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STRUCTURE, FUNCTION AND STABILITY OF FUNGAL CELLULASES

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LIST OF ABBREVIATIONS

T	absolute temperature
A	absorbance
$E^{1\%}_{1cm, 280nm}$	absorption coefficient of 1% solution in 1cm path length
$(NH_4)_2SO_4$	ammonium sulphate
ANS	8-anilino-1-naphthalene sulphonic acid
Å	Angstrom
Asp	asparatic acid
AU	arbitrary units
BSA	bovine serum albumin
$CaCl_2$	calcium chloride
CBD	carbohydrate binding domain
CBH	cellobiohydrolase
CMC	carboxy methyl cellulose, sodium salt
cm	centimeter
CD	circular dichroism
C	concentration of protein
$CuCl_2$	copper chloride
$CuSO_4$	copper sulphate
$^{\circ}C$	degree centigrade
EG	endoglucanase
$^{\circ}K$	degree Kelvin
ρ_p	density of protein solution (mg/mL)
ρ_o	density of solvent buffer (mg/mL)
DEPC	diethyl pyrocarbonate
DEAE	diethylaminoethyl
DNS	2, 4-dinitrosalicylic acid
DTT	dithiotreitol
DTNB	5, 5´-dithiobis -2-nitrobenzoic acid

$[\theta]_{MRW}$	ellipticity corrected for mean residue weight
EGTA	ethylene glycol bis(β-aminoethyl ether) N, N-tetraacetic acid
EDTA	ethylenediamine tetra acetic acid
FPLC	fast protein liquid chromatography
$FeCl_2$	ferrous chloride
Fig.	figure
F_u	fraction unfolded
Glu	glutamic acid
GME	glycine methyl ester
g	gram
GuHCl	guanidine hydrochloride
HPLC	high performance liquid chromatography
h	hour(s)
HCl	hydrochloric acid
pI	isoelectric pH
ϕ_2^o	isomolal partial specific volume (mL/g)
ϕ'°_2	isopotential partial specific volume (mL/g)
kDa	kiloDalton
L	liter
mA	milliampere
MRW	mean residue weight
MRE	mean residue ellipticity
MW	molecular weight
m	meter
μL	microliter
μM	micromolar
μg	microgram
mg	milligram
mL	milliliter

mm	millimeter
mM	millimolar
min	minutes
M	molar concentration
ε	molar extinction coefficient (M^{-1} cm^{-1})
mol	mole
EDAC	N- (3-Dimethylaminopropyl)-N-ethylcarbodiimide
TEMED	N, N, N, N-tetramethylethylene diamine
ng	nanogram
nm	nanometer
NBS	N-bromosuccinimide
pH	negative logarithm of hydrogen ion concentration
N	normality
NMR	nuclear magnetic resonance
$\bar{v}$	partial specific volume (mL/g) extrapolated to zero
$\bar{v}_{app}$	apparent partial specific volume (mL/g) at single protein concentration
%	percent
PITC	phenylisothiocyanate
H_3PO_4	phosphoric acid
pNPG	*p*-nitrophenyl β-D-glucopyranoside
PAGE	polyacrylamide gel electrophoresis
K_2SO_4	potassium sulpahte
ξ_3	preferential interaction parameter of protein
$(\delta g_3/\delta g_2)$	preferential interaction parameter on g/g basis
$(\delta m_3/\delta m_2)$	preferential interaction parameter on mol/mol basis
Pro	proline
s	second

SeO_2	selenium oxide
Ser	serine
NaCl	sodium chloride
SDS	sodium dodecyl sulphate
NaOH	sodium hydroxide
K_{sv}	Stern-Volmer constant
R_s	Stokes radius
H_2SO_4	sulphuric acid
T_m	thermal denaturation temperature
SH	thiol
$\times$ g	times gravitational force
TEA	triethylamine
TFA	trifluoroacetic acid
Tris	tris (hydroxy methyl) amino methane
Trp	tryptophan
Tyr	tyrosine
UV	ultraviolet
v	volts
w/v	weight /volume
v/v	volume/volume
λ_{max}	wavelength maximum
$ZnCl_2$	zinc chloride

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Title of Ph.D Thesis: STRUCTURE, FUNCTION AND STABILITY OF FUNGAL CELLULASES

The knowledge of protein structure-function and stability relationship is very important from both academic and commercial point of view. Enzymes despite their potential role as catalysts, find wide applications in various industrial processes which are limited due to instability in solution. The limitation for their application is the instability under operational conditions due to exposure to extreme temperatures, pressure, pH, denaturants and organic solvents. Thus, in order to utilize these enzymes to its full potential, the structure-function stability relationship of enzymes needs to be understood. Thus, a detailed elucidation of the mechanism of its action and factors affecting stabilization and destabilization is important. A well defined three-dimensional structure is essential for an enzyme to perform its biological function, for which the enzyme structural stability and catalytic activity are interlinked.

The polymers cellulose and hemicellulose constitute the major portion of the organic matter on our planet. They form the cell walls of plants and are consequently the principal components of wood, cotton and grass. Cellulose, as a renewable raw material, is an ideal resource for the industrial production of paper, textiles and synthetic products. Cellulases from fungal origin are known to be most powerful in cellulose hydrolysis. These microorganisms produce a multi-component enzyme system consisting of endoglucanase, exoglucanase and β-glucosidase. A combination of these three types of enzymes is necessary for the complete hydrolysis of crystalline cellulose. Endoglucanase and exoglucanase are known to act synergistically in cellulose hydrolysis, while β-glucosidase is needed for removal of cellobiose, a strong inhibitor of both endoglucanase and exoglucanase. The diversity of endoglucanase, exoglucanases and β-glucosidases from fungal origin is emphasized by many investigators. In the present study, an attempt is made to purify and characterize the enzyme from crude cellulases

produced from *Aspergillus aculeatus* obtained commercially. *A. aculeatus* being large producer of enzyme endoglucanase, which was taken up for further isolation and characterization.

The structural stability of proteins is affected by a number of factors like water and salts. Cofactors play an important role in the catalytic activity and structural stability of proteins. They serve a variety of functions in proteins like enhancing the catalytic activity and some of them have the ability to inhibit. These influence the structural stability by altering the conformation of the proteins. The mode of action of these is very specific and depends on the nature of each cofactor. The pH and denaturants like urea and GuHCl are known to unfold the protein molecules and alter the hydrophobic interactions leading to the significant conformational changes with modified functionality. Cosolvents play an important role in the structural stability of the proteins which is very specific and dependent on the nature of the cosolvent used. The effect of cofactors, pH, denaturants and cosolvents on the structure, function and stability of the cellulase has been carried out. The mechanism of stabilization was studied by measuring the catalytic activity, fluorescence spectroscopy, circular dichroic spectroscopy and partial specific volume measurements.

Based on the background of literature available on the above subject, the present investigation has been undertaken and the results and conclusions are brought out in the form of a thesis entitled "**Structure, function and stability of fungal cellulases**". The thesis has been organized into introduction, scope and objectives, materials and methods, results and discussion, summary and conclusions and references along with tables and figures. The summary of results under each chapter is as indicated below.

Chapter 1: 1.1 Purification and Characterization of Endoglucanase from commercial source
1.2. Effect of Cofactors on Structure and function of endoglucanase

This chapter describes the isolation, purification and characterization of endoglucanase produced from *Aspergillus aculeatus* obtained commercially and effect of cofactors on the optimization of catalytic activity has been worked in greater detail. Endoglucanase was purified to homogeneity and the purity was checked by SDS-PAGE and FPLC. The amino acid composition of the isolated endoglucanase was in correlation with the literature values of other related endoglucanases. The optimum temperature and pH were found to be 40°C and 5.0, respectively. The isoelctric point was found to be 4.3. Lineweaver-Burk analysis showed that K_m was 0.060 ± 0.001% and V_{max} was 0.08 units (mg of protein)$^{-1}$ min^{-1}. The end product analysis was carried out by HPLC. The release of glucose and cellobiose as the end products during incubation with carboxymethyl cellulose as substrate confirmed it as endoglucanase. The secondary structure measurements carried out by far-UV CD spectra, showed that endoglucanase has 64% beta-sheets and 14% α-helical structures. The purified endoglucanase lost 50% of its activity at 90°C.

In the presence of dithiotreitol, endoglucanase activity was reduced to 60% compared to the control, indicating that disulfide bonds play an important role at the catalytic site. N-bromosuccinimide did not show any effect on the endoglucanase activity, showing that tryptophan is not involved in the catalytic activity. Carboxymethylation of the histidyl residues of the enzyme with diethylpyrocarbonate showed a 70% loss in the activity suggesting that these residues participated mechanistically and also in the maintenance of the conformation necessary for the active enzyme. It may be playing a role in the binding of the substrate to the enzyme and proper orientation of the catalytic site of the enzyme for its hydrolysis. The participation of specific carboxyl groups in the active site of the enzyme were indicated by

N- (3-Dimethylaminopropyl)-N-ethylcarbodiimide modification of endoglucanase where it was completely inhibited.

The catalytic activity of endoglucanase was not affected by the presence of metal ions (calcium, zinc, iron and copper). The fluorescence emission spectra of endoglucanase in presence of cofactors did not show any change, indicating that there was no change in the microenvironment of the aromatic chromophores. The far-UV CD spectra of endoglucanase in presence of cofactors did not show any change in the secondary structures. The enzyme activity was found to increase with increase in the concentration of ethylenediamine tetra acetic acid. The catalytic activity of endoglucanase was 1.5 fold in presence of 5 mM ethylenediamine tetra acetic acid. In presence of ethylenediamine tetra acetic acid, K_m decreased to 0.040 ± 0.001 % compared to control and had a K_{cat} of 1.9 ± 0.2 min^{-1}. The decrease in the K_m and increase in the K_{cat} shows that the endoglucanase has attained the maximum catalytic efficiency in presence of ethylenediamine tetra acetic acid. The fluorescence emission spectra of endoglucanase in presence of ethylenediamine tetra acetic acid showed no changes. The endoglucanase showed 20% increase in the ellipticity value at 217 nm in presence of ethylenediamine tetra acetic acid. The apparent thermal transition temperature (T_m) of native endoglucanase increased from a control value of 57°C to 76°C in presence 5 mM ethylenediamine tetra acetic acid. The endoglucanase isolated from *A. aculeatus* is subjected to further detailed investigations in the next chapters to understand the structure-function and stability relationship of the enzyme.

Chapter 2: Effect of pH, urea and GuHCl on the folding and unfolding of endoglucanase

This describes the effect of pH, urea and guanidine hydrochloride on the folding and unfolding of endoglucanase. The pH induced denaturation of endoglucanase was followed over a pH range of 2.0 - 5.0. The pH denaturation is irreversible and the enzyme loses 50% of its activity at pH 2.0. Far-UV CD spectra of endoglucanase did not show

any change in the secondary structure at pH 2.0. The 8-anilino-1-naphthalene sulphonic acid binding studies showed increased fluorescence intensity at pH 2.0, indicating the exposure of hydrophobic amino acids to the solvent. The pH induced denaturation leads to the formation of a partially folded intermediate at low pH with characteristic features of a molten globule.

In order to further characterize unfolding process, the effect of urea and guanidine hydrochloride on the structure and function were followed at pH 5.0. In presence of 2 M urea, the K_m was 0.035 ± 0.001% and K_{cat} was 3.0 ± 0.3 min^{-1}, where as in the presence of guanidine hydrochloride, the K_m was found to be 0.041 ± 0.001% and K_{cat} was 2.5 ± 0.2 min^{-1}. The decrease in the K_m and increase in the catalytic constant suggests that endoglucanase is having more affinity towards the substrate and has maximal catalytic efficiency in low concentrations of the denaturants. Further, the activity decreased with increasing concentrations of these denaturants. The T_m value increased to 60 and 61°C in presence of 2 M urea and guanidine hydrochloride, respectively compared to control value of 57°C, which indicates stabilization of endoglucanase at low concentrations. The urea and GuHCl induced equilibrium unfolding of endoglucanase was followed by changes in the fluorescence emission maximum and ellipticity values at 217 nm as probes indicating that the transition is sigmoidal and follows a simple two-state transition. The results obtained from folding and unfolding studies showed differences in stability towards denaturation by urea and guanidine hydrochloride.

Chapter 3: Effect of cosolvents on structure and stability of endoglucanase

In this chapter, the structural stabilization of endoglucanase in presence of cosolvents such as glycerol, sorbitol and sucrose has been described. The endoglucanase showed four-fold increase in the activity at 40% concentration of the cosolvents. In presence of these cosolvents, the endoglucanase was able to maintain its original activity at 90°C. The apparent T_m value of the endoglucanase was found to be 57°C. All the

cosolvents are able to elevate the apparent T_m to different extents in a concentration dependent manner. The structural integrity of endoglucanase was monitored by fluorescence and circular dichroic spectral measurements. Fluorescence spectra did not show any changes in presence of cosolvents. Far-UV CD spectra of endoglucanase showed no significant conformational changes in the protein molecule in presence of these cosolvents.

In order to understand the mechanism of cosolvent-induced stabilization of endoglucanase, the partial specific volume and preferential interaction parameters were determined in presence of cosolvents. The apparent partial specific volume of endoglucanase under isomolal and isopotential condition is 0.723 ± 0.001mL/g in sodium acetate buffer at pH 5.0. The preferential interaction parameters showed that for all the cosolvents the preferential hydration parameter is positive and the preferential interaction parameter is negative indicating preferential hydration or exclusion of cosolvents from the protein domain. The preferential interaction parameter was found to be maximum with -0.126 ± 0.06 g/g in 40% glycerol and minimum value of -0.033 ± 0.01 g/g in 10% glycerol. These results indicate that stabilization of endoglucanase is due to preferential hydration. Thus, these cosolvents stabilize the enzyme against thermal denaturation which is dependent on the concentration and nature of the cosolvent used.

The above investigation is comprehensively documented in the form of a thesis for Ph.D. degree in the subject area of Biochemistry of the University of Mysore, Mysore, India.

List of research articles published:

1. **Gajendra S Naika**, Purnima Kaul and Prakash V. (2007). Purification and characterization of a new endoglucanase from *Aspergillus aculeatus*. *J. Agric. Food Chem*. **55**, 7566-7572.

2. **Gajendra S Naika**, Prakash, V. and Purnima Kaul Tiku (2009). Effect of cosolvents on the structural stability of endoglucanase from *Aspergillus aculeatus*. Accepted in *J. Agric. Food Chem*.

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INTRODUCTION

Proteins are the most important and dynamic macromolecules of the cell. They are involved in catalyzing various chemical reactions and also form part of the structural components. Proteins being long polymers of amino acids linked by peptide bonds constitute the largest fraction (besides water) of cells. Some proteins have catalytic activity and function as enzymes; others serve as structural elements, signal receptors or transporters that carry specific molecules into or out of the cells. A well defined three-dimensional structure is a pre-requisite for the biological functions of a protein. The three-dimensional structure of protein in a folded state with a well defined compact configuration is known as native state.

