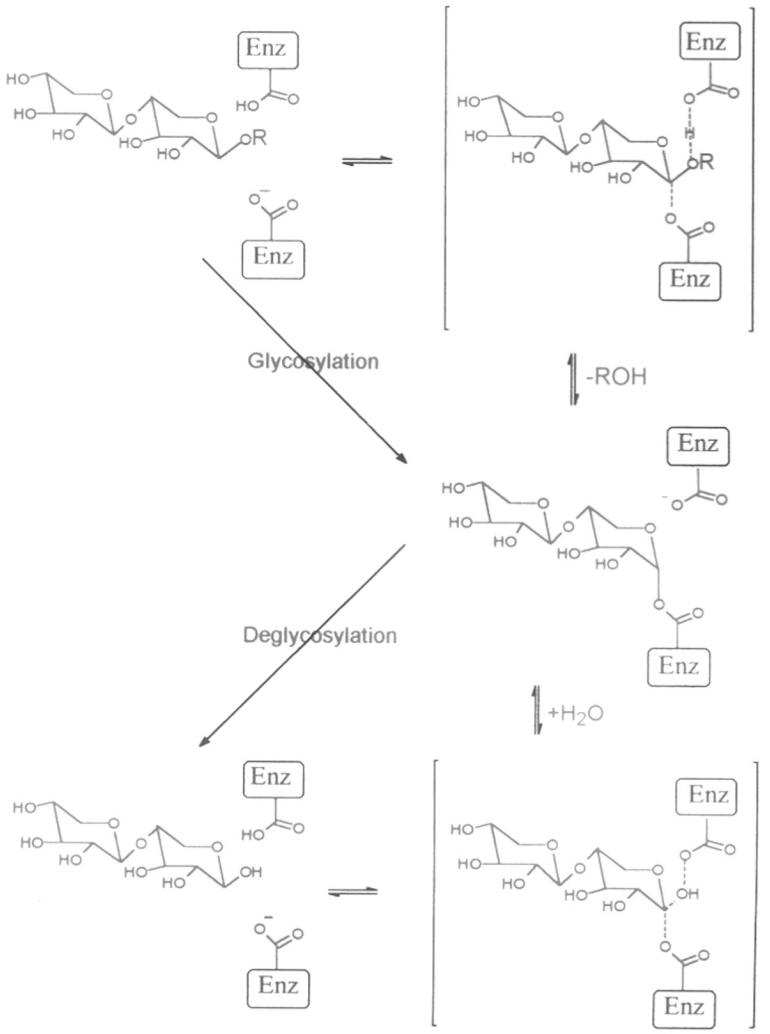
Among the family 11 xylanases, three-dimensional structure of six enzymes has been resolved *viz.* endoxylanases from *B. pumilus* [288,289], *B. circulans* [264], *Trichoderma harzianum* [290], *A. niger* [291] and two xylanases Xyn I [292] and Xyn II [293] from *Trichoderma reesi*. Family 11 xylanases fold into a "β-sandwich" consisting of two pleated antiparallel sheets folded against each other in a parallel manner forming a cleft on one side (Fig 2). The fold is unique and thus far has been observed only in family 11 xylanases. Overall shape of these enzymes is similar with a few minor structural differences. The catalytic residues are properly oriented either by salt linkages (*B. pumilus* xylanase) or by hydrogen bonding. The active site cleft is dominated by aromatic residues and the amino acid residues seen in the active site cleft are all highly conserved. Two catalytic Glu residues are placed at a distance of 5-6 °A, which is typical of configuration retaining glycosyl hydrolases. *B. pumilus* xylanase has an ellipsoidal shape (40x35x35 °A). The catalytic cleft of *A.niger* xylanase contains a large number of aromatic amino acid residues and can accommodate 4 xylosyl residues [291]. An enzyme-tetrasaccharide complex of *B. circulans* xylanase revealed some information on substrate binding [264]. Two Tyr residues (Tyr 69 and Tyr 80), one Trp (Trp 9) and an Arg (Arg 112) were shown to be involved either in stacking interaction or hydrogen bonding with the xylose ring of the substrate. The three dimensional structures of *B. pumilus* and *T. harzianum* xylanases are

similar [289]. The active site residues are completely conserved and occupy nearly identical positions. Overall structure of Xyn I and Xyn II of *T. reesi* [292] is similar but the subsite architecture seems to be different *i.e.* the active site of Xyn I is tighter than Xyn II. The location and orientation of catalytic Glu residues confirm previously identified or suggested nucleophile and acid/base residues.

Lysozyme-type of hydrolytic mechanism in xylanases

The involvement of two acidic residues in glycosidase catalysis was first described for hen egg white lysozyme (HEWL) [294,295]. Since then, studies on glycosyl hydrolases have suggested that the mechanism of hydrolysis resemble that of lysozyme for acid catalyst [246]. Chemical labeling and site-directed mutagenesis studies on xylanases have indicated lysozyme type of action involving two acidic residues. These two residues are totally conserved in both the xylanase families and are surrounded by highly homologous region specific to each family [278]. Experimental evidences from stereochemical studies suggest that xylanases catalyze the hydrolysis of glycosidic bonds through double-displacement mechanism similar to HEWL [278]. Recent progress in establishment of three-dimensional structure of xylanases confirmed the previous results and provided valuable information on the hydrolytic mechanism of xylanases. Hydrolysis by double-displacement mechanism requires a nucleophile to assist the catalytic acid/base residue during proton transfer. The nucleophile and the acid/base catalyst, in order to play their role, would have to be in an ionized and uncharged protonated state respectively, under catalytic conditions. Crystallographic data on xylanases show that the proposed nucleophile and acid/base catalyst Glu residues are located and oriented to fulfill the requirement. The position and orientation of proposed nucleophile (Glu 78) and acid/base catalyst (Glu 172) of *B. circulans* xylanase relative to hydroxyl group of second xylosyl residue was found to be similar to that of Asp 52 and Glu 35 respectively, of HEWL with a trisaccharide. In configuration inverting enzymes, where access of a water molecule is necessary to attack the substrate, a distance of ~10-11°A is observed between the two acidic

residues [285,296,297]. But in configuration retaining enzyme's mechanism, involving formation of a covalent intermediate, the acidic residues are closer to each other (4.5 - 5.5°A) [236,298]. The distance between the proposed catalytic residues (5-6 °A) for xylanases is in agreement with the retaining mechanism [264,284,285, 277,287,291]. The distance between Glu 35 and Asp 52 of HEWL are 5.5 °A [299]. Based on these information, it was proposed that xylanases act through a general double-displacement mechanism involving two acidic residues for catalysis [278] (Fig 3). The fact that the ionization states and the distance between two catalytic carboxyl residues play a critical role in double displacement mechanism, has been demonstrated in case of *B. circulans* xylanase [300-302]. Glu 78 of the enzyme acts as nucleophile while Glu 172 acts as a general acid catalyst during glycosylation (protonation of departing aglycone) and then as general base during deglycosylation. ^{13}C-NMR titration of the enzyme labeled with [δ-^{13}C] Glu have revealed the pKa values of 4.6 and 6.7 for Glu 78 and Glu172 respectively, which is in agreement with the apparent pK_a values obtained from pH dependence of K_{cat}/K_m [301]. At the enzyme's pH optimum of 5.7, Glu 78 is deprotonated and Glu 172 is initially protonated. The pK_a of Glu172 drops to 4.2 in a trapped glycosyl intermediate [274]. This large drop in pK_a, is consistent with the role of Glu 172 as a general base catalyst in deglycosylation and appears to be the consequence of both electrostatic repulsion and conformational change [300]. Furthermore, an increase in the distance between catalytic carboxyls by site-directed mutagenesis of nucleophile Glu 78 (E78D) resulted in 1600 - 5000 fold decrease in the rate of glycosylation (first step of hydrolysis) [301]. Decrease in the distance (by carboxymethylation of E78C mutant) resulted in less reduction of activity (16-100 fold) and upon removal of carboxyl group (E 78 C) the enzyme did not even undergo the first step [301]. Whereas increase and decrease in the distance by site-directed mutagenesis of acid/base catalyst (E 172 D and carboxymethylation of E172C mutant, respectively) resulted in 400 and 25 fold decrease in K_{cat}, respectively [302].



R is an aryl group or one or more xylose residues

Fig 3: Proposed double displacement hydrolytic mechanism of xylanases involving two carboxylate residues with the retention of anomeric configuration. Based on *Bacillus circulans* xylanase (From Reference 302).

Complete removal of carboxyl (acid/base) group resulted in total loss of activity on xylan and phenyl xylobiosides-which require acid catalyst. However, the mutant hydrolyzed aryl xylobiosides, which need little protonic assistance and sodium azide increased the hydrolysis significantly [302]. Absence of acid/base catalyst was partially compensated by anionic nucleophile. Based on the above observations it was concluded that the positional requirements for proton transfer are less demanding than those for carbon oxygen bond formation [302].

Protein unfolding and thermodynamic studies

The study of protein unfolding is of importance both in academic as well as applied fields and the thermal unfolding can give valuable information on the conformational stability of the protein [303]. Differential Scanning Calorimetry (DSC) is a thermal analysis technique that is most commonly used to generate the information regarding the presence of domains, the strength of interaction between the domains, effect of substrate binding on domain stability and interactions and to characterize the thermostability of the proteins [304-307]. Xylanases have been studied in terms of their production, expression, isolation, mode of action, biotechnological exploitation and more recently in terms of their three dimensional structure and mechanism of action, but very little is known about their structural stability, folding and unfolding. DSC studies on two xylanases from *S. halstedii* [308] and exoglucanase/xylanase Cex from *C. fimi* [309] have revealed some valuable information on these aspects.

S. halstedii produces two xylanases *viz.* Xys 1L (45 kDa) and Xys 1S (33-35 kDa) which originates from proteolytic cleavage of Xys 1L on the C-terminal end. Thermal denaturation of Xys 1L revealed that the enzyme has 3 thermodynamically independent domains whereas Xys 1S has two. Xys 1L showed three transitions and Xys 1S showed two, which is the consequence of unfolding of each discrete domain through single step. Enzyme activity measurements showed that the loss of enzyme activity takes place strictly in the temperature region of last high temperature transition. The conformational

stability of Xys 1L domains at 30°C (physiologically relevant temperature) is 5-9 kcal/mol, which seemed to be adequate. Estimation of molecular mass of Xys 1L domains from the calorimetric data (on the assumption of constant mean heat sorption value per amino acid residue) gave the mass of 10, 14 and 21 kDa for 1st 2nd and 3rd domains respectively, which is in good agreement with Xys 1S.

Cex from *C. fimi* has two domains *viz.* a catalytic domain and a CBD. The enzyme had a melting temperature (T_m) of 64.2°C at pH 7.0 and was independent of scan rate. The T_m of isolated CBD and catalytic domains were 64°C and 64.7°C respectively, suggesting that neither domain significantly influences the stability of the other and the interaction between them are weak if any [309]. Additivity of denaturation enthalpy (ΔH) gave a stronger verification of the lack of significant interdomain interactions. An inactivation model was developed for accurate prediction of thermostability and inactivation kinetics [309].

Protein unfolding studies on hyperthermophilic bacterium *T. maritima* xylanase A and its CBD suggested that the domains fold independently [310]. Renaturation of the unfolded CBD could be obtained up to 80% and it showed high intrinsic stability in presence of glucose as a ligand. Guanidine hydrochloride induced unfolding of CBD leads to biphasic transitions but the differences in transition profiles monitored by fluorescence emission and dichroic absorption indicate multi-state behavior of xylanase A. At acid pH, xylanase A exhibits increased stability and the temperature induced increase in negative ellipiticity of CBD, at 217 nm was attributed to alterations in the environment of aromatic residues [310].

Xylanase A from *P. fluorescens* contains a single calcium binding site and isothermal titration calorimetry revealed that the enzyme binds calcium with a K_a of 4.9 X 10^4 M^{-1} [311]. Presence of calcium protected the enzyme from proteolytic and thermal inactivation. Removal of calcium did not significantly influence the catalytic activity of the xylanase, suggesting the role of calcium binding domain is to increase the stability against the thermal inactivation and to

protect against proteolytic attack. Site-directed mutagenesis studies revealed that Asp 256, Asn 261 and Asp262 play a pivotal role in calcium binding [311].

Protein engineering of xylanases

Biotechnological applications of xylanases demand the identification of enzymes that are optimally active at extremes of pH and temperature. This objective can be achieved either by screening for novel xylanolytic thermophiles (as they are expected to act optimally under the optimal growth conditions) or by engineering the protein genetically or chemically to meet the requirements. A comprehensive understanding of the structure–function relationship of the enzyme is an essential prerequisite for protein engineering studies. Recently, three-dimensional structures of a few xylanases from both the families have been solved and the knowledge about the enzyme structure-function relationship should make the protein engineering studies more meaningful.

Random mutagenesis of the cloned fragment from *B. pumilus* [291] resulted in alteration of specific activity and thermal stability of the mutants. Four mutants, each with single amino acid substitution, were selected on the basis of activity at 60°C. The mutant G38S showed 5-fold decrease in specific activity whereas G38D showed 80% increase. Site specific mutagenesis of conserved Arg156 and Asn173 into R156E, R156K and N173D of *S. lividans* xylanase A resulted in increased specific activity and thermal half life at 60°C. Double mutant R156E and N173D showed increased half-life but a decreased specific activity [312,313]. The replacement of Asn173 by Asp also resulted in alteration of cleavage mode and transglycosylation reactions. Xylanase A yielded almost equal amounts of X_2 and X_3 when X_5 was used as the substrate whereas, the mutant yielded 90% of X_2 and 10% of X_3. The transglycosidation products of xylanase A with X_5 were X_6, X_7 and X_8 but the mutant produced very low levels of these oligosaccharides. Based on these results it was proposed that the negative charge introduced by mutation greatly affected the transglycosylation reactions catalyzed by the enzyme [304]. Substitutions were made for 23 conserved residues and the mutant enzymes were

studied [305]. The mutants H86K, S87A, Q88E, N173D, H86W, D50N, F155Y, R156K showed more activity as compared to the wild type enzyme. However, a few mutants showed marked decrease in the specific activity. Replacement of conserved H207 with Arg resulted in decrease in the catalytic activity but showed a different pH-profile as compared to the wild type enzyme.

In case of *B. circulans* xylanase, the thermostability of the enzyme was increased by the introduction of both intramolecular and intermolecular disulfide bonds [312]. Favorable geometry for the introduction of disulfide bonds was chosen by 3-D structure and computer modeling. Single intramolecular and intermolecular, double intramolecular, double intermolecular and triple intramolecular bonds were introduced to the enzyme. The 3-D structure of the mutant containing single intramolecular disulfide bond (S100C, N148C) was solved and the structure was found to be completely isomorphus with the wild type. Mutants with intermolecular disulfide bonds were less active than the wild type enzyme. Double and triple intramolecular disulfide mutants were less active but were stable even at 69°C whereas, the wild type enzyme was stable up to 55°C. One of the mutants, TS6 (A1C, G187C), containing a disulfide bond that joins the N and C terminus of the enzyme showed increased activity at 60°C. It was also noted that the buffers used had a great influence on the thermostability of the mutants. In presence of ammonium acetate buffer the mutants got inactivated rapidly at 65°C whereas, they were stable at same temperature in MES buffer.

Biotechnological application of xylans and xylanases

Purified xylan is an excellent additive in tableting, which has a potential for delayed drug release [315]. Dietary xylan has been shown to play a role in the regulation of human immune system [316]. Xylose is presently being used in food and pharmaceutical industry and could also serve as starting material for the synthesis of speciality products. Xylo-oligosaccharides have potential application as food thickeners, which could substitute fats [150], and as antifreeze food

additive [317]. Hydrolytic products of xylan can be converted into liquid fuel, single cell protein and solvents [318] and low calorie sweeteners [319].

Traditionally, the application of xylanases was viewed from the point of bioconversion [71,320]. Bioconversion is one possibility being considered for dealing with the massive accumulation of agricultural, forestry and municipal solid waste [321]. The enzymic hydrolysis of xylan is extremely useful in the production of oligosaccharides from the isolated xylans [322,323]. The oligosaccharides thus produced (mainly X_2 and X_3) are used as functional food additives or alternative sweeteners with certain beneficial properties [71]. From the hydrolytic products of xylan, either higher xylo-oligomers can be prepared or other sugar residues can be added by using transglycosylation activity of some of the xylanolytic enzymes [324, 325]. These sugars may be useful for research as well as for their rheological properties [150,317,318,320]. Xylanases, along with pectinases and cellulases can be used in food industry for the maceration of fruit and vegetable materials [59,78,86,326-329] and for the clarification of juices and wines [59,76,150,327]. Xylanases can also be used in extraction of coffee [59,330], plant oils [331,332] and starch [86,317,329]. The use of xylanases has been suggested in bakery [317, 333,334] and they were found to increase the specific volume, textural properties and shelf life of the product [334]. Xylanases can be used to modify the staling properties of the bakery product [335]. The use of xylanases has also been suggested in improving the nutritional properties of agricultural silage [336-338] and nutritive properties of grain feed [339-342]. Except for ruminant organisms, dietary hemicellulose has little nutritional value. These undigested fibers increase the viscosity of the food in the gut and may support pathogenic conditions especially in broiler chicks [343-348]. These problems can be corrected by the use of xylanase in poultry feed which increases the nutritional value of the feed [339,340,342]. Xylanases can be used in the production of dissolving pulps (dissolving pulps are purified celluloses used for making viscose rayons, cellulose esters and cellulose ethers) [349] to remove

undesired hemicellulose content as these hemicelluloses can lead to color, haze, thermal instability, and may hamper the derivatization of cellulose [350,351].

Recent studies have shown that the xylanases from microbial origin, preferably free of cellulase activity, hold tremendous promise and potential for use in pulp and paper industry. The potential application of xylanases in pulp and paper industry has been discussed by several authors and related information has been reviewed [3,320,352-364].

Xylanases can be employed in paper industry for debarking, enzymatic beating of pulp for enhancing fibrillation, swelling of the fiber matrix to disrupt adsorptive interaction, solublization of redissolved xylan, breakdown of lignin-carbohydrate interaction, deinking of recycled fibers and prebleaching of kraft pulp. Pulp bleaching is a multistage process, which involves elemental chlorine, ClO_2 or both. Use of chlorinated compounds results in chlorinated hydrocarbons and chloroorganics, which are hazardous. Studies have shown that the use of xylanases in pulp bleaching helps in reducing the kappa number and requirement of bleach chemicals such as elemental chlorine and ClO_2. Enzyme aided prebleaching was also found to be useful in increasing the brightness of the paper. The state of art as well as future prospects in this area has been critically reviewed by Viikari *et al.* [363]. Replacement of ClO_2 by xylanase was found to increase the productivity of the bleaching plant.

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Objectives of the present investigation

Xylanases have drawn increasing attention in recent years, because of their potential biotechnological application in industrial processes. Some of these processes can tolerate or at times demand the presence of other cell wall degrading glycosidases, while others need the xylanase preparations strictly devoid of other enzymes, especially cellulases. The demand for cellulase-free xylanases, which are stable at alkaline conditions and elevated temperatures, is growing in recent years. Use of xylanases (of defined substrate specificity and mode of action) in plant cell wall research, structural elucidation of complex cell wall polysaccharides, synthesis of higher oligosaccharides (by the use of enzymes showing transglycosylation activity) and the role of microbial xylanases in plant pathogenesis is of academic and scientific interest. Actinomycetes are good source of xylanolytic enzymes and the studies are concentrated mainly on *Streptomyces* and other thermophilic actinomycetes. *Chainia* sp. (NCL-82-5-1) - a sclerotium forming actinomycete produces cellulase-free extracellular xylanases. The present investigation were carried out to a) purify and characterize cellulase-free xylanases b) study the active site nature and structure-function relationship and c) study the subsite architecture and thermodynamic parameters of substrate binding and thermostability of extracellular endoxylanase.

CHAPTER 2

Purification and characterization of extracellular endoxylanase(s) from *Chainia* sp.

SUMMARY

An extracellular cellulase-free endo β-1,4 xylanase from *Chainia* sp. has been purified to homogeneity by ammonium sulfate precipitation, successive chromatography on CM-Sephadex C-50 and Sephadex-G-50 followed by FPLC (Mono S). The molecular mass of the enzyme as determined by SDS/PAGE and Laser desorption mass spectroscopy was 21,000 and 21,485, respectively. The purified xylanase is non-glycosylated protein with a pI of 9.4-9.5. The enzyme is low in basic, aromatic and sulfur containing amino acids and the partial N-terminal sequence exhibits a remarkable homology with other actinomycete and family G/11 xylanases and thus belongs to low Mr/basic, family G/11 glycosyl hydrolases. The pH and temperature optima for the enzyme are 6.0 and 50°C, respectively. The xylanase is stable over a wide range of pH (3-10, 120h) and is stable at 50°C for 24h in pH 6.0. The enzyme has a higher affinity for the oat spelt arabinoxylan as compared to birch wood (acetylated) xylan and the mode of action on xylo-oligosaccharides and xylan suggests that *Chainia* xylanase is a non-debranching endoxylanase with a minimum of four xylose units (xylotetraose) required for the initiation of hydrolytic action.

INTRODUCTION

Major application of xylanases in industrial processes such as prebleaching of the Kraft pulp or in dissolving pulp (which are purified celluloses used for making viscose rayon, cellulose ether and cellulose esters [1]) demands xylanases free from cellulase activity. In order to obtain cellulase-free xylanases attempts have been made to inactivate cellulases with mercurial compounds [2,3], cloning and expression of xylanase genes in homologous and heterologous hosts [4-9], bulk scale purification of xylanases [10] and screening for naturally occurring cellulase negative microbes [11-15]. As would be expected from the structural complexity and heterogeneity of xylans, xylanases vary in their size, shape, substrate and positional specificities as well as catalytic rates [16-20]. A comprehensive understanding of the substrate specificity, mode and mechanism of action and the factors that affect the interactions of these enzymes is an essential pre-requisite for their optimum use in industrial process [21]. Production of oligosaccharides, oligosaccharide intermediates and controlled digestion/ degradation of plant cell wall material by xylanases are the areas of scientific interest. Despite their biotechnological promise only a few xylanases have been studied with respect to their structure-function relationship.

Actinomycetes are emerging as an important source of xylanolytic enzymes [22,23] and studies have been concentrated on *Streptomyces* and other genera. *Chainia* sp. (NCL 82-5-1 also NCIM 2980), a sclerotium forming cellulase negative actinomycete producing extracellular endoxylanases, was isolated in our laboratory [11]. As a pre-requisite for either gene manipulation or engineering the protein for better adaptability in industrial processes, it is essential to study the structure-function relationships of the enzyme. The studies on the purification and extensive characterization of extracellular xylanase(s) from *Chainia* sp. are reported in this chapter.

MATERIALS AND METHODS

CM-Sephadex C-50, 3,5-dinitrosalicylic acid (DNSA), β-mercaptoethanol (β-ME), dithiothreitol (DTT), SDS-PAGE and gel filtration molecular weight markers, Pharmalytes, Sephadex G-10, G-50, G-75, Coomassie Brilliant Blue R-250, Tris, SDS, BSA, xylans (oat spelt and birchwood), xylose, xylobiose, trypsin, 3-(cyclohexylamino-1-propanesulfonic acid) (CAPS), polyvinylidinedifluoride (PVDF) membrane, acrylamide, trifluoroacetic acid (TFA), Tricine and ammonium bicarbonate were obtained from Sigma Chemical Company, USA. Standard xylo-oligosaccharides (from xylotriose to xyloheptaose) were the kind gift of Dr. Peter Reilly (Iowa State University, Ames, USA). Bio-Gel P-2 and P-30 was from Bio-Rad Laboratories, USA. Malt extract, yeast extract, glucose and bactopeptone were from Hi-Media Chemicals, India. All the other chemicals and reagents used were of analytical grade.

Preparation of Remazol brilliant blue (RBB) xylan

RBB xylan was prepared according to Biely *et al.* [24]. To a solution of soluble oat spelt xylan (10 g in 250 ml water), 10 g of the dye was added under constant stirring. The dark solution was first mixed with 50 ml of sodium acetate (2.7 g) and then with 100 ml of 6% NaOH and stirred at room temperature for 1h. The product was precipitated with two volumes of ethanol, collected by centrifugation and washed several times with a mixture of 0.05 M sodium acetate-ethanol, 1:2 (v/v) until the filtrate was practically colorless. The product was then redissolved in 0.05M sodium acetate, reprecipitated as above, desalted by washing with ethanol-water (4:1), dissolved in water and lyophilized.

Preparation of Xylo-oligosaccharides

Soluble oat spelt xylan (5 g in 500 ml water) was hydrolyzed with 5 ml of trifluoroacetic acid for 20 minutes in a boiling water bath. The reaction was terminated by adjusting the pH to 5, with 5N NaOH and the hydrolysate was centrifuged to remove any precipitated material. The clear supernatant was then

passed through Dowex 1-X_8 column (1.5x25 cm, acetate form) to remove acidic sugars. The neutral oligosaccharides eluted, in the void volume, were concentrated to 15 ml on a rotavapor. Individual xylo-oligosaccharides (X_2 - X_8) were then separated by chromatography on Bio-Gel P-2 column (1.5 x 180 cm) at room temperature, using deionised water as the eluant, at a flow rate of 15 ml/h. Each oligosaccharide fraction was pooled, concentrated and rechromatographed twice under the similar conditions. The carbohydrate content in the fractions was monitored by phenol-sulfuric method [25]. The fractionated xylo-oligosaccharides were checked for its purity by HPLC, on a Bio-Rad Aminex 42A column, using deionised water as eluant at 70°C, at a flow rate of 0.5 ml/min. The standard or the sample (20 µl) was injected (Rheodyne model with 20 µl loop) onto the column and the xylo-oligosaccharides were detected with a RI detector (Waters 410). The standard xylo-oligosaccharides were eluted in the order of X_8, X_7, X_6, X_5, X_4, X_3, X_2, X and arabinose with a retention time of *ca.* 12.35, 13.10, 14.05, 15.10, 16.25, 17.70, 19.60, 21.70 and 23.60 min, respectively.