Almost all enzymes are proteins which have various roles in physiological functions of a cell. They are the biological catalysts, specialized for each biochemical processes. Enzymes are the most superior and highly specialized protein molecules. They have extraordinary catalytic power, high degree of specificity for their substrates and they accelerate chemical reactions at a greater rate than that of chemical catalysts. They function under very mild conditions of temperature and pH. Acting in organized sequences, they catalyze hundreds of stepwise reactions that degrade nutrient molecules, conserve and transform chemical energy and make biological macromolecules from simple precursors. The action of enzymes in metabolic pathways is highly coordinated to yield a harmonious interplay among the many activities necessary to sustain life. Enzymes have numerous applications in a number of industries such as pharmaceutical, food and fruit processing, brewing, dairy, bakery, chemical industries and also in agriculture.

In spite of their potential as catalyst, the overall application and utility in industries is limited. A major limitation to industrial use is their instability under operational conditions, like exposure to high temperatures, pressure, pH, denaturants and organic solvents. Thus, in order to achieve good results and to design enzymes with tailor made properties, relationship between its structure and function has to be well understood. Detailed investigation of the mechanism of stabilization and destabilization of enzyme is of prime importance from both academic and commercial point of view.

1. Cellulose

Celluloses and hemicelluloses constitute a major portion of the organic matter on earth. They constitute cell walls of plants and are principal components of wood. Cellulose is one of the most abundant renewable raw material and main source for the production of energy by biomass conversion, industrial production of paper, textiles and synthetic products. Cellulose is a linear chain of several hundreds of thousands of glucose residues linked by β-1, 4 glycosidic bonds which are stabilized by hydrogen bonds. Aggregates of about 40-60 chains are aligned in parallel and linked through hydrogen bonds. Such lengthy, crystalline aggregates known as microfibrils are in turn subunits of macro-fibrils that have a cross-sectional diameter of about 0.5 nm. The cellulose in native cotton has a degree of polymerization (DP) of about 10,000 which means that about 10,000 glucose residues are linked to each molecule by glycosidic bonds, where as wood pulp has a DP of only 600-1000 and regenerated cellulose has about 200-600 DP.

Cotton consists of approximately 90% cellulose and wood approximately about 60%. Due to its complex nature, the degradation of cellulose is not easily accomplished and requires the action of many enzymes. The degradation of cellulose is mainly through the action of micro-organisms which produces extracellular enzymes known as cellulases. Because of the complex nature of both substrate and enzyme, analysis of cellulases is not simple. Cellulase preparations from different microorganisms show different enzyme profiles. The composition of an enzyme preparation from the same organism depends mainly on the conditions under which it is grown and the substrate complexity (Helmut, 1998). Increasing demand for eco-friendly processes has lead to the increasing use of hydrolytic and other enzymes instead of chemicals and cellulase is one such enzyme which occupies a prime position.

2. Cellulases

Cellulases are the enzymes which hydrolyse the β-1, 4 linkages in celluloses. Cellulases from fungal origin are known to be most powerful enzyme in cellulose hydrolysis. These microorganisms produce a multi-component enzyme system, which mainly includes the 1, 4-β-D-glucan glucanohydrolase (endoglucanase; EC 3.2.1.4), 1, 4-β-D-glucan-cellobiohydrolase (exoglucanase; EC 3.2.1.91) and β-D-glucoside glucohydrolase (β-glucosidase; EC 3.2.1.21). A combination of these three principal types of enzymes is necessary for the complete hydrolysis of crystalline cellulose. Endoglucanase and exoglucanase are known to act synergistically in cellulose hydrolysis (Wood and McCrae, 1978). The enzyme β-glucosidase is needed for removal of cellobiose, a strong inhibitor of both endoglucanase and exoglucanase in the hydrolysis of cellulose (Woodward

and Wiseman, 1982). The diversity of endoglucanases, exoglucanases and β-glucosidases from fungal origin is emphasized by many reviewers (Ryu and Mandels, 1980; Shewale, 1982; Ladish *et al*., 1983).

Endoglucanases are often called CM cellulases or Cx enzymes or CMCases. They attack carboxymethyl cellulose or phosphoric acid swollen cellulose in a random fashion, resulting in a rapid decrease in chain length together with slow increase in reducing sugars (Wood and McCrae, 1979). Water soluble cello-oligosaccharides are intermediate products of attack, which are hydrolyzed to glucose and cellobiose.

2.1 Classification of cellulases

Cellulases have been classified based on several criteria. Traditional method is based on the mode of action. Exoglucanases cleave sugar units from the end of the cellulose chain, whereas endoglucanases makes random cuts along the chain. Exoglucanases and endoglucanases act synergistically on crystalline cellulose (Nidetzky *et al*., 1993 & 1994; Irwin *et al*., 1993; Beguin and Aubert, 1994). Endoglucanases provides new chain ends for the action of exoglucanases. Exo and endo synergism has been demonstrated with a few bacterial and fungal cellulases (Wood and McCrae, 1978; Nidetzky *et al*., 1993; Irwin *et al*., 1993; Medve *et al*., 1994). Due to a large number of possible combinations between carbohydrates, there are large numbers of glycosidases of varying substrate specificity. They are conventionally classified based on amino acid sequence similarity (Henrissat, 1991; Henrissat and Bairoch, 1993; Henrissat and Bairoch, 1996) which reflects the structural features of these enzymes better than their sole substrate specificity and it also reveals evolutionary relationships between cellulases of different specificity.

Glycosidases catalyze the hydrolysis of glycosidic bonds of their carbohydrate substrates via mechanism leading to either (a) retention or (b) inversion of the anomeric configurations at the cleavage point. With no known exception, the mechanism is conserved among all the members of a given family and stereochemistry of the hydrolysis reaction is presently known for 42 families (Henrissat and Davies, 1997). Comparison of amino acid sequences in the catalytic domains of cellulases by hydrophobic cluster analysis has led to the proposed classification of cellulases into 13 families (Parsiegla *et al*., 2002). The enzymes are grouped according to similarities of their catalytic domain apart from function. Enzymes produced from the same organism despite sharing the same type of CBD appear in different families.

2.2. Fungal cellulases

Cellulolytic fungi like *Trichoderma, Humicola* and *Penicillium* species are among the most studied cellulolytic micro-organisms. They produce all the enzymes needed for the complete hydrolysis of cellulose. They normally produce one or more exoglucanases, several endoglucanases and at least one β-glucosidase, all are secreted into the culture medium (Wood *et al*., 1989; Wood and Gracia-Campayo, 1990; Covert *et al*., 1992, Schulein *et al*., 1993). The structure of cellulose is prone to changes during the degradation process and requires different enzymes at different stages. The exact number of enzymes reported also varies, hence the purification of cellulases is difficult (Wood *et al*., 1989, Reinikainen *et al*., 1995). The cellulase diversity is attributed to post translational modifications (glycosylation) and proteolysis (Beguin, 1990; Srisodsuk *et al*., 1993) and protein-protein interactions are also known to cause further problems in purification (Sprey and Bochem, 1993; Wood *et al*., 1989). The overall domain structure of fungal cellulases is same for

exoglucanases and endoglucanases. All these enzymes consist of a bigger catalytic domain and a smaller CBD separated by an O-glycosylated linker peptide. The CBD can be either N-or C-terminal. There are reports of exoglucanase (Covert *et al*., 1992) and several endoglucanases (Ward *et al*., 1993; Schulein *et al*., 1998) having only catalytic domain. Some cellulases are active against other polymeric substrate, e.g. *Trichoderma reesei* endoglucanase I (EGI) can degrade both xylan and cellulose (Biely *et al*., 1991).

The crystallization of fungal cellulases has been difficult, probably due to the linker peptide which is flexible and/or heterogeneously glycosylated. The two techniques commonly used for determining the spatial relationship between the individual domains of fungal cellulases are small-angle X-ray scattering (Abuja *et al*., 1988a & b) and dynamic light scattering (Boisset *et al*., 1995). The results have revealed that both *Trichoderma reesei* cellobiohydrolases and *Humicola insolens* endoglucanase have an elongated tadpole like shape. The catalytic domain forms the ellipsoidal head and the linker, forms the connection between the catalytic domain and the CBD. The role of (cellobiohydrolase I) CBH I linker has been studied (Srisodsuk *et al*., 1993). The two domains are separated by a linker, allowing certain amount of flexibility in the conformation which is needed for the efficient function of CBH I on crystalline cellulose. The linker is a proline–glycine rich sequence functioning as a hinge which is also O-glycosylated.

Of the 13 cellulase families classified (Henrissat and Bairoch, 1993; Tomme *et al*., 1995b), fungal cellulases are found in six families. Two exoglucanase structures are solved for cellobiohydrolases from *T. reesei*. The 3D structure of the catalytic domain of *T. reesei* CBH II of family 6 was the first

cellulase crystal structure to be solved (Rouvinen *et al*., 1990). The polypeptide folds into α/β barrel structure similar to triose phosphate isomerase (TIM), except that it has seven beta strands. The active site was situated in a tunnel formed by stable surface loops. This could explain the exo-mode action of CBH II. The cellulose chain could only enter the active site tunnel by one end which was missing in the endoglucanase from the same family. This shows that related endoglucanases have similar overall folds but with a more open active site structure allowing the random binding and breakdown of the cellulose chain (Rouvinen *et al*., 1990). This hypothesis was confirmed with the 3D structure of the *Thermomonospora fusca* endoglucanase, E2 (Spezio *et al*., 1993) belonging to the same family 6 and family 7 (*T. reesei* CBH I) had a completely different fold, but the active site was similarly situated in a tunnel formed by stable loops (Divne *et al*., 1998). It appears to be common feature of true cellobiohydrolases, with an exception of bifunctional exoglucanase/ xylane Cex from the bacterium *Cellulomonas fimi* which has an open active site structure (White *et al*., 1994).

The fungal CBDs have 36 amino acid residues with high sequence identity, suggesting that they are similar (Hoffren *et al*., 1995), belonging to the CBD family I (Tomme *et al*., 1995a). The 3D structure of *T. reesei* CBH I CBD by NMR reveals a wedge-shaped structure consisting of three antiparallel beta-strands and has two disulfide bridges which stabilize the structure. One side of the wedge is flat, containing three highly conserved aromatic residues (Kraulis *et al*., 1989). Site directed mutagenesis studies and synthetic CBDs show that the aromatic ring structures (tyrosine or tryptophan) is the primary interaction surface with cellulose (Reinikainen *et al*., 1992; 1995; Linder *et al*., 1995 a & b).

2.3. Bacterial cellulases

Bacterial cellulases show more diversity in their structure than fungal cellulases. Best studied is the one produced by anaerobic bacterium *Clostridium thermocellum*, cellulase complex is called cellulosomes, active against crystalline cellulose. Cellulosome can be more than 2000 kDa in molecular mass (Beguin and Aubert, 1994). Cellulosomes can form polycellulosomal clusters on the cell surface (Bayer *et al*., 1994). The largest subunit called scaffolding is a non catalytic domain which forms the core of the cellulosome. It consists of a single CBD and many distinct, but closely related domains, cohesions, which bind the catalytic subunits. Catalytic domains contain conserved duplicated segments called dockerins, which mediate the attachment of the catalytic domains to scaffolding. The dockerin domains are interconnected by linking segments or linkers. Some scaffolding and catalytic domains contain other domains, currently their function is not known (Bayer *et al*., 1994, 1995).

Cellulomonas fimi cellulases are secreted into the medium. It produces four endoglucanases (CenA, CenB, CenC and CenD) and two cellobiohydrolases (cbhA and cbhB) which have been characterized (Din *et al*., 1995). The cellulolytic system of *C. fimi* is similar to the enzyme system of *T. reesei*. All *C. fimi* cellulases have a modular structure which comprises of two to six modules or domains, all having two common domains, the catalytic and CBD, which functions independently. The catalytic domains belong to six different families and the CBDs, except that of CenC which belongs to family II (Tomme *et al*., 1995b). *C. fimi* CBDs from family II are the most extensively studied bacterial CBDs. They adsorb to both crystalline and amorphous cellulose, which is dependent on aromatic amino acid residues (Din *et al*.,

1994). The 3D structure of *C. fimi* Cex CBD of family II has been solved and found to have a β-barrel fold consisting of 9 anti-parallel beta strands (Xu *et al*., 1995).

2.4. Mechanism of catalysis of cellulases

The work of Reese *et al*. (1950) helped to elucidate an understanding of the complex action of cellulases involving two-step degradation process which requires an activation reaction, followed by hydrolytic cleavage. The activating enzyme was named "C1 activity". According to this concept, microorganisms that are capable of degrading crystalline cellulose have C1 activity. This activity is not present in enzymes that attack only substituted cellulose (CMC). These have only CMCase or Cx activity as shown below illustrating the enzyme hydrolysis of cellulose (Reese *et al*. 1950).

Hydrolysis of β 1, 4 bonds of cellulose may give rise to either inversion or retention of anomeric configuration. In 1953, Koshland proposed a simple scheme where by 'inverting' enzymes would function by a single displacement reaction in which nucleophilic attack by water from under the sugar ring with departure of the leaving group. 'Retaining' enzymes however were proposed to operate by a double displacement mechanism in which the covalent glycosyl enzyme intermediate is formed and subsequently hydrolysed via oxocarbenium ion transition state.

In 1994, McCarter and Withers showed that for enzymes operating with inversion of anomeric configuration the two catalytic carboxylates are normally separated by approximately 9-10Å in order to provide a general acid-catalyzed assistance to leaving group departure and a base-catalyzed assistance to nucleophilic attack from the opposite side of the sugar ring.

Double displacement mechanism involves two discrete chemical steps. Both of these individual chemical steps involve an inversion at the anomeric centre, the two inversion giving rise to a net retention of the anomeric configuration. In the first step, nucleophilic carboxylate attacks the anomeric centre resulting in the formation of glycosyl-enzyme intermediate. In the second step, the deprotonated carboxylate acts as a base and assists a nucleophilic water to hydrolyse the glycosyl-enzyme intermediate giving the hydrolysed product (Withers, 2000). It has been demonstrated that retaining enzymes have their two catalytic residues functionally separated by approximately 5.5 Å this allows the enzymatic acid/base to provide protonic assistance to leaving group departure, simultaneously with nucleophilic attack by the second carboxylate to form the glycosyl enzyme intermediate, the catalytic acid/base is now able to function as a bronsted base, activating the incoming nucleophile for the attack.

Crystalline cellulose is the major structural component of plant cell walls and is thus poorly amenable to enzymatic degradation. The long polymeric chain of glucose attached to one another by network of H-bonds leads to eventual crystallization of cellulose. Among cellulases CBH's are the enzymes with highest apparent activities on crystalline cellulose. Recent structure and function studies of microbial CBH's are beginning to shed light on the molecular basis of their efficiency on this demanding substrate (Teeri *et al.*, 1998).

The catalytic machineries of both CBH I and CBH II are completely buried with in their active site tunnels. In CBH II, the catalytic site is furnished by amino acid side chains required for a single displacement reaction resulting in the inversion of the configuration of the anomeric carbon (Davies *et al*., 1997). The catalytic machinery of CBH I provide an acid/base catalyst and a nucleophile which are required for a double displacement reaction resulting in net retention of the configuration of the anomeric carbon. Both reactions seem to proceed via formation of an oxocarbenium ion with intermediates.

2.5. Role of cofactors on endoglucanase

Cofactors have a variety of functions in proteins. In some proteins cofactors are required for the catalytic activity and other biological functions. And some of these can also function as inhibitors. Many proteins undergo structural changes in presence of divalent metal ions. Studies on structure-function relationship of endoglucanase in absence and presence of metal ions are of significance. The effect of these on conformation of proteins has been studied (Goto *et al*., 1990, Antony *et al*., 1996). Some of these metal ions have been shown to inhibit the catalytic activity of few cellulases (Eriksson and Pettersson, 1968; Highley, 1975; Ferchak and Pye, 1983).

The metal ions like Cu^{2+}, Hg^{2+}, Mn^{2+} have been shown to inhibit the enzymes from fungi, *A. niger*, *T. viride* and *P. notatum* (Iwasaki *et al*., 1965). It has been shown to interact with tryptophan residues in the protein (Eriksson and Pettersson, 1968; Clarke and Adams, 1987) and also have the capability to bind between the two catalytic carboxylic acid residues (Perkins *et al*., 1979). Some of these divalent cations like Ca^{2+}, Co^{2+}, Mg^{2+} and Zn^{2+} caused partial

inhibition (10-20%). Sami and Akhtar (1993) have shown that heavy metals like Ag^{2+} and Fe^{2+} inactivated the endoglucanase from *C. flavigena*. But the mechanism of inhibition is still not clearly understood. Hence, the effect of these cofactors on endoglucanase differs widely.

3. Industrial applications of cellulases

Since 1990 cellulases have achieved significant industrial importance. They are commercially used in the textile industries as biopolishing agents and for reducing fuzz and pilling of fabrics and substituting in stonewash (Lange, 1993). They have a great potential in the paper and pulp industries (Mansfield *et al*., 1999) and in detergent industries. Continued research on cellulases have revealed their biotechnological potential in various industries like food, brewery and wine, animal feed and agriculture (Bayer *et al*., 1994; Beguin and Aubert, 1994; Bhat and Bhat, 1997, 1998).

Cellulases with multi-activities are used in industries due to their low cost, but they cause the deterioration of the fibre strength due to degradation of the crystalline cellulose. To minimize this, only the effect of endoglucanase is often preferred (Mohlin and Pettersson, 2002; Lenting and Warmoeskerken, 2001; Belghith *et al*., 2001). They have tremendous potential to convert the lignocellulosic wastes into glucose and other soluble sugars which can be utilized for the production of bio-fuel, ethanol. Table 1 summarizes the industrial applications of cellulases.

4. Cellulases from *Aspergillus aculeatus*

Cellulolytic microorganisms normally produce several cellulases differing with respect to substrate specificities, mode of action in order to

Table 1: Important industrial applications of cellulase

Sl. No	Industry	Applications
1	Food Technology	Improvement in pressing and extraction of juice from fruits and oil from seeds, releasing of flavor, enzymes, proteins, polysaccharides, clarification of fruit juices, improvement of texture, quality and shelf life of bakery products. Controlling coronary heart diseases and atherosclerosis, production of water soluble dietary fibres to enrich the fibre content of foods.
2	Brewery and Wine biotechnology	Improvement in skin maceration and color extraction of grapes, clarification of wines, improving aroma of wines.
3	Animal feed biotechnology	Improvement in nutritional quality of animal feed, improved feed digestion and absorption, weight gain in chickens and hens.
4	Textile and laundry biotechnology	Bio-stoning of denim fabrics, production of high quality and environment friendly washing powders, bio-polishing of cotton and non denim fabrics.
5	Pulp and paper biotechnology	Bio-mechanical pulping, modification of fibre properties, de-inking of recycled fibres, bio-characterization of pulp fibres.
6	In R & D and agriculture	Production of plants or fungal protoplasts, hybrid and mutant strains, bio-control of plant pathogenesis and diseases, production of designer cellulosomes, affinity purification, immobilization and fusion of proteins.

(From Bhat, 2000)

achieve synergism in the degradation of crystalline cellulose. The cellulolytic fungus *Aspergillus aculeatus* produces many types of cellulases with a high degradation capacity for crystalline cellulose (Murao *et al*., 1988). The isolation of multiple cellulases from this fungus has revealed nine distinct enzymes including three endoglucanases also known as CM-cellulases (Murao *et al*., 1979; Sakamoto *et al*., 1985). The three CM-cellulases which differ in substrate specificity are all important for the enhancement of efficiency in the enzymatic reaction.

Three cellulase genes coding for an endoglucanase, FI-CMCase, a cellobiohydrolase, CBH I and a β-glucosidase BGL I, all of which are major cellulase components were cloned and sequenced (Ooi *et al*., 1994; Takada *et al*., 1998). The molecular weight varies from 25 – 66 kDa. Endoglucanase is the most abundant cellulase of the nine cellulases produced by *A. aculeatus*. *A. niger*, *A. ustus*, *A. fumigatus* and *A. japonicus* are also known cellulase producers (Hrmova *et al*., 1989). The CMCase is shown to be composed of three domains, a core domain that contains the catalytic domain, a highly conserved cellulose binding domain that is involved in binding to crystalline cellulose and connecting these two domains, a hinge that is glycosylated (Takada *et al*., 2002). For FI-CMCase, two acidic amino acids in the cleft namely, Glu 118 and Glu 202 are shown to be involved in the catalytic activity (Ohnishi *et al*., 1999).

5. Stability of proteins/enzymes

Protein stability is one of the most important criteria for their commercial applications. A well defined structure is pre-requisite for an enzyme to carry out its biological functions. The enzyme's structure, stability

and catalytic activity are interlinked. The native, catalytically active structure of an enzyme is maintained by a delicate balance of different covalent and non-covalent forces like hydrogen bonds, van der Waals, hydrophobic and ionic interactions. Upon exposure to extreme conditions, these forces diminish and the protein molecule unfolds, resulting in the alterations in the active site conformation and hence inactivation of the enzyme (Anfinsen and Scheraga, 1975).

Protein stability can be assessed by two different ways, one is conformational stability of the structure and the other kinetic stability of the folded state. The conformational stability deals with the resistance of the folded protein conformation to denature and kinetic stability measures the resistance to irreversible inactivation. The stability of protein is not only determined by its three-dimensional structure but also by its sensitivity to environmental perturbations. Studies have revealed that temperature and pH changes cause alterations in the protein's mechanical stability (Schlierf and Rief, 2005; Paci and Karplus, 2000).

The stability of a native protein is a function of external variables like pH, temperature, and solvent composition as they disrupt the different kinds of bonds that are responsible for the stability. There are three major approaches to enhance the enzyme stabilization: (i) screening and isolation of enzymes from extremophiles, (ii) production of stable enzymes in genetically manipulated mesophilic organisms and (iii) stabilization of unstable enzymes by methods like protein engineering, chemical modifications and employing additives (Iyer and Anantanarayan, 2008). Most of the reports on enzyme stabilization are focused on effect of additives on protein stability showing that it is one of the popular methods of enzyme stabilization.

6. Stabilization of proteins by cosolvents

Resistance to irreversible inactivation defines protein stability (Wetzel, 1987). For the protein to be in its functional native conformation, a particular pH, temperature and salt concentrations are required and any changes on either side of these conditions causes destruction of the native conformations and hence, function of the molecule. The stability of proteins is affected by a variety of substances that can act at higher concentrations. Substances referred to as cosolvents are known to stabilize the proteins native structure. They affect the stability of the protein by direct interaction or by indirect action through effects on the structure and nature of the solvent or by both the mechanism (Lee and Timasheff, 1981). In the cosolvent-protein interaction, the water is structured around the protein molecule leading to the phenomenon known as preferential hydration which in turn stabilizes the protein. Here, the cosolvents are also known as thermodynamic booster molecules (Lee *et al*., 1979; Gekko and Timasheff, 1981a).

The stabilization of proteins by sugars and polyhydric alcohols has been studied (Lee and Timasheff, 1981; Radha *et al*., 1998; Rajendran *et al*., 1995; Rajeshwara and Prakash, 1996). According to these studies the reason for the increased thermal stability was preferential hydration of the protein in presence of cosolvents. The cosolvents are preferentially excluded from the domain of the protein leading to unfavorable interactions between proteins and solvent due to ordered structure of water around the protein. Thus, favoring the native structure of the protein (Lee and Timasheff, 1981; Arakawa and Timasheff, 1982; Gekko and Timasheff, 1981b). Different cosolvents tend to stabilize the proteins in different ways depending on their nature. Amino acids tend to stabilize proteins by increasing the hydrogen bonds at low concentrations (Arakawa and Timasheff, 1983).

The measurement of partial specific volume and related parameters give an idea into the mechanism of stabilization of proteins. The negative value of preferential interaction parameter signifies that there is excess water in the domain of the protein and that it is preferentially hydrated and cosolvent is preferentially excluded from the protein domain. The use of such additives is due to their structure stabilizing effect. Many industrially important enzymes and proteins have been stabilized against thermal denaturation in presence of different cosolvents based on the preferential hydration mechanism (Satish *et al*., 2007; Rajendran *et al*., 1995).

7. Denaturation of proteins: chemical denaturants, pH and temperature

Understanding the structure-function relationships of an enzyme under different conditions is fundamentally important for both theoretical and application aspects. Such studies provide an insight into the molecular basis of stability of the enzyme which can be used to design protocols and/or the proteins with special properties for biotechnological applications. The stability of protein is a function of external variables like pH, temperature, ionic strength and solvent compositions as they can disrupt the different kinds of interactions that are responsible for the protein instability. A simple method for the analysis of the roles for these variables in protein stability is to monitor the conformational changes due to perturbation of a protein molecule by various reagents like acid, GuHCl, urea and temperature. Even though the mechanism of denaturation of proteins by urea and GuHCl have been studied extensively (Tanford, 1968, 1970; Pace, 1975), our understanding of the mechanism of denaturation of proteins by these denaturants is still limited.

Prakash and Timasheff (1981, 1985) have well documented the interaction of these denaturants with several proteins in terms of partial specific volume measurements showing that these denaturants may interact directly with proteins. At higher concentrations they can cause change in the behavior of the solvent itself (Breslow and Guo, 1990). A general mechanism of binding to proteins shows that two urea molecules bind to a single peptide bond and one each to aromatic amino acids (Prakash *et al*., 1981) and one GuHCl molecule will form a hydrogen bond with two peptide bonds forming a cyclic structure (Robinson and Jencks, 1965). It is understood that the denaturants unfold the protein molecule by migrating into the interior of the protein and forming hydrogen bond with back bone (Hedwig *et al*., 1991) and also shown to act by decreasing the hydrophobic effect (Roseman and Jencks, 1975). Now it's well known that urea denatures proteins by decreasing the hydrophobic effects by displacing the water in the salvation shell and by directly binding to the amide bonds (Zou *et al*., 1998).

The function of a protein depends on its ability to acquire a unique three-dimensional structure and any change in its structure alters its function and stability. Proteins accumulate in different conformational states during their unfolding by various denaturants. For understanding the protein stability and protein folding phenomenon, all the conformational states should be described with respect to their structure and function, because such conformational states might resemble the intermediate state along the *in vivo* protein-folding pathway. Thus, play an important role in understanding the mechanism of protein folding (Arai and Kuwajima, 2000; Ahmad *et al*., 2007). Though, a large number of reports on the interaction of denaturants with proteins are available in literature, there are limited reports on the interaction

of these denaturants with endoglucanase. Endoglucanase being the first to initiate the cellulose degradation assumes significant importance from the application point of view.

In view of the above literature, the studies are undertaken in the area of cellulases to initially isolate the endoglucanase from a fungal source, Aspergillus aculeatus *which is known as an efficient producer of cellulases. The enzyme is purified and characterized to elucidate their enzymatic and bio-physical properties. Further, endoglucanase is subjected to detailed study by changing the solution conditions in order to understand the structure-function relationship and stability in presence of various stabilizing and destabilizing agents. The effect of pH and chemical denaturing agents like urea and GuHCl on the structure and stability of endoglucanase is followed to better understanding of the folding and unfolding pattern of the protein molecule. Further, these studies on the interaction of endoglucanase with denaturants are of biochemical and biotechnological importance revealing the mechanism of inactivation and providing avenues for stabilization. The effect of different cosolvents like glycerol, sorbitol and sucrose on the structure and functional stability of endoglucanase are investigated to understand the basis of thermal stabilization due to microenvironment changes brought about by these cosolvents. These studies would help in better understanding of the structure-function relationship of endoglucanase, which is of significant importance both scientifically and industrially. The main objectives of the present study are discussed in detail in the section scope and objectives.*

SCOPE AND OBJECTIVES

The characterization of specific enzyme helps in better understanding of their structure-function relationships. Cellulases from microbial origin have gained tremendous importance from industrial and scientific community owing to bio-mass conversion and its applications in food industry. Fungi produce the complete set of cellulases required for the complete hydrolysis of cellulose. Cellulases from *Trichoderma* and *Humicola* species are most extensively studied. Although, *Aspergillus* species are good producers of cellulases, detailed investigation in terms of structure-function relationship are not known much. In the present study, an attempt has been made to purify endoglucanase from the crude mixture of cellulase from *Aspergillus aculeatus*. The purified enzyme is characterized for their bio-physical properties, which would help in better understanding of their structure-function relationship and for providing insights for newer methods of stabilization which is of both scientific and industrial importance.

One of important problems for the limitations of enzymes in industrial applications is their relative instability under operational conditions. Therefore, understanding the structure and functional relationship of enzymes in various environmental conditions is important. Equilibrium studies provide a detailed knowledge concerning the structure, stabilization and folding of proteins. Further, the structure of intermediate that is formed in the folding/unfolding pathways of proteins will provide valuable information about folding pathways. The studies on interaction of denaturants such as pH and denaturing agents like urea and GuHCl with endoglucanase will help us in understanding the mechanism of denaturation

of proteins in general and cellulase in particular. Knowledge of enzyme structure and its active site topology is very important for the design of tailor made enzymes for various applications. Therefore, it is important for the determination of the structure-function relationship of the enzyme from biotechnological point of view.