Organism and culture conditions

Chainia sp. (NCL- 82-5-1) was maintained on MXYP (malt extract 0.3%, xylan 1%, yeast extract 0.3% and bactopeptone 0.5%, all w/v) agar slants. The inoculum was developed in MGYP (malt extract 0.3%, glucose 1%, yeast extract 0.3% and peptone 0.5% all w/v) medium by inoculating 10 ml of the medium with a seven day old slant followed by incubation under shaking conditions (200 rpm) at 30°C for 48 h. The fermentation was carried out by transferring 10 ml of the inoculum into 500 ml Erlenmeyer flasks containing 100 ml medium (4% w/v wheat bran and 0.5% w/v yeast extract) and incubating under shaking conditions (200 rpm) at 30°C for 96 hours. The enzyme production was also carried out in a 15 liter-fermenter (New Brunswick Scientific). The inoculum was developed by inoculating 100 ml of wheat bran (4% w/v) and yeast extract (0.3% w/v) medium with a 48h old MGYP liquid culture followed by incubation under shaking conditions (200 rpm) for 48h at 30°C. Ten liters of wheat bran-yeast extract

medium was inoculated with 1 liter of the inoculum in a 15-liter capacity fermenter. The fermentation was carried out at 30°C for 72h at an agitation speed of 600 rpm. A positive air pressure of 3-5 psi was maintained throughout and the maximum enzyme activity was obtained after 64-68h and it remained constant till 80h.

Xylanase Assay

The total reaction mixture of 1 ml contained 0.5 ml of 1% (w/v) soluble oat spelt xylan in 50 mM citrate-phosphate buffer, pH 6.0 and 0.5 ml of appropriately diluted enzyme. The reaction mixture was incubated at 50°C for 30 min and then terminated by the addition of 1 ml of 1% DNSA reagent. The reducing sugars liberated were estimated spectrophotometrically, at 540 nm, according to Miller [26] by using D-xylose as the standard. One unit of xylanase activity is defined as the amount of enzyme that produced one μ mole of xylose equivalents/minute under the assay conditions

Protein determination

Protein concentration, in the culture broth and during purification steps was determined according to Lowry *et al.*[27] using BSA as standard. The molar absorption coefficient of the purified enzyme was determined by weighing out salt-free lyophilized powder of the enzyme (1-5 mg) and dissolving it in 1 ml of deionised water. The protein solutions were diluted (1/2, 1/4, 1/6) and the absorption at 280 nm of the diluted solutions were determined. The protein content of the diluted solution was determined by Lowry's method. The average value of $A^{0.1\%}$ cm, $_{280\ nm}$ from three sets of such experiments was taken as the molar absorption coefficient.

Purification of xylanase

Unless otherwise stated all steps were carried out at 4°C. The cell-free broth (1liter) was brought to 0.65 saturation by the addition of solid ammonium sulfate under constant stirring and left for 30 min. The precipitated protein was

collected by centrifugation (10,000x *g*, 10 min), dissolved in minimum volume of 25 mM sodium phosphate buffer, pH 6.7 and dialyzed extensively against the same buffer. The precipitated material obtained after dialysis was removed by centrifugation (10,000 x *g*, 10 min) and the supernatant was used for the next step.

CM-Sephadex chromatography : To the supernatant obtained from the above step, β-ME was added to a final concentration of 10 mM and adsorbed onto a CM-Sephadex C-50 column (5 x 45 cm), pre-equilibrated at pH 6.7 with the same buffer, at a flow rate of 60 ml/h. The column was then washed with the same buffer, till A_{280} nm of the flow-through fractions was less than 0.01. Subsequently, the bound enzyme was eluted with a linear gradient, 1000 ml total volume, of NaCl (0-500 mM) in sodium phosphate buffer (25-200 mM), pH 6.7. Fractions of 12 ml were collected at a flow rate of 60 ml/h and the active fractions were pooled, concentrated using an Amicon ultrafiltration unit having YM-3 membrane and used for gel filtration.

Gel filtration on Sephadex G-50 : 5 ml of the concentrated enzyme solution (7 mg/ml) was chromatographed on a Sephadex G-50 column (2.5 x 115 cm) equilibrated with 50 mM sodium phosphate buffer, pH 6.7, at a rate of 30 ml/h. Active fractions were collected, concentrated by ultrafiltration and used for further purification.

FPLC : The enzyme sample obtained from the above step was dialyzed extensively against 10 mM sodium acetate buffer, pH 5.2 and subjected to FPLC on a Mono S column (Pharmacia gradient programmer GP-250 plus), pre-equilibrated with the same buffer. Elution of the bound enzyme was carried out, at room temperature, with a gradient of 0-1M NaCl in above buffer. The active fractions were pooled, concentrated by ultrafiltration, dialyzed against distilled water, lyophilized and stored at 0°C till further use.

Electrophoresis

Native PAGE was carried out on 9% (w/v) polyacrylamide slab gel, at pH 4.3, using methylene blue as marker. SDS-PAGE was carried out either according to Laemmli [28] or according to Schagger and von Jagow [29]. Protein bands were visualized either by Coomassie Brilliant Blue R-250 or by silver staining. Analytical isoelectric focusing was carried out in polyacrylamide tube gels using wide range (pH 3-10) as well as narrow range (pH 9-11) Pharmalytes. The pI of the enzyme was also determined by density gradient single column isoelectricfocussing [30].

Relative molecular mass (Mr) determination

Gel filtration : Mr of the enzyme was determined on a Sephadex G-75 as well as Bio-Gel P-30 column (1.5 x 60 cm) equilibrated with 50 mM sodium phosphate buffer, pH 6.7, containing 0.1M NaCl. For calibration, Sigma low molecular weight markers (GF-70) were used.

SDS/PAGE : This was carried out according to Laemmli using 11.5% (w/v) gel [28]. Sigma low molecular weight markers (SDS 70L) were used to calibrate the gel.

Laser desorption mass spectra (LDMS) : LDMS of the purified enzyme was performed on a Reflek/II-2387 mass spectrometer on a DHB matrix using water as the solvent.

Carbohydrate content

Carbohydrate content was determined both for native and denatured enzyme. The enzyme was denatured with 6M guanidine hydrochloride for 24h and dialyzed against distilled water to remove any non-covalently adsorbed sugars. 500 μg of xylanase (in 400 μl water) was incubated with 400 μl of 5% (w/v) phenol for 10 min at room temperature. 2 ml of sulfuric acid was added and the mixture was allowed to cool for 20 min at room temperature. The total carbohydrate was then estimated spectrophotometrically, at 490 nm, using D-glucose as the standard[25].

Amino acid analysis

Salt-free lyophilized enzyme sample (200 μg) was hydrolyzed with 6N HCl (200 μl) containing 0.05% phenol and 0.025% β-ME, *in vacuo,* at 110°C for 22 h. Analysis was carried out on a Shimadzu amino acid analyzer with a fluorescent detector. Post column derivatization was done by orthophthalaldehyde (OPA). Tryptophan [31] and cysteine [32] contents were determined spectrophotometrically.

Electroblotting and N-terminal sequence

Electroblotting of the enzyme, onto PVDF membrane, was carried out according to LeGendre *et al.*[33]. The purified xylanase fractions were separated on SDS/PAGE (10% w/v gel, pH 8.3). After the electrophoresis, the gel and PVDF membrane were sandwiched between 3 mM Whatman paper and placed in the blotting cassette. The tank was filled with 10 mM CAPS buffer, pH 11.0, containing 10% methanol and the electrotransfer was carried out under a constant current of 250 mA for 40 min. The PVDF membrane was then washed several times with Milli-Q water and stained with Coomassie Brilliant Blue R-250. The N-terminal amino acid sequence of the first 30 residues of the enzyme was determined by subjecting the blot to Edman degradation on an automated protein sequencer (Shimadzu model PSQ-1).

Zymogram analysis

The purified enzyme and culture filtrate were separated on native PAGE at pH 4.3 and visualized according to Beily *et al.* [24]. After the electrophoresis, the gel was overlaid on a preheated (50°C) RBB-xylan-agar gel (0.2% w/v RBB-xylan and 1% w/v agar in 50 mM citrate-phosphate buffer, pH 6.0) and incubated at room temperature (30°C) for 45 min. The clearance zones were visualized by washing the RBB-xylan-agar gel with ethanol: buffer (2: 1).

Preparation of anti-serum

The homogenous enzyme preparation obtained from FPLC (major xylanase) was used as antigen. The enzyme, in Freund's complete adjuvant was subcutaneously injected into six month old New NewZeland white rabbits. Booster doses were given on the 21st and 30th days. The rabbits were bled when the titer reached 1:64 (40th day) and subsequent isolation of the anti-xylanse polyclonal antibodies from the serum was performed according to Dunbar and Schwoebel [34]. The blood was allowed to clot for 4h at room temperature and the serum was decanted and centrifuged (2000 x g, 10 min) to remove blood cells. To 30 ml of serum, 20 ml of saturated ammonium sulfate solution (~78% w/v) was added slowly under constant stirring and the mixture left at 25°C for 30 min. The precipitate was collected by centrifugation (8000 x g, 10 min), resuspended in 25 ml of 20 mM sodium phosphate buffer, pH 7.4, dialyzed extensively against the same buffer and loaded onto a DEAE-cellulose column (1.5 x 20 cm) equilibrated with the same buffer. The column was washed with the above buffer till the flow-through fractions showed no detectable absorbance at 280 nm. The flow-through fractions containing IgG were pooled, dialyzed against 50 mM sodium phosphate buffer, pH 7.0, concentrated by Amicon ultrafiltration (YM-5 membrane) and stored at -20°C till further use. These antibodies were used for Ouchterlony immunodiffusion studies.

Peptide mapping

Tryptic digest of the xylanase sample was prepared according to Stone and Williams [35]. The enzyme (250 µg) was dissolved in 50 µl of 8M urea, prepared in 400 mM ammonium bicarbonate (pH 8.0). The solution was incubated with 10 µl of 5 mM DTT at 50°C for 15 min and after lowering the temperature to 30°C, 5 µl of 10 mM iodoacetamide was added. The mixture was incubated for 15 min and 1 ml of 100 mM ammonium bicarbonate (pH 8.0) was added to reduce the urea concentration to 0.4M. The protein was digested with trypsin (1:50 w/w) for 24h at 37°C. Reverse phase FPLC (Pharmacia gradient programmer GP 250 plus) of

the tryptic digest was carried out on Pep Rpc C_{18} column (Pharmacia) by monitoring at 220 nm. The digest was injected into the column equilibrated with 0.1% TFA and the peptides were eluted with a linear gradient of 0 - 50% acetonitrile, in 0.1% TFA, over 70 min at a flow rate of 0.6 ml/min.

Mode of action

This was carried out by incubating the xylo-oligosaccharides (X_3 to X_6, 1.0 mg each) and oat spelt xylan (2 mg) with 1U (~2μg) of the purified enzyme, at 50°C, and pH 6.0 (50 mM citrate-phosphate buffer) for 24 h. Aliquots (100 μl) were withdrawn at different time intervals (2 min to 24 h) and the reaction was terminated by incubating the mixture in boiling water bath for 3 min. The products were then analyzed by HPLC, as described earlier. The peaks were identified by comparing the retention times with those of standard xylo-oligosaccharides.

RESULTS

Purification of xylanase: The elution profile of the enzyme from CM-Sephadex and FPLC is shown in Fig 1. The xylanase obtained after FPLC was found to be homogeneous by PAGE (Fig 2A), isoelectric focusing (Fig 2B), SDS/PAGE and LDMS. The specific activity of the enzyme increased from an average of 3.75 U/mg of protein in the crude broth to 550 U/mg after FPLC. Thus the resultant purification is 150 fold with 50% overall recovery (Table I). The $A^{0.1\%}$ cm, $_{280\ nm}$ of the purified *Chainia* xylanase was 1.40

Physicochemical properties : Determination of Mr of the purified xylanase by gel filtration using Sephadex G-75 and Bio-Gel P-30 gave a value of 5400 (Fig 3a) and 2000 (Fig 3b), respectively. However, by SDS/PAGE (Fig 4) and Laser desorption mass spectroscopy (Fig 5) it was 21,000 and 21,485, respectively. The pI of the enzyme was 9.4-9.5. The native enzyme contained 1-2% of neutral

sugar. The amino acid composition of the xylanase is listed in Table II. The xylanase had Ala as the N-terminus amino acid and Fig 6 shows the N-terminal sequence of the enzyme.

Optimum pH and pH stability : The xylanase had an optimum pH of 6.0 and showed 75% of the optimum activity at pH 4.5 and 8.0, respectively (Fig 7). The enzyme showed high pH stability and retained significant amount of its activity (>70%) between pH 3-9 for 120h, at 30°C (Fig 7).

Optimum temperature and temperature stability : The temperature optimum of the enzyme was 50°C (Fig 8) and it showed 80% of the activity at 60°C. Purified xylanase was fully stable up to 24 h at 50°C but lost 50% of the activity at 55°C (Fig 8). The plot of log (V_{max}/K_m) versus pH (pH-activity profile) was bell shaped implying the involvement two ionisable groups with pK_a of pH 5.1 and pH 7.3 in the catalytic activity of the enzyme (Fig 9A). The affinity of the enzyme towards the substrate decreased with temperature and the energy of activation of the enzyme was 35 kcal/mole (Fig 9B).

Kinetic parameters

The xylanase showed a K_m of 8.19 and 3.58 mg/ml and V_{max} of 1.99 and 0.59 mmole/mg/min, for soluble oat spelt and birch wood xylan respectively. In case of xylo-oligosaccharides, the enzyme could not hydrolyze X_2 and X_3 even after prolonged incubation (24h) but detectable hydrolysis of X_4 was observed only after 60 min of incubation. The kinetic parameters like, K_m, turnover number (k_{cat}) and the specificity constant (k_{cat}/K_m) for xylan as well as xylo-oligosaccharides are listed in Table III.

Substrate specificity and mode of action

The purified xylanase could not release any arabinose from oat spelt xylan even after prolonged incubation (24 h) suggesting it to be a non-debranching enzyme. Under the conditions of assay the enzyme had very little or no activity on starch, cellulose and CM-cellulose. The HPLC analysis of hydrolytic products of

soluble xylan, yielded intermediate size fragments at early stage of enzymatic action while lower oligosaccharides (X_3, X_2 and a small amount of X with traces of higher oligosaccharides) were obtained as the end products (Fig 9). During the hydrolysis of xylo-oligosaccharides, the enzyme was unable to hydrolyze X_2 and X_3 while almost complete hydrolysis was observed for X_4 X_5 and X_6. The main cleavage products of X_5 and X_6 were X_3, X_2 and a small amount of X and X_4 while the major hydrolytic products of X_4 were X_2 and X_3 along with small amount of X (Fig 9). No higher oligosaccharides other than those used for hydrolysis could be detected under the experimental conditions even after extensive (90%) hydrolysis of the substrate.

Table I . Summary of purification of xylanase from *Chainia* sp.

Step	Protein (mg)	Activity (Units)	Sp.activity (unit/mg)	Fold purification	Recovery (%)
Culture filtrate	3250	12,000	3.7	—	100
$(NH_4)_2SO_4$ precipitation	760	9,900	13	3.5	82.5
CM-Sephadex chromatography	35	8,700	248	67	72.5
Sephadex G50 chromatography	14	7,300	520	140	60.8
FPLC (Mono S)	11	6,100	550	150	50.8

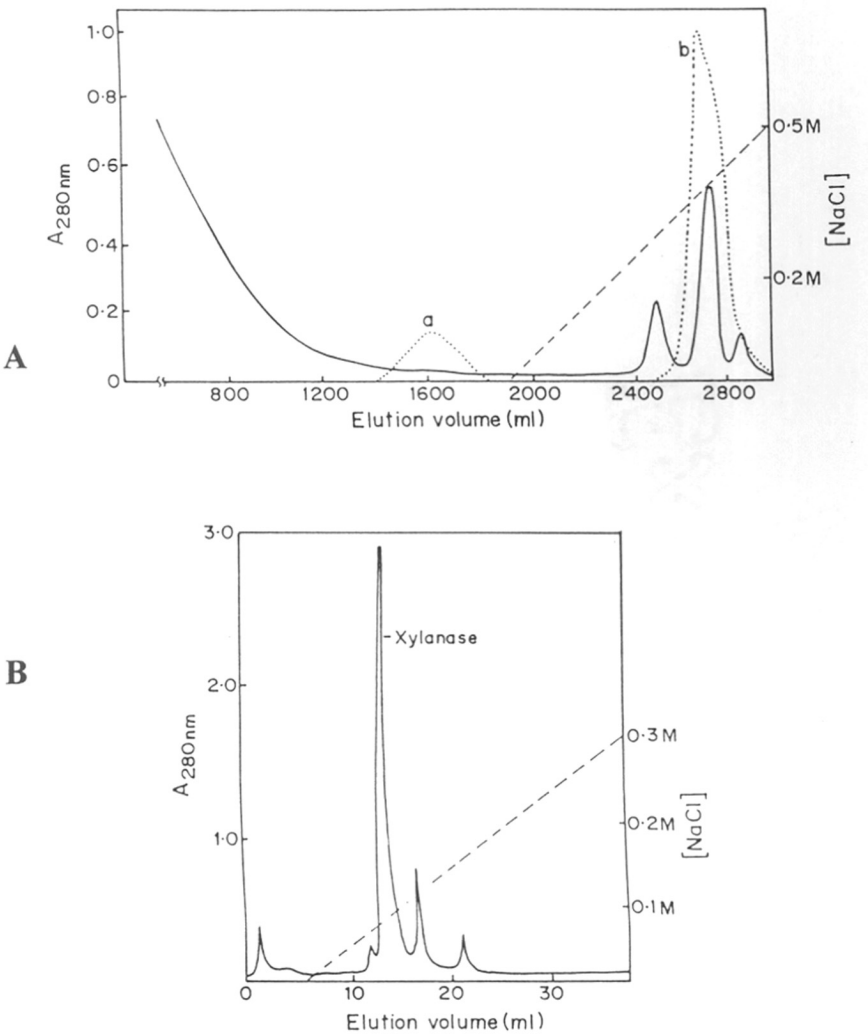


Figure 1 . Elution profile of *Chainia* xylanase

A) **From CM-Sephadex :** —— A_{280} nm, ... xylanase activity (*a* - minor xylanase fraction and *b*- xylanase) and ---- NaCl concentration

B) **From FPLC :** —— A_{280} nm and --- NaCl concentration

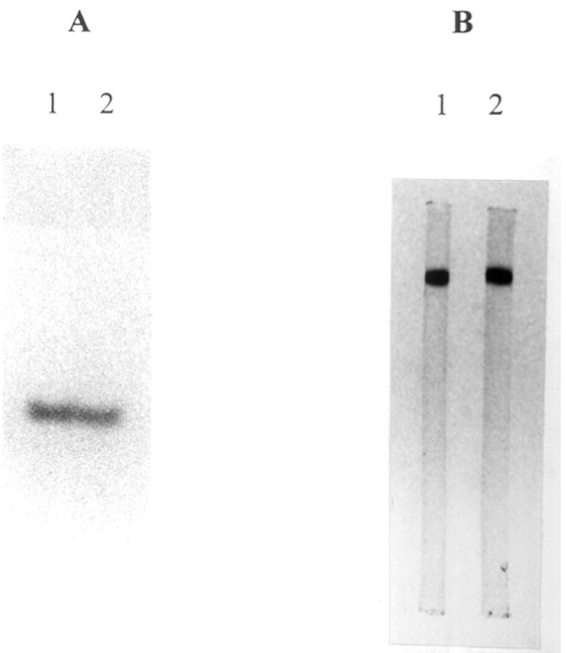


Figure 2 : Electrophoresis of purified xylanase

A) Xylanase (25 μg) was electrophoresed in 9% (w/v) polyacrylamide gel at pH 4.5, and the protein was visualized with Coomassie Brilliant Blue R-250. Lane 1 – xylanase and lane 2 – minor xylanase fraction

B) The enzyme (50 μg) was focused in 7.5% (w/v) polyacrylamide tube gel using wide range Pharmalytes (pH 3-10) and the gels were stained with Coomassie Brilliant Blue R-250. Lane 1 – xylanase and lane 2 – minor xylanase fraction.

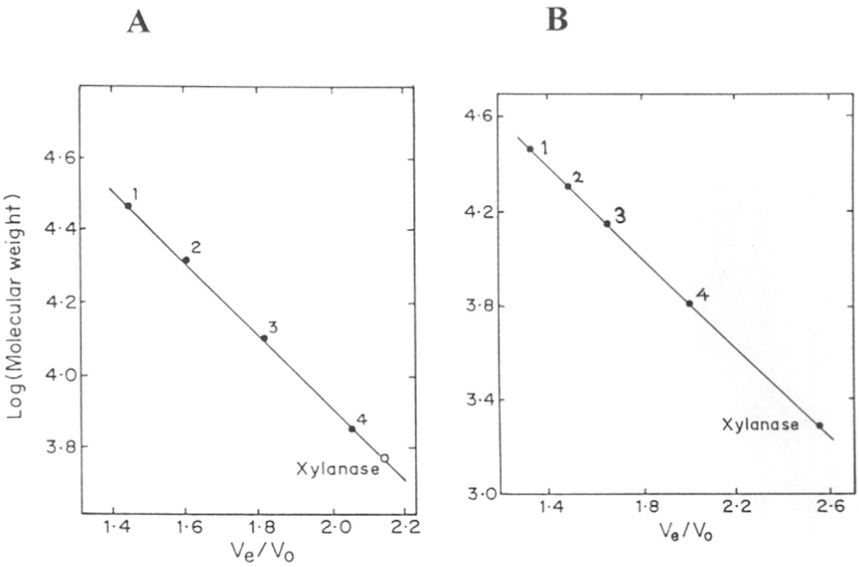


Figure 3 . Molecular weight determination of *Chainia* xylanase by Gel filtration

A. Sephadex G-75 column (1.5 x 60 cm) was equilibrated with 50 mM sodium phosphate buffer, pH 6.7, containing 100 mM NaCl and calibrated with (1) carbonic anhydrase [29,000], (2) soyabean trypsin inhibitor [20,400], (3) cytochrome C [12,400] and (4) approtinin [6,600]. V_o = void volume and V_e = elution volume

B. Bio-Gel P-30 column (1.5 x 60 cm) was equilibrated with 50 mM sodium phosphate buffer, pH 6.7, containing 100 mM NaCl and calibrated with (1) carbonic anhydrase [29,000], (2) soyabean trypsin inhibitor [20,400], (3) cytochrome C [12,400] and (4) approtinin [6,600]. V_o = void volume and V_e = elution volume

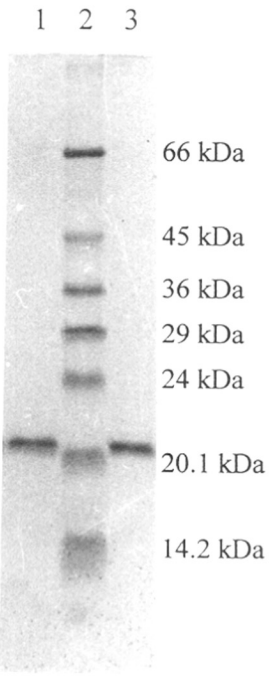


Figure 4: Determination of molecular weight by SDS-Polyacrylamide gel electrophoresis :

The xylanase was electrophoresed using 11.5% (w/v) polyacrylamide gel containing 0.1% SDS according to Laemmli at a constant 200 V for 4h at 25°C and stained with Coomassie Brilliant Blue R-250. Lane 1 and 3 xylanase and minor xylanase fraction respectively. Lane 2 – Molecular weight markers : BSA (66,000), ovalbumin (45,000), glyceraldehyde-3-phosphate dehydrogenase (36,000), carbonic anhydrase (29,000), chymotrypsinogen (24,000), soyabean trypsin inhibitor (20,100) and α-lactalbumin (14,200)

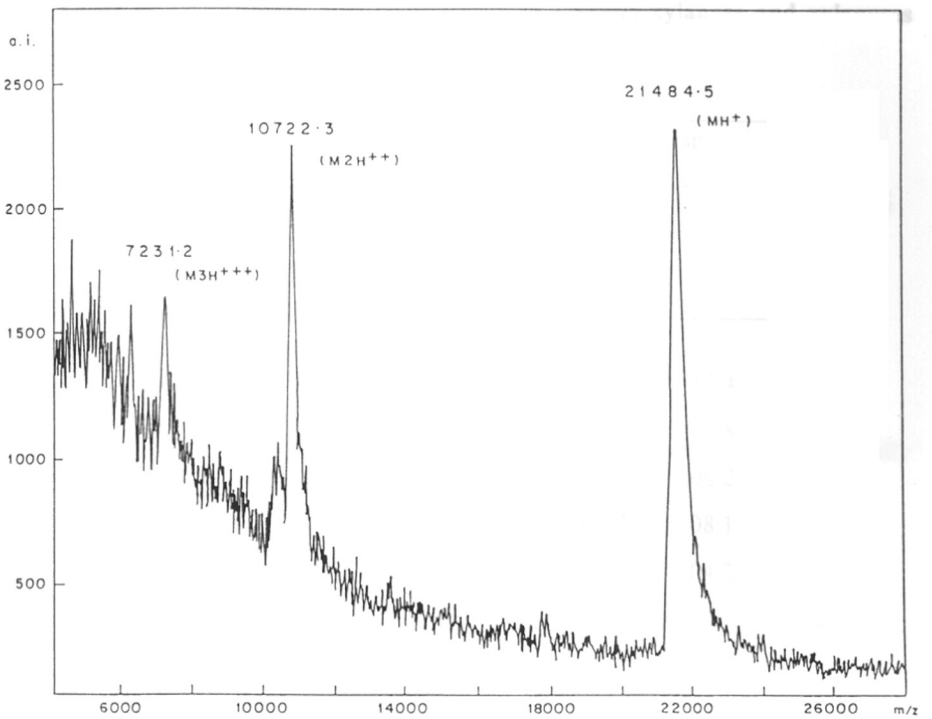


Figure 5. Laser Desorption Mass Spectrum (LDMS) of the xylanase from *Chainia* sp. The mass analysis showed a single initial spectral line corresponding to 21485 mass units.

Table II . Amino acid composition of *Chainia* xylanase and xylanases from *Streptomyces* sp.

Amino Acid	*Chainia* sp.	*Streptomyces* sp.				
		Strain 3137			Strain E-86	Strain KT-23
		X-1	X-IIA	X-IIB		
Asx	**26**	13.1	13.7	11.5	12.4	09.5
Thr	**09**	07.7	14.0	11.6	07.8	09.5
Ser	**38**	07.9	13.9	10.4	08.2	17.5
Glx	**37**	08.8	04.5	05.7	08.1	14.9
Pro	**06**	02.5	02.1	03.3	04.2	02.8
Gly	**32**	12.4	13.3	15.9	11.7	16.3
Ala	**14**	09.4	03.3	03.8	09.4	09.0
Val	**06**	06.0	05.2	06.1	05.4	04.8
Met	**02**	01.3	01.2	01.6	01.5	n.d
Ile	**03**	03.5	02.5	02.2	04.0	02.8
Leu	**04**	05.9	04.3	04.0	05.6	04.3
Tyr	**06**	03.4	08.6	08.2	03.9	01.2
Phe	**02**	02.8	03.1	03.3	04.1	02.0
His	**04**	01.9	01.0	01.5	02.2	03.1
Lys	**03**	03.1	02.3	02.7	03.8	03.1
Arg	**03**	05.1	03.4	03.5	05.8	02.5
Trp	**03**[a*]	02.6*	02.9*	03.1*	n.d	n.d
Cys	**01**[b*]	02.7	00.9	01.3	01.9	0.60

* Determined spectrophotometrically

a) Spande and Witkop [31] b) Ellman [32]

Figure 6 . N-terminal sequence of *Chainia* xylanase

a) A T T I T T N Q T G Y ? G M Y Y S F W T----

b) A T T I T T N Q T G Y D G M Y Y S F W T D G G G S V S M T L N ---

a) minor xylanase fraction and b) xylanase

Comparison of N-terminal sequence of *Chainia* xylanase with Family G/11 xylanases.

Ss36a.	1	ATTIT NETGYD GMYY SFWTD GGG SVSMTLN-	30
Sl C	1	ATTITTNQTGTD GMYY SFWTD GGG SVSMTLN-	31
C sp.	1	ATTITTNQTGYD GMYY SFWTD GGG SVSMTLN-	31
Bp	1	RTITNNEMGNHS GYDY ELWKD YGN TSMTLN-	30
Sl B	1	DTVVTTNQEGTNNGYYY SFWTD SQG TVSMNMG-	32
Ca	1	PKTITSNEIGVNGGYDY ELWKD YGN T SMTLK-	31
Tr 2	1	QTIQPGT GYNNGYFY SYWND GHG GVTYTNG-	30
Th	1	QTIGPGT GYSNGYYY SYWND GHG GVTYTNG-	30
Sc	1	SGTPSSTGTDGGYYY SWWTD GAG DATYQNN-	30
Bc	1	ASTDYWQNWTD GGG IVNAVNG-	20
An	1	SASTDYWQNWTD GGG IVNAVNG-	21

Ss – *Streptomyces* species 36a, Sl C– *Streptomyces lividans* xylanase C, C sp. –*Chainia* sp., Bp – *Bacillus pumilus*, Sl B - *Streptomyces lividans* xylanase B, Ca – *Clostridium acetobutylicum*, Tr 2 – *Trichoderma reesi* xylanase 2, Th – *Trichoderma harzianum*, Sc – *Schizophyllum commune*, Bc – *Bacillus circulans* and An – *Aspergillus niger*. (sequences are taken from Reference 47)

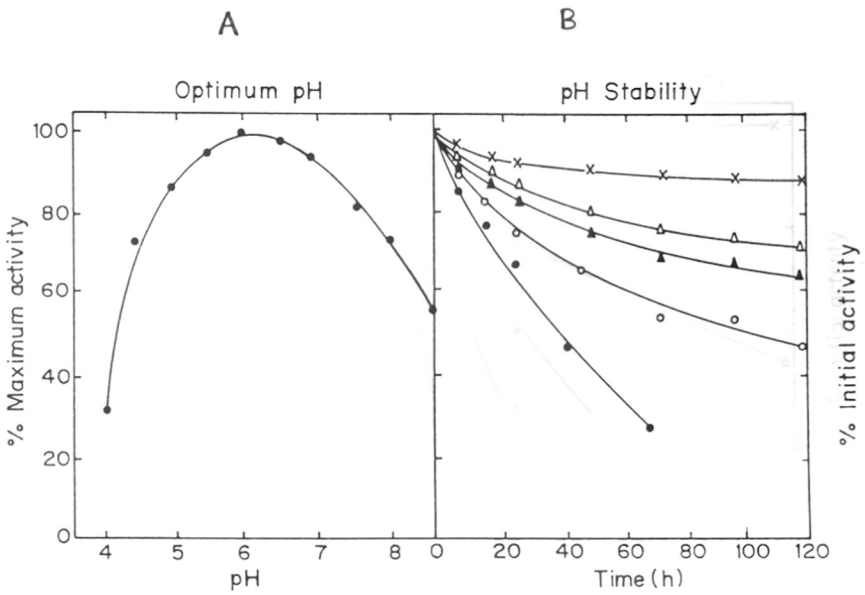


Figure 7 . Optimum pH and pH stability

A) Optimum pH : Purified xylanase (0.1U) was assayed in a series of pH (4-8.5) at 50°C as described in Methods and the maximum enzyme activity obtained was taken as 100%.

B) pH stability : Purified xylanase (10 μM, 1 ml) was incubated in a series of pH (2-10) at room temperature for 120h. Aliquots were removed at different time intervals, appropriately diluted and assayed under standard assay conditions. Symbols used are; —●— pH 2.0, —▲— pH 3.0, —×— pH 6.0, —△— pH 9.0 and —○— pH 10.0

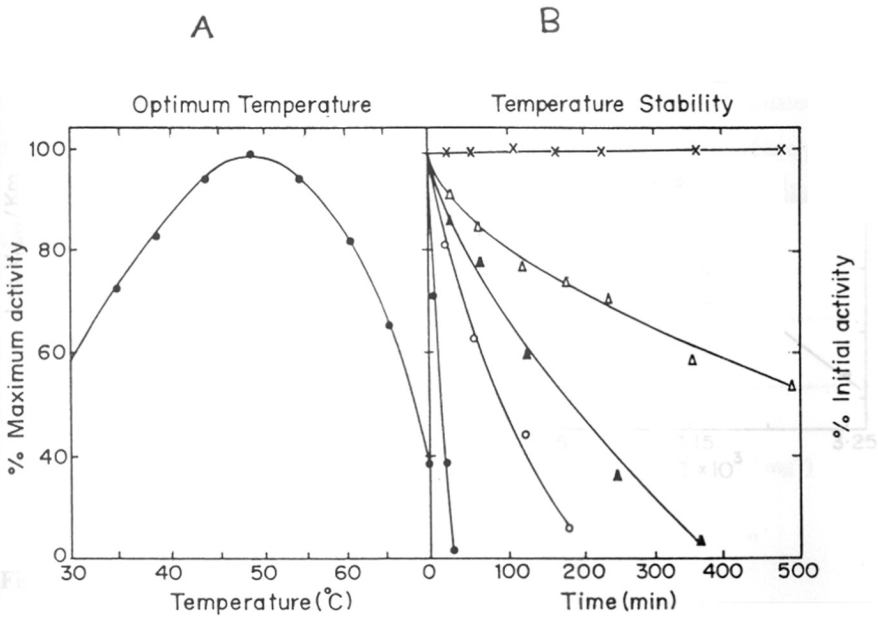


Figure 8 . Optimum temperature and temperature stability

A) **Optimum temperature** : Purified xylanase (0.1U) was assayed at various temperatures ranging from 35-65°C at pH 6.0, as described in Methods. The maximum enzyme activity obtained was taken as 100%.

B) **Temperature stability** : The enzyme (20 μM, 1 ml) was incubated different temperatures (50-70°C) for 24h at pH 6.0. Symbols used are; —●— 70°C, —○— 65°C, —▲— 60°C, —△— 55°C and —×— 50°C

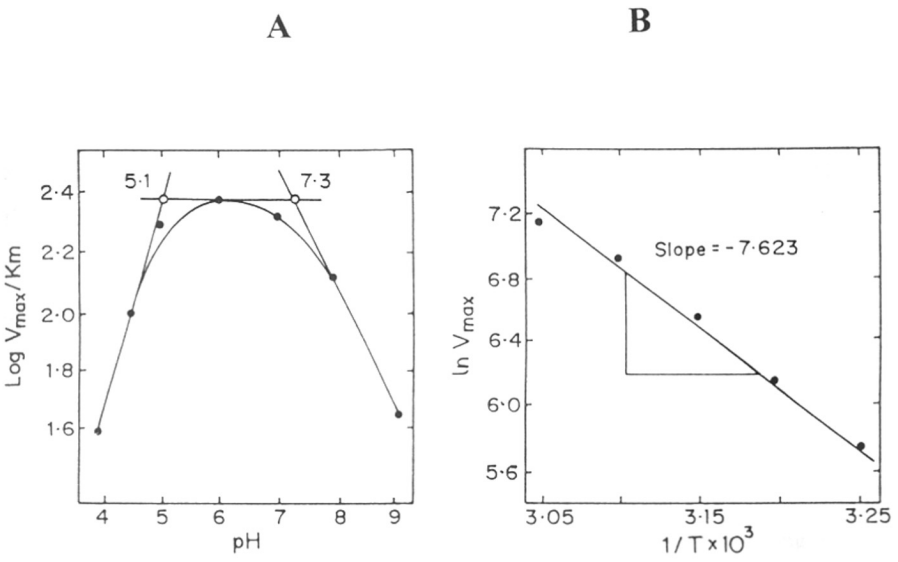


Figure 9.

A) **pH activity profile of *Chainia* xylanase :** The K_m and V_{max} values were determined at different pH (4-9) by using the substrate concentration in the range of 1-5 mg of oat spelt xylan and by fitting the data to linear regression using Lineweaver-Burk plots.

B) **Arrhenius plot :** The K_m and V_{max} values were determined at different temperature in the range of 30-55°C using the substrate concentration in the range of 1-5 mg of oat spelt xylan and by fitting the data to linear regression using Lineweaver-Burk plots. Either ln V_{max} or ln $1/K_m$ was plotted against 1/T and the energy of activation was calculated by using the equation;

E_a = -0.219 x slope where, E_a is energy of activation in calories.

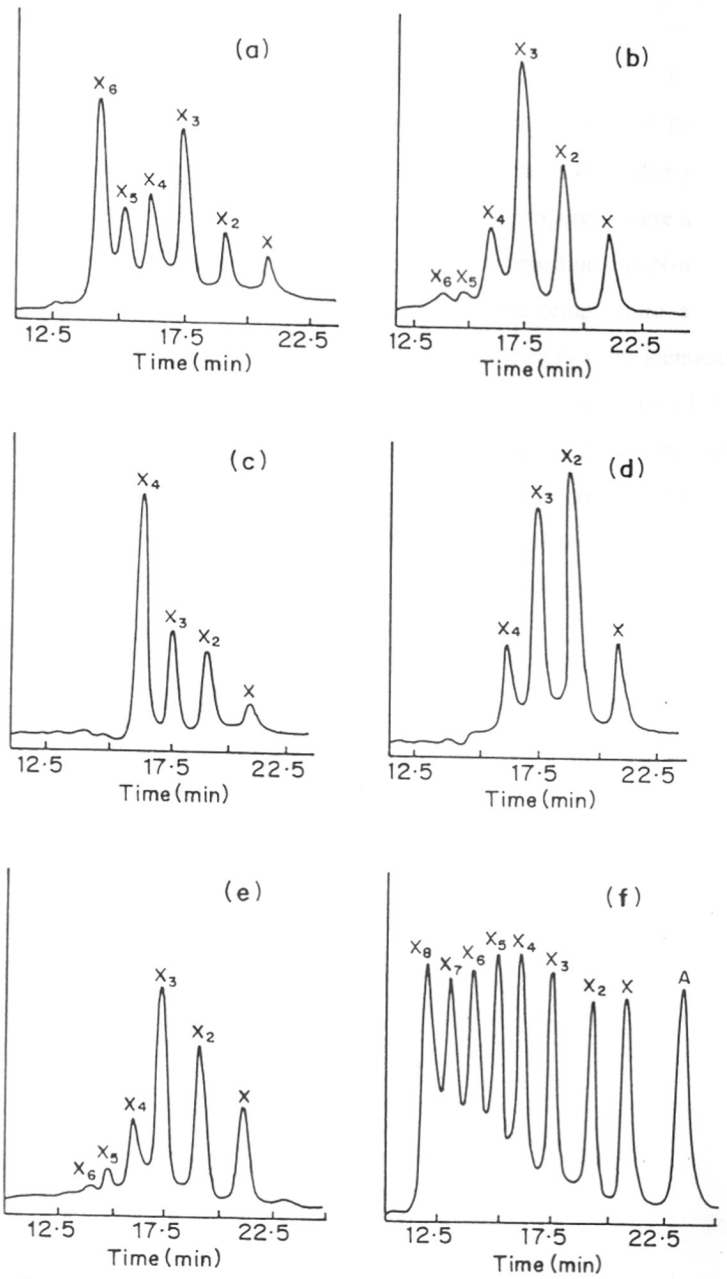
Table III . Kinetic constants for different substrates of *Chainia* xylanase

The values were determined under standard assay conditions by using the substrate concentration in the range of 1-5 mg for xylan and 2.8-5.0 mM for xylo-oligosaccharides. The kinetic constants (K_m and k_{cat}) were calculated by fitting the data to linear regression using Lineweaver-Burk or Eadie-Hofstee plots as well as by fitting them to non-linear least square analysis with Enzfitter (Biosoft) and Origin 4.0 (MicroCal).

Substrate	k_{cat} (min^{-1})	K_m	k_{cat}/K_m
Oat spelt xylan	1988.8	8.19 mg/ml	242.8 (min^{-1} mg^{-1})
Birch wood xylan	595.8	3.58 „	166.4 „
Xylo-oligosaccharides [a]			
X_4	54.9	6.60 mM	8.32 (min^{-1} mM^{-1})
X_5	61.5	6.36 „	9.67 „
X_6	279.8	14.76 „	19.00 „
X_7	202.4	7.23 „	28.00 „

[a] Reducing sugar estimation was done by the method of Somogyi [51].

Figure10 : Hydrolysis of xylan and xylo-oligosaccharide by xylanase from *Chainia* sp. The substrates (1 mg each of xylo-oligosaccharides and 2 mg oat spelt xylan) were incubated with 1 unit of purified xylanase in 50 mM citrate-phosphate buffer pH 6.0 at 50°C and subjected to HPLC as described in Materials and Methods. a) X_6, 2 min, b) X_6, 1 h, c) X_4, 1h, d) X_4, 24 h e) xylan, 1h and f) separation and retention times of standard xylo-oligosaccharides


(a)
X6
X5
X4
X3
X2
X
12·5
17·5
22·5
Time (min)
(b)
X3
X2
X4
X
X6 X5
12·5
17·5
22·5
Time (min)
(c)
X4
X3
X2
X
12·5
17·5
22·5
Time (min)
(d)
X2
X3
X4
X
12·5
17·5
22·5
Time (min)
(e)
X3
X2
X4
X
X6
X5
12·5
17·5
22·5
Time (min)
(f)
X8
X7
X6
X5
X4
X3
X2
X
A
12·5
17·5
22·5
Time (min)

Characterization of minor xylanase fraction

A minor xylanase activity peak (10-15% of total activity) was observed in the initial washings of CM-Sephadex column (Fig 1) in the absence of β-ME or DTT, which merged into the main peak in the gradient in presence of β-ME or DTT. This minor fraction was collected separately, concentrated, further purified to homogeneity by subjecting to FPLC (as above) and the properties were studied. The physical properties viz. Mr (Fig 4), pI, amino acid composition and N-terminal sequence (Fig 6) as well as the catalytic properties like temperature and pH optimum and stabilities, mode of action and kinetic parameters were identical to that of the major xylanase. This minor xylanase fraction showed total immunological cross-reactivity with the antibodies raised against the major xylanase (Fig 11a). Activity staining of crude extracellular broth and purified xylanase fractions showed only one clearance zone at identical positions (Fig 11b). Tryptic digestion followed by reverse phase FPLC of major xylanase and the minor xylanase yielded a similar peptide fragmentation pattern (Fig 12).

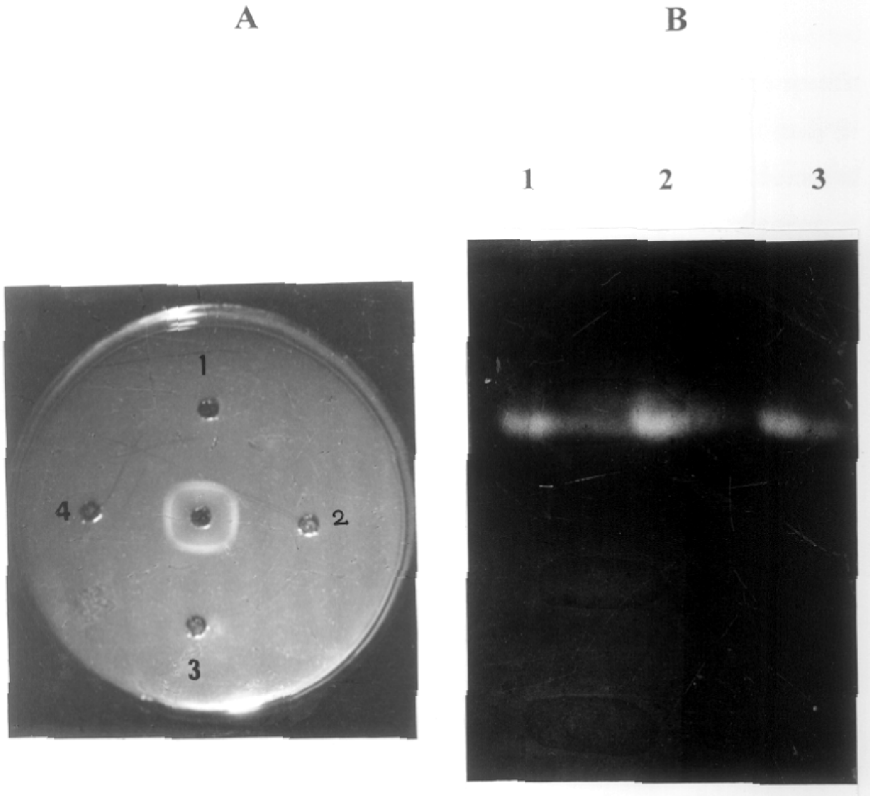


Figure 11

A) **Ouchterlony immunodiffusion** : The purified xylanase preparations (200 µg) was cross-reacted with purified anti-xylanase antibodies raised against purified xylanase (center well, 1 mg) in a 3 mm thick 1% agarose plate (prepared in 50 mM sodium phosphate buffer, pH 7.0, containing 100 mM NaCl) for 60 h at 4°C. Wells 1and 3 : xylanase and 2and 4 : minor xylanase fraction.

B) **Zymogram :** *The enzyme was separated on 9% PAGE at pH 4.5 was* overlaid on a preheated (45°C) RBB-xylan - agar gel and incubated for 45 min at 30°C and the xylanase activity was visualized by washing the gel with ethanol : buffer (2:1, v/v). Lane 1: purified xylanase (5 units), lane 2 : minor xylanase fraction (5 units) and lane 3 : culture broth (5 units)

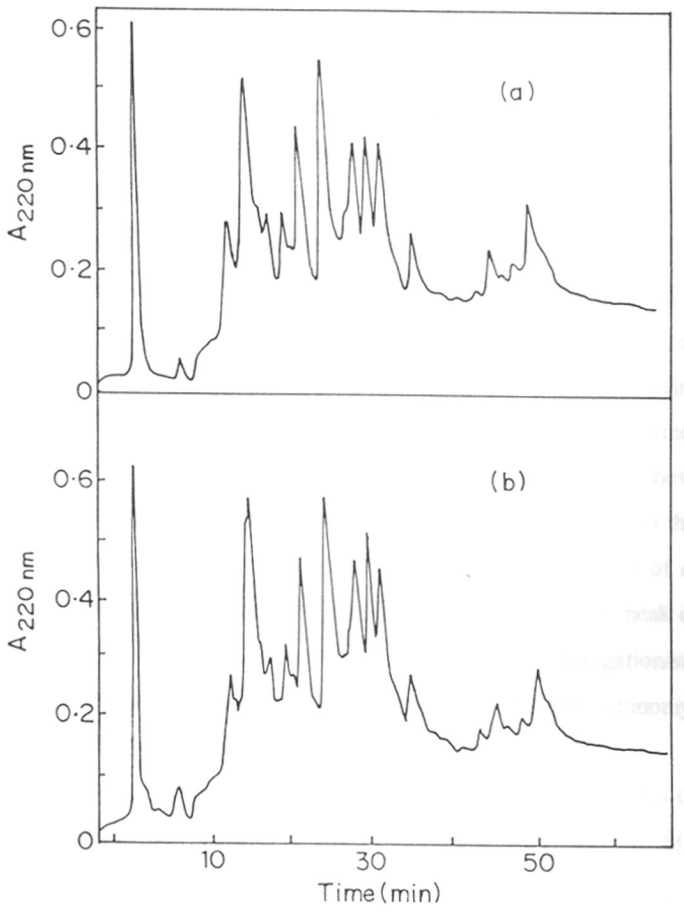


Figure 12 . Peptide mapping

Purified xylanase (250 µg) was digested with trypsin (1:50 w/w) at 37°C for 24h and the peptides were separated by reverse phase FPLC as described in Methods. a) tryptic digest of purified xylanase and b) tryptic digest of minor xylanase fraction

DISCUSSION

The extracellular endoxylanase purified from *Chainia* had a high specific activity of 550 U/mg and could be obtained in good yield. In the present study β-ME was added prior to CM-Sephadex chromatography because in the absence of β-ME, 10-15% of the total enzyme activity came out unadsorbed. However, in presence of β-ME this fraction could not be detected and only one enzyme species could be obtained after CM-Sephadex chromatography. Interestingly the physical and functional properties of this minor peak were similar to that of major xylanase suggesting that both the species are same. The total immunological cross-reactivity of this minor xylanase with the antibodies raised against the major xylanase (Fig 11a) and a similar peptide fragment pattern (Fig 12) of both the enzyme species substantiates the above view. Furthermore, the crude culture broth showed only one xylanase activity band and the position of the band was identical to that of the purified xylanase fractions (Fig 11b) pointing towards the presence of only one extracellular xylanase in *Chainia* sp. The appearance of the minor peak observed in absence of β-ME can be correlated to nonspecific aggregation/sulfhydryl interaction(s). Our studies show that *Chainia* differs from other actinomycetes in that it produces only one extracellular endoxylanase [22].

The Mr determined by gel filtration on Sephadex G-75 and Bio-Gel P-30 was 5,400 (Fig 3a) and 2000 (Fig 3b), respectively. However, the Mr of the enzyme when determined by SDS/PAGE was 21,000 (Fig 4). A more accurate determination by LDMS gave a molecular mass of 21485 (Fig 5) consistent with SDS/PAGE. Such anomalous behavior on gel filtration is not uncommon for xylanases [36-41] and it has been suggested that ionic or hydrophobic interactions with the gel matrices could lead to increased retention times and there by leading to underestimation of mass [39]. The pI of the purified xylanase is 9.4-9.5 suggesting that it belongs to low Mr/basic, family G/11 glycosyl hydrolases. The enzyme is not a glycoprotein since the denatured enzyme did not reveal the presence of carbohydrate moiety. This data suggests that the low amount of carbohydrate found in the native enzyme was non-covalently bound saccharides.

Amino acid composition revealed that the enzyme is rich in Gly and Ser while low in sulfur containing (Cys and Met), basic (His, Arg and Lys) as well as in aromatic (Trp, Tyr and Phe) amino acids (Table II). Compared to xylanases from *Streptomyces* species [42], *Chainia* xylanase showed higher amount of Gly, Ser and Ala. The xylanase from *Bacillus subtilis* and *Schizophyllum commune* of the same family too possess a high content of Gly and Ser and this reflects in 3D structure of all known family G/11 xylanases, which consists largely of β-sheets [43-47]. Comparison of N-terminal sequence of *Chainia* xylanase with other actinomycete and family G/11 xylanases showed a considerable homology (Fig 6). The N-terminal sequence of *Streptomyces lividans* xylanase C [48] is exceptionally similar, with just Thr 11 being replaced by Tyr in *Chainia* xylanase. Despite their N-terminal sequence homology, the enzymes from *S. lividans* and *Chainia* differ with respect to their pI, temperature stability and xylan hydrolytic end products [49].

The pH activity profile of the enzyme revealed the participation of two ionisable groups with a pK_a of pH 5.1 and 7.3 (Fig 9A) in the catalytic activity of the enzyme. The pH optimum (6.0) of the enzyme is similar to other actinomycete endoxylanases [22]. The enzyme from *Chainia* showed good activity at neutral and alkaline pH, retaining 75% of the activity at pH 8.0. The enzyme is considerably stable over a wide range of pH (pH 3-10, 120h) which is similar to endoxylanases from other actinomycete and *Streptomyces* species. The xylanase is stable at 50°C for 24h and this is significant from biotechnological point of view.

Chainia xylanase showed a less affinity (K_m) for oat spelt xylan as compared to birchwood xylan. However, assuming only one active site, the turnover rate (k_{cat}) and the catalytic efficiency (k_{cat}/K_m) for soluble oat spelt xylan were 3.3 and 1.4 fold, higher than the birchwood xylan (Table III). The higher efficiency observed could be due to the nature of these xylans, one being an arabinoxylan (oat spelt) and the other acidic (birch wood) [17]. Thus, the substitution by acetyl esters in birchwood xylan may be limiting the access of the enzyme to the substituted xylopolymer chain more than the 1,3-arabinofuranoside

found in oat spelt xylan. A higher (k_{cat}/K_m) would also reflect the ability for multiple attacks on the arabinoxylan polymer chain [49]. Amongst the xylo-oligosaccharides the enzyme was unable to hydrolyze X_3 and had the best and weakest affinities for X_5 and X_6, respectively. As expected, the rate increased from X_4 to X_6 and then decreased slightly for X_7. The catalytic efficiency increased by a factor of 3.5 with an increase in the oligomer size from X_4-X_7 (Table III). X_4 was the minimal chain length degraded after prolonged incubation (16-24h) suggesting it to be the smallest possible substrate. The relative rates of hydrolysis of X_4 - X_7 indicate that a chain of 4 xylosyl residues is required for initiation of the attack. It also suggests that the active site of the enzyme could have four subsites, similar to that of xylanase from *Clostridium thermolacticum* [36] and lysozyme [50]. Higher oligosaccharides, other than those used for hydrolysis, could not be detected under the experimental conditions used, implying the absence of xylosyl transferase activity.

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CHAPTER 3

Active site characterization of *Chainia* xylanase

SUMMARY

Chemical modification of the endoxylanase from *Chainia* sp. with group specific chemical modifiers in absence and presence of substrate and kinetics of modification revealed the involvement of a thiol, a carboxylate and a guanidinium group in the catalytic function of the enzyme. Sequence alignment of the chemically labeled peptide with other G/11 family xylanases showed that the carboxylate of *Chainia* xylanase is located in a highly homologous region and may function as acid/base catalyst while thiol of the Cys may function as a nucleophile. The failure of the substrate (xylooctaose) to quench the fluorescence of the Arg modified enzyme indicates the involvement of Arg in substrate binding. Modification of Trp by N-bromosuccinimide leads to inactivation of the enzyme through peptide cleavage although substrate binding quenches the enzyme fluorescence. Analysis of quenching of enzyme fluorescence by acrylamide, potassium iodide and cesium chloride suggest that all the Trp residues are located in a similar moderately hydrophobic environment.