Even though the effect of cosolvents on the thermal stability of proteins is very well studied, the mechanism of this thermal stability is not clearly understood. Studies on the effect of cosolvents on cellulase are sparse in literature. The present study on the effect of cosolvents such as glycerol, sucrose and sorbitol on the structural stability of endoglucanase would give more information on the mechanism of stabilization based on the individual nature of cosolvents. These studies also indicate the conditions for maximum stability of cellulase and also the hierarchy of effectiveness of different cosolvents on the structural stabilization of cellulase.

Following are the specific objectives of the present study:

1. Purification and characterization of endoglucanase from commercial source and effect of cofactors on structure and function of endoglucanase

2. Effect of pH, urea and GuHCl on the folding and unfolding of endoglucanase

3. Effect of cosolvents on structure and stability of endoglucanase

The present investigation elucidates the characterization of the endoglucanase isolated from *Aspergillus aculeatus*. The study involves purification of endoglucanase using various chromatographic and electrophoretic methods. The purified enzyme will be characterized for their

biochemical properties using different analytical techniques. The effect of various chemical reagents on the structure and function of endoglucanase is investigated. The effect of pH and chemical denaturing agents like urea and GuHCl on structure and function is studied. The effect of pH and urea and GuHCl-induced equilibrium unfolding of endoglucanase is followed to elucidate the folding mechanism to understand the molecular basis of protein stability. The conformational changes of the enzyme are monitored using several biophysical techniques to evaluate the same from various approaches.

The purified endoglucanase is studied for the mechanism of thermal inactivation and stabilization using cosolvents. The different cosolvents used in the study are chosen based on the knowledge of their stabilizing effect after preliminary screening. The stabilizing effect of cosolvents on endoglucanase is also followed by various analytical techniques. The mechanism of cosolvent –induced structural and functional stabilization is being determined by partial specific volume measurements and other related parameters are evaluated.

With these above objectives, studies are conducted to understand the endoglucanase molecule in depth, particularly with respect to structure-function relationship of the enzyme. The data generated would help in understanding the enzymatic properties of endoglucanase and its potential in the industrial applications. The folding and unfolding studies elucidate the mechanism of stability and define the conformational states during denaturation process. The interaction of cosolvents with endoglucanase gives a deeper insight for evaluating the mechanism of thermal inactivation and stabilization of the enzyme.

MATERIALS AND METHODS

1. Materials

Viscozyme, a commercial cellulase preparation of *A. aculeatus*, was obtained from Novozymes, Bangalore, India. Avicel PH 101, carboxymethyl cellulose (CMC, medium viscosity), *p*-nitrophenyl-β-D-glucopyranoside (pNPG), 5, 5-dithiobis(2-nitrobenzoic acid) (DTNB), *N*-bromosuccinimide (NBS), calcium chloride, ferrous chloride, copper chloride, DL-dithiothreitol (DTT), 2,4-dinitrosalicylic acid (DNS), ethylenediamine tetra acetic acid (EDTA), ethyleneglycol bis (β-aminoethyl ether) N, N-tetra acetic acid (EGTA), diethylpyrocarbonate (DEPC), N-(3-dimethylaminopropyl)-N-ethylcarbodiimide (EDAC), 8-anilino-1-naphthalene sulphonic acid (ANS), sodium chloride, zinc chloride, potassium sodium tartarate, glycine, urea, guanidine hydrochloride (GuHCl), glycerol, sorbitol, sucrose, acrylamide, N, N-methylene bis acrylamide, N,N,N´,N´ tetra methyl ethylene diamine (TEMED), coomassie brilliant blue R-250, 2-mercaptoethanol, lauryl sulphate (SDS), ammonium persulphate, N-acetyl tryptophan amide and a cellulose nitrate dialysis membrane having a 10 kDa molecular weight cut-off (MWCO) were purchased from Sigma-Aldrich, Co., St. Louis, MO, USA. The Diafuro YM10 membrane was purchased from Amicon Co., Beverly, MA, USA. Chromatography media such as Sephadex G-25, blue dextran 2000, DEAE-Sephadex A-50 were purchased from Amersham Pharmacia Biotech, Ltd., U.K. Phosphorous pentaoxide, citric acid, sodium citrate, trifluoroacetic acid, glacial acetic acid, sulphuric acid, phosphoric acid, hydrochloric acid, methanol, phenol, Tris-buffer, ammonium sulphate, sodium hydroxide, ethanol, sodium acetate, monosodium and disodium salts of phosphate, copper sulphate, potassium sulphate, bromo-cresol green, methyl red,

selenium dioxide were obtained from E-Merck Ltd, Mumbai, India. Folin Ciocalteu's phenol reagent was procured from Sisco Research Laboratory, Mumbai, India. All of the chemicals used were of analytical grade. Quartz triple distilled water was used in all the experiments.

2. Methods

2.1. Purification of endoglucanase from commercial source

2.1a. Ammonium sulphate precipitation

Endoglucanase was purified by the procedure as described below. The crude cellulase mixture of *Aspergillus aculeatus* obtained commercially was diluted in the ratio 1:5 (v/v) with 20 mM sodium acetate buffer at pH 5.0, centrifuged for 20 min at 8000 × *g* to remove the solids and desalted on Sephadex G-25 column equilibrated with 20 mM sodium acetate buffer at pH 5.0. The clear solution was subjected to ammonium sulfate precipitation to obtain 80% saturation with constant stirring, allowed to stand for 1 h and kept at 4°C overnight and then centrifuged at 8000 × *g* for 15 min. The precipitate was discarded and the supernatant was dialyzed against the sodium acetate buffer pH 5.0, 20 mM overnight with three changes.

2.1b. Anion-exchange chromatography

The endoglucanase was further purified by ion exchange chromatography on DEAE-Sephadex A-50 column and size exclusion chromatography on G-25 column as follows: The dialyzed fraction was loaded onto the pre-equilibrated DEAE-Sephadex A-50 column (50 × 1.9 cm). The protein was eluted with 20 mM sodium acetate buffer at pH 5.0 containing a linear gradient of sodium chloride from 0 to 0.5 M at a flow rate of 25 mL/h at 4°C and 2 mL fractions were collected. The active enzyme

eluted as a single peak. The pooled fraction, which showed maximum enzyme activity, was loaded onto Sephadex G-25 column pre-equilibrated with sodium acetate buffer, pH 5.0 at 4°C and the peak obtained was pooled and concentrated by ultra filtration to its minimum volume. Protein absorbance was measured at 280 nm. Purified enzyme thus obtained was stored at 4°C and used for further studies.

2.2. Determination of protein concentration

2.2a. UV absorption method

The protein concentration of endoglucanase in solution was estimated by ultraviolet absorbance at absorption maximum, 280 nm in Shimadzu UV-160A double beam UV-Visible recording spectrophotometer against the buffer blank. These absorbance values were converted to concentration units (mg/mL) using an extinction coefficient, $E^{1\%}_{1cm,\ 280nm}$ values. The extinction coefficient value of endoglucanase was obtained from the plot of absorbance values versus protein concentration in mg/mL. A value of 10.25 ± 0.05 at 280 nm was used for the endoglucanase for 10 mg/mL concentration.

2.2b. Lowry's method

The protein content of the sample was measured according to the procedure of Lowry's (Lowry *et al.*, 1951). The reaction mixture was made of the following reagents: Reagent A; 2% sodium carbonate in 0.1 N NaOH; Reagent B: 0.5% $CuSO_4.5H_20$ in 1% sodium potassium tartarate; Reagent C: Mix 50 mL of reagent A with 1 mL of reagent B. Reagent D: 1:1 diluted Folins reagent with water. To a sample of 20 to 100 μg of protein in 0-1 mL in a 10 mL test tube, 5 mL of reagent C was added. Mixed well and allowed to stand for 10 min. 0.5 mL of reagent D was added, mixed rapidly and allowed

to stand for 30 min. The absorbance of the solution was recorded at 660 nm and concentration of protein was determined using BSA as standard protein (Lowry *et al*., 1951).

2.2c. Micro-Kjeldhal method

The total nitrogen was estimated by the method of micro-kjeldhal (AOAC, 1984). The estimation was carried out in three steps namely digestion, distillation and titration. For digestion, 0.5 g of sample was mixed with 100 mg of digestion mixture (K_2SO_4: $CuSO_4$: SeO_2: 10:1:0.25). 15 mL of sulphuric acid was added and digested in a Gerhardt digestion system by increasing the temperature gradually to 400°C. The digestion was continued for 4-6 h until the solution in the tubes turned colorless. The digested sample was made up to 50 mL in a volumetric flask with triple distilled water. During distillation of 5 mL sample, 20 mL of 40% NaOH was added and allowed the steam to pass through. The steam distillate liberated was trapped in 10 mL of 2% boric acid solution containing 2-3 drops of mixed indicator (0.033% (w/v) methyl red and 0.167% (w/v) bromocresol green). The distillate was titrated against N/70 HCl. Ammonium sulphate was used as standard to determine the acid factor. The total protein in the samples was determined by multiplying the total nitrogen obtained, by a factor of 6.25 (AOAC, 1984).

2.3. Enzyme assay

2.3a. Assay for avicelase activity

The avicelase activity was carried out according to the procedure of Beldman *et al*., (1985). The reaction mixture consisted of 0.9 mL of 1% Avicel suspension in 20 mM sodium acetate buffer at pH 5.0 and 0.1 mL of the enzyme solution, incubated at 37°C for 1 h with shaking. Reducing sugar was

analyzed by the method of Nelson- Somogyi (Somogyi, 1952), using glucose as the standard. One unit of activity is defined as the amount of enzyme required to liberate 1 μmol of the reducing sugars per minute.

2.3b. Assay for carboxymethyl cellulase (CMCase) activity

CMCase activity was measured in reaction mixture containing 0.9 mL of 0.5% CMC in 20 mM sodium acetate buffer at pH 5.0 and 0.1 mL of the enzyme solution. After incubation at 37°C for 1 h, the reaction was stopped by adding 3 mL of DNS reagent and reducing sugars were determined by DNS method, using glucose as standard. Absorbance was measured at 540 nm. One unit of enzyme activity was defined as the amount of enzyme required to produce 1 μmol of reducing sugars per minute.

2.3c. Assay for β-glucosidase activity

The assay was performed by the addition of 0.1 mL of the enzyme solution to 0.9 mL of 0.1% pNPG in 20 mM sodium acetate buffer at pH 5.0 and incubated at 37°C for 1 h. The reaction was stopped by the addition of 1 mL of 0.5 M glycine buffer at pH 9.0 containing 2 mM EDTA. The concentration of *p*-nitrophenol was measured at 400 nm, using an absorption coefficient of 13,700 $M^{-1}cm^{-1}$ (Beldman *et al*., 1985).

2.4. Effect of pH and temperature on endoglucanase

The effect pH and temperature on endoglucanase were determined by measuring the activity as described earlier using the following buffers of 20 mM each: glycine-HCl buffer (pH 1-2), sodium acetate buffer (pH 3-5.6), phosphate buffer (pH 6.0-8.0), and borate buffer (pH 9.0) and in the temperature range of 25-90°C, respectively. A plot of relative activity versus

pH and temperature respectively was employed to obtain the pH and temperature optimum. To test the pH stability, the enzyme in different buffers was incubated for different time intervals and activity measured as described in assay methods.

2.5. Determination of degree of randomness of cellulose hydrolysis

The degree of randomness of cellulose hydrolysis was carried out using viscometric method to identify the endoglucanase activity of the purified enzyme. The activity of endoglucanase on CMC was measured viscometrically at 37°C after 1 h of incubation using 0.5% substrate. The ratio of the activity of endoglucanase on CMC measured viscometrically to the activity on CMC measured by the increase of reducing sugar is an indication of the degree of randomness in cellulose hydrolysis (Almin and Eriksson, 1967).

2.6. Electrophoretic methods

2.6a. Non-denaturing polyacrylamide gel electrophoresis

Non-denaturing PAGE (Native-PAGE) was carried out according to the procedure of Laemmli (1970) on a mini vertical slab gel electrophoresis unit at 25 ± 1°C. From the stock solution of acrylamide-bisacrylamide (29.2:0.8%), a stacking gel of 5% and separating gel of 10% was prepared. The samples were prepared in Tris buffer pH 8.3 containing 10% glycerol, and 0.02% bromophenol blue. After an initial pre-run for 30 min, the samples were loaded and electrophoresed at a constant voltage (100 volts) for 2-3 h. The gels were stained with 0.5% Coommasie Brilliant Blue R-250 prepared in 50% methanol, 10% acetic acid for 2h and destained in 50% methanol and 10% acetic acid and water.

2.6b. Sodium dodecyl sulfate-polyacrylamide gel electrophoresis

Sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) was carried out on a slab gel electrophoresis using the discontinuous buffer system of Laemmli (1970).The polyacrylamide gel of 12% containing 0.1% SDS was cast in slab gel apparatus. The gel buffer was 0.025 M Tris, 0.3 M glycine, pH 8.3 having 0.1% SDS. The samples were prepared in sample buffer, pH 6.8 containing 5% (v/v) 2-mercaptoethanol, 10% (w/v) glycerol, 2% SDS (w/v) and 0.1% (w/v) bromophenol blue. Samples were heated in boiling waterbath for 5 min before subjecting it to electrophoresis at 100 volts. The gels were stained with 0.5% Coommasie Brilliant Blue R-250 prepared in 50% methanol, 10% acetic acid for 2 h and destained in 50% methanol and 10% acetic acid and water. The SDS-PAGE molecular weight markers were used as standard.

2.6c. Isoelectric focusing of protein

Analytical thin-layer gel isoelectric focusing was performed in the pH range of 3-10 using ready-made gels on Multiphor II system (Amersham Biosciences, Uppsala, Sweden). 1 M NaOH and 1 M H_3PO_4 were used as cathode buffer and anode buffer, respectively. The samples were prepared in triple distilled water and 20-40 µL sample was loaded. Focussing was carried out at 1500 volts and 200 mA for 1 h at 10°C. After electrofocusing, the gel was fixed in solution containing a methanol/acetic acid solution. The gels were stained with 0.5% Coommasie Brilliant Blue R-250 prepared in 50% methanol, 10% acetic acid for 2 h and destained in 50% methanol and 10% acetic acid and water. The pI of endoglucanase was determined using the plot of relative mobility of standard protein markers versus their pI.

2.7. Fast protein liquid chromatography of protein

The homogeneity of the purified enzyme was determined by fast protein liquid chromatography (FPLC). The protein sample was loaded to a Superdex-75 gel-filtration column (10 mm × 30 cm; Amersham Bioscience, Uppsala, Sweden) pre-equilibrated with 20 mM sodium acetate buffer pH 5.0. Sample of 20 µL was injected into the column and eluted in the same buffer with a flow rate of 1 mL/min. The eluted protein fractions are detected using absorbance at 280 nm.