INTRODUCTION

Identification of specific amino acid residues involved in the catalytic activity of the enzymes and the specific role played by them is important in understanding the mechanism of action and structure-function relationship of enzymes. The effect of chemical modifiers on enzyme activity, kinetic measurements of inactivation process and determination of amino acid residues involved, can lead to important information on active site and structure-function relationship of enzymes. Though xylanases have been extensively studied with respect to their functional properties and potential industrial application, the structure-function relationship of this class of enzymes has not been studied in detail. Although the catalytic mechanism of lysozyme and cellulases, which are functionally related to xylanases, has been studied in detail [1-5], very few reports exist on active site characterization of xylanases. The involvement of carboxyl group [6-10], Cys [11,12], Trp [12,13], Arg [10] and Tyr [14] in the catalytic activity of xylanases has been reported. Hence modification of these residues were carried out to assess their role in the structure-function relationship of *Chainia* xylanase.

MATERIALS AND METHODS

Oat spelt xylan, phenylglyoxal, N-ethylmaleimide (NEM), 2,2'-dithiobis nitrobenzoic acid (DTNB), phenylmethylsulfonylfluoride (PMSF), p-hydroxymercurybenzoate (PHMB), diethylpyrocarbonate (DEP), 3-nitro-L-tyrosine ethylester (NTEE), 1-ethyl-3-(3-dimethylaminopropyl) carbodiimide (EDC), 2-hydroxy-5-nitrobenzylbromide (HNBB), iodoacetamide, ascorbic acid, 2,4,6-trinitrobenzenesulfonic acid (TNBS), N-bromosuccinimide (NBS), citraconic anhydride, N-acetylimidazole, 2,3,butanedione (diacetyl), trypsin, ammonium bicarbonate, trifluoroacetic acid (TFA), trichloroacetic acid (TCA), dithiothreitol (DTT), HEPES and MES were from Sigma Chemical Company, USA. All other chemicals and reagents used were of analytical grade.

Preparation of Reagents.

Para-nitrophenylglyoxal (pNPG) : This was synthesized according to the procedure of Steinbach and Becker [15]. A solution of selenium dioxide (3.9 g) in 2.4 ml of distilled water and 15 ml of glacial acetic acid was refluxed, at 120°C, for 1h with 5 g of p-nitroacetophenone. Subsequently, the reaction mixture was cooled and selenium was removed by filtration. The filtrate was then distilled to remove water and acetic acid. The pNPG was then crystallized and recrystallized by cooling the solution of glyoxal. The purity of the product was checked by melting point and IR spectroscopy.

Tetranitromethane (TNM) : TNM was prepared according to Liang [16]. A 250 ml Erlenmeyer flask (with a two-holed stopper) containing 31.5 g (0.5 mole) of anhydrous HNO_3 (sp. gr. ~ 1.60) was cooled to 0°C by ice-salt mixture and 51 g (0.5 mole) of freshly distilled acetic anhydride was added slowly in portions of 0.2-0.3 ml at a time. The temperature of the reaction mixture was always maintained below 5°C. After the addition of about 5 ml, larger portions of acetic anhydride (increasing gradually from 1-5 ml) was added at a time under constant shaking. Subsequently, the mixture was allowed to come to room temperature and allowed to stand for 7 days. TNM was separated by pouring the mixture into 300 ml of water in 500 ml round-bottom flask followed by steam distillation. The heavy product was separated from the upper layer of water, washed first with dilute alkali, then with water and dried over anhydrous Na_2SO_4.

N-(2,4-dinitroanilino) maleimide (DAM) : DAM was synthesized according to Clark-Walker and Robinson [17]. Maleic anhydride (4 g) and 2,4-dinitrophenyl hydrazine (7.5 g) were refluxed in chloroform (80 ml) with occasional shaking, for 90 min. The orange solid obtained after cooling was repeatedly crystallized (4 times) from acetone-benzene to get the yellow needles of dinitroanilinomaleimic acid. The compound (1 g) was refluxed with acetic acid (20 ml) for 10 min and cooled to get yellow imide crystals. The imide crystals were recrystallized

repeatedly (5 times) from acetone-benzene to get pale yellow needles of DAM. The purity of the reagent was checked by melting point and IR spectroscopy.

Glycine methyl ester (GME): To 3.75 g of glycine (0.05 mole) in 30 ml of dry methanol, freshly distilled thionyl chloride (3.65 ml, 0.06 mole) was added drop wise, at 0°C, with constant stirring. The mixture was allowed come to room temperature and stirred for 16h. The solid glycine methylester hydrochloride was collected by filtration and dried in *vacuo*. The purity of the product was checked by melting point.

Organism and culture conditions

Growth, maintenance of *Chainia* sp. (NCL 82-5-1), production and the purification of xylanase were carried out as described in the Chapter 2.

Enzyme assay

The total reaction mixture of 1 ml contained 0.5 ml of 1% (w/v) soluble oat spelt xylan in 50 mM citrate-phosphate buffer, pH 6.0 and 0.5 ml of appropriately diluted enzyme. The reaction mixture was incubated at 50°C for 30 min and then terminated by the addition of 1 ml of 1% DNSA reagent. The reducing sugars liberated were estimated spectrophotometrically, at 540 nm, according to Miller [18] by using D-xylose as the standard (Chapter 2).

Determination of protein concentration

Protein concentration determination of the purified enzyme was routinely performed by using the relationship *viz.* $A^{0.1\%}$ cm, 280 nm = 1.40.

Chemical modification studies

Modification of carboxylate groups

Reaction with 1-ethyl-3-(3-dimethylaminopropyl) carbodiimide (EDC): The enzyme solution (20 μM, 1 ml) in 50 mM MES/HEPES buffer (75:25 v/v), pH 6.0, was incubated with varying concentrations of EDC (10-40 mM)at 30°C. Aliquots were removed at suitable intervals and after terminating the reaction by the addition of 100 μl of 1M acetate buffer (pH 4.5), the residual activity was determined under standard assay conditions. The K_m and k_{cat} values were also determined for partially modified enzyme (residual activity 62, 50 and 31%). Enzyme sample incubated in the absence of EDC served as control. EDC modification was also carried out in presence of glycine methyl ester.

Reaction with EDC / Nitrotyrosine ethylester (NTEE) : The enzyme solution (50 μM, 1 ml) in 50 mM MES/HEPES buffer (75:25 v/v), pH 6.0, was incubated with a total of 50 mM EDC and 30 mM NTEE at 30°C for 45 min. Subsequently the reaction was arrested by the addition of 10%(w/v)TCA and the precipitated protein was collected by centrifugation, washed extensively with chilled acetone, air-dried and dissolved in 100 mM sodium hydroxide. The number of nitrotyrosyl groups incorporated was determined spectrophotometrically, at 430 nm, using a molar absorption coefficient of 4600 M^{-1} cm^{-1} [19].

Isolation of modified carboxylate containing peptide and sequencing : The enzyme solution (250 μM, 2 ml) was treated with EDC/NTEE as above, precipitated by 10%(w/v)TCA, washed with chilled acetone and air-dried. The pellet was dissolved in 250 μl of 8M urea prepared in 400 mM ammonium bicarbonate (pH 8.0). The solution was incubated with 10 μl of 50 mM DTT at 50°C for 15 min and 5 μl of 100 mM iodoacetamide was added to it after cooling to room temperature. The mixture was incubated for 15 min and 2 ml of 100 mM ammonium bicarbonate (pH 8.0) was added. The treated protein was then digested with trypsin (1:50 w/w) for 24h at 37°C [20] and the peptides were

separated by Reverse phase HPLC (LKB LCC 2252) on a C_{18} column (Waters Deltapak, 7.8 x 300 mm). The peptides were eluted with a linear gradient (0-80%) of acetonitrile, in 0.1% TFA over a period of 60 min, at a flow rate of 0.5 ml/min, by monitoring at 220 nm and 430 nm (Pharmacia variable wavelength monitor 2141). The labeled peptide was rechromatographed on the same column and sequenced on an automated gas phase sequencer (Shimadzu PPSQ-10).

Modification of Cysteine residues

Reaction with N-ethylmaleimide (NEM) : The xylanase solution (10 μM, 1 ml) in 50 mM sodium phosphate buffer, pH 7.0, was incubated with varying concentrations of NEM (3-12 mM) at 30°C [21]. Aliquots were removed at different time intervals, passed through Sephadex G-10 and the residual activity was determined under standard assay conditions. Enzyme sample incubated without NEM served as control.

Reaction with p-hydroxymercurybenzoate (PHMB) : The enzyme solution (10 μM, 1 ml) in 50 mM sodium acetate buffer, pH 5.5, was incubated with varying concentrations of PHMB (2-8 μM) at 25°C. Aliquots were removed at different time intervals, passed through Sephadex G-10 and the residual activity was determined under standard assay conditions. Enzyme sample incubated without PHMB served as control. Reactivation of PHMB inactivated enzyme was carried out by incubating the enzyme solution with 1 mM cysteine at pH5.5, at 25°C for 30 min followed by estimating the enzyme activity. The K_m and k_{cat} values of the partially modified enzyme (residual activity 71, 42 and 26%) were also determined.

Reaction with 2,2'-dithiobisnitrobenzoic acid (DTNB) : The xylanase solution (20 μM, 1 ml) in 100 mM phosphate buffer, pH 8.0, was incubated with 0.5 mM of DTNB(effective concentration) at 30°C for 45 min. Aliquots were removed at different time intervals and the residual activity was determined. The modification reaction was followed by monitoring the absorbance at 412 nm and the number of

sulfhydryl groups modified was calculated by using a molar absorption coefficient of 13,600 M^{-1} cm^{-1} [22].

Alkylation of Cys with DAM and isolation of modified Cys containing peptide.

The xylanase solution (50 μM, 10 ml) in 50 mM sodium acetate buffer, pH 5.5, was incubated with 5 mM DAM (effective concentration) for 2h at 30°C and the residual enzyme activity was determined under standard assay conditions. The modified enzyme was precipitated by the addition of 10%(w/v) TCA. The precipitate was collected by centrifugation, washed extensively with chilled acetone and air-dried. The protein was digested with trypsin (1:50 w/w) for 24h at 37°C [20]. The tryptic peptides were then separated by Reverse phase HPLC (LKB LCC 2252) on a C_{18} column (Waters Deltapak, 7.8 x 300 mm). The peptides were eluted with a linear gradient (0-80%) of acetonitrile, in 0.1% TFA, over a period of 60 min, at a flow rate of 0.5 ml/min, by monitoring at 220 nm and 340 nm (Pharmacia variable wavelength monitor 2141). The labeled peptide was rechromatographed on the same column and sequenced on an automated gas phase sequencer (Shimadzu PPSQ-10).

Modification of Arginine

Reaction with 2,3, butanedione : Purified xylanase (10 μM, 1 ml) in 50 mM borate buffer, pH 7.8, was incubated with varying concentrations of butanedione (14-58 mM) at 30°C [23]. Aliquots were removed at suitable intervals and the excess reagent was removed by passing through Sephadex G-10. Subsequently, the residual activity was determined under standard assay conditions. Enzyme samples incubated in the absence of butanedione served as control.

Reaction with phenylglyoxal : The enzyme (10 μM, 1 ml) in 50 mM sodium bicarbonate buffer, pH 8.0, was incubated with varying concentrations of phenyl glyoxal (0.5-2.5 mM) at 30°C [24]. Aliquots were removed at suitable intervals and the excess reagent was removed by passing through Sephadex G-10. Subsequently, the residual activity was determined under standard assay

conditions. The K_m and k_{cat} values of the partially modified enzyme (residual activity 78, 67 and 48%) were also determined. Enzyme samples incubated in the absence of phenyl glyoxal served as control. Reactivation of the phenylglyoxal treated enzyme was achieved by incubating the desalted enzyme in 50 mM Tris buffer, pH 8.5, for 4h.

Reaction with p-nitrophenylglyoxal (pNPG) : The enzyme (20 μM, 2 ml), in 100 mM sodium pyrophosphate buffer, pH 8.5, containing 150 mM sodium ascorbate was incubated with 0.1% (w/v, effective concentration) of pNPG in methanol, at 30°C. Samples were removed at different time intervals and the residual activity was determined under standard assay conditions. The number of Arg residues modified was determined according to Yamasaki *et al.* [25].

Modification of Tyr residues

Reaction with N-acetylimidazole (NAI) : The enzyme (20 μM, 1 ml) in 50 mM sodium borate buffer, pH 7.5, was incubated with 1 mM NAI for 30 min, at room temperature (25°C), followed by the estimation of the residual activity under standard assay conditions. The enzyme incubated in the absence of NAI was taken as control. The number of Tyr residues modified was calculated by using a molar absorption coefficient of 1160 M^{-1} cm^{-1} at 278 nm [26].

Reaction with tetranitromethane (TNM) : TNM was diluted with 95% ethanol to a concentration of 10 mM. The modification was carried out by incubating the enzyme (20 μM, 1 ml) in 50 mM Tris/HCl buffer, pH 8.5, with a total of 20-50 μM of TNM under shaking, at 25°C, for 1h and measuring the residual activity under standard assay conditions. The enzyme incubated in the absence of TNM served as the control. The number of Tyr residues modified was estimated spectrophotometrically, by using a molar absorption coefficient of 4200 M^{-1} cm^{-1} for nitrotyrosine at 428 nm [27].

Modification of Lys residues

Reaction with citraconic anhydride : The purified xylanase (10 mM, 1.9 ml) in 200 mM sodium bicarbonate buffer, pH 8.4, was treated with 100 µl of 200 mM citraconic anhydride (diluted in dioxane) at 25°C. The reagent was added in 5 installments and the pH of the reaction mixture was maintained at 8.4 by the addition of 1N NaOH. Aliquots were removed after each addition and the residual activities were determined under standard assay conditions.

Reaction with 2,4,6, trinitrobenzoic acid (TNBS) : The enzyme (20 µM, 1 ml) in 4% sodium bicarbonate buffer, pH 8.4, was incubated with 1 mM TNBS in dark at 30°C for 4h. Aliquots were withdrawn at suitable time intervals and the reaction was terminated by adjusting the pH to 4.6. Subsequently, the residual activities were determined under standard assay conditions. The number of amino group modified was determined spectrophotometrically, by assuming a molar absorption coefficient of 9950 M^{-1} cm^{-1} at 335 nm for the trinitrophenylated lysine [28].

Modification of His residues

Reaction with diethylpyrocarbonate (DEP) : DEP was diluted with absolute alcohol and the concentration of the DEP was determined by mixing an aliquot of the sample 3 ml of 10 mM imidazole buffer, pH 7.0, followed by monitoring the change in absorbance at 230 nm. The amount of N-carbethoxyimidazole formed was calculated by using a molar absorption coefficient 3000 M^{-1} cm^{-1} [29]. The concentration of the diluted DEP was adjusted to 100 mM and the enzyme solution (20 µM, 1 ml) in 50 mM sodium phosphate buffer, pH 7.0, was incubated with 2 mM of DEP at 28°C for 30 min. Aliquots were removed at different time intervals and the residual activities were determined under standard assay conditions. The modification of the enzyme was also monitored spectrophotometrically, by measuring the change in absorbance at 240 nm as described by Ovadi *et al.* [30].

Modification of Ser residues

Reaction with phenylmethylsulfonyl fluoride (PMSF) : The xylanase (10 μM, 1 ml) in 50 mM sodium phosphate buffer, pH 7.5, was incubated with 1 mM PMSF at 30°C for 2 h. Aliquots were removed at different time intervals and the residual activities were determined under standard assay conditions.

Modification of Trp residues

Reaction with N-bromosuccinimide (NBS) : This was carried out by titrating 1 ml of the enzyme solution (10 - 40 μM) with freshly prepared NBS, at different pH (4.5-6.5). The NBS mediated reaction was followed by monitoring the change in absorbance at 280 nm. The reagent was added in 10 installments (10 μl each) till the protein : NBS ratio reached 1: 10. After each addition, an aliquot (10μl) was removed and quenched by the addition of 20μl of L-Trp (25 mM) and checked for residual enzyme activity under standard assay conditions. The K_m and k_{cat} values were also determined for partially inactivated enzyme. The NBS treated samples were analyzed by Tricine SDS-PAGE [31], RPFPLC (on PepRpc C_{18} column) and Ouchterlony immunodiffusion. The NBS modification was also carried out under denaturing conditions (8M urea, pH 5.5) and the number of Trp residues modified were determined spectrophotometrically, by assuming a molar absorption coefficient of 5500 M^{-1} cm^{-1} for the modified Trp at 280 nm [32].

Modification with 2-hydroxy-5-nitrobenzylbromide (HNBB) : The xylanase (10-25 μM, 400-500 μl) in 50 mM sodium acetate buffer, pH 4.0, was incubated with a total of 20 mM of HNBB freshly prepared in dry acetone. The reaction mixture was rapidly mixed and allowed to react for 10-20 min and the residual activity was measured under standard assay conditions. The number of Trp residues modified was determined spectrophotometrically, at 410 nm, using a molar absorption coefficient of 18,450 M^{-1} cm^{-1}[33]. HNBB reaction was also carried out under denaturing conditions (8M urea, pH 2.7).

Substrate protection studies

In all chemical modification reactions, the effect of substrate protection was studied by incubating the enzyme with excess amount of xylan followed by treatment with various modifying reagents.

CD measurements

The CD spectra of native and chemically modified enzyme samples (5 μM, 2 ml) in 5 mM sodium acetate buffer pH 5.6, were recorded on a JASCO-710 Spectropolarimeter from 190 - 250 nm using 1 cm path length at 25°C.

Fluorescence measurements

Fluorescence measurements were performed on a Perkin Elmer spectrofluorimeter LS 5B, at 25°C, using an excitation and emission slit width of 5 nm. The fluorescence spectrum of native as well as chemically modified enzyme (5 μM, 2 ml) in 50 mM sodium acetate buffer, pH 5.6, both in absence and presence of xylooctaose (X_8) were recorded from 300 - 400 nm using an excitation wavelength of 280 nm. The enzyme solution (5 μM, 2 ml) in the same buffer was titrated with 0.5 mM NBS solution (10 μl aliquot) both in absence and presence of X_8. An excitation wavelength of 295 nm was used and emission recorded from 300 - 400 nm. In a series of parallel experiments, the enzyme solution (5 μM, 2 ml) in the above buffer was titrated with acrylamide (8M), KI (5M) and CsCl (5M). Excitation wavelength of 280 nm was used and the emission was recorded at 338 nm. The quenching data was analyzed by the Stern-Volmer and modified Stern-Volmer equation [34,35] using MicroCal Origin 4.0.

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

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

Where, F_o and F are the fluorescence intensities in the absence and presence of the quencher, f_a is effective fractional accessible fluorescence, K_{sv} is effective quenching or Stern-Volmer constant, [Q] is the quencher concentration and δF is the difference in fluorescence intensity in the absence and presence of quencher

RESULTS

The effect of various chemical modifiers on the activity of *Chainia* xylanase is shown in the Table I. Treatment of the enzyme with DEP resulted in the modification of one His residue with retention of 90% of the original activity. Modification of the enzyme with TNBS resulted in the modification of two Lys residues with a loss of only 10% of its initial activity whereas; citraconic anhydride did not affect the enzyme activity. Similarly, treatment of the enzyme with PMSF did not result in any loss of enzyme activity. Above results suggest that His, Lys and Ser residues do not have a role in the catalytic activity of the enzyme. N-acetylimidazole modification decreases the absorbance at 278 nm of the enzyme, leading to modification of two Tyr residues with only 20% loss of initial activity. The treatment of the enzyme with a more specific reagent, TNM, resulted in the modification of two Tyr residues but led to protein aggregation and precipitation even at low molar ratio (1:5) of the reagent. Carboxylate, Cys and Arg specific reagents inactivated the enzyme indicating the possible involvement of these residues in activity.

Modification of carboxyl group : The purified xylanase when incubated with 50 mM EDC at pH 6.0 and 30°C, for 60 min lost approximately 90% of its activity. However, no loss of activity was observed in the control samples. The log of residual activity plotted as the function of time at various EDC concentrations was linear up to 10% of the initial activity. EDC mediated inactivation followed pseudo-first-order kinetics at any fixed concentration of the reagent (Fig 1). The pseudo-first-order rate constants were calculated from the slopes of the plots of log (percent residual activity) versus reaction time and the order was determined from the plots of log (K_{app}) against log [EDC][36]. These plots (inset of Fig 1) indicated that the loss of activity occurred as result of modification of a single carboxyl group. Similar results were obtained when EDC mediated inactivation was carried out in the presence of glycinemethylester. Modification of the enzyme with EDC in presence of NTEE resulted in the incorporation of 1.15 nitrotyrosyl

residue per molecule of the enzyme suggesting that the inactivation of the enzyme could be due to the modification of single carboxyl group. Though EDC is specific to carboxyl group, it also reacts with sulfhydryl groups. However, the native as well as carboxylate modified enzyme did not show any difference with respect to sulfhydryl content suggesting that the EDC mediated enzyme inactivation was due to the modification of an essential carboxylate and not due to sulfhydryl modification. Inactivation of the enzyme by EDC could be prevented to a considerable extent by incubating the enzyme with excess xylan prior to modification (Table IIA). No change in the K_m values of the partially inactivated enzyme with a concomitant decrease in k_{cat} as compared to the control suggests that the inactivation is due to modification rather than structural changes (Fig 2A, Table IIB). Moreover, the CD spectra of native and EDC modified enzyme (Fig 2B) were almost identical confirming that the modification does not lead to any gross change in the enzyme conformation.

Peptide isolation and amino acid sequencing : The RP HPLC of tryptic digest of carboxyl modified enzyme showed only one peak upon monitoring at 430 nm (Fig 3). N-terminal sequence of the labeled peptide was Ile-Val-Xxx-Gly-Tyr-Phe-Asp-Thr-- (Fig 4). The absence of phenylthiohydantoin derivative in the modified peptide at position three is designated as Xxx and represents the modified Asp/Glu.

Table I : Effect of chemical modifiers on the activity of xylanase from *Chainia* sp. Enzyme (10 μM, 1 ml) was incubated with various reagents at room temperature and after terminating the reaction, residual activities were measured under standard assay conditions.

Reagent	Residual activity(%)	Concen tration.	Buffer
None	100		
TNBS	90	1 mM	Sodium bicarbonate, 100 mM, pH 8.5
Citraconic anhydride	95	5 mM	Sodium bicarbonate, 100 mM, pH 8.5
DEP	90	1 mM	Sodium phosphate, 50 mM, pH 7.5
PMSF	100	1 mM	Sodium phosphate, 50 mM, pH 7.5
DTNB	0	1 mM	Sodium phosphate, 50 mM, pH 8.0
PHMB	0	10 μM	Sodium acetate, 50 mM, pH 5.5
NEM	5	10 mM	Sodium phosphate, 50 mM, pH 7.5
Butanedione	0	0.5%	Sodium borate, 50 mM, pH 7.8
Phenylglyoxal	0	3 mM	Sodium bicarbonate, 50 mM, pH 8.0
pNPG	0	2 mM	Sodium pyrophosphate, 50 mM, pH 8.5
NBS	0	50 μM	Sodium acetate, 50 mM, pH 5.5
HNBB	80	20 mM	Sodium acetate, 50 mM, pH 4.0
NAI	80	10 mM	Sodium borate, 200 mM, pH 7.5
TNM	0	50 μM	Tris-HCl, 50 Mm, pH 8.5
EDC	10	40 mM	MES/HEPES, 50 mM, pH 6.0

Table II A : Influence of carboxyl group modification on the activity of xylanase : Substrate protection studies.

Incubation mixture	Residual activity (%)
Control	100
Enzyme + EDC(40 mM)	10
Enzyme + xylan (5 mg) + EDC	60

Table IIB : Effect of carboxyl group modification on the K_m and k_{cat} values of *Chainia* xylanase: The values were determined under standard assay conditions by using the substrate concentration in the range of 1-5 mg of oat spelt xylan. The kinetic constants were calculated by fitting the data to linear regression using Lineweaver-Burk plots and Origin 4.0 (MicroCal).

Percentage activity	Residue modified	Reagent used	K_m (mg/ml)	k_{cat} (min^{-1})
100	None	---	8.19	1960
62	Carboxyl	EDC	8.25	1175
50	group		8.33	780
31			8.00	610

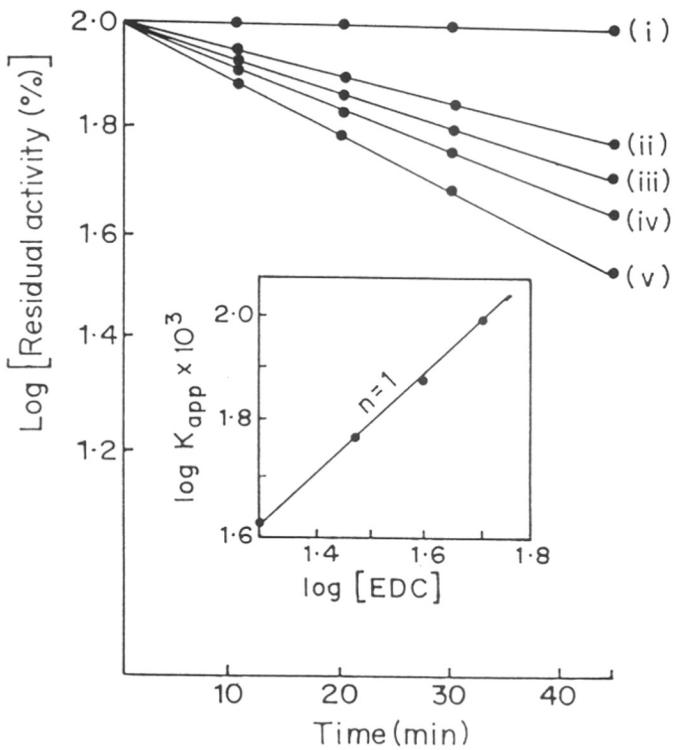


Figure 1.

Pseudo first-order plot for inactivation of *Chainia* xylanase by EDC : Concentrations of EDC were (i) control (ii) 10 mM (iii) 20 mM (iv) 30 mM and (v) 40 mM. Inset : second order plot of pseudo-first order rate constants (K_{app}) (min^{-1}) as a function of log EDC concentration.