2.7a. Determination of Stokes radius

Stokes radius of endoglucanase at different conditions were determined using size exclusion chromatographic method. Gel filtration chromatographic measurements were carried out using Superdex-75 HR column (10 mm × 30 cm; Amersham Bioscience, Uppsala, Sweden) on a FPLC system (Pharmacia Biotech AB, Uppsala, Sweden). For monitoring the elution profile at different pH, the enzyme solution at the test pH was injected into the column at 25°C after equilibrating the column at the test pH. Samples were centrifuged and filtered before injection. The flow rate was adjusted to 0.5 mL/min with detection at 280 nm. The standard proteins used for the calibration of the column with known molecular mass and Stokes' radii (R_S) were alcohol dehydrogenase (45.35 Å) (Horiike *et al.*, 1983) bovine serum albumin (35.5 Å) ovalbumin (30.5 Å) carbonic anhydrase (24.4 Å) myoglobin (21.4 Å) and ribonuclease (19.3 Å) (Uversky, 1993). Blue dextran was used to determine the void volume.

2.8. Analysis by HPLC

The end products released by the activity of purified endoglucanase on CMC were determined by HPLC method (Beldman *et al*., 1985). The HPLC was carried out using a Waters Associate HPLC system consisting of binary gradient pumping system with a Millennium data processor. The enzyme was incubated with 0.5% CMC at 37°C for 1 h in sodium acetate buffer at pH 5.0. The enzyme was inactivated by placing the tubes in a boiling water bath for 10 min. The hydrolysed products were analyzed by HPLC using an Aminex (0.25 × 5 cm) column with a light-scattering detector and acetonitrile/water (60:40) as the solvent with a flow rate of 1 mL/min.

2.9. Amino acid analysis

Amino acid analysis of endoglucanase was performed according to the method of Bidlingmeyer *et al*., (1984) using a Waters Associate Pico-Tag amino acid analysis system. The analysis was carried out using a three-step procedure. In the first step, protein samples were acid hydrolyzed to free amino acids. Amino acids were modified by PITC in the second step and the last step included the separation of the modified amino acids by RP-HPLC.

An aliquot of the purified enzyme (~30 μg of protein) was dried under vacuum and 200 μL of constant boiling HCl (6 N) containing phenol 1% (v/v) was added. After hydrolysis, the residual HCl was removed under vacuum. Standard free amino acid mixture containing u p to 25 nmol of each amino acid was placed in the tubes (6 × 50 mm) and dried under vacuum. Standard free amino acid mixture and the hydrolyzed samples were dried down under vacuum after the addition of re-drying solution (20 μL) containing ethanol:

water: triethylamine (TEA) in the ratio of 2:2:1 at 50-60 mtorr vacuum. The derivatization reagent consisted of ethanol: triethylamine: water: PITC (7:1:1:1). PITC was stored at –20° C under nitrogen to avoid its degradation. To make 300 µL reagent, 210 µL of ethanol was mixed thoroughly with 30 µL each of PITC, TEA and water. PTC amino acids were formed by adding 20 µL of the reagent to the dried samples. The excess of reagents were then removed under vacuum using the workstation. When the vacuum reached 50-60 mtorr, the samples were ready for analysis by RP-HPLC.

The HPLC was carried out using a Waters Associate HPLC system consisting of binary gradient pumping system and photodiode array detector (Model 2996) with a Millennium data processor. The temperature was controlled at 38 ± 1°C with a column heater. Samples were injected in volumes ranging from 5-50 µL. The column was an application specific Pico-Tag column (150 × 3.9 mm). The solvent system consisted of two eluents, (A) an aqueous buffer (0.14 M sodium acetate containing 0.5 mL/L of TEA) titrated to pH 6.4 with glacial acetic acid: acetonitrile (94:6) and (B) 60% acetonitrile in water. The gradient run for the separation at a flow rate of 1mL/min consisted of: 100% A and 0% B (initial), 54% A and 46% B (10 min), 0% A and 100% B (11 min), 0% A and 100% B (13 min), 100% A and 0% B (14 min) and 100% A and 0% B (25 min). The PTC amino acids were detected at 254 nm.

2.10. Estimation of thiol groups

Free cysteine and disulfide content of endoglucanase were determined by assaying for sulfhydryl content using DTNB. Measurements were made in

1 mL of protein solution containing 6 M guanidine hydrochloride in 0.1 M Tris-glycine buffer at pH 8.0 having 0.01 M EDTA and 50 μL of DTNB (10 mM) was added to the reaction mixture and kept for 15 min before measuring the absorbance at 412 nm. For free cysteine estimation protein in Tris-glycine buffer pH 8.0 without guanidine hydrochloride was used. The number of free cysteine residues was calculated using a molar extinction coefficient value of 13,600 $M^{-1}cm^{-1}$ according to the procedure of Ellman (1959).

2.11. Estimation of tryptophan

The number of trptophan residues in endoglucanase was estimated by titrating the protein with N-bromosuccinimide (NBS) using a Shimadzu 160A UV-vis spectrophotometer. To 1 mL of endoglucanase in 20 mM sodium acetate buffer at pH 5.0, 10 μL of 10 mM NBS solution was added to the sample and reference cuvette having buffer. Addition of NBS results in the oxidation of indole chromophore of tryptophan which has a strong absorbance at 280 nm, to oxindole, a much weaker chromophore at this wavelength. The decrease in the absorbance was measured at 280 nm upon the addition of NBS in a stepwise manner until further addition of NBS lead to an increase in the absorbance (Spande and Witkop, 1967). The number of tryptophan residues per mole of protein was calculated by equation:

$$n = \Delta A \times 1.31 \times MW \ / \ A_i \times a_f \times 5500 \qquad ------ (1)$$

where ΔA is the corrected absorbance decrease at 280 nm, A_i is the initial absorbance of protein, a_f is the absorptivity factor to convert absorbance of

endoglucanase to mg protein, MW is the molecular weight of the protein and 5500 is the molar extinction coefficient of tryptophan at 280 nm.

2.12. Estimation of carbohydrate

The total carbohydrate content of endoglucanase was estimated by the phenol-sulfuric acid method using glucose as the standard according to Dubois *et al*., (1956). Sample of 30 to 150 μg of carbohydrate in 0-1 mL taken in 10 mL test tubes, 1 mL of 5% phenol and 5 mL of sulphric acid was added. Mixed well and allowed to stand for 30 min. The absorbance of the solution was recorded at 490 nm and concentration of carbohydrate was determined using glucose standard curve.

2.13. Chemical modification of endoglucanase

The effect of various specific reagents, such as dithiotreitol (DTT) for cysteine residues, N-bromosuccinimide (NBS) for tryptophan residues, N-(3-dimethylaminopropyl)-N-ethylcarbodiimide (EDAC) for acidic amino acids and diethylpyrocarbonate (DEPC) for histidine residues at different concentrations on the endoglucanase activity in 20 mM sodium acetate buffer, pH 5.0 was studied. The extent of modification was measured through enzyme activity. The decrease in the activity was expressed as percentage remaining activity as compared with the control.

2.13a. Modification of histidine residues by DEPC

Histidine residues were modified using DEPC, which reacts with histidine residues to form mono-N-carbethoxyhistidyl derivatives. This can be monitored by the increase in the absorbance maxima between 230-250 nm spectrophotometrically, using an extinction coefficient for the modified

residue of 3200 $M^{-1}cm^{-1}$ at 240 nm. Treatment with hydroxylamine decarbethoxylates the monosubstituted histidine allowing the reversal of the activity, where as the disubstituted histidine undergoes a ring cleavage and thereby reversal of the activity is not possible (Miles, 1977).

2.13b. Modification of carboxyl groups by EDAC

Carboxyl groups of proteins are converted into amides by water soluble carbodiimide. The modification of carboxyl groups takes place in two steps: the activation of the carboxyl group by water soluble carbodiimide (EDAC) to form the O-acylisourea which in turn reacts with a nucleophile glycine methyl ester (GME) to form the amide bond (Hoare and Koshland, 1967). Due to its high solubility in water and high specificity for acidic amino acids, it is commonly used to modify the enzymes which probably have the carboxy groups in their catalytic sites (Clarke and Yaguchi, 1985; Yoshida *et al*., 1994).

2.14. Spectroscopic methods

2.14a. Absorption spectroscopy

Absorption spectra of protein were recorded in a Shimadzu UV 160A, a double beam recording spectrophotometer using 1 cm quartz cell at 25°C in the range of 230-360 nm with respective buffers as the reference solution.

The extinction coefficient of the enzyme in different concentrations of the third component was determined by UV spectroscopy. For the determination of $E^{1\%}$ in presence of third component, identical aliquot of native enzyme stock solution were diluted volumetrically to identical extent with native buffer as well as with different solvents. The UV spectra were

recorded in Shimadzu UV 160A double beam spectrophotometer between 260-340 nm. Using the extinction coefficient of enzyme in native and its absorbance ratio measured at given wavelength (Absorbance of native/Absorbance in mixed solvent) with corresponding blanks, extinction coefficient of the enzyme in different concentrations of third component was calculated.

2.14b. Fluorescence spectroscopy

(i) Intrinsic fluorescence of protein

Fluorescence measurements of endoglucanase under various conditions were done using a Shimadzu RF-5000 automatic recording spectrofluorimeter at 25°C. The temperature of the cell was maintained by circulating the water through the thermo-jacketed holder from a circulating water bath. The protein was excited at 280 nm and emission-scanned from 300 to 400 nm. The bandwidths for excitation and emission monochromators were fixed at 5 and 10 nm, respectively. The readings either relative fluorescence intensity or change in the wavelength of maximum emission were plotted against denaturant/cosolvent concentrations.

(ii) ANS binding studies of protein

Endoglucanase was treated with different concentrations of denaturants/salts for 24 h. The protein thus equilibrated with different concentrations of denaturants/salts was then treated with freshly prepared ANS. The protein was incubated with a 100 fold molar excess of ANS for more than 30 min in the dark at room temperature and ANS fluorescence was measured. The excitation wavelength was 380 nm and the emission spectra

were measured between 400 to 600 nm. Slit widths of excitation and emission were 5 and 10 nm, respectively. Assays in absence of the protein were performed in order to correct for the unbound ANS emission fluorescence intensities.

(iii) Acrylamide quenching studies of protein

The protein in sodium acetate buffer, pH 5.0 was treated with different concentrations of urea/GuHCl/salts were taken in a cuvette and the spectra was recorded between 300-400 nm after exciting at 295 nm. Aliquotes of (5-10 μL) of the acrylamide stock (8 M) solution was added, mixed well and again spectra were recorded. The addition of acrylamide was continued till the fluorescence was almost completely quenched. The decrease in fluorescence intensity at λ_{max} was analyzed by Sterm-Volmer equation (Eftink and Ghiron, 1981):

$$F_o / F = 1 + K_{sv} [Q] \qquad \text{---------} (2)$$

where F_o and F are the fluorescence intensities at an appropriate wavelength in the absence and presence of quencher (acrylamide) respectively, K_{sv} is the Stern-Volmer constant and $[Q]$ is the concentration of the quencher.

2.14c. Circular dichroism (CD) spectroscopy

CD spectra were obtained using a Jasco J-810 automatic recording spectropolarimeter (Japan Spectroscopic Co., Tokyo, Japan) fitted with a 250 W xenon lamp and calibrated with d-10-camphorsulfonic acid. All samples were previously centrifuged and filtered through Millipore filters (0.45 μm pore diameter). Sample was analyzed in 0.1 cm optical path length cells in the far UV (200-260 nm) region. Near-UV CD measurements in the

region of 240-320 nm were determinded with the 10 mm path length cell. The protein solution was dialyzed exhaustively for 24 h versus different concentrations of denaturants/cosolvents/metal salts, centrifuged and the supernatant was used for the CD spectral measurements. The molar ellipticity values were calculated using a mean residue weight of 115. The secondary structure of endoglucanase was analyzed using the computer program of Yang *et al*., (1986). The reported CD values were the average of at least three independent scans.

2.15. Thermal denaturation studies of protein

(i) Thermal Stability Studies

Activity loss of endoglucanase as a function of the temperature was followed in acetate buffer (20 mM, pH 5.0) by incubating the enzyme for 30 min at different temperatures in the range of 20-90°C. Thermal inactivation of endoglucanase in presence of third component was determined by incubating the enzyme at 90°C. After cooling, the residual activity was measured by transferring an aliquot to the assay mixture as mentioned earlier. The activity of the without heat treated enzyme was taken as 100%.

(ii) Determination of apparent thermal transition temperature

The apparent T_m of endoglucanase in native state and in presence of third component was determined by using Cary100 Bio UV-Visible spectrophotometer (Varian, Victoria, Australia) with six position thermostat cuvette holder. Measurements were done at different temperatures ranging from 20-90°C with 1°C per min increment and the spectra were recorded at 287 nm. A protein concentration of 2.2 $\times 10^{-6}$ M was used for all the experiments. The apparent thermal transition temperature (apparent T_m) was

calculated by monitoring the progress of denaturation followed by changes in the absorbance or van't Hoff plot (Pace and Scholtz, 1997). The fraction of protein in the unfolded state (F_u) is given by

$$F_u = (Y_n - Y) / (Y_n - Y_u) \qquad \text{--------} (3)$$

where, Y_n is the absorbance of the protein in the native state, Y_u is the absorbance of the protein in the unfolded state and Y is the absorbance of the protein at different temperatures. The apparent thermal transition is defined as the temperature at which the value of F_u is 0.5.

2.16. Acid denaturation experiments

The samples of endoglucanase for acid denaturation studies were carried out using the following buffers of 20 mM each: glycine-HCl buffer (pH 1-2), sodium acetate buffer (pH 3-5.6), phosphate buffer (pH 6.0-8.0). The enzyme samples were incubated with buffers of desired pH at 25°C and allowed to equilibrate for 3 h before taking all the spectroscopic measurements. In order to assess the reversibility of acid induced unfolding, endoglucanase at various pH values was extensively dialyzed at 4°C against 20 mM sodium acetate buffer pH 5.0. This dialyzed sample was compared to native endoglucanase using fluorescence and CD measurements.