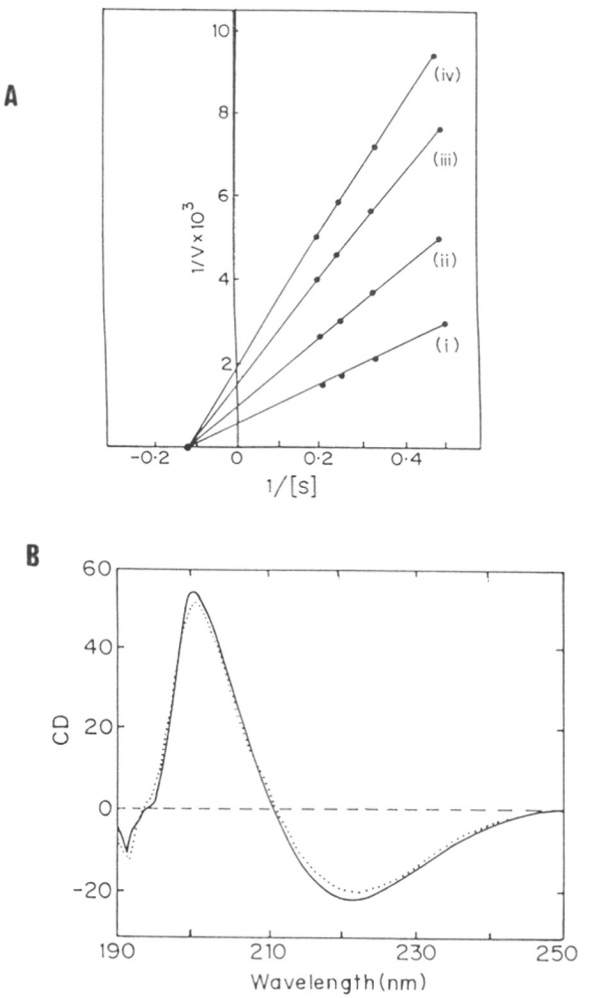


Figure 2.

A) **Determination of kinetic constants of partially EDC inactivated xylanase:** The kinetic constants were calculated by fitting the data to linear regression using Lineweaver-Burk plots and Origin 4.0 (MicroCal). (i) native enzyme, (ii) 38% inactivated, (iii) 50% inactivated and (iv) 69% inactivated

B) **The CD spectra of native and EDC treated xylanase :** The spectra were recorded on a JASCO-710 spectropolarimeter from 190-250 nm using 1 cm path length at 25°C at an enzyme concentration of 5 μM (2 ml) in 5 mM sodium acetate buffer, pH 5.6, at a scan speed of 100 nm/min. Native enzyme (——) and EDC treated enzyme (-----)

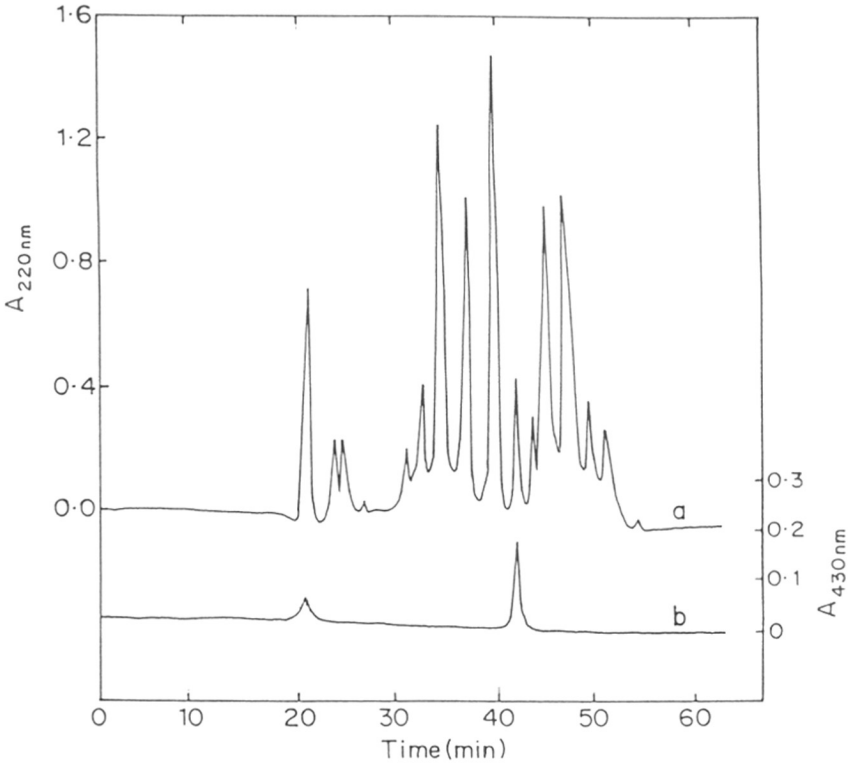


Figure 3.

Reverse phase HPLC of tryptic digest of carboxylate modified enzyme: Tryptic digestion of the modified enzyme was carried out as described in Materials and Methods. The column was eluted with a linear gradient of 0-80% acetonitrile in 0.1% TFA over 60 min at a flow rate of 0.5 ml/min. The effluent was monitored at 220 nm (a) and 430 nm (b)

Figure 4. Alignment of amino acid sequences containing acid/base catalyst of family G/11 xylanases with NTEE labeled peptide from *Chainia* xylanase.

1		–	–	–	–	–	–	–	I	V	**X**	G	Y	F	D	T	–	–
2	–	–	H	Y	Y	Q	I	V	A	T	**E**	G	Y	F	S	T	G	–
3	–	M	Y	E	–	T	A	F	T	V	**E**	G	Y	Q	S	S	G	–
4	–	M	Y	E	–	V	A	L	T	V	**E**	G	Y	Q	S	S	G	–
5	–	M	–	D	Y	Q	I	V	A	V	**E**	G	Y	F	S	S	G	–
6	–	M	H	E	–	T	A	F	N	I	**E**	G	Y	Q	S	S	G	–
7	–	T	L	Y	E	V	S	L	N	I	**E**	G	Y	R	S	N	G	–
8	–	M	–	N	Y	Q	V	V	A	V	**E**	G	W	G	G	S	G	–
9	–	E	H	N	Y	Q	I	V	A	T	**E**	G	Y	Q	S	S	G	–
10	–	L	Y	E	–	A	K	V	L	G	**E**	A	Y	V	K	N	G	–
11	–	N	W	A	Y	Q	V	M	A	T	**E**	G	Y	Q	S	S	G	–
12	–	N	W	A	Y	Q	V	M	A	T	**E**	G	Y	Q	S	S	G	–
13	–	S	W	S	Y	Q	V	M	A	T	**E**	G	Y	Q	S	S	G	–
14	–	F	R	Y	Y	M	I	M	A	T	**E**	G	Y	Q	S	S	G	–
15	–	F	S	Y	Y	M	I	M	A	T	**E**	G	Y	Q	S	S	G	–
16	–	F	–	N	Y	Q	V	M	A	V	**E**	A	W	S	G	A	G	–

The sequences are obtained from SWISS-PROT, PIR (and also Reference 68) and the accession numbers are indicated in the bracket. 1–*Chainia* sp., 2–*Cochliobous carbonum* SB 111 Xyn (L13596), 3–*Bacillus pumilus* IPO XynA (P00694), 4–*Clostridium stercorarium* F9 XynA (P33558), 5- *Trichoderma reesi* Xyn2 (P36217), 6–*Clostridium acetobutylicum* P262 XynB (P17137), 7–*Ruminococcus flavefaciens* N-terminal XynA (P29126), 8–*Trichoderma reesi* Xyn1 (P36218), 9–*Schyzophyllum commune* XynA (P35809), 10–*Fibrobacter succinogenes* S 85 XynC (P35811), 11–*Bacillus circulans* XynA (P09850), 12–*Bacillus subtilis* PAP15 Xyn A (P18429), 13– *Aeromonas caviae* XynA (D32065), 14–*Streptomyces lividans* XynC (P26220), 15–*Streptomyces lividans* XynB (P26515) and 16 – *Aspergillus kawachii* IFO4308 Xyn C (P33557).

Modification of cysteine : The xylanase activity was inhibited up to 100% by sulfhydryl reagents *viz.* PHMB, DTNB and NEM. The xylanase when incubated with 12 mM NEM at pH 7.8, for 45 min or 10 μM PHMB at pH 5.5 for 20 min lost approximately 90% of its initial activity. However, no loss of activity was observed in the control samples. The log of residual activity plotted as the function of time at various concentrations of NEM (Fig 5A) and PHMB (Fig 5B) were linear up to 10% of the initial activity. NEM and PHMB mediated inactivation followed pseudo-first-order kinetics at any fixed concentration of the reagent. The pseudo-first-order rate constants were calculated from the slopes of the plots of log (percent residual activity) versus reaction time and the order was determined from the plots of log (K_{app}) against log[NEM] and log[PHMB] [36]. These plots (inset of Fig 5A and 5B) indicated that at least one molar equivalent of the inhibitor binds to one molecule of enzyme. Modification of the enzyme with DTNB was accompanied by an increase in the absorbance of modified protein at 412 nm. Based on a molar absorption coefficient of 13,600 M^{-1} cm^{-1} and molecular weight of *Chainia* xylanase to be 21,000, the total number of Cys residues modified was found to be one. Moreover, the plot of percent residual activity against the number of Cys residues modified revealed that the loss of activity resulted from the modification of one Cys residue (Fig 6A). Inclusion of β-ME or DTT during enzyme assay resulting in three-fold increase in specific activity and three-fold decrease in K_m, also suggested the role of sulfhydryl group in the enzyme activity.

NEM, PHMB and DTNB mediated inactivation could be prevented by incubating the enzyme with excess of xylan prior to the modification reaction (Table IIIA). Additionally, the PHMB inactivated enzyme regained 97% of its original activity upon incubation with 1 mM cysteine. The K_m values of the partially inactivated enzyme remained unaltered while a concomitant decrease in k_{cat} as compared to the control was observed, suggesting that inactivation is due to modification rather than a structural change (Table IIIB). Almost identical CD

spectra of native and NEM modified enzyme indicated that the modification did not result in any gross conformational change of the enzyme (Fig 6B).

Peptide isolation and amino acid sequencing

Modification of Cys residues was also carried out by DAM to facilitate the isolation of the active peptide. RPHPLC of tryptic digest of the DAM modified enzyme showed a single peak when monitored at 340 nm (Fig 7). The N-terminal sequence of the modified peptide was Gln-Val-Phe-Xxx-Tyr-Thr-Ser--. The Xxx at position four indicates the absence of phenylthiohydantoin derivative at the modified Cys.

Table III A . Influence of cysteine modification on the activity of xylanase : Substrate protection studies.

Incubation mixture	Residual activity (%)
Control	100
Enzyme + NEM(12 mM)	15
Enzyme + xylan (5 mg) + NEM	50
Enzyme + PHMB (10 μM)	0.0
Enzyme + xylan (2 mg) + PHMB	95

Table IIIB . Effect of cysteine modification on K_m and k_{cat} values of *Chainia* xylanase: The values were determined under standard assay conditions by using the substrate concentration in the range of 1-5 mg of oat spelt xylan. The kinetic constants were calculated by fitting the data to linear regression using Lineweaver-Burk plots and Origin 4.0 (MicroCal).

Percentage activity	Residue modified	Reagent used	K_m mg/ml	k_{cat} (min^{-1})
100	None	---	8.19	1910
71	Cys	PHMB	8.45	1210
42			8.19	790
26			8.25	410

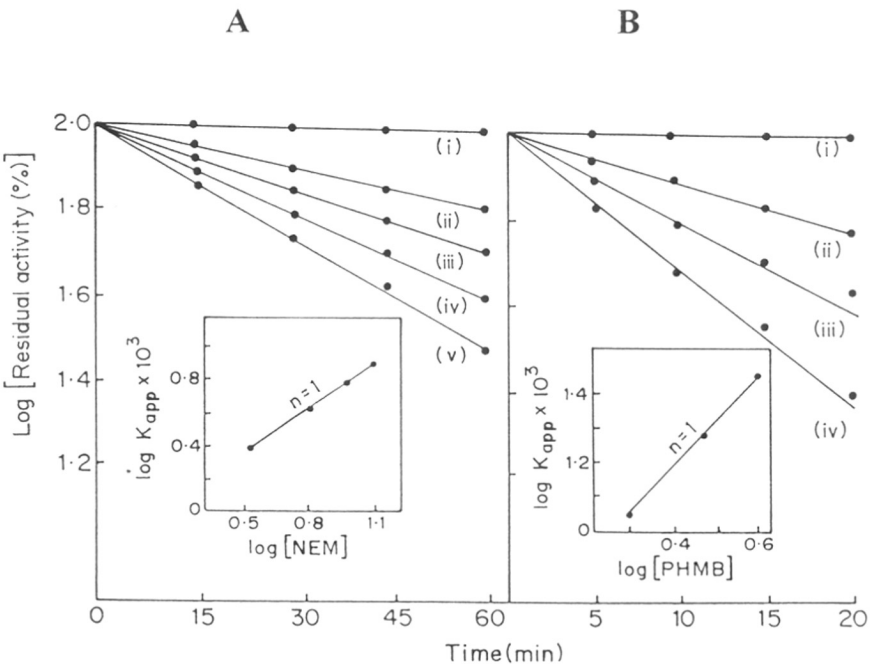


Figure 5.

A) Pseudo first-order plot for inactivation of *Chainia* xylanase by NEM: Concentrations of NEM were (i) control (ii) 3 mM (iii) 6 mM (iv) 9 mM and (v) 12 mM. Inset : second order plot of pseudo-first order rate constants (K_{app}) (min^{-1}) as a function of log NEM concentration.

B) Pseudo first-order plot for inactivation of *Chainia* xylanase by PHMB : Concentrations of PHMB were (i) control (ii) 4 μM (iii) 6 μM and (iv) 8 μM. Inset : second order plot of pseudo-first order rate constants (K_{app})(min^{-1}) as a function of log PHMB concentration.

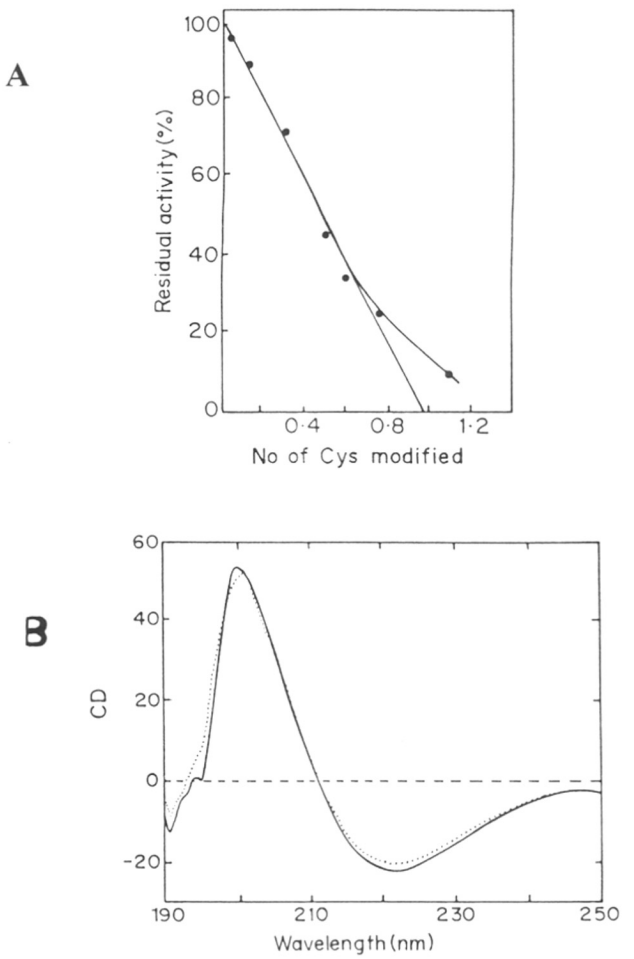


Figure 6.

A) Effect of DTNB on *Chainia* xylanase: Number of Cys residue modified was estimated as described in the Materials and Methods.

B) The CD spectra of native and NEM treated xylanase : The spectra were recorded on a JASCO-710 spectropolarimeter from 190-250 nm using 1 cm path length at 25°C at an enzyme concentration of 5 μM (2 ml) in 5 mM sodium acetate buffer, pH 5.6, at a scan speed of 100 nm/min. Native enzyme (——) and NEM treated enzyme (-----)

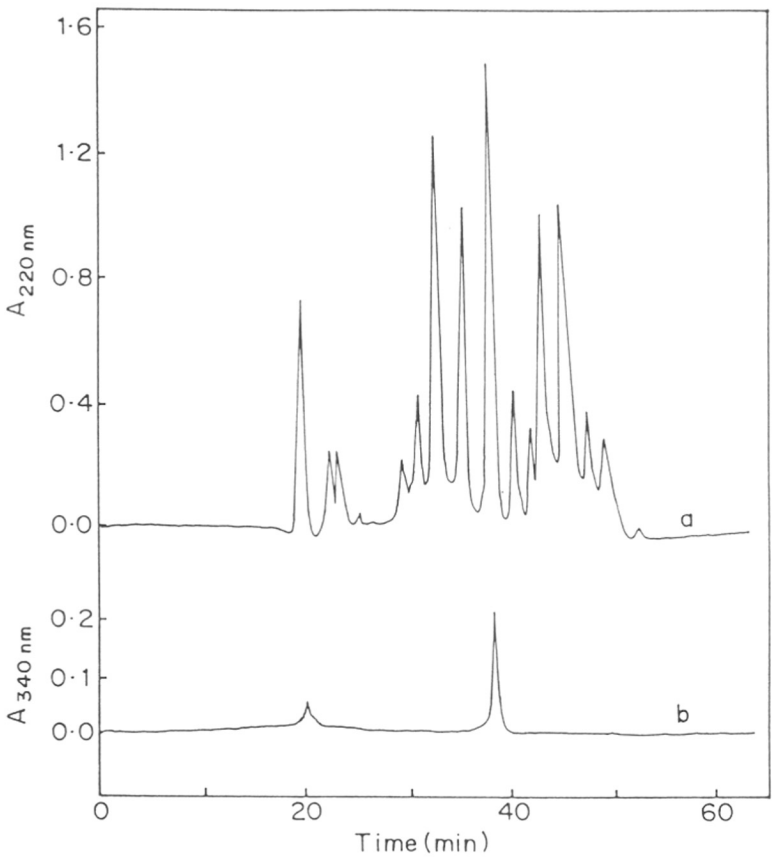


Figure 7.

Reverse phase HPLC of tryptic digest of Cys modified enzyme : Tryptic digestion of the modified enzyme was carried out as described in Materials and Methods. The column was eluted with a linear gradient of 0-80% acetonitrile in 0.1% TFA over 60 min at a flow rate of 0.5 ml/min. The effluent was monitored at 220 nm (a) and 340 nm (b)

Modification of arginine : The purified xylanase when incubated with 2,3, butanedione (50 mM) at pH 7.8, and phenyl glyoxal (3 mM), at pH 8.0, for 45 min lost approximately 90% of its activity. However, no loss of activity was observed in the control samples. The log of residual activity plotted as the function of time at various butanedione and phenylglyoxal concentrations was linear up to 10% of the initial activity. Butanedione and phenylglyoxal mediated inactivation followed pseudo-first-order kinetics at any fixed concentration of the reagent. The pseudo-first-order rate constants were calculated from the slopes of the plots of log (percent residual activity) versus reaction time (Fig 8A and 8B) and the order was determined from the plots of log (K_{app}) against log[butanedione] and log[phenylglyoxal] [36]. These plots (inset of Fig 8A and 8B) indicated that the loss of activity occurred as a result of modification of a single Arg residue. Modification of Arg residues by treating the enzyme with pNPG resulted in 80-90% loss in its initial activity and the inactivation was dependent on the concentration of the reagent. The plot of percentage residual activity against the number of Arg residues modified (Fig 9A) revealed that the loss of activity resulted from the modification of a single Arg residue.

Butanedione, pNPG and phenylglyoxal mediated inactivation could be prevented to a significant extent by incubating the enzyme with excess xylan prior to the modification reaction (Table IVA). Reactivation (65%) of phenylglyoxal modified enzyme upon incubation at pH 8.5, shows that the enzyme inactivation is due to the modification of an Arg residue. No change in the K_m values of the partially inactivated enzyme coupled with a concomitant decrease in k_{cat} as compared to the control, suggests that inactivation is due to modification rather than structural changes (Table IVB). Moreover, the CD spectra of both native and butanedione modified enzyme were almost identical (Fig 9B) indicating that the modification does not result in a gross change in the conformation of the enzyme.

Table IVA . Influence of arginine modification on the activity of xylanase ; Substrate protection studies.

Incubation mixture	Residual activity (%)
Control	100
Enzyme + butanedione (0.5% v/v)	05
Enzyme + xylan (5 mg) + butanedione	60
Enzyme + phenylglyoxal (2.5 mM)	10
Enzyme + xylan(5 mg) + phenylglyoxal	50

Table IVB . Effect of arginine modification on the K_m and k_{cat} values of *Chainia* xylanase: The values were determined under standard assay conditions by using the substrate concentration in the range of 1-5 mg of oat spelt xylan. The kinetic constants were calculated by fitting the data to linear regression using Lineweaver-Burk plots and Origin 4.0 (MicroCal).

Percentage activity	Residue modified	Reagent used	K_m (mg/ml)	k_{cat} (min^{-1})
100	None	---	8.19	2020
78	Arg	Phenylglyoxal	8.00	1305
67			8.33	1060
48			8.00	605

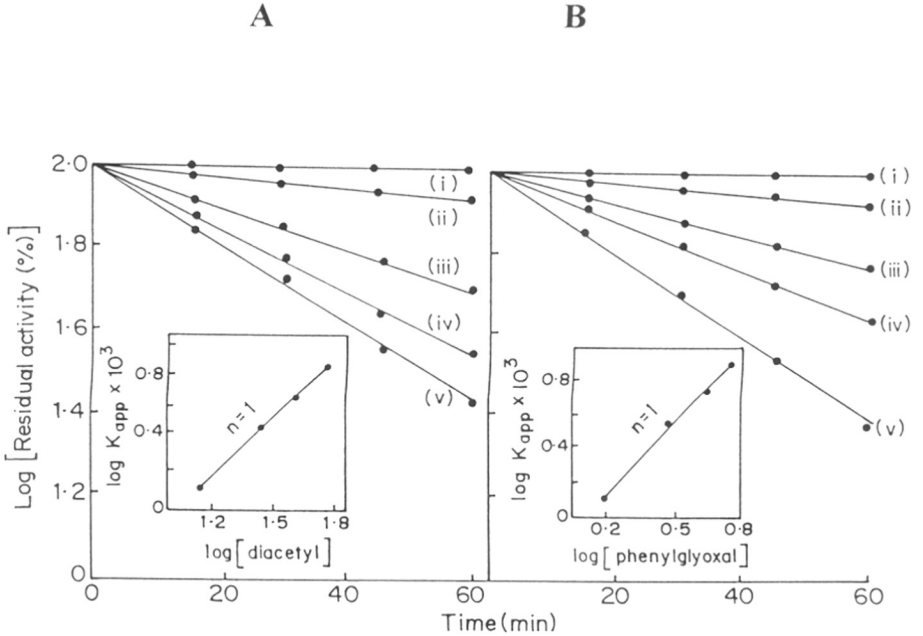


Figure 8.

A) **Pseudo first-order plot of inactivation of *Chainia* xylanase by butanedione (diacetyl) :** Concentrations of butanedione were (i) control (ii) 14.4 mM (iii) 28.8 mM (iv) 43.2 mM and (v) 57.6 mM. Inset : second order plot of pseudo-first order rate constants (K_{app}) (min^{-1}) as a function of log butanedione (diacetyl) concentration.

B) **Pseudo first-order plot of inactivation of *Chainia* xylanase by phenyl glyoxal :** Concentrations of phenyl glyoxal were (i) control (ii) 1.5 mM (iii) 3.0 mM (iv) 4.5 mM and (v) 6.0 mM. Inset : second order plot of pseudo-first order rate constants (K_{app}) (min^{-1}) as a function of log phenyl glyoxal concentration.

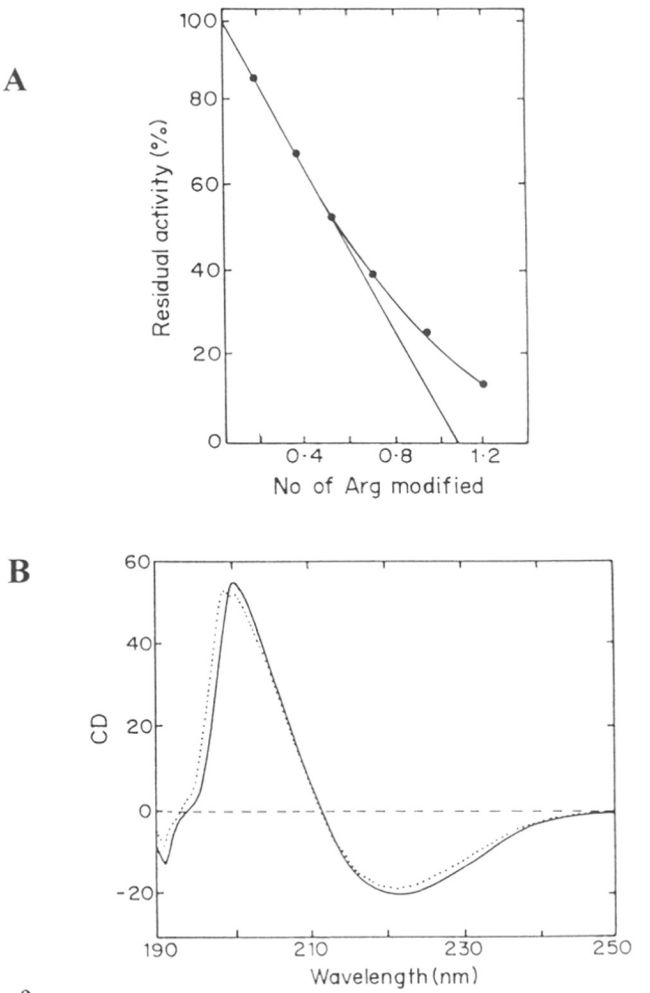


Figure 9.

A) **Effect of pNPG on *Chainia* xylanase:** Number of Arg residue modified was estimated as described in Materials and Methods.

B) **The CD spectra of native and butanedione treated xylanase :** The spectra were recorded on a JASCO-710 spectropolarimeter from 190-250 nm using 1 cm path length at 25°C at an enzyme concentration of 5 μM (2 ml) in 5 mM sodium acetate buffer, pH 5.6, at a scan speed of 100 nm/min. Native enzyme (——) and butanedione treated enzyme (-----)

Modification of Trp residues : The titration of xylanase with NBS exhibited a concentration dependent and time independent inactivation of the enzyme. Spectrophotometric analysis of NBS mediated inactivation showed an increase in absorbance at 280 nm (Fig 10A) suggesting either cleavage of the protein or modification of Tyr residues [14,37,38]. The analysis of NBS treated enzyme by reverse phase FPLC (Fig 10B) as well as SDS-PAGE (Fig 11A) showed multiple species indicating that the NBS mediated inactivation is due to cleavage of the peptide bond at Trp residues. In Ouchterlony immunodiffusion, no precipitin arc was observed for the samples with [NBS]:[enzyme] ratio of seven and above (Fig 11B) indicating a loss in antigenic determinant. The presence of substrate (2 mM X_8 or 2 mg xylan) could not protect against the cleavage (Fig 10A). Moreover, the K_m of partially NBS inactivated enzyme increased with a concomitant decrease in the k_{cat} values (Table V). However, the treatment of the denatured enzyme (8M urea) with NBS resulted in the modification of three Trp residues. Moreover, modification of Trp residues by a Trp specific reagent *viz.* HNBB resulted only in 20% loss of enzyme activity with no significant modification of Trp residues (0.2-0.3 residue per molecule), confirming that Trp has no role in the catalytic activity of *Chainia* xylanase. Modification of three Trp residues, with HNBB, under denaturing conditions (8M urea, pH 2.7) in comparison to 0.2-0.3 residue in the native enzyme indicates that the Trp residues are not readily accessible for modification.

Fluorescence studies : The protein exhibits a fluorescence emission maxima(λ_{em}) at 338 nm upon excitation either at 280 nm or 295 nm. Up to 95% of the fluorescence of the enzyme was quenched by NBS at a concentration of 50 μM (i.e. at a molar ratio of NBS : enzyme 10 : 1) with a large blue shift in the fluorescence maxima, from 338 nm in the native enzyme to 329.5 nm (Fig 12) and the presence of substrate could not protect the enzyme against quenching. Ionic and neutral quenchers quenched the fluorescence of the protein. The percentage of quenching by acrylamide, KI and CsCl were 95%, 20% and 15%, respectively

(Table VI). The effective Stern-Volmer or quenching constant $K_{sv(eff)}$ and effective fractional accessible fluorescence $f_{a(eff)}$ were obtained from the slope and intercept of $F_o/\delta F$ vs $1/[Q]$ plots at low [Q] values (Table VI). The plots of F_o/F versus [Q] (Stern-Volmer plots) were linear for KI and CsCl and upward for acrylamide (Fig 13A). The modified Stern-Volmer plots were linear for acrylamide, KI and CsCl (Fig 13B).

Fluorescence quenching by the substrate (X_8) of native and chemically modified enzyme showed difference with respect to the extent of quenching. Quenching of fluorescence of native, carboxylate and Cys modified enzyme was 15%, while the Arg modified enzyme was quenched only by 3% (Table VII). However, no significant change in the emission maxima was observed for the modified enzymes.

Table V . K_m and k_{cat} values for partially inactivated (modified) *Chainia* xylanase: The values were determined under standard assay conditions by using the substrate concentration in the range of 1-5 mg of oat spelt xylan. The kinetic constants were calculated by fitting the data to linear regression using Lineweaver-Burk or Eadie-Hofstee plots and Origin 4.0 (MicroCal).

Percentage activity	Residue modified	Reagent used	K_m (mg/ml)	k_{cat} (min^{-1})
100	None	—	8.20	1980
66	Trp	NBS	10.0	1380
36			12.25	890

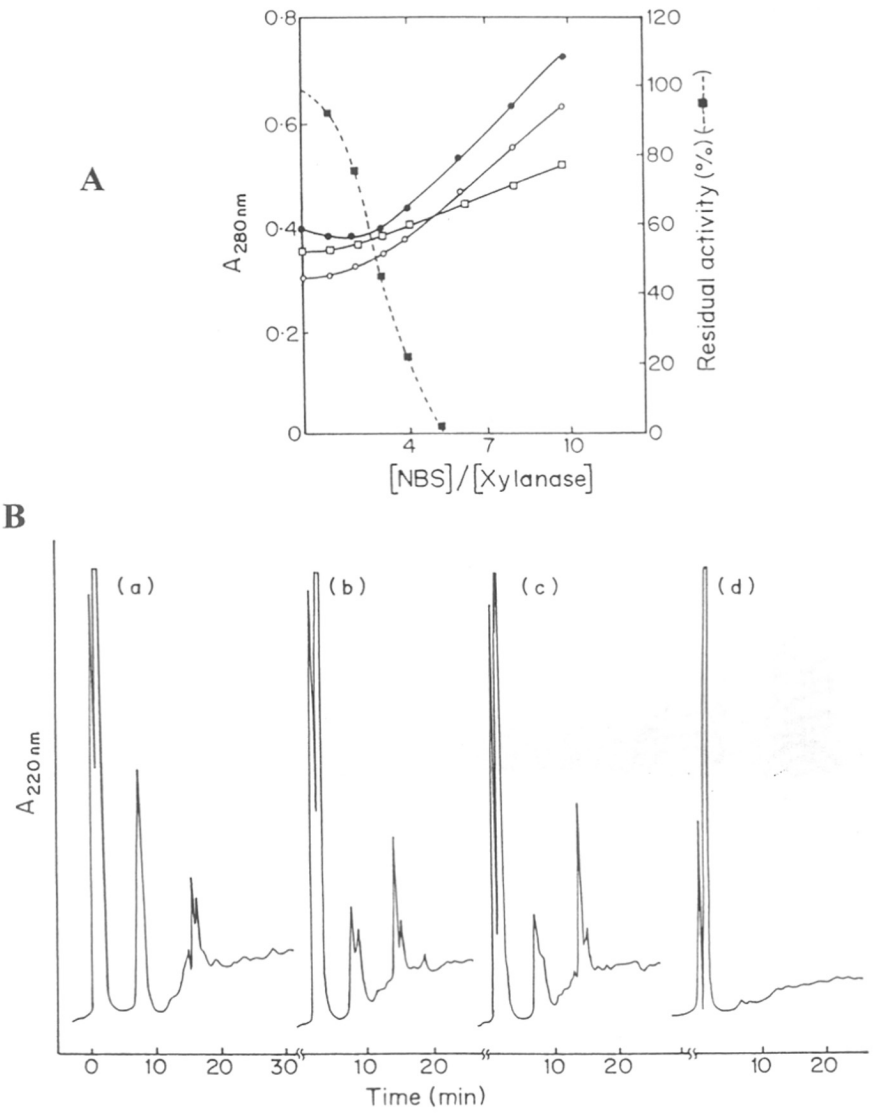


Figure 10. Titration of *Chainia* xylanase with NBS ; The enzyme in 50 mM acetate buffer, pH 5.5, was titrated with NBS at 25°C as described in Materials and Methods.

A) A_{280} nm of xylanase upon titrating with NBS ; xylanase (o——o), xylanase + 2 mg xylan (□——□) and xylanase + 2 mM X_8 (●——●)

B) Reverse-phase FPLC elution profile of NBS treated xylanase; Molar ratio of [NBS] : [xylanase] are ; a) 2 , b) 4 , c) 7 and d) untreated xylanase

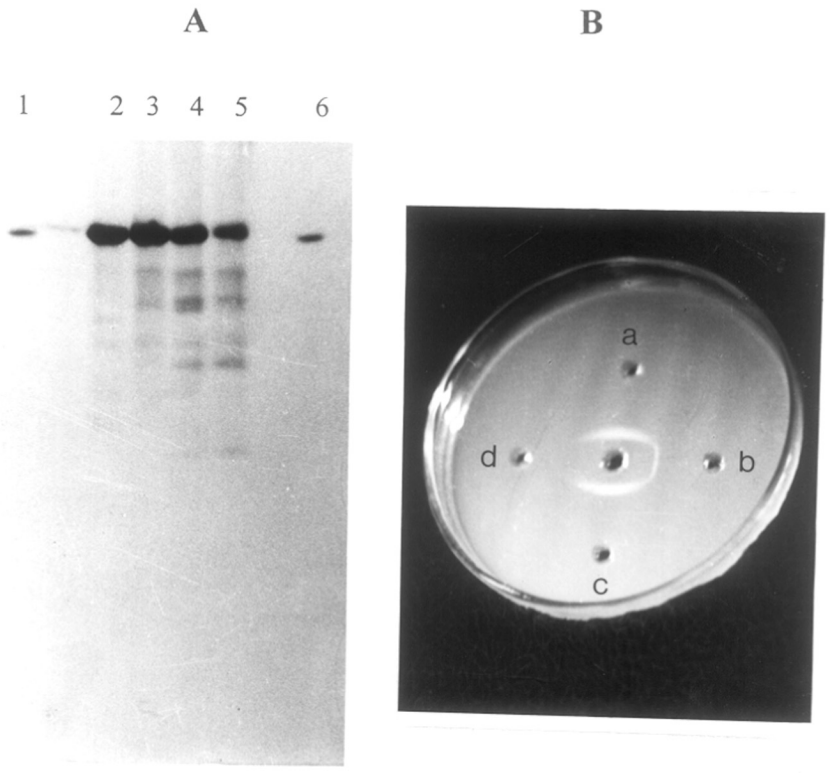


Figure 11. Titration of *Chainia* xylanase with NBS ; The enzyme (10 μM, 1 ml) in 50 mM acetate buffer, pH 5.5, was titrated with NBS (1 mM) at 25 °C as described in Materials and Methods.

A) **Tricine SDS-PAGE :** The enzyme (40 μg) was electrophoresed on 16.5% w/v gel and stained with silver nitrate. The molar ratio of [NBS] : [xylanase] are; lane 2 - 2 : 1, lane 3 - 4 : 1, lane 4 - 7 : 1, lane 5 - 10 : 1 and lane 1 and 6 - untreated xylanase

B) **Ouchterlony immunodiffusion :** The NBS treated xylanase (200 μg) was cross-reacted with purified anti-xylanase antibodies (center well, 1 mg) in a 3 mm thick 1% (w/v) agarose plate (prepared in 50 mM sodium phosphate buffer, pH 7.0, containing 100 mM NaCl) for 60 h at 4°C. (a and c) native xylanase, (b) [NBS] : [xylanase] :: 4:1 and (d) [NBS] : [xylanase] :: 7:1

Table VI . Effect of neutral and ionic quenchers on fluorescence of xylanase from *Chainia* sp: The protein (5 μM, 2 ml) was excited at 280 nm and the emission was recorded at 338 nm (λ_{max}). The $K_{sv\ (eff)}$ values were calculated at low concentration [Q] of acrylamide, KI and CsCl

Quencher	15% quenching	$K_{sv(eff)}$	$f_{a(eff)}$
Acrylamide (0-0.54M)	0.001M	13.47	1.10
KI (0-0.84M)	0.241M	0.20	0.03
CsCl (0-1.0M)	0.788M	1.02	0.10

Table VII . Fluorescence quenching of native and chemically modified samples: 2 ml of enzyme solution (5 μM) in 50 mM sodium acetate buffer, pH 5.6, was quenched with 100 μl of xylooctaose (25 mg/ml) at 25°C and the values were corrected for dilution effect.

Sample	λ_{max}	% quenching
Native	338 nm	15.0
Carboxylate Modified	339 nm	15.0
Cys modified	338 nm	15.0
Arg modified	335 nm	03.2

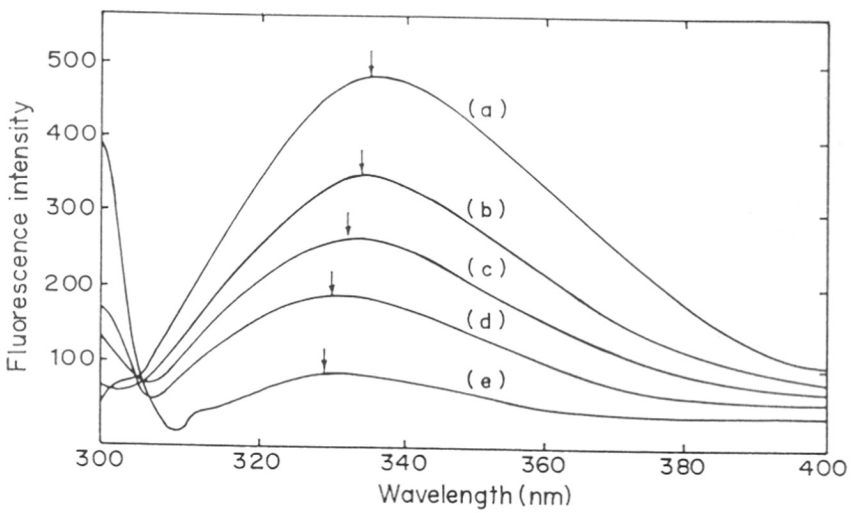


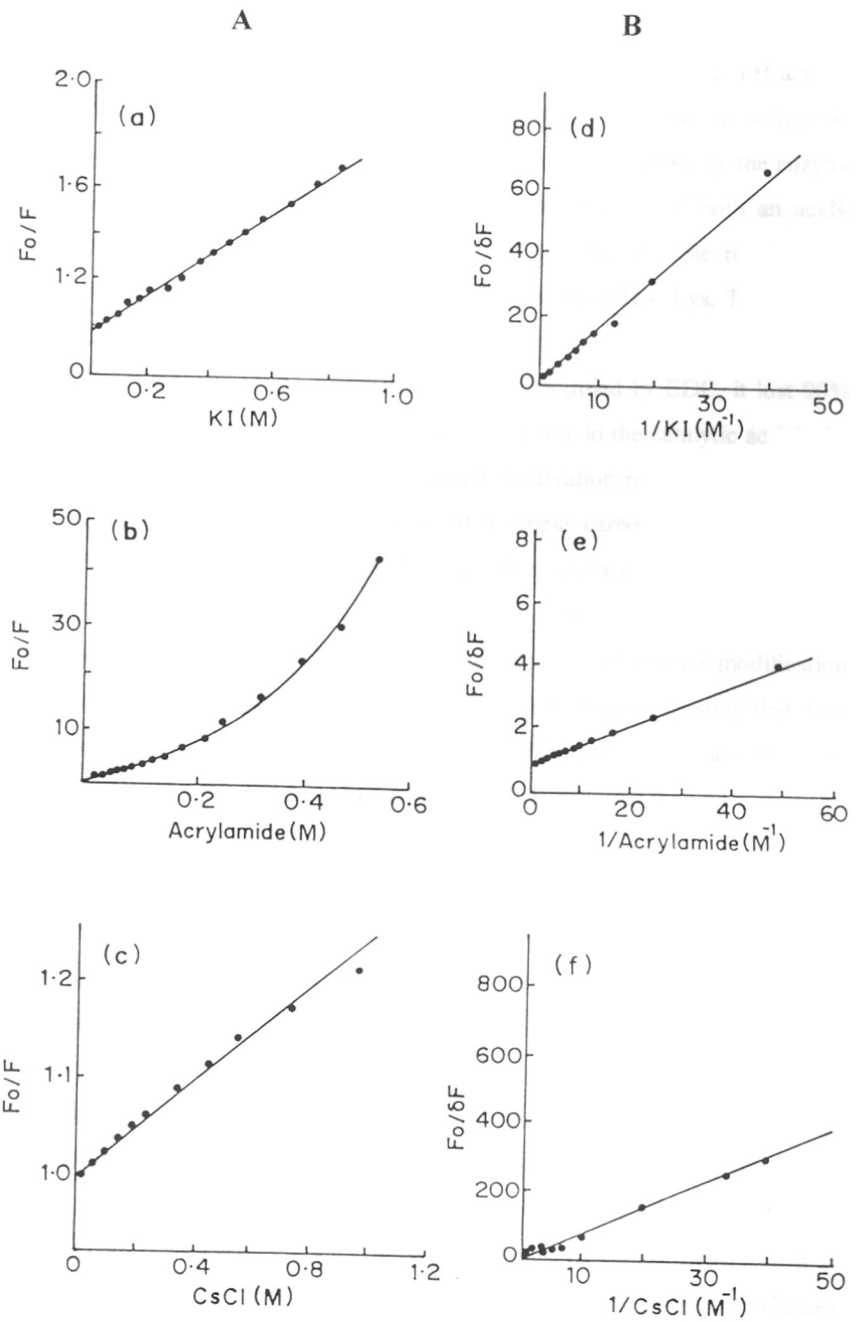
Figure 12. Fluorescence quenching of *Chainia* xylanase by NBS : The emission spectra at 25°C were recorded from 300 - 400 nm after excitation of the enzyme (5 μ M, 2 ml) at 295 nm. The molar ratios of NBS to xylanase are, (a) untreated xylanase, λ_{em} 338 nm (b) [NBS] : [xylanase] : : 1 : 1, λ_{em} 335.5 nm (c) [NBS] : [xylanase] : : 2.5 : 1, λ_{em} 333 nm (d) [NBS] : [xylanase] : : 5 : 1, λ_{em} 331.5 nm and (e) NBS : xylanase : : 10 : 1, λ_{em} 329.5 nm

Fig 13.

Stern-Volmer and modified Stern-Volmer plots of fluorescence quenching of *Chainia* xylanase by KI, CsCl and acrylamide: The native protein (5μM, 2 ml) in 50 mM acetate buffer, pH 5.6, was excited at 280 nm and the emission read at 338 nm.

A) Stern-Volmer plots ; a) KI, b) acrylamide and c) CsCl

B) Modified Stern-Volmer plots ; d) KI, e) acrylamide and f) CsCl


A
B
(a)
Fo/F
2·0
1·6
1·2
0
0·2
0·6
1·0
KI (M)
(d)
Fo/δF
80
60
40
20
0
10
30
50
1/KI (M⁻¹)
(b)
Fo/F
50
30
10
0·2
0·4
0·6
Acrylamide (M)
(e)
Fo/δF
8
6
4
2
0
0
20
40
60
1/Acrylamide (M⁻¹)
(c)
Fo/F
1·2
1·1
1·0
0
0·4
0·8
1·2
CsCl (M)
(f)
Fo/δF
800
600
400
200
0
10
30
50
1/CsCl (M⁻¹)

DISCUSSION

The *Chainia* endoxylanase has an optimum pH of 6.0 and the pH-activity profile was bell shaped implying the presence two ionisable groups, one with a pK_a of pH 5.1 and the other with a pK_a of 7.3, essential for the catalysis by the enzyme (page 75). The pH activity profile indicates the participation of both an acidic residue and possibly a thiol group in its catalytic mechanism. The results from various modifications eliminate the possible involvement of His, Lys, Tyr and Ser residues in the catalytic activity of the enzyme.

When carboxyl groups of the enzyme were modified by EDC, it lost 90% activity suggesting that carboxyl groups may have a role in the catalytic activity of the enzyme. Kinetic analysis of EDC mediated inactivation revealed that the loss of activity occurred due to modification of a single carboxyl group (Fig 1). Substrate protection studies on EDC modified enzyme showed that the inactivation could be prevented to a considerable extent by preincubating the enzyme with excess of xylan (Table IIA). Additionally, carboxylate modification did not bring about any gross change in the enzyme structure suggesting that loss of enzyme activity could be due to carboxylate modification rather than structural changes. Absence of Asp/Glu in the modified peptide confirms the involvement of carboxylate in the catalytic activity of *Chainia* xylanase.

Participation of carboxyl groups in the catalytic function has been reported in a variety of glycosyl hydrolases including lysozyme [1,2], cellulase [3-5,39-43], cellobiohydrolase [44], glucoamylase [45-48], α-amylase [49], α-galactosidase [50], β-galactosidase [51], cyclodextrin glycosyl transferase [52-54], xylanase [6-10] etc. and the carboxylate seems to be an integral part of the catalytic site of these enzymes where acid-base catalysis is involved. The possibility of all glycosidases sharing a common mechanism, with the classic paradigm of hen egg white lysozyme, has been intensively investigated for a variety of glycosidases and many subsequent mechanistic studies have been reviewed [55,56]. Involvement of two glutamate residues in catalysis of *Bacillus pumilus* [8], *Bacillus circulans* [10], *Streptomyces lividans* [9], *Thermoanaerobacterium saccharolyticum* [57],

Pseudomonas fluorescens [58] xylanases and a non-specific cellulase from *Cellulomonas fimi* [59] was shown by site-directed mutagenesis. The three dimensional structure of family G/11 xylanases from *B. circulans* [10], *B. pumilus* [60], *Aspergillus niger* [61], *T. reesi* (xyn I and xyn II) [62] and *T. harzianum* [63] as well as family F/10 xylanases from *C. fimi* [64], *P. fluorescens* [58,65], *S. lividans* [66] and *Clostridium thermocellum* [67] reveal the involvement of two glutamate residues (one acting as a nucleophile and the other as an acid/base catalyst). These two glutamate residues (catalytic nucleophile and acid/base catalyst) are totally conserved and are surrounded by a highly homologous region specific to each family [68]. The results of chemical modification, chemical labeling and N-terminal sequencing of the labeled peptide suggest that *Chainia* xylanase has only one catalytic carboxyl group rather than two observed for other xylanases. The sequence comparison and alignment of carboxylate modified peptide from *Chainia* xylanase (Fig 4) with other family G/11 xylanases (SWISS-PROT, Release 34, Dec 1996 and Ref. 68) showed striking similarity with the amino acid sequence around the catalytic glutamate residue acting as the acid/base catalyst.

When Cys of the enzyme was modified by sulfhydryl reagents it lost 90-100% activity suggesting that Cys may have a role in the catalytic activity of the enzyme. Kinetic analysis of NEM and PHMB mediated inactivation revealed that the loss of activity occurred due to the modification of a single Cys residue (Fig 5A and 5B). The PHMB inactivated enzyme regained 97% of its original activity upon incubation with 1 mM cysteine. Substrate protection studies on NEM and PHMB modified enzyme showed that the inactivation could be prevented to a considerable extent by preincubating the enzyme with excess of xylan (Table IIIA). Additionally, Cys modification did not bring about any gross change in the enzyme structure (Fig 6B) suggesting that the loss of enzyme activity is due to Cys modification rather than structural changes. The involvement of Cys was further ascertained by modifying the enzyme with DTNB and DAM. Incubation of the enzyme with DTNB led to its rapid inactivation. The DTNB mediated inactivation of the enzyme was accompanied by an increase in absorbance of the enzyme at 412

nm. Determination of number of essential Cys residues following DTNB modification indicates that the modification of the single Cys residue is responsible for the loss of enzyme activity (Fig 6A).

Though many xylanases are inhibited by sulfhydryl reagents *viz.* Hg^{+2}, PHMB, NEM, DTNB, cysteine etc. [69,70], only a few have been worked out quantitatively. DAM, a chromophoric derivative of NEM which carries a reporter group absorbing at 340 nm has been effectively used to isolate the active cysteinyl peptide in *Streptomyces* T_7 xylanase [71]. Reaction of DAM with *Chainia* xylanase resulted in the modification of a single Cys residue (Fig 7). Studies on *Thermomyces* xylanase [72, 73] have indicated the presence of a Cys residue at the active site and the probable role of Cys residue in the catalytic activity of cellulases has also been suggested [74]. The active cysteinyl peptide sequenced here does not show much sequence similarity with that reported by Keskar *et al* [71] from *Streptomyces* T_7 xylanase.

In case of *B. circulans* xylanase, it has been demonstrated that the enzyme catalyses the hydrolysis of glycosidic bonds like lysozyme *viz.* via a double-displacement mechanism involving two carboxyl residues [10,75,76]. Glu 78 acts as the nucleophile while Glu 172 acts as the acid catalyst during the first step *i.e.* glycosylation (protonation of departing aglycone) and then as a general base during deglycosylation [75, 76] (page 25). However, replacement of Asp 20 with Cys in T_4 lysozyme (similar to Asp52 of hen egg white lysozyme and Glu 78 of *B.circulans* xylanase) yielded a variant with pH activity profile and specific activity similar to wild type enzyme but with a high degree of sensitivity to thiol reagents [77]. The -SH of Cys is a more powerful nucleophile than the oxygen of a carboxylate, which can form a covalently linked intermediate by nucleophilic attack and can thus act as a catalytic nucleophile [77]. Hence, it is reasonable to assume that the Cys residue in the active site of *Chainia* xylanase may function as the catalytic nucleophile.

Treatment of the enzyme with 2,3, butanedione and phenylglyoxal brought about 90% loss in its initial activity indicating that Arg may have a role in the catalytic activity of the enzyme. Kinetic analysis of butanedione and phenylglyoxal

mediated inactivation showed that the loss of activity occurred due to the modification of a single Arg residue (Fig 8A and 8B). Similar results were obtained when the modification was carried out using pNPG (Fig 9A).

Substrate protection studies on butanedione and phenylglyoxal modified enzyme revealed that the inactivation could be prevented by preincubating the enzyme with excess amount of xylan (Table IVA). Phenylglyoxal modified enzyme when incubated at pH 8.5, could recover significant amount of activity (65-70%). Additionally Arg modification did not bring about any gross change in the enzyme structure indicating that the loss of enzyme activity is due to Arg modification rather than structural changes (Fig 9B). The above results point towards the involvement of Arg in the catalytic activity of *Chainia* xylanase.

Addition of substrate to Arg modified enzyme resulted in only 3% quenching of its fluorescence as compared to 15% of Cys and carboxylate modified and native enzyme (Table VII). Similar degree of quenching of carboxylate and Cys modified enzyme to that of native enzyme indicated that the modification of these residues does not affect the substrate binding. On the other hand, the low level of quenching of fluorescence of the Arg modified enzyme by the substrate suggests that it fails to bind to the active site of the enzyme, pointing towards the role of Arg in substrate binding. Indeed, the three dimensional structure of enzyme-substrate complex (at 1.8 A° resolution) of *B. circulans* xylanase [10] indicates the presence of an Arg at the active site, with the $-NH_2$ of Arg 112 forming a hydrogen bond with xylose.

The treatment of *Chainia* xylanase with NBS resulted in total loss of enzyme activity at a NBS: xylanase ratio of five and above. The loss of activity was independent of time and was dependent on the concentration of the reagent. The increase in $A_{280\ nm}$ upon titration with NBS (instead of decrease generally observed when indole chromophore of Trp is oxidized by NBS to oxindole) has been correlated to cleavage of the peptide bond at Trp residues and this is evident from SDS/PAGE (Fig 11A) and FPLC (Fig 10A) of the NBS treated enzyme. Absence of any precipitin arc for the enzyme sample with a NBS: enzyme ratio of

seven and above in Ouchterlony immunodiffusion (Fig 11B) indicates the gross conformational change in the enzyme leading to the loss of antigenic determinant. The increase in K_m of partially NBS inactivated enzyme suggests that the inactivation led to a decrease in affinity of the enzyme towards substrate. A large blue shift in emission maxima, associated with the quenching of fluorescence by NBS (Fig 12) and increase in K_m also indicates major structural changes in the enzyme. It should be noted that NBS was initially used to cleave the peptide bond at Trp residues [78]. Trp specific reagent HNBB failed to bring about any significant Trp modification or a loss in enzyme activity under native conditions. All the three Trp residues were modified under denaturing conditions suggesting that these residues are not readily accessible in the native enzyme. However, the quenching of fluorescence by the substrate suggests that tryptophans might be located near the active site. To probe such a possibility, the microenvironment of tryptophans was further investigated by fluorescence quenching studies.