2.17. Folding and unfolding experiments

Unfolding of endoglucanase was induced by incubating or dialyzing with different concentrations of denaturants in sodium acetate buffer, pH 5.0 till equilibrium is attained. The extent of unfolding of endoglucanase was monitored either by the measurement of enzyme activity, change in emission

maximum or change in the ellipticity values in the far-UV region by circular dichroism measurements. Refolding experiments were carried out using endoglucanase that had already been denatured by 8 M urea/6 M GuHCl. The denatured endgolucanase was either dialyzed or diluted with the appropriate concentration of urea/GuHCl and the mixture was incubated till the reaction reached equilibrium or dialyzed against the desired concentration of denaturants with several changes. The extent of unfolding and refolding of endoglucanase induced by denaturants was analyzed either by changes in the ellipticity values at 217 nm and fluorescence emission at 336 nm for each concentration of denaturant used. This was calculated with an assumption that the change observed at the highest concentration of denaturant was 100 percent. The data are expressed in terms of the fraction unfolded (F_u) calculated from the standard equation (Pace and Scholtz, 1997).

2.18. Measurement of partial specific volume

The partial specific volume measurements of endoglucanase were carried out according to the standard procedure (Lee *et al*., 1979; Prakash, 1982; Prakash and Timasheff, 1985). The densities of the sample solutions were determined using Anton Paar DMA-5000 precision densitymeter (Anton Paar GmbH, Graz, Austria) with a built-in thermostat at constant molality and constant chemical potential at 20.00 ± 0.01°C. The densities of the solutions were converted into apparent partial specific volume; ϕ(mL/g) based on the standard method using the following equation (Casassa and Eisenberg, 1961, 1964)

$$\phi = 1/\rho_0[1-\{(\rho_P - \rho_0)/C\}] \qquad ------ (4)$$

where ρ_p and ρ_0 are the densities of the protein solution and solvent respectively and C is the concentration of endoglucanase in g/mL. The apparent partial specific volume of the protein ($\bar{v}_{app}$) was calculated by extrapolating the obtained ϕ values at different concentrations of protein to zero protein concentration. The partial specific volume of proteins was obtained under isomolal (ϕ_2^o) and isopotential ($\phi_2'^o$) conditions and related preferential interaction parameters were calculated using the established procedures (Lee *et al.*, 1979; Prakash and Timasheff, 1985). Using standard notations of Scatchard (1946) and Stockmayer (1950), the data were analyzed for thermodynamic parameters using three-component system namely water (component 1), protein (component 2) and the additive (component 3). The preferential interaction parameters (ξ_3) for this three component system can be calculated using the following equation (Casassa and Eisenberg, 1961; 1964)

$$\xi_3 = (\delta g_3/\delta g_2)_{T,\mu_1,\mu_3} = 1/\rho_0 (\phi_2^o - \phi_2'^o) / (1 - \bar{v}_3 \rho_0) \qquad ------ (5)$$

where g is the concentration of component 'i' in gram per gram of water, μ is its chemical potential, ρ_0 is the density of the solvent and T is the thermodynamic temperature. The ϕ_2^o and $\phi_2'^o$ are the partial specific volume of the protein at isomolal and isopotential conditions, respectively and $\bar{v}_3$ is the partial specific volume of the component three.

1.1. Purification and Characterization of Endoglucanase from commercial source

Microbial cellulases have attracted considerable research and commercial interest due to their enormous potential applications in biotechnology (Bhat, 2000). Many cellulolytic microorganisms, especially fungi, produce extracellular cellulases that catalyze the hydrolysis of insoluble cellulose to glucose. They are composed of endoglucanase (EC 3.2.1.4), exoglucanase (EC 3.2.1.91) and β-glucosidase (EC 3.2.1.21) in free and often multiple forms (Wood, 1992). Cellulases are bimodular proteins with a large catalytic and a small cellulose binding module linked by a short highly glycosylated protein sequence rich in Ser and Pro (Gilkes *et al*., 1991). The substrate for these enzymes is cellulose, a heterogeneous supra molecular structure which requires enzymes of widely differing properties and mechanism of action for degradation. Endoglucanases preferentially hydrolyze the internal β-1, 4 linkages of amorphous cellulose, while exoglucanase acting synergistically with endoglucanase hydrolyse the crystalline cellulose by cleavage of cellobiose from the chain ends (Beguin and Aubert, 1994).

Aspergillus species are an important commercial source of inexpensive cellulase, not only in the food and textile industries but also in pharmaceutical industries (Bakalova *et al*., 1996). Endoglucanase is the main component for cellulose degradation produced by *Aspergillus aculeatus*. Endoglucanases play an important role in increasing the oil extraction, improving the nutritive quality of bakery products and animal feed, increasing the yield of fruit juices, beer filtration, enhancing the brightness, smoothness and quality of cellulosic garments (Bhat, 2000). Specification of these applications requires a wide

range of endoglucanases with varying pH and temperature optimum, stability and substrate specificities. There is very little information available on the biochemical and enzymatic properties of endoglucanases in general. In this study, we have purified and characterized the endoglucanase from *Aspergillus aculeatus* for its enzymatic and chemical properties in order to understand the structure-function relationship of cellulases.

1.1a. Purification and characterization of endoglucanase from commercial source

The crude cellulase from *A. aculeatus* obtained commercially was screened for various cellulase activities. *A. aculeatus* being the producer of endoglucanase in large quantities was isolated, purified and characterized for its biochemical characteristics. Purification of endoglucanase was carried out in 20 mM sodium acetate buffer pH 5.0 from the crude cellulase mixture obtained from *A. aculeatus* as described in the following section. The cellulase mixture was desalted using Sephadex G-25 column and fractions were pooled and subjected to partial purification using 80% ammonium sulphate precipitation method. The supernatant having CMC activity was dialyzed using a cellulose nitrate dialysis membrane (6-8 kDa cut off) versus 20 mM sodium acetate buffer at pH 5.0 and then loaded onto a DEAE-Sephadex A-50 ion-exchange column equilibrated in 20 mM sodium acetate buffer at pH 5.0 and fractionated using a sodium chloride gradient of 0-0.5 M in sodium acetate buffer pH 5.0. The elution profile of ion exchange column and activity profile of the enzyme are shown in Fig. 1. The yield and purity of endoglucanase are shown in Table 2. The total protein content was reduced by about 72% and the specific activity was increased by 100%, indicating 2-fold purification. After DEAE-Sephadex A-50 chromatography purification, the total protein content of the ion-exchange fraction was reduced by 94%, but the specific activity was increased by about 4-fold compared to the crude.

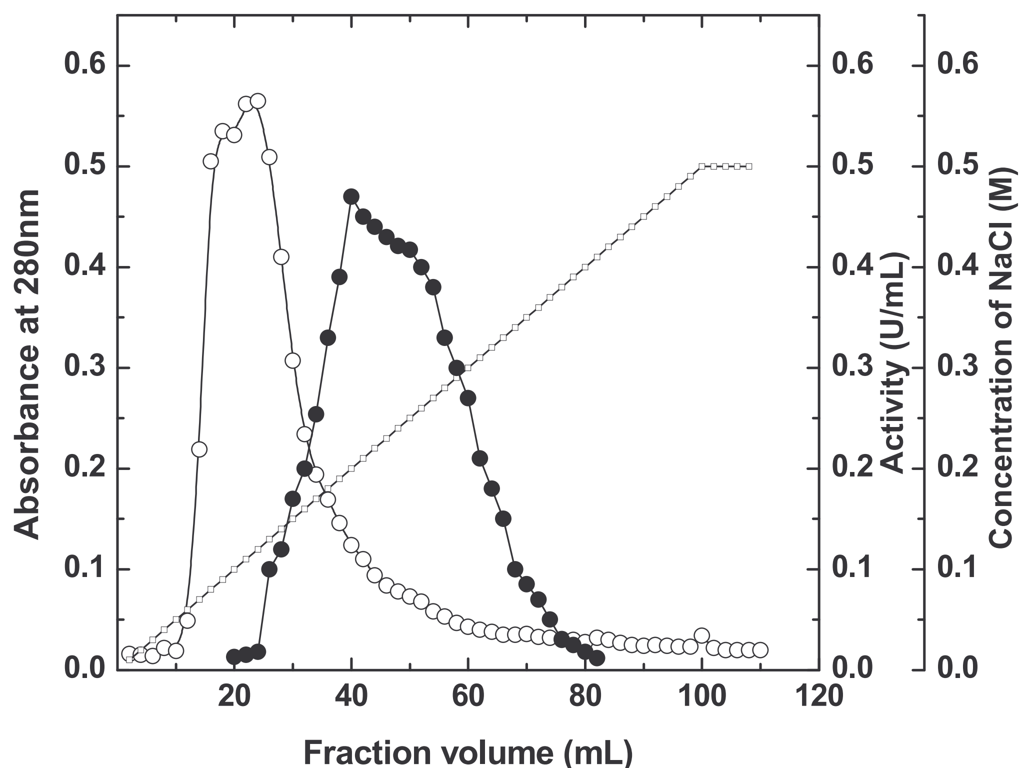


Fig. 1: DEAE-Sephadex A-50 column chromatography of the ammonium sulphate fraction. The concentrated ammonium sulphate fraction was applied to the pre-equilibrated column in 20 mM sodium acetate buffer pH 5.0. The column was eluted with a linear gradient of NaCl in the same buffer. The flow rate was 25 mL/h and the fraction volume was 2 mL. (●) endoglucanase activity; (○) absorbance at 280 nm; (□) NaCl gradient 0 - 0.5 M.

Table 2: Purification steps of endoglucanase (EC 3.2.1.4) from *Aspergillus aculeatus**

Steps	Total protein (mg)	Total activity (U)	Specific activity (U/mg)	Yield (%)	Purification factor
Crude	157 ± 4	54.40 ± 3.1	0.35 ± 0.05	100	1
80% $(NH_4)_2SO_4$ Precipitation	44 ± 2	34.66 ± 1.5	0.80 ± 0.08	64	2.2
DEAE-Sephadex A-50 chromatography	10 ± 1	13.83 ± 1.0	1.40 ± 0.1	25	4.0

* *These are the results of purification from 25 mL of the crude cellulase mixture and are reproducible*

The peak obtained having cellulase activity was typical endoglucanase, showing a high specific activity towards CMC. The fractions were pooled and loaded on to Sephadex G-25 column equilibrated in 20 mM sodium acetate buffer at pH 5.0, the peak obtained was pooled and concentrated by ultra filtration. The FPLC profile of crude cellulase and PAGE pattern are shown in Fig. 2A. The homogeneity of the preparation was ascertained by polyacrylamide gel electrophoresis (PAGE). The FPLC profile of purified enzyme obtained using Superdex-75 gel filtration column along with the PAGE patterns under denaturing conditions is shown in Fig. 2B. Single sharp peak obtained signifies the purity of the endoglucanase. The molecular mass of endoglucanase was found to be 45 kDa. The preparation had over 95% purity. Araujo *et al*., (1986) reported that cellulases produced by *Aspergillus terreus* were resolved in SDS-PAGE into four components, with a molecular weight range of 16-90 kDa.

The activity of endoglucanase with different substrates is given in Table 3. The purified enzyme was identified as endoglucanase, showing high activity towards CMC and low specific activity towards Avicel. The specific activity towards Avicel and *p*NPG was 30 times lower. The amino acid composition of endoglucanase is shown in Table 4, where the data is presented as gram percent. The amino acid composition is comparable with related endoglucanases from other species (Olama *et al*., 1993). From the table, it is apparent that, the endoglucanase is rich in acidic amino acids like asparatic and glutamic acids which is true with other cellulases.

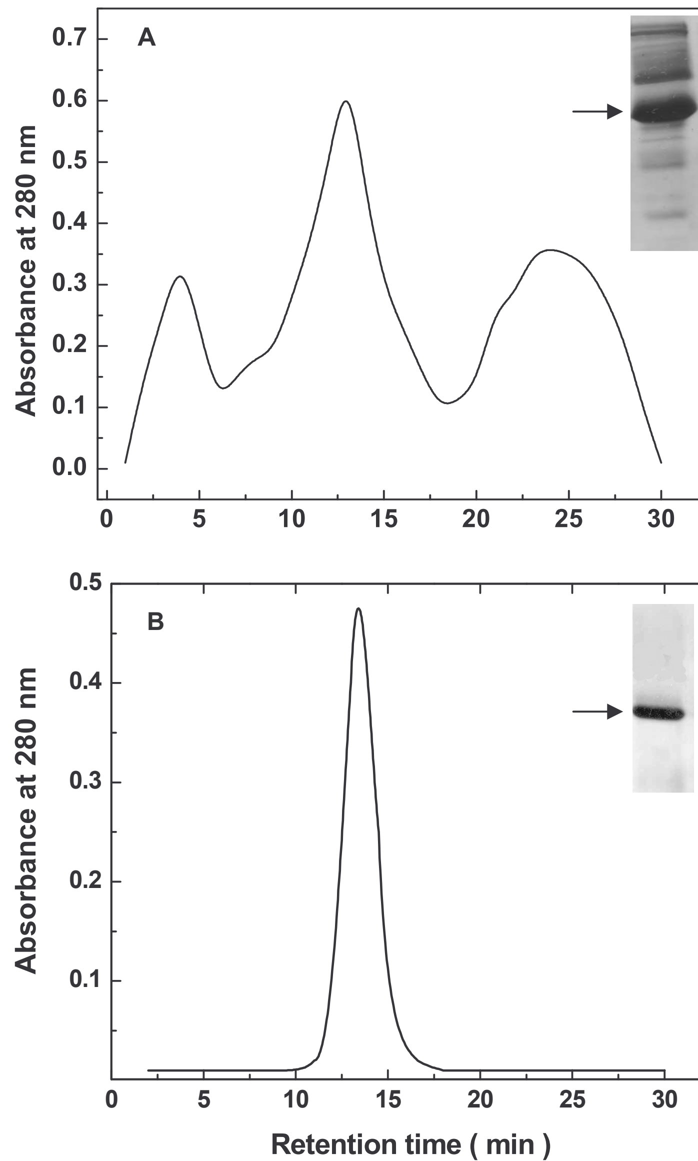


Fig. 2: (A) Size-exclusion chromatography profile of the crude cellulase on Superdex-75 column on FPLC. The eluant used was 20 mM sodium acetate buffer, pH 5.0 at a flow rate of 1 mL/min and the fractions were detected at 280 nm. Inset: SDS-PAGE showing endoglucanase in crude cellulase from *A. aculeatus*. (B) The homogeneity of the purified endoglucanase from crude cellulase from *A. aculeatus*. Inset: SDS-PAGE of purified endoglucanase from *A. aculeatus.*

Table 3: Specific activities of endoglucanase purified from *A. aculeatus* using different substrates in 20 mM sodium acetate buffer, pH 5.0.

Substrate	Specific activity (units / min)
CMC	1.40 ± 0.10
Avicel	0.047 ± 0.001
*p*NPG	0.037 ± 0.001

Table 4. Amino acid composition of endoglucanase from *A. aculeatus*

Amino acid	Gram percent
Asp	17.0 ± 0.50
Glu	6.0 ± 0.40
Ser	14.0 ± 0.30
Gly	8.0 ± 0.10
His	1.0 ± 0.10
Arg	2.0 ± 0.10
Thr	11.0 ± 0.30
Ala	5.0 ± 0.10
Pro	3.0 ± 0.05
Tyr	4.0 ± 0.10
Val	5.0 ± 0.10
Met	1.0 ± 0.05
Cys	1.0 ± 0.05
Ile	3.0 ± 0.10
Leu	6.0 ± 0.15
Phe	4.0 ± 0.10
Lys	4.0 ± 0.10
Trp*	5.0 ± 0.10

**Determined independently by NBS method (Spande and Witkop, 1967)*

The purified enzyme had an optimum pH of 5.0. Effect of temperature on the activity of the endoglucanase was investigated by incubating the enzyme for 1 h at different temperatures ranging from 30 to 90ºC. The activity profile is shown in Fig.3. From the figure, it is evident that the enzyme loses its activity with increase in temperature and at 90ºC the enzyme lost 50% of its activity. To further check the stability of the enzyme, the incubation time was increased to 5 h, the enzyme was able to maintain its 50% activity with no further loss. These results show that the endoglucanase is susceptible to high temperatures and is thermally inactivated with increase in temperature.

The optimum temperature determined was 40ºC. The calculated Michaelis-Menton constant (K_m) and velocity maximum (V_{max}) for the purified endoglucanase were 0.060 ± 0.001% and 0.08 unit (mg of protein)$^{-1}$ min^{-1}, respectively. The isoelectric focusing of the purified enzyme on polyacrylamide gel having a pH range of 3-10 showed a sharp band, confirming the purity of the enzyme. The pI of the purified endoglucanase was found to be 4.3. The carbohydrate content of the purified endoglucanase was around 25 ± 2. The carbohydrate content of the endoglucanase is significantly higher than that reported by Berghem *et al*., (1976).

The HPLC profile of the end-product analysis by the action of endoglucanase is shown in Fig. 4. The release of glucose and cellobiose as the end products during incubation with CMC confirmed it as endoglucanase. The ratio of the activity of endoglucanase on CMC measured viscometrically to the activity on CMC measured by the increase of reducing sugars is an indication of the degree of randomness in cellulose hydrolysis. The degree of randomness for the isolated enzyme was found to be 0.527, which is in

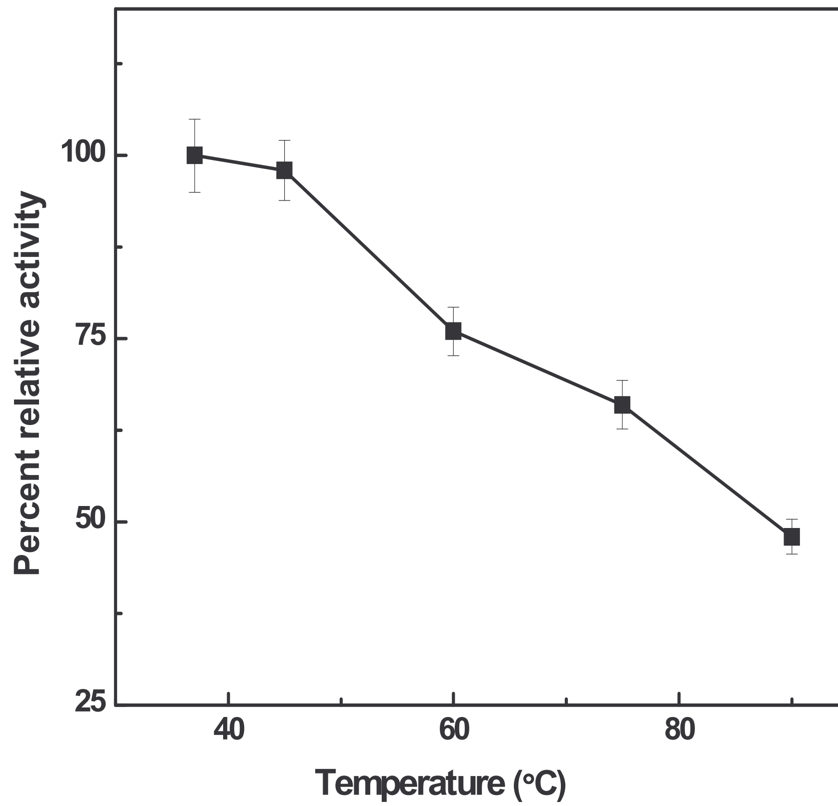


Fig. 3: Effect of temperature on activity of endoglucanase. Samples of the enzyme were incubated for 1h at different temperatures (30-90°C) and their activities then determined as described under materials and methods.

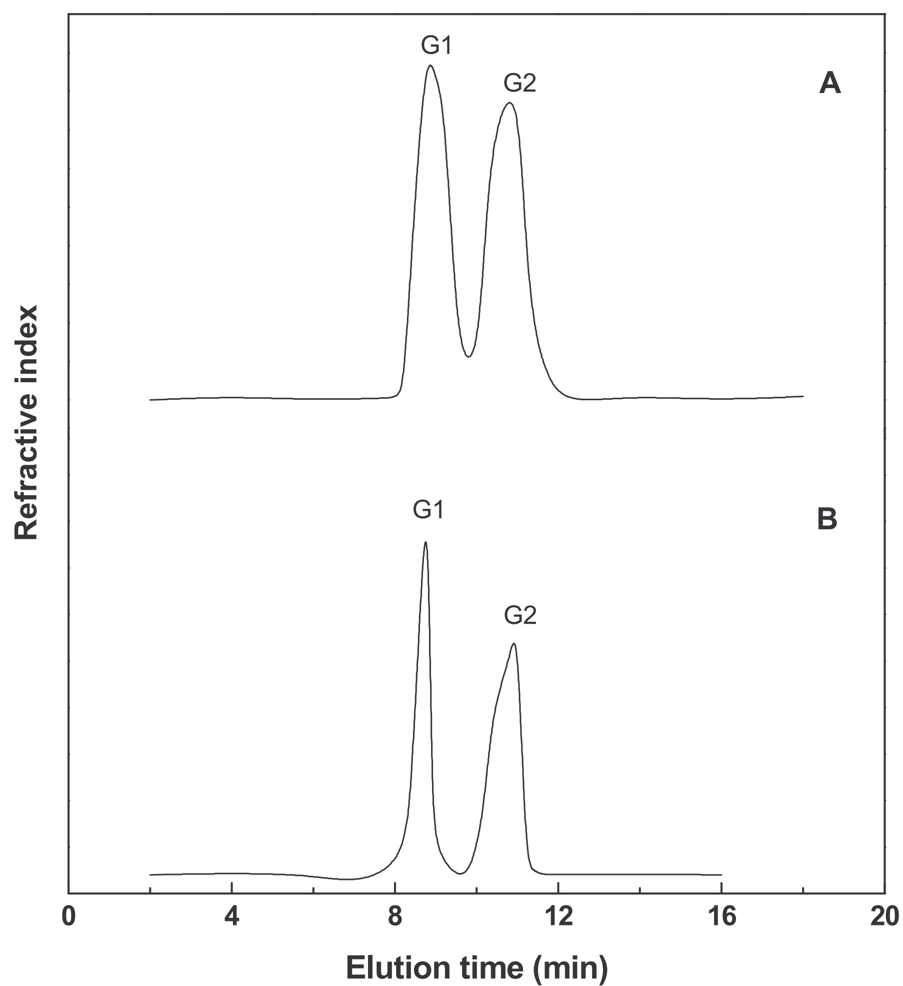


Fig. 4: HPLC analysis of end products released from CMC by the action of endoglucanase in 20 mM sodium acetate buffer, pH 5.0 at 40°C for 1 h. (A) Hydrolysed sample after incubating for an hour; (B) Standards: G1, glucose and G2, cellobiose.

accordance with other endoglucanases (Beldman *et al*., 1985). These two properties are generally accepted to distinguish endoglucanase from exoglucanase and β-glucosidase. Schoemaker *et al*., (1978 a & b) reported two endoglucanases with different degrees of randomness in their action on CMC, Endo III and Endo IV. The less random type of enzyme referred to by these authors as Endo III was similar to the isolated enzyme, as it showed decrease in viscosity compared to the increase in reducing sugars during incubation with CMC and has the same molecular weight and pI similar to Endo II (45 000) and Endo V (pI 4.4), respectively, isolated by Beldman *et al*., (1985) thus, it was determined as endoglucanase.

The intrinsic fluorescence emission spectrum of the purified endoglucanase was determined in sodium acetate buffer, pH 5.0 (20 mM). Tryptophan residues show an emission maximum at 330 nm when in the hydrophobic environment and around 350 nm, when present in a hydrophilic environment (Eftink and Ghiron, 1981). The purified enzyme showed an emission maximum at 336 nm showing that the tryptophan residues are partially exposed to solvent as shown in Fig. 5A.

Circular dichroism is a sensitive method to study the secondary and tertiary structures of protein solution. The far-UV CD spectrum of endoglucanase in 20 mM sodium acetate buffer, pH 5.0 is shown in Fig. 5B. The spectrum is characterized by minima at 217 nm with a mean residue ellipticity value of -3450 ± 30 $degcm^2d.mol^{-1}$ with a characteristic positive absorption band starting from 202 nm. This relatively weak magnitude (mean residue ellipticity) is consistent with a predominance of β-sheet structure (β-sheet ~ 62%, and α-helix ~14%) in endoglucanase, which is true with other endoglucanases.

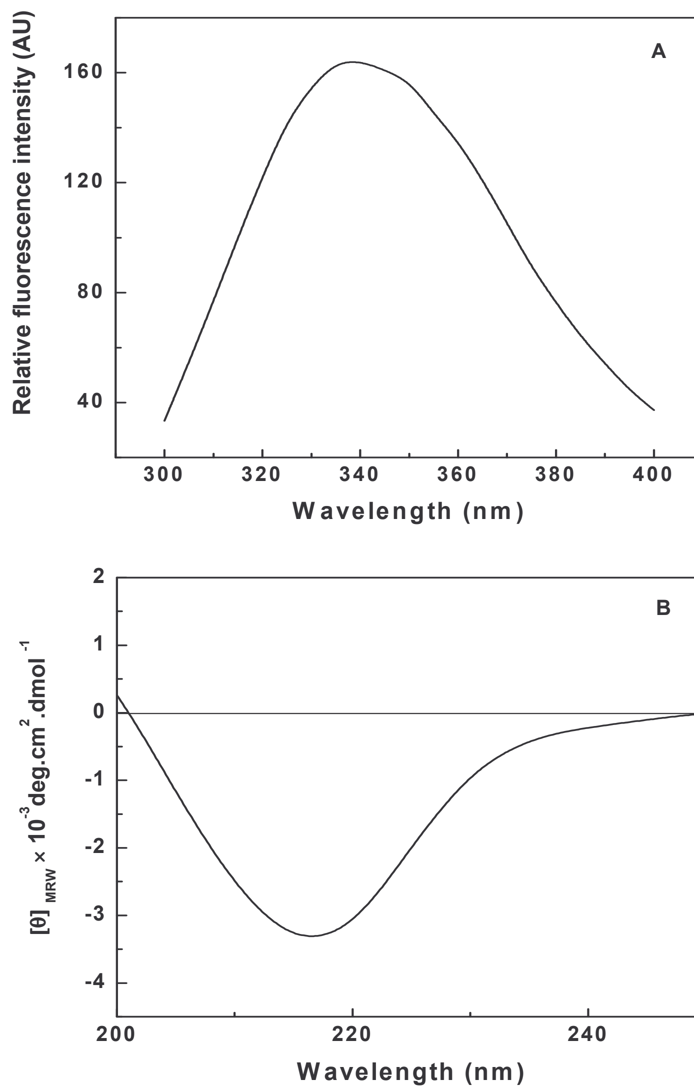


Fig. 5: (A) The intrinsic fluorescence emission spectrum of purified endoglucanase measured in 20 mM sodium acetate buffer, pH 5.0 at 25°C. (B) Far-UV CD spectrum of the endoglucanase.

1.1b. Effect of chemical modification on endoglucanase

Chemical modification is a very useful tool for identification of the functional groups of a protein (Lundblad and Noyes, 1984). There are three methods to alter the stability of proteins: (i) genetic engineering method to replace the specific amino acid residues; (ii) altering the environment of the protein using additives and (iii) chemical modification of the amino acid residues. Modification of specific amino acid residues results in either stabilization or destabilization of the protein (Klibanov, 1983; Cupo and Pace, 1983). Changes in the enzyme structure through selective modification can lead to alterations in the catalytic activity. Specific reagents are available for aspartic acid, glutamic acid, histidine, arginine, tryptophan and cysteine residues (Imoto and Yamada, 1997). The identification of essential groups has been classically relied on the kinetic studies, chemical modification using group specific reagents (Glazer *et al.*, 1976) and affinity labelling (Colman, 1989). Use of modern techniques like site directed mutagenesis, nuclear magnetic resonance and X-ray crystallography have complemented the data obtained by classical means for several enzymes like histidine carboxylase (Gallagher *et al.*, 1989), chloramphenicol acetyl transferase (Lewendon *et al.*, 1994) and cytochrome B5 (Altman *et al.*, 1989). Recently crystal structures of cellobiohydrolases from *T. reesei* (Divne *et al.*, 1994) endoglucanase from *T. reesei* (Kleywegt *et al.*, 1997) and endoglucanase from *A. niger* (Khademi *et al.*, 2002) were elucidated. All these studies have revealed the presence of two acidic amino acid residues in the catalytic site acting as acid-base mechanism. In this connection, an attempt is made to identify the amino acid residues namely glutamic acid, aspartic acid, histidine, tryptophan and cysteine and their role in the catalytic activity.

In Fig. 6A is shown the activity profile of endoglucanase as a function of DTT concentration. As seen from the figure, activity decreased over a range of 0-5 mM concentration. Endoglucanase retains 80% activity in the concentration range of 1- 4 mM and was reduced to 60% at 5 mM compared to the control, indicating that disulfide bonds perhaps play a role in the catalytic activity.

NBS is a potent oxidizing agent and a specific reagent for the modification of tryptophan. In Fig. 6B is shown the activity profile of endoglucanase as a function of NBS concentration. As seen from the figure, NBS did not have any effect on the activity of endoglucanase. It has been shown by Clarke (1987) that modification of the tryptophan residue with NBS led to inactivation of cellulase, showing that tryptophan is involved in the binding of the substrate and is also a part of the catalytic site. In the present study, it is shown that NBS did not have any effect on the endoglucanase activity, indicating that tryptophan is not involved in the catalytic activity.