The fluorescence characteristic of Trp residues depends on the microenvironment and this provides a sensitive method to probe the conformational state of the protein. The interpretation of fluorescence data for multi-Trp proteins is complicated by factors such as heterogeneous emission and varied accessibility of individual Trp residues to the quenchers. Therefore in the analysis, the effective Stern-Volmer constant $K_{sv(eff)}$; the parameter that represents the weighted average of the quenching constant of individual Trp residues and may contain the contributions from both dynamic and static quenching process, has been used. Addition of small molecules to the native protein has been used to evaluate the accessibility of Trp residues, *viz.* buried Trp residues which are less effectively quenched than the solvent exposed ones. The efficiency of the quenching is determined by the nature of the quencher and the accessibility of Trp residues. Acrylamide is a small apolar, neutral molecule capable of penetrating the protein matrix and has been known to quench Trp fluorescence from exposed to moderately buried Trp residues. The emission maximum at 338 nm, indicates that the Trp residues are present in a moderately hydrophobic environment. The fluorescence quenching of *Chainia* xylanase by acrylamide was 95% with no

change in the λ_{em} of 338 nm. It can therefore be inferred that the quenching is due to collision and not because of a conformational change in the protein. The upward curvature of F_o/F versus [Q] plot (Stern-Volmer plot) of acrylamide (Fig 13A) indicates the presence of heterogeneously emitting multiple fluorophores and occurrence of both dynamic and static quenching. The maximum $K_{sv(eff)}$ was obtained for acrylamide with all Trp residues being available for quenching. Iodide (I^-) and Cesium (Cs^+) are hydrated ionic quenchers and are able to extinguish the fluoresence of the fluorophores located at or near the protein surface. Low quenching by KI and CsCl (20% and 15%, respectively) suggest that the tryptophans are not accessible to ionic quenchers and may be solvent masked. The linear Stern-Volmer plots for KI and CsCl are the results of the inefficiency in both static and dynamic quenching process [34] and linearity of such plots for weak quenchers does not indicate the absence of static quenching [79]. The fraction of Cs^+ accessible Trp (0.1) was slightly larger as compared to I^- (0.03) and the $K_{sv(eff)}$ values for KI and CsCl were also modest (Table VI). The linearity of the modified Stern-Volmer plots for acrylamide, KI and CsCl (Fig 13B) suggests that all the three tryptophans are contributing equally to the fluorescence of the protein and are equally accessible to the quenchers. The result of quenching studies indicate that the tryptophans are not located on the protein surface as the ionic quenchers are unable to quench them to any significant degree (Table VI) and that all the three tryptophans present in the protein are partially buried in a similar moderately hydrophobic environment.

Several reports exist regarding the presence of Trp in the active site of glycosyl hydrolases including lysozyme [2,80], glucoamylase [42,46,81],cellulases [43,82],xylanases [12,13,83,84] etc. On the other hand, it has also been shown that Trp are not involved in the catalytic activity of xylanases from *Schizophyllum commune* [14] and *Streptomyces sp.*[85,86]. The above results clearly suggest that Trp has no role in catalytic function of *Chainia* xylanase. Based on the results of modification it is clear that *Chainia* xylanase contains a catalytic carboxylate and thiol of cysteine and guanidinium of arginine in its active site.

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CHAPTER 4

Equilibrium and thermodynamic parameters of xylo-oligosaccharide binding and thermostability of *Chainia* xylanase

SUMMARY

The binding of xylo-oligosaccharides to *Chainia* xylanase resulted in a decrease in fluorescence intensity of the enzyme with the formation of 1:1 complex. The percentage quenching of the enzyme fluorescence increased from 5% for xylobiose (X_2) to 12% for xylopentaose (X_5). Equilibrium and thermodynamic parameters of substrate / inhibitor binding were determined by following the fluorescence changes and by titration calorimetry. The standard free energy changes ($-\Delta G°$) at 20°C were 17.84, 19.45, 20.21 and 20.50 kJ mol^{-1} for X_2, X_3, X_4 and X_5, respectively. The Gibb's free energy changes as determined from titration calorimetry were 13.10 kJ mol^{-1} for X_3 at 10°C, 16.50 kJ mol^{-1} for X_4 at 10°C and 17.30 kJ mol^{-1} for X_5 at 7°C. Contribution from the enthalpy towards the free energy decreased with increasing chain length from X_2 to X_4 whereas, an increase in entropy was observed. The change in enthalpy and entropy of binding are compensatory and entropically driven binding process suggests that hydrophobic interactions play a predominant role in substrate/inhibitor binding. The difference between ΔH and ΔS for X_4 and X_5 were marginal as compared to X_2 and X_3 indicating that the enzyme has 4 subsites. The xylanase showed a single irreversible transition peak with the T_m of the enzyme varying with protein concentration, scan rate and pH indicating that the thermal unfolding of the enzyme may be kinetically determined.

INTRODUCTION

The knowledge about the nature of interactions of the substrate molecule with specific site on an enzyme molecule is one of the major factors in elucidating the mechanism of enzyme action. The variation of binding constants with systematic changes within a series of substrates and substrate analogues may provide considerable information about such interaction [1]. An accurate determination of the association constants is necessary for evaluating the thermodynamic parameters associated with the binding, which in turn would yield valuable information regarding the forces involved in interaction as well as the nature and dimensions of the combining region. Enzyme-saccharide interaction of glycosidases have been measured primarily for lysozyme using UV difference spectroscopy, fluorescence spectroscopy and equilibrium dialysis [1-4]; which were consistent with and further supported the mechanism deduced by X-ray crystallographic studies [5-7]. Each of the above techniques provide some unique advantage but suffer from the drawback that the thermodynamic parameters characterizing the binding event viz. ΔH (change in enthalpy), ΔS (change in entropy) and ΔC_p (heat capacity change) are obtained indirectly from van't Hoff analysis of the temperature dependence of binding energies. Calorimetric study is a powerful tool for investigating these processes and yield a complete characterization of the binding reaction in terms of single-site thermodynamic parameters ΔG_b, ΔH_b, ΔS_b and ΔC_b; where ΔG_b, ΔH_b, ΔS_b and ΔC_b are free energy change, enthalpy change, entropy change and heat capacity change of binding, respectively. Titration calorimetry measures the heat changes accompanying the isothermal binding reaction and has been used in the present studies to determine the single-site binding constants and binding enthalpies of xylo-oligosaccharides to *Chainia* xylanase.

The xylanases from *Bacillus circulans* [8] and *Pseudomonas fluorescens* [9] have been crystallized with xylotetraose and xylopentaose respectively, and some of the enzyme-substrate interactions have been shown. However, the exact nature and strength of these interactions and the role played by the individual

subsites are not yet clear. There are only few reports on the structural studies and thermal stability of family F/10 xylanases (multi domain proteins) [10-13] but family G/11 xylanases (single domain proteins) have not been studied with respect to the above aspects. The present investigation was carried out to study the nature of interactions of xylo-oligosaccharide binding to xylanase from *Chainia* sp.; determine the equilibrium and thermodynamic parameters of substrate/inhibitor binding by using the fluorescence spectroscopy and titration microcalorimetry (ITC) and study the thermal stability of the xylanase by UV spectroscopy and differential scanning calorimetry (DSC).

MATERIALS AND METHODS

The enzyme and saccharides were prepared as described in the chapter 2. Sodium acetate, sodium phosphate (monobasic and dibasic), acetic acid and citric acid were from Sigma Chemical Company, USA. Any other chemicals and reagents used were of analytical grade.

Organism and culture conditions

Growth, maintenance of *Chainia* sp. (NCL 82-5-1), production and the purification of xylanase were carried out as described in chapter 2.

Enzyme assay

The xylanase activity was assayed at pH 6.0 and 50 °C by incubating 0.5 ml of 1% (w/v) soluble oat spelt xylan and 0.5 ml of appropriately diluted enzyme for 30 min followed by measuring the reducing sugars liberated spectrophotometrically, at 540 nm, using D-xylose as standard (Chapter 2).

Protein Determination

Protein determination of the purified enzyme was routinely performed by using the relationship *viz.* $A^{0.1\%}$ cm, 280 nm = 1.40.

Preparation of enzyme solution

The enzyme solution (for fluorescence measurements) was prepared in 30 mM sodium acetate buffer (prepared in Milli-Q water), pH 5.6, and dialyzed against the same buffer overnight, filtered through 0.45 μm filter and protein concentration was determined. For ITC, DSC and UV spectroscopic studies, the protein solution was prepared as above in 20 mM citrate-phosphate buffer, pH 6.0, and the concentration was determined.

Preparation of xylo-oligosaccharide solutions

Xylo-oligosaccharide solutions were prepared (X_2, X_3, X_4 and X_5) by weighing out the dry powder and dissolving in the respective dialyzate to minimize the buffer mismatch of the protein and ligand solution. The solutions were filtered through 0.45 μm filter before use. The saccharide concentration was also determined by reducing sugar estimation [14] as well as total carbohydrate estimation [15].

Fluorescence measurements

Fluorescence measurements were performed on Perkin-Elmer Spectrofluorimeter LS 5B, using an excitation and emission slit width of 5 nm. An excitation wavelength of 280 nm was used and the emission was monitored at 338 nm (λ_{max} of the enzyme). The fluorescence quenching measurements were made by titrating 2 ml of enzyme solution (4 μM in 30 mM sodium acetate buffer, pH 5.6) with saccharide solution (30 mM, 5-30 μl aliquots) followed by monitoring the change in fluorescence at 338 nm. The fluorescence of the buffer and saccharide solution (if any) were measured at appropriate wavelength and used to correct the observed fluorescence. Corrections were also made to compensate the dilution upon addition of the sugar and at the highest concentration of the saccharide the volume change was less than 5% of the enzyme solution. The temperature of the enzyme and saccharide solutions was maintained at ± 0.1°C of the specified temperature by using a circulating cryobath. The relative fluorescence intensity of the xylanase saturated with

saccharide (F_∞) was obtained from the experimental data by plotting $1/F_o$-F versus 1/[S] and extrapolating to Y-axis, where, F_o is the fluorescence intensity of the enzyme alone and F is the fluorescence intensity of the enzyme at a saccharide concentration [S]. Log (F_o-F/F-F_∞) was plotted against Log [S] and the association constant (K_a) was determined from the plot by assuming the relation that the pK_a of the complex equals the value of [S] when Log (F_o-F/F- F_∞) = 0 [1]. Free energy changes of association were determined by the equation;

$$\Delta G° = -RT \ln K_a. \quad (1)$$

The thermodynamic parameters *viz.* change in enthalpy (ΔH) and entropy (ΔS) were calculated by van't Hoff analysis from the temperature dependence of K_a by using the equation;

$$\ln K_a = (-\Delta H/RT) + \Delta S/R. \quad (2)$$

The slope of the plot of ln K_a versus 1/T equals ($-\Delta H/R$), from which the enthalpy change was calculated. The entropy change was then obtained from the equation;

$$\Delta G° = \Delta H - T\Delta S. \quad (3)$$

Titration microcalorimetry studies

The calorimetric titrations were performed on a MicroCal Omega titration calorimeter as described by Wiseman *et al.* [16]. The titration calorimeter consists of a matched reference cell containing the buffer and a solution cell (1.38 ml) containing the protein solution. Microliter aliquots of the ligand solution were added to the protein solution through a rotating stirrer-syringe operated with a plunger driven by a stepping motor. After a stable base line was achieved at a given temperature, the automated injections were initiated and the accompanying heat change per injection was determined by a thermocouple sensor between the cells. A sharp exothermic peak appeared after each injection and was allowed to return to the base line before the next injection. The successive injections were repeated at an interval of 3 minutes. The area of each peak represents the amount of heat accompanying binding of the added ligand to the protein. The xylanase

concentrations, in a typical calorimetric experiment ranged from 0.306 to 0.383 mM while the ligand concentrations were 10, 20 and 30 mM for X_5, X_4 and X_3, respectively. The results in each titration were independent of the number of injections, which ranged from 16-25. The enthalpy change (ΔH_b) and association constant (K_b) were determined by the following equations.

As described by Wiseman *et al.* [16], for a reaction of 1:1 stoichiometry :

$$M + X \rightleftharpoons MX$$

the single-site binding constant K_b is defined as

$$K_b = [MX] / [X][M] \tag{4}$$

and total ligand X_t and protein M_t concentration by

$$X_t = [X] + [MX] \tag{5}$$

$$M_t = [MX] + [M] = [MX] + [MX] / K_b[X] \tag{6}$$

The change in MX concentration can be related to the heat change as

$$dQ = d\,[MX]\,\Delta H_b V \tag{7}$$

Where, ΔH_b is the molar enthalpy of binding, dQ is the heat change and V is the cell volume.

The total heat evolved or absorbed, Q_t, for a reaction with stoichiometry 1:1 is given by the equation ;

$$Q_t = V\Delta H_b/2 \left\{ X_t+M_t+1/K_b - \left[(X_t+M_t+1/K_b)^2 - 4M_t X_t \right]^{1/2} \right\} \tag{8}$$

The total heat, Q_t in a calorimetric method can be fitted via non-linear least square minimization method [16] to the total ligand concentration, X_t, using the general equation [17],

$$Q_t = n\, M_t\, \Delta H_b\, V / 2 \left\{ 1 + X_t / n\, M_t + 1 / n\, K_b\, M_t \right.$$

$$\left. - \left[(1 + X_t / nM_t + 1/nK_b\, M_t)^2 - 4\, M_t X_t \right]^{1/2} \right\} \qquad (9)$$

where, n is the number of ligand binding sites.

The temperature dependent values of the change in binding enthalpies obtained from the reaction calorimetry performed at different temperatures, the change in heat capacities can be obtained according to the equation :

$$\Delta C_p = (\delta \Delta H / \delta T)_p \qquad (10)$$

or

$$\Delta C_p = R\, (\delta / \delta T) \left\{ - \delta \ln K_{app} / \delta\, (1/T) \right\} \qquad (11)$$

The thermodynamic quantities $\Delta G°_b$ and ΔS_b were obtained from the basic equations of thermodynamics,

$$\Delta G°_b = - RT \ln K_b \quad \text{and} \quad \Delta G°_b = \Delta H_b - T\Delta S_b$$

UV spectroscopy and Differential scanning calorimetry

Thermal unfolding of the *Chainia* xylanase was studied using UV spectroscopy. The transition temperature (T_m) was determined using a Perkin-Elmer Lambda UV-visible spectrophotometer connected with temperature programmer. Xylanase solution (5 μM, 2 ml) in 20 mM citrate-phosphate buffer, pH 6.5, both in presence and absence of 0.5 mM X_2 and 0.3 mM X_3 was heated at a rate of 30°C h^{-1} and monitored at 280 nm. The data was fitted to the equation ;

$$f_u = A_N - A / A_N - A_u. \qquad (12)$$

where, f_u is the fraction unfolded and A_N, A and A_u are $A_{280\ nm}$ of native, intermediate and unfolded fraction. The transition temperature, T_m, was determined from the fitted curve. In a series of parallel experiments, the T_m of the

enzyme was also determined using different enzyme concentrations *viz.* 5-25 μM in pH 6.0 and at different pH at a concentration of 2.5 μM.

The thermal denaturation of the xylanase was also monitored using DSC. DSC measurements were performed with a MicroCal MC-2 DSC heat conduction scanning microcalorimeter. The calorimeter consisted of two fixed cells (1.18 ml), a reference cell and a solution cell. The enzyme solution was prepared by dialyzing extensively against 20 mM citrate-phosphate buffer, pH 6.0. The solution was filtered through 0.45 μm filter and degassed in an evacuated chamber for 10 min and after determining the concentration loaded into the calorimeter cell and the final dialysis buffer (also degassed) was loaded into the reference cell. The measurements were made at varying scan rates *viz.* from 20 – 60 K h^{-1} with different protein concentrations (1 – 4 mg / ml) in 20 mM citrate-phosphate buffer, pH 6.0, from 283 – 363 K. Reversibility of the thermal transitions was checked by performing a rescan immediately after cooling the sample from first scan. The temperature dependence of molar heat capacity was analyzed using software package (Origin) supplied by MicroCal.

RESULTS

Fluorescence measurements : Addition of xylo-oligosaccharides to xylanase resulted in quenching of fluorescence associated with a small blue shift in fluorescence maxima from 338 nm to 335 nm. The maximum quenching of intrinsic fluorescence of the xylanase was 5, 8, 10 and 12% by X_2, X_3, X_4 and X_5 respectively, and higher oligosaccharides yielded a maximum quenching of 15%. The slope of the plot of log F_o-F/F-F_∞ versus log [S] was unity for all the oligosaccharides used, indicating the formation of one to one complexes (Fig 1B). The association constants of binding calculated are 1245, 2670, 3730 and 3926 for X_2, X_3 X_4 and X_5, respectively, at 25°C (Table I, Fig 1B). The change in entropy increased with the increase in oligosaccharide chain length whereas, a decrease in

van't Hoff enthalpy was observed (Fig 2, Table I). Temperature dependence of the association constants showed a gradual decrease for all the saccharides with increasing temperature. The plot of ΔH versus $T\Delta S$ (Fig 3) gave a straight line with a slope of 0.9 indicating the enthalpy-entropy compensation.

Titration calorimetry : A typical calorimetric titration curve of the addition of xylo-oligosaccharides to 1.38 ml of xylanase is shown in Figure 4. The titration curve exhibits a monotonic decrease in exothermic heat of binding for each successive injection. Binding constant was very low for X_3 and moderate for X_4 and X_5. The enthalpies range from -5.4 ± 0.8 kJ mol^{-1} for X_3 and 1.2 ± 0.18 kJ mol^{-1} for X_4 at 283 K and 1.79 ± 0.29 kJ mol^{-1} for X_5 at 293 K. The thermodynamic parameters of binding are given in Table II. The standard deviation of the K_b values range from 5-20% while that of ΔH_b values range from 5-15%. The standard deviation in K_b and ΔH_b values can be attributed to the uncertainty in protein and ligand concentrations, uncertainty in fitting Eq. 8 to the calorimetric measurements at low K_b values and a buffer-saccharide mismatch of about 0.6 µcal/sec (Fig 4B inset) observed for all the sugars. Moreover, because of the low K_b values and protein solubility, the measurements were made at C values from 0.2 to 0.4 (C value - a unit less constant is determined by total protein concentration, $M_{tot} \times K_b$. As described by Wiseman *et al.* [16] ideal C values for calorimetric measurements are from 1- 1000).

UV spectroscopic and DSC studies : *Chainia* xylanase showed a single irreversible transition upon heating. The T_m of the enzyme varied with pH and enzyme concentration (Table III). Presence of either X_2 or X_3 did not have any effect on the transition curve (Fig 5). The enzyme also showed irreversible single transition in DSC and the T_m of the enzyme showed a small variation with change in concentration and scan rate. Temperature induced transition of the enzyme was irreversible as rescans showed a stable base line. The T_m of the xylanase at a concentration of 2 mg/ml was 68.77°C at a scan rate of 45°C h^{-1} and 70.28°C at a scan rate of 60°C h^{-1} (Fig 6). The transition enthalpies ΔH_{Tm} were 68.3 ± 0.95 and 95.2 ± 1 kcal mol^{-1} at a scan rate of 45 and 60°C h^{-1}, respectively.

Table I : Thermodynamic parameters of xylo-oligosaccharide-*Chainia* xylanase binding from fluorescence titrations and van't Hoff analysis. K_a, $\Delta G°$, ΔH and ΔS are association constant, free energy change, van't Hoff enthalpy change and entropy change of binding, respectively.

Saccharide	K_a				$-\Delta G°$ *	$-\Delta H$	$-\Delta S$
	—— Temperature (K) ——				(kJ mol^{-1})	(kJ mol^{-1})	(J $mol^{-1}K^{-1}$)
	293	298	303	308			
X_2	1513	1245	935	—	17.84	36.69 ± 1.9	63.8
X_3	2935	2670	2401	1930	19.45	20.97 ± 2.3	4. 77
X_4	4010	3730	3320	1915	20.21	16.51 ± 1.3	-12.61
X_5	4315	3926	3540	—	20.51	14.94 ± 0.5	-18.60

* - values are determined at 293 K.

Table II : Thermodynamic parameters of xylo-oligosaccharide-*Chainia* xylanase binding from the calorimetric titrations. K_b, $\Delta G_b°$, ΔH_b and ΔS_b are single-site binding constant, Gibb's energy change and binding enthalpy change and binding entropy change, respectively.

Saccharide	Temperature K[(a)]	K_b (M^{-1})	$-\Delta G_b°$ (kJ mol^{-1})	$-\Delta H_b$ (kJ mol^{-1})	ΔS_b (J $mol^{-1}K^{-1}$)
X_3	283	260	13.10	5.40 ± 0.80	26.80
X_4	283	1110	16.50	1.20 ± 0.18	54.10
	293	982	16.80	1.02 ± 0.30	53.80
X_5	280.1	1260	16.62	2.44 ± 0.32	50.60
	293	1210	17.30	1.79 ± 0.20	52.93

[(a)] uncertainty in the temperature is + 0.1 K.

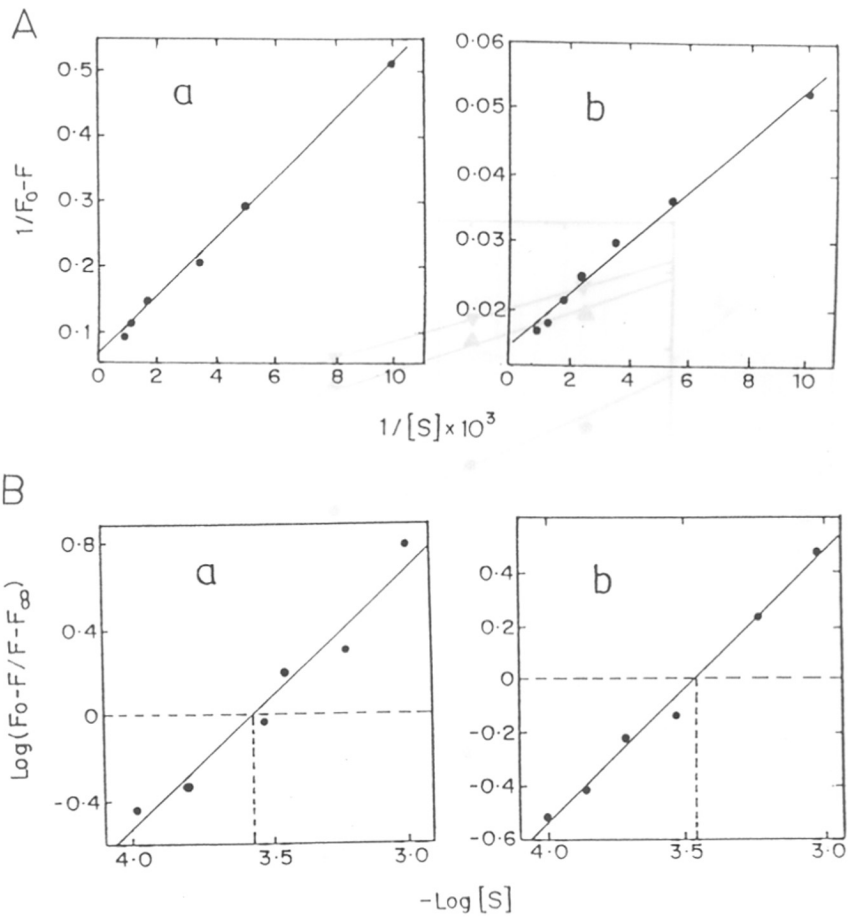


Fig 1. Titration of xylanase with xylo-oligosaccharides at 25°C. The xylanase solution (4 μM, 2 ml) in 30 mM sodium acetate buffer was titrated with 30 mM solution of xylo-oligosaccharides.

A. Determination of F_∞ : (a) X_2 and (b) X_5. The relative fluorescence intensity of the xylanase saturated with saccharide (F_∞) was obtained from the reciprocal of $1/F_o$-F at X = 0.

B. Graphical representation for the determination of association constant. (a) X_3 (K_a = 2670 mol^{-1} at 25°C) and (b) X_4 (K_a = 3730 mol^{-1} at 25°C)

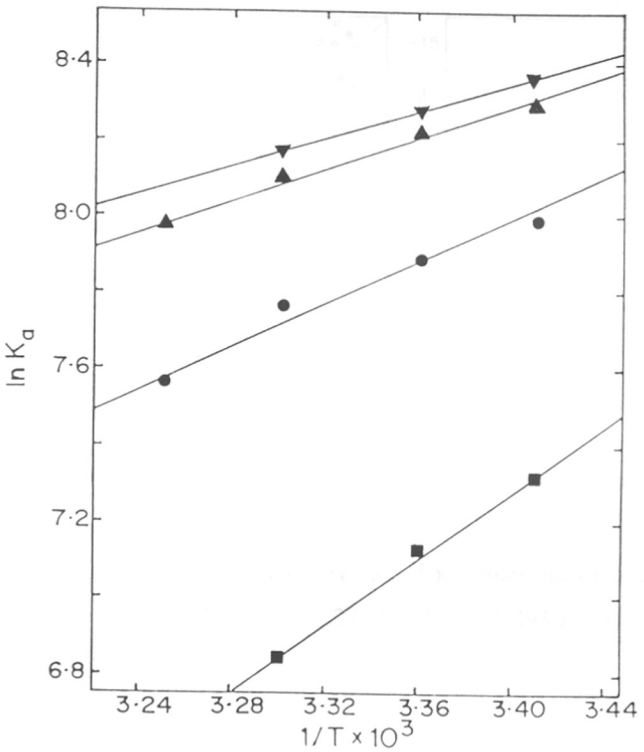


Fig 2. Van't Hoff plots for the association of xylo-oligosaccharides to *Chainia* xylanase. Symbols used are; —■— X_2, —•— X_3, —▲— X_4 and —▼— X_5

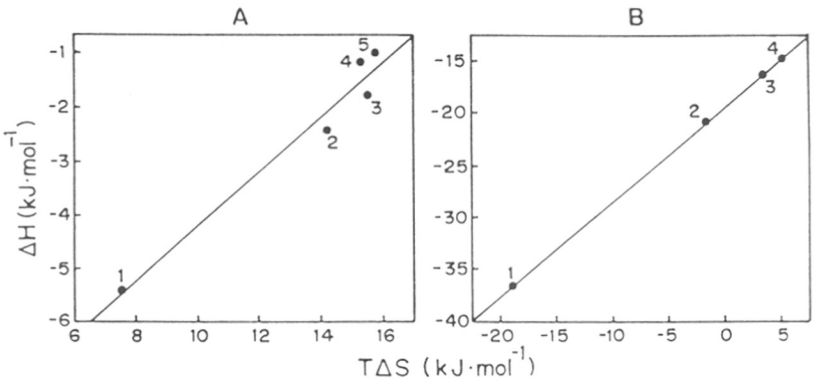


Fig 3. Plot of ΔH versus TΔS for the binding of xylo-oligosaccharides to *Chainia* xylanase.

A. *From calorimetric measurements* : The values are calculated from table II. 1) X_3 at 283 K, 2) X_4 at 283 K, 3) X_4 at 293 K, 4) X_5 at 280 K and 5) X_5 at 293 K.

B. *From fluorescence titrations* : The values at 293 K are calculated from the experimental data from the table I. 1) X_2, 2) X_3, 3) X_4 and 4) X_5.

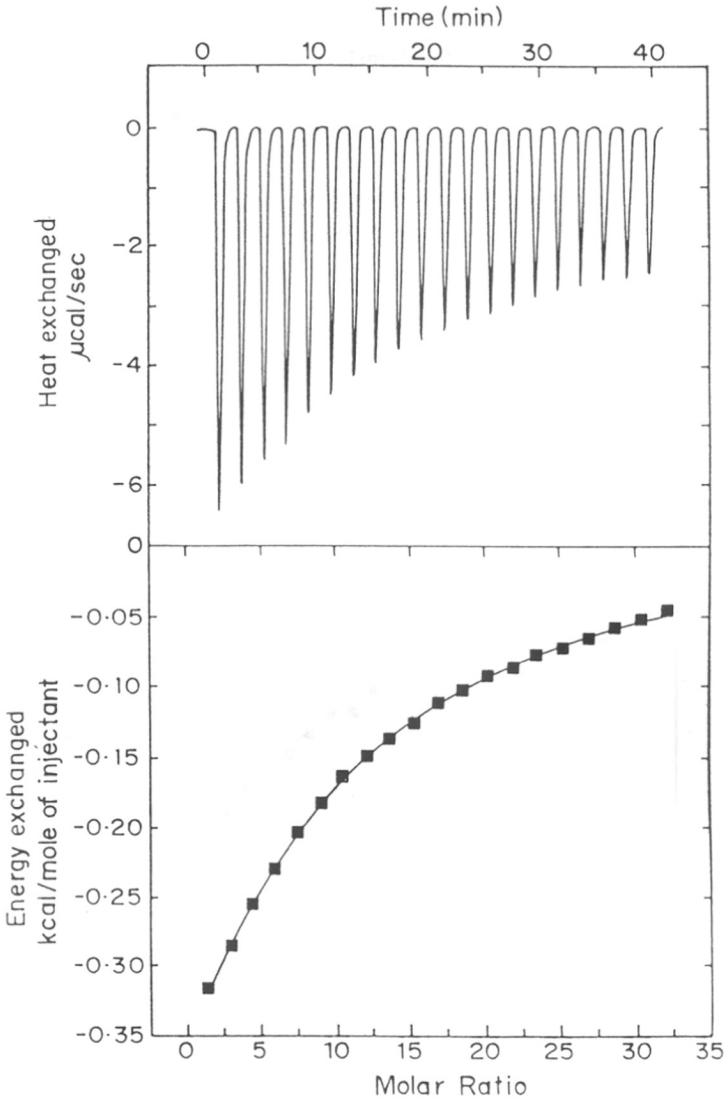
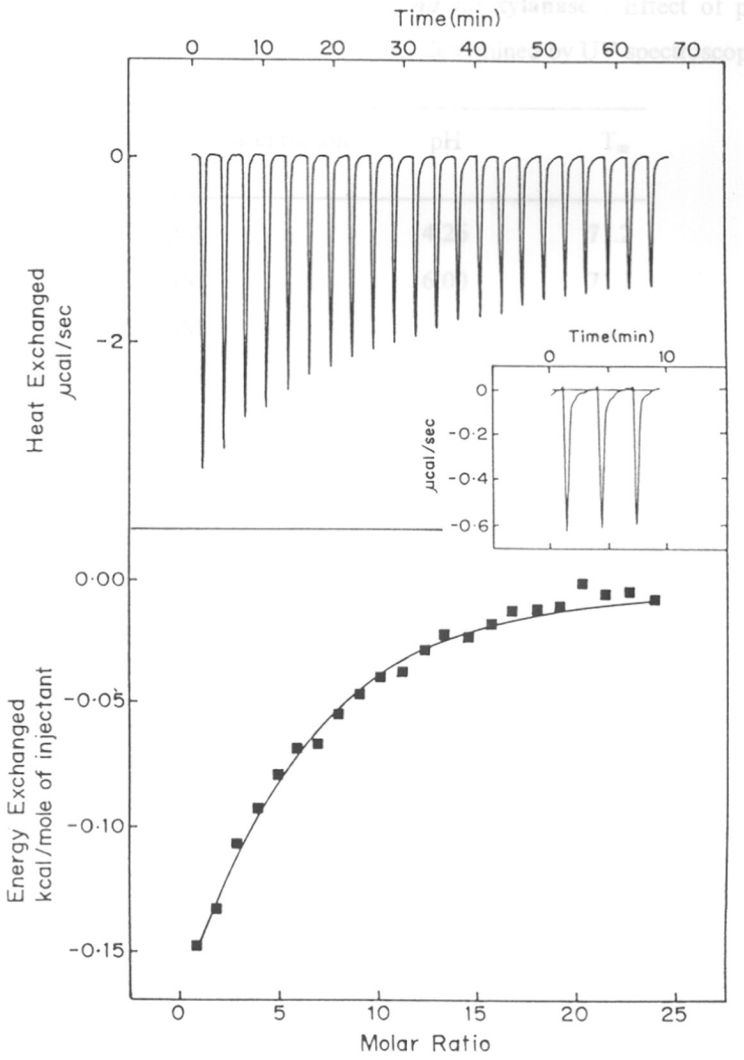


Fig 4. Calorimetric titrations

A. Calorimetric titration of 15 µl aliquots of 30 mM X_3 into 0.306 mM of xylanase in 20 mM citrate-phosphate buffer, pH 6.0, at 283 K.



B. Calorimetric titration of 15 μl aliquots of 20 mM X_4 into 0.306 mM of xylanase in 20 mM citrate-phosphate buffer, pH 6.0, at 283 K. Inset : buffer-saccharide mismatch (observed for all the sugars used)

Table III . Thermal denaturation of *Chainia* xylanase : Effect of pH and enzyme concentration on thermal unfolding, determined by UV spectroscopy.

Enzyme concentration	pH	T_m
2.5 μM	4.26	71.2
2.5 μM	6.00	71.1
2.5 μM	7.40	68.2
2.5 μM	8.25	66.0
2.5 μM	9.52	65.7
2.5 μM	9.80	63.9
2.5 μM	10.03	62.9
5 μM	6.00	69.9
10 μM	6.00	68.9
25 μM	6.00	67.3
5 μM	6.50	69.4
5 μM + X_2(0.5 mM)	6.50	69.6
5 μM + X_3(0.3 mM)	6.50	69.4

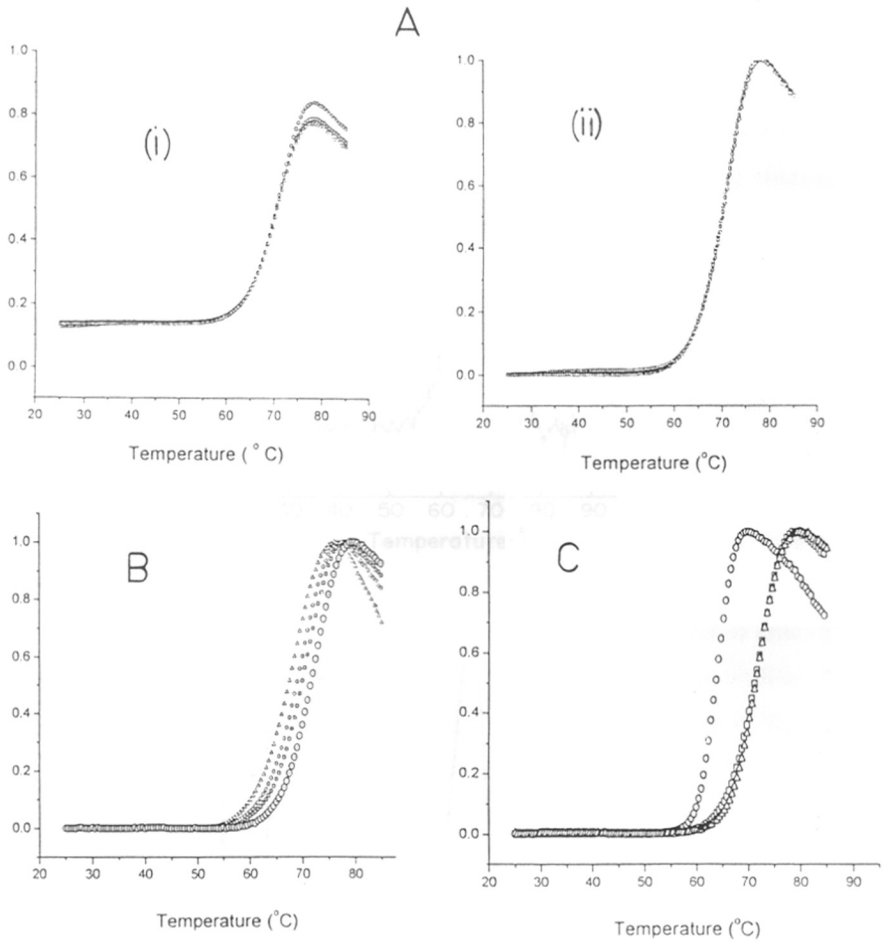
Fig 5. UV spectrum of thermal unfolding of *Chainia* xylanase.

A. The enzyme 5 μM in 20 mM citrate-phosphate buffer, pH 6.5, was heated at a rate of 30°C h^{-1} and the T_m was calculated from the fitted data

(i) Observed data ; —□— xylanase, —o— xylanase + 0.5 mM X_2 and —Δ— xylanase + 0.3 mM X_3. (ii) Fitted data ; —□— xylanase, —o—xylanase + 0.5 mM X_2 and —Δ— xylanase +0.3 mM X_3

B. Concentration dependent thermal unfolding of xylanase. The enzyme in 20 mM citrate-phosphate buffer, pH 6.0, was heated at a rate of 30°C h^{-1} and the T_m was calculated from the fitted data; —⊙— 2.5 μM, —□— 5 μM, —o—10 μM and —Δ— 25 μM

C. pH dependent thermal unfolding of xylanase. The enzyme 2.5 μM was heated at a rate of 30°C h^{-1} and the T_m was calculated from the fitted data. —Δ— pH 4.3, —□— pH 6.0 and —0— pH 9.8


A
(i)
(ii)
B
C
Temperature (° C)
Temperature (°C)
Temperature (°C)
Temperature (°C)

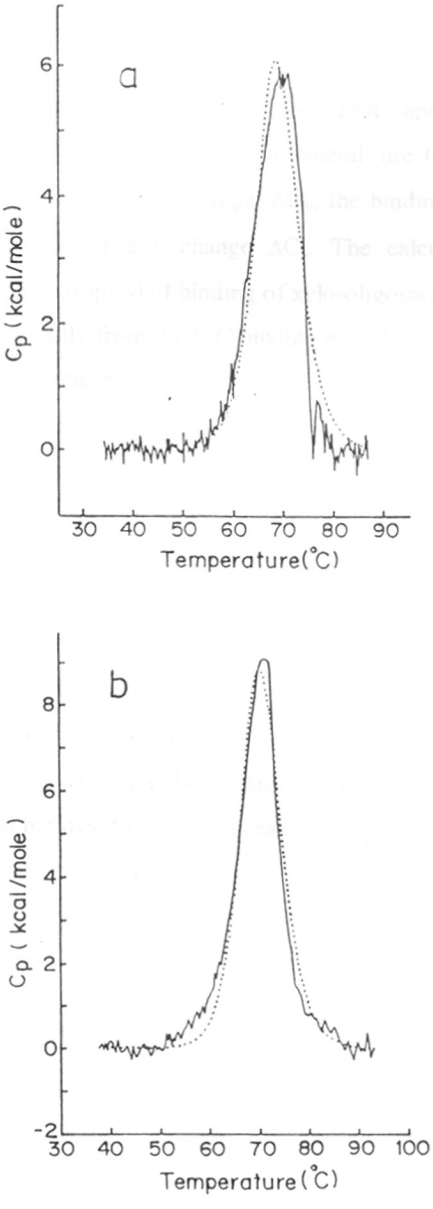


Fig 6. **DSC thermogram of *Chainia* xylanase:** The enzyme (2 mg/ml) in 20 mM citrate-phosphate buffer, pH 6.0, was scanned at rate of (a) 45°C h^{-1} and (b) 60°C h^{-1}

DISCUSSION

The thermodynamic quantities that are most appropriate for the characterization of ligand binding equilibria, in general, are the Gibb's binding energy change, ΔG_b, binding enthalpy change, ΔH_b, the binding entropy change, ΔS_b and the binding heat capacity change ΔC_b. The calculated van't Hoff enthalpies (and hence the entropies) of binding of xylo-oligosaccharide to *Chainia* xylanase differed substantially from the ITC binding enthalpies. Such differences between the ITC binding enthalpies and van't Hoff enthalpies are not uncommon and have been described in detail [18]. Both in van't Hoff analysis and ITC measurements, the enthalpies decreased with the increasing chain length while an increase in entropy was observed. The ITC data could not be obtained for X_2 with certainty because of very low exothermic heat signals, which in turn gave a large standard deviation. The enzyme displayed a very low affinity towards X_2 and such low affinities towards xylo-oligosaccharides have been reported in case of other xylanases also [19,20]. The fluorescence data suggests that the binding of X_2 is enthalpically driven. The thermodynamic parameters of binding of X_3, X_4 and X_5 (van't Hoff analysis as well as ITC measurements) reveal that the binding of these xylo-oligosaccharides to the xylanase is entropically driven. Direct microcalorimetric measurements of the binding reaction enthalpy, at different temperatures, are the most reliable source of heat capacity data. The binding enthalpies of xylo-oligosaccharides do not vary appreciably with the temperature ($dH_b / dT = 0$) and together with the linearity of van't Hoff plots indicate that very little, if any, change in the heat capacities accompany the reaction. Van der Waals interaction, such as hydrogen bonding would essentially be enthalpically driven, with little change in the heat capacity, while hydrophobic interactions would be essentially entropically driven. Lack of heat capacity change upon binding of the xylo-oligosaccharides to *Chainia* xylanase implies that little structural change in the enzyme takes place on oligosaccharide binding. The three-dimensional structure of xylanases (from both families, G/11 and F/10) reveal that the active site cleft is dominated by highly conserved aromatic residues which are perfectly positioned to stabilize at least three to four xylose residues

[21,22]. The structure of xylanase-X_4 complex of *B. circulans* xylanase [8] indicated some of these interactions. The stacking interaction of Trp 9 with the face of the xylose ring was clearly observed and such stacking interaction have been shown for a variety of glycosidases [23,24] including lysozyme [25,26] and appears to be common among the glycosidases [8]. The pyranose rings of sugars with axial hydroxyls present a hydrophobic surface that interacts with aromatic side chains [27]. Tyr residues form hydrogen bonds with the hydroxyl group of xylose ring. In xylanase A from *P. fluorescens*, the conserved aromatic residues (Trp 83, Trp 305, Trp313, Phe 181, Tyr 220 and Tyr 225) line the substrate binding cleft and make contacts with X_5.

The changes in the enthalpy and entropy for the binding process of xylo-oligosaccharides to *Chainia* xylanase are compensatory, indicating that these interactions which are primarily entropically driven, are accompanied by reorganization of water molecules around both the enzyme and xylo-oligosaccharides. This compensatory effect is displayed in the plots of ΔH versus $T\Delta S$ whose slope is close to 1. Indeed, substrate binding studies of inactive xylanase A E246C mutant from *P. fluorescens* [9,28] have shown that X_5 is bound in the cleft in such a way that some or all of a chain of 20 water molecules that runs along the substrate binding cleft, are expelled upon binding of X_5. Such enthalpy-entropy compensation is common in lectin-saccharide interaction [29-32] and has also been observed for several other protein-ligand interactions [33-35] and has been rationalized by implicating a unique role of water in mediating protein-carbohydrate recognition [36].

Temperature induced unfolding of the xylanase led to a single irreversible transition indicating that the enzyme is a single domain protein. The T_m of the enzyme showed variation with pH, scan rate and enzyme concentration. The enzyme was stable at 65°C in pH 6.0, for 30 min with the retention of 90 % of its initial activity but was inactivated totally within 10 minutes at 70°C which is in agreement with the observed T_m of the enzyme. As would be expected from the low binding constants of X_2 and X_3, they did not have any effect on the melting which is reflected in the identical transition curve of the enzyme (without any

change in T_m) in presence of X_2 and X_3. Linearity of van't Hoff plot suggests that X_2 and X_3 would not bind to the enzyme above the temperatures 45°C and 60°C, respectively. *Chainia* xylanase is more susceptible to denaturation in alkaline pH at elevated temperatures as compared to acidic conditions which is reflected in lower T_m of the enzyme at alkaline pH. DSC measurements also revealed an irreversible scan rate dependent transition for the enzyme. The scan rate dependence, concentration dependence and the irreversibility of the DSC thermogram suggest that the denaturation process of the enzyme may be kinetically determined.

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CHAPTER 5

General discussion and conclusions

DISCUSSION

Xylans are the most plentiful of the hemicelluloses (from major renewable sources) produced by photosynthesis, amounting to several million tonnes per annum globally. Microbial xylanolytic enzymes have received a great deal of attention because of their biotechnological application in paper and pulp industries. Recent findings of xylanases as elicitors of plant defense mechanism have opened a new area of research. Xylanases appear to play an important role in host-pathogen interaction in plants.

Based on sequence homology and hydrophobic cluster analysis, glycosyl hydrolases have been classified into 45 families; xylanases and cellulases falling into families 1,2,5, 10, 11 and 17 [1]. Recently, conserved catalytic and structural (common fold) features among the members of different family have led to a proposal for glycosyl hydrolase super families [2,3]. The enzymes from families 1,2,5,10 and 17 have a common fold (8-fold α/β barrel resembling triosephosphate isomerase) and the structure of the active site is highly conserved. [4]. The pectin hydrolyzing enzymes form yet another super family [4]. Xylanases have been grouped mainly into two families (family 10 and 11). Family 11 xylanases are relatively low molecular weight proteins (20-25 kDa) and have a unique β-sandwich type fold resembling plant legume lectins [5] and share some similarities with cellobiohydrolase I from *Trichoderma reesi* (Family 7) [6]. Family 10 enzymes on the other hand, show structural similarities with other glucanases and amylases. In evolutionary terms, the microbes producing these enzymes were diverged prior to the appearance of their substrate, xylan, in plants [7,8]. It is speculated that the binding and hydrolysis of three main plant cell wall carbohydrates has been achieved by both convergent and divergent evolution of different protein structural motifs [4].

Among the microorganisms, fungi, bacteria and actinomycetes have been studied in detail for xylanolytic enzymes. *Chainia* sp. (NCL 82-5-1) - a sclerotium forming actinomycete was isolated in our laboratory from soil samples from Rajastan, India [9]. The genus *Chainia* was first proposed by Thirumalachar

[10] to accommodate an actinomycete isolated from soil samples from Poona, India. Since then several species of *Chainia* have been described [11-13]. Taxonomic status of the genus *Chainia* is a subject of controversy with some workers considering that it should be regarded as a synonym of *Streptomyces* [14-18] and others giving reasons for the retention of the taxon [19-21]. The transformation of vegetative mycelium into lobed multilocular cerebriform sclerotic granules [22] and the production of L-2,3 diamino propionic acid in the cell wall [19] clearly distinguish *Chainia* from *Streptomyces* and other actinomycetes. However, *Chainia* shows some similarities with *Streptomyces*, such as, presence of high G + C content in its DNA [23,24], high DNA homology with *Streptomyces* [25,26], presence of streptomycete like fatty acids [24], menaquinones [27,28] and polar lipids [29], susceptibility towards polyvalent streptomycete phages [25,30,31] etc. Based on these properties, Goodfellow *et al.* [32] have proposed that the genus *Chainia* become a junior synonym of the genus *Streptomyces* and that the species it contains be named accordingly.

Chainia sp.(NCL 82-5-1) produces intra/extracellular xylose isomerase [9,33,34] and an extracellular endoxylanase but no detectable extracellular cellulase even when grown on CM-cellulose. The results of our studies clearly show that *Chainia* produces a single extracellular endoxylanase of Mr ~21,000, in contrast to the reports of the presence of two xylanases *viz.* xylanase I and xylanase II of molecular mass 21,000 and 5,000-6,000, respectively, from the same strain of *Chainia* [35,36]. It had been previously suggested that xylanase I with an apparent molecular weight 4 to 6 times that of xylanase II, is a cluster of xylanase II molecules [37]. We did observe a minor xylanase peak in the initial washings of CM-Sephadex column, showing identical physicochemical and functional properties to that of major xylanase and could not be detected in presence of either β-ME or DTT, indicating it to be an aggregate of the same enzyme. Indeed, Srinivasan *et al.* had earlier reported the presence of only one extracellular xylanase from *Chainia* sp. [38]. The xylanase II (Mr 5,000-6,000) reported by the earlier group has the same temperature and pH optima, mode of

action and its N-terminal sequence is identical to our enzyme. The pI of the xylanase determined by us to be 9.4-9.5, in contrast to that reported earlier i.e., 8.5 [37] and 8.1-8.2 [35]. The protein does not contain any covalently associated carbohydrate while the earlier work classifies the enzyme to be a glycoprotein. These differences could probably due to a heterogeneous preparation of the enzyme used earlier. This was reflected in the significant amount of low molecular weight peptides which were found associated with the enzyme on reverse phase HPLC and matrix assisted laser desorption spectroscopy [37]. Recently we came across a report of purification of xylanase II from *Chainia* reporting homogeneous enzyme preparation with a specific activity of 41 U/mg [39] whereas; our enzyme preparation had a specific activity of 550 U/mg. In the absence of an accurate determination of molecular mass of xylanase II by the earlier group, using another approach such as mass spectrum or a complete amino acid sequence, it is reasonable to assume that the xylanase reported in the present study and xylanase II reported formerly are the same.

The results of chemical modification studies indicates that *Chainia* xylanase has only one catalytic carboxyl group instead of two reported for the other xylanases. The sequence alignment of the chemically labeled peptide with the conserved sequence of acid/base Glu of other xylanases (Family G/11) clearly indicates that the carboxylate of *Chainia* xylanase act as acid/base catalyst. Our studies indicate the involvement of arginine in substrate binding and thiol of cysteine functioning as catalytic nucleophile. However, the Cys residue is not conserved in family G/11 xylanases and some of the enzymes are devoid of cysteine. It has also been suggested that the cysteine residue could play an important role in maintaining the active site geometry. The exact role played by cysteine residue in xylanases will have to wait for further studies like site-directed mutagenesis and X-ray crystallography.

Chainia xylanase contains 3 Trp residues, which are located in a similar moderately hydrophobic environment. From our studies it is clear that N-bromosuccinimide inactivates the enzyme through peptide cleavage and tryptophan residues are not directly involved in the substrate binding.

As evidenced by crystallographic studies, the active site of xylanases is dominated by aromatic residues (conserved tryptophans and tyrosines and to a lesser extent phenylalanines), it is possible that Trp residues of *Chainia* xylanase are located in the active site crevice but not adjacent to catalytic residues: They may play a minor role in stacking interaction with extended xylan chain.

Thermodynamic parameters of xylo-oligosaccharide binding suggest that the substrate binds to the enzyme through van der Waals forces (hydrogen bonding) and hydrophobic interactions with little structural change in the enzyme. The binding affinity of X_2 to the enzyme is very low, which is in agreement with the experimental observation that X_2 does not cause any end product inhibition even at higher concentrations. Fluorescence quenching data reveals that the binding of X_2 takes place through van der Waals interactions. Entropically driven binding of X_3, X_4 and X_5 imply that the hydrophobic interaction play a predominant role in the process of binding which is obvious from the presence of a large number of aromatic residues in the active site crevice and the ability of pyranose rings with axial hydroxyls to form a hydrophobic surface that could interact with these aromatic side chains.

CONCLUSIONS

1. *Chainia sp. (NCL-82-5-1) produces a single extracellular endoxylanase of molecular mass ~21,000.*

2. *Chainia xylanase is a monomeric, non-glycosylated basic protein with a pI of 9.4-9.5 and belongs to family G/11 of glycosyl hydrolases.*

3. *The enzyme is a non-debranching endoxylanase with a minimum of four-xylose units required for initiation of hydrolytic action and does not show transglycosylation reaction. End products of xylan and xylo-oligosaccharide hydrolysis are mainly xylotriose and xylobiose, a small amount of xylose and traces of higher xylo-oligosaccharides.*

4. *Chainia xylanase contains a single catalytic carboxylate. By sequence homology of the labeled carboxylate peptide it has been proposed that the carboxylate act as the acid/base catalyst while the thiol of Cys may function as a nucleophile. Arginine is involved in substrate binding.*

5. *N-bromosuccinimide inactivates the enzyme through peptide cleavage and Trp does not play any significant role in the catalytic function. All three tryptophan residues are present in a similar moderately hydrophobic environment.*

6. *Xylo-oligosaccharides bind to the enzyme with very low affinities and the binding is entropically driven with little structural change, indicating the reorganization of water molecules around both the enzyme and xylo-oligosaccharides, to be the driving force in substrate binding.*
   Entropically driven binding also implies the predominant role of hydrophobic interactions in the process of binding.

7. *The thermal unfolding of the enzyme is concentration and scan rate dependent indicating that the unfolding process may be kinetically determined.*

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List of publications

1. Purification and characterization of an extracellular endoxylanase from an actinomycete *Chainia* sp. Hegde S. S., Kumar A. R., Suresh C. G., Kotwal S. M., Ganesh K. N. and Khan M. I. (1997) J. Biochem. Mol. Biol. Biophys. (in Press)
2. Endoxylanase from *Chainia* sp.: Involvement of a cysteine and carboxylate in catalysis and arginine in substrate binding. Hegde S. S., Kumar A. R., Ganesh K. N. and Khan M. I. (communicated).
3. Binding of xylo-oligosaccharide inhibitors/substrates to the endoxylanase from *Chainia* sp.: Evaluation of equilibrium and thermodynamic parameters. Hegde S. S., Kumar A. R., Ganesh K. N. and Khan M. I. (communicated).