The modification of histidyl residues of the enzyme was achieved by treatment with different concentrations of DEPC, in 20 mM sodium acetate buffer at pH 5.0 and 37°C for 2 h. The residual activity was determined with CMC as the substrate. Carboxymethylation of the histidyl residues of the enzyme showed 70% loss in the activity (Fig. 6C), suggesting that these residues participated mechanistically in catalysis and also in the maintenance of the conformation necessary for the active enzyme. It may have a role in the binding of the substrate to the enzyme and proper orientation of the catalytic site of the enzyme for hydrolysis.

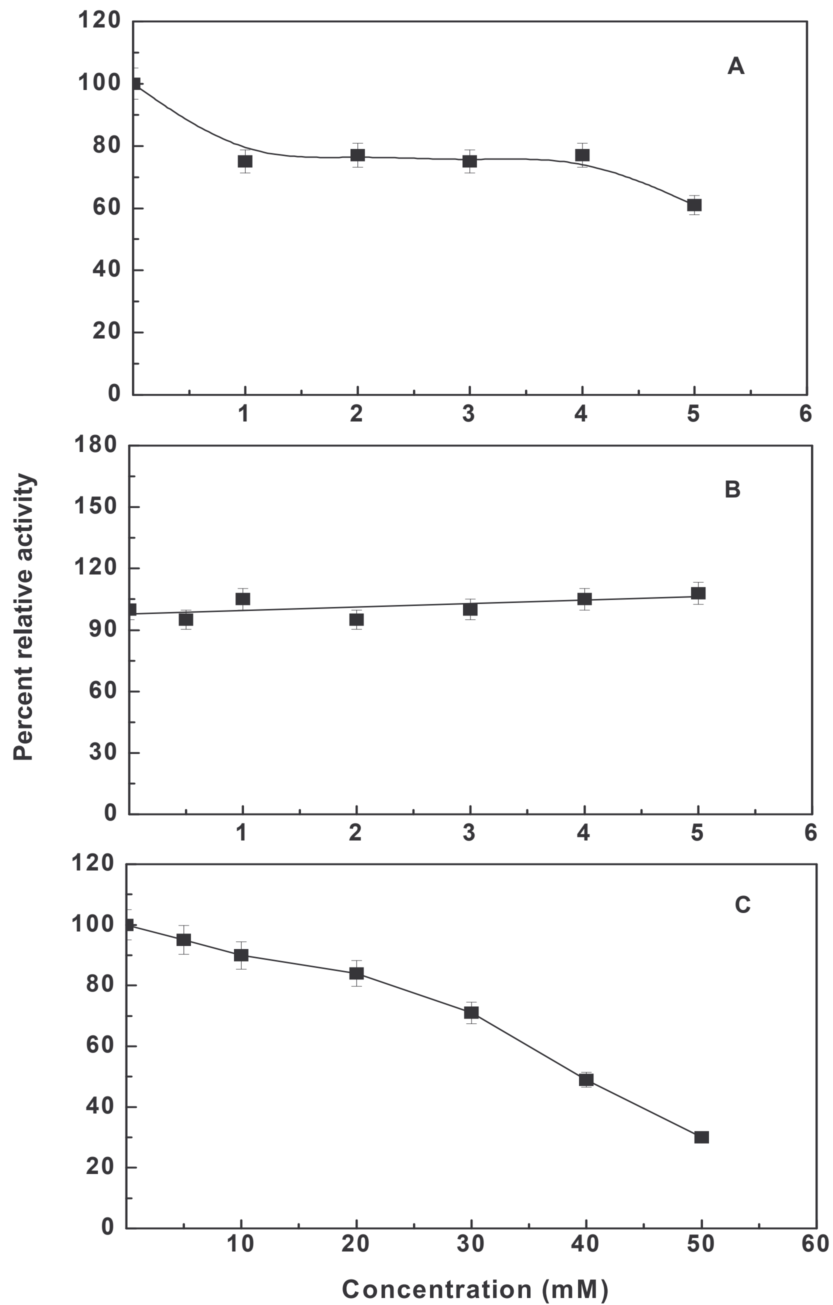


Fig. 6: Effect of chemical modification on endoglucanase activity. The enzyme in 20 mM sodium acetate buffer pH 5.0 was treated with different concentrations of (A) DTT, (B) N-Bromosuccinimide and (C) DEPC for 2 h at 40°C.

The carboxyl groups of Asp/Glu residues of endoglucanase have been modified with EDAC in 20 mM sodium acetate buffer pH 5.0. Under these conditions (pH 4-5), EDAC is known to modify proteins most effectively (Takagi *et al*., 1989). In Fig. 7 is shown the inactivation of endoglucanase as a function of EDAC concentration in the absence and presence of the nucleophile, 0.1 M glycine methyl ester (GME) at 40°C for 3 h. As seen from the figure, the presence of EDAC showed a concentration dependent effect on the enzyme activity and the enzyme retained 100% activity up to 40 mM EDAC concentration. However, at 70 mM EDAC concentration, the activity is reduced to 75% with further loss of 90% of its activity at 100 mM. On the other hand, in presence of 0.1 M GME, the endoglucanase lost its activity in linear fashion with increasing concentration of EDAC. This clearly shows that the modification of endoglucanase takes place in two steps, first forming *O*-acylisourea in the presence of carbodiimide and forms an amide bond in presence of nucleophile, GME. The enzyme becomes totally inactive at 100 mM EDAC concentration.

To assess the nature of inhibition by EDAC, the time course of inactivation of endoglucanase in absence and presence of different concentration of EDAC was carried out and the results are shown in Fig. 8A. It can be seen from the figure, that the inactivation of endoglucanase by EDAC, is competitive in nature with K_m values of 0.060 ±0.001% in absence of EDAC and 0.074 ± 0.002, 0.12 ± 0.003 and 0.17 ± 0.003% in presence of 20, 40 and 60 mM EDAC concentrations, respectively. This clearly shows that the inhibitor competes for the active site along with the substrate.

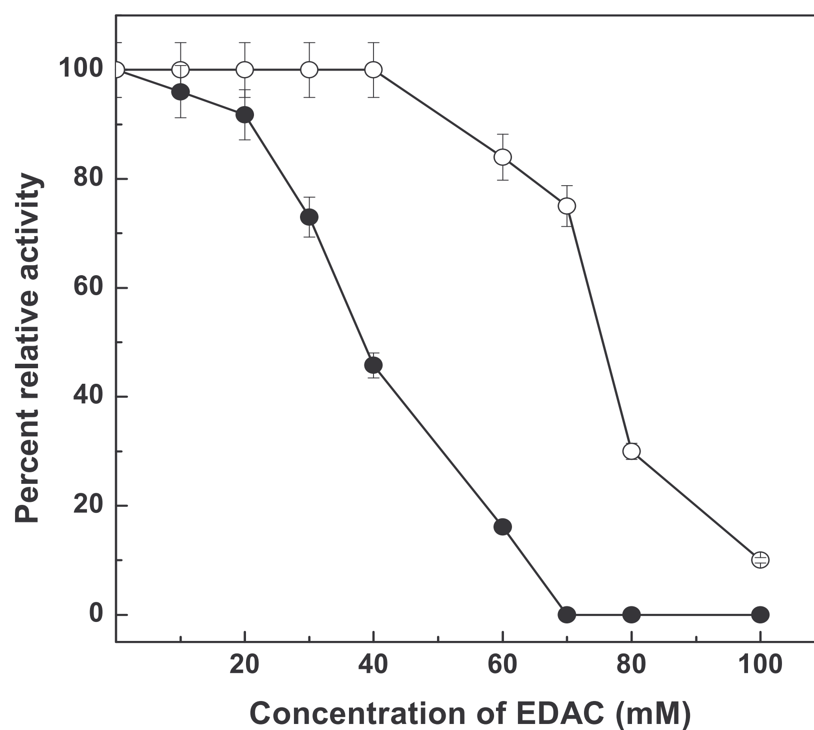


Fig. 7: Effect of EDAC on endoglucanase activity in presence and absence of nucleophile, 0.1% GME in 20 mM sodium acetate buffer, pH 5.0 at 40ºC. (○) in absence of GME and (●) in presence of GME.

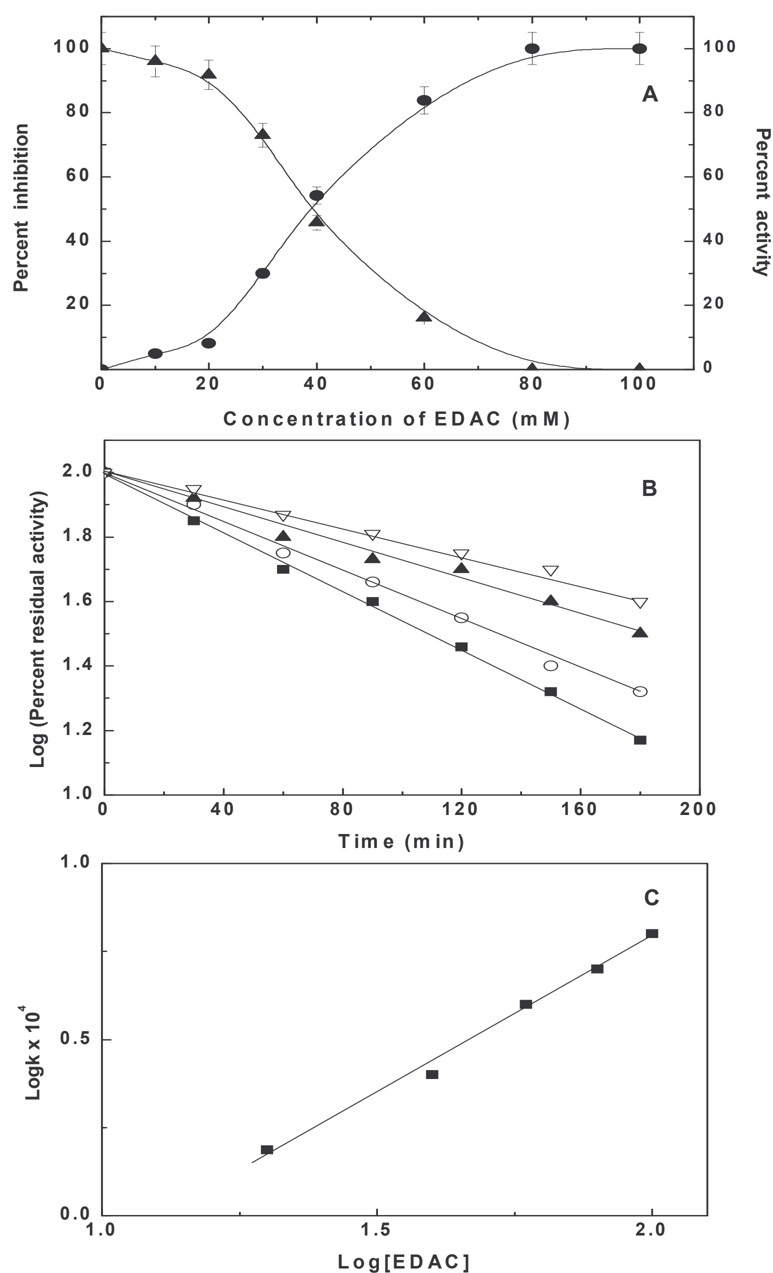


Fig. 8: (A) Effect of EDAC on endoglucanase activity. The enzyme in 20 mM sodium acetate buffer pH 5.0 was treated with different concentrations of EDAC for 3 h at 40°C. From these samples the residual activity was determined by reducing-sugar assay with CMC as substrate. (▲) Activity; (●) Inhibition. (B) Inactivation of endoglucanase by EDAC. Final concentrations of EDAC were (■) 80 mM; (○) 60 mM; (▲) 40 mM and (∇) 20 mM. (C) Apparent order of the reaction with respect to the reagent concentration, the pseudo first order rate constant (k) were calculated from the slopes of the data.

EDAC has been successfully employed for the inactivation of yeast enolase (George & Borders, 1979) and identification of specific carboxyl residues involved in the binding of cytochrome *c* to cytochrome oxidase (Millett *et al*., 1982a & b). In the present study, the kinetics of the reaction with endoglucanase indicated that the essential carboxyl group of the enzyme is modified during inactivation. Semi-logarithmic plots of the residual activity as a function of time of inactivation in presence of different concentrations of EDAC (Fig. 8B) were linear and obey pseudo-first-order kinetics. A plot of the apparent rate of inactivation determined from the slope of log residual activity versus time as a function of the EDAC concentration (Fig. 8C) revealed that the inactivation is first-order with respect to the carbodiimide concentration (Clarke and Yaguchi, 1985).

The activity of purified endoglucanase in presence of various modifying agents reveals the nature and characteristics of the enzyme. The modification of the enzyme with DTT reduced its activity. Disulfide bridges are known to be important in stabilizing proteins at high temperatures and apparently play a role in the structural stability of the enzyme. It appears that the inaccessible disulfides have an important role in maintaining a stable active site, whereas reduction in the accessible disulfides had some effect on the activity. This may be due to the fact that the inaccessible disulfides may be located in the catalytic domain, while the accessible disulfides are in the binding domain. The stability and active conformation of the catalytic core of the Cellobiohydrolase-I are reported to be maintained by six disulfide bridges (McGinnis and Wilson, 1993).

The modification of histidine residues in proteins using DEPC is well documented (Miles, 1977). DEPC can also interact with other amino acid residues like lysine, cysteine, tyrosine and arginine (Muhlard *et al*., 1967). The problem whether the inactivation of the enzyme is the result of the modification of the histidine residues in endoglucanase by DEPC can be explained from the results. There was no absorbance at 278 nm, indicating that O-carbethoxylation of tyrosine residues is not formed. It is reported that DEPC in carboxylate buffer of pH range 4-6 reacts with sulphydryl groups which show increase in absorbance at 240 nm that can be reversed by hydroxylamine (Garrison and Himes, 1975). Hydroxyl amine removes the carbethoxy group from modified histidine, tyrosine and cysteine residues except for lysine residues (Miles, 1977). Endoglucanase inactivation by DEPC showed an increased absorbance at 240 nm, with no change in the absorbance at 278 nm indicating that the O-carbethoxylation of tyrosine residues is not formed.

Modification of the carboxyl groups of Asp/Glu residues of endoglucanase by water soluble EDAC, takes place in two steps. The presence of the nucleophile, GME at 0.1% concentration has been shown to be essential for the effective modification of the carboxy group by carbodiimide. But there are evidences to show that inactivation could also take place in the absence of nucleophile like in RNase A (Riehm and Scheraga, 1996), rabbit muscle phosphorylase b (Ariki and Fukui, 1978) and Rhizopus glucoamylase (Iwama *et al*., 1984). All these data provides an evidence that the residues, viz., histidine, cysteine and aspartic acid/glutamic acid are essential for the catalytic activity of endoglucanase.

1.2. Effect of cofactors on the structure and function of endoglucanase

Cofactors have variety of functions in proteins and metal ions are the commonly used cofactors. They can make proteins more active and also affect their stability by altering the molecular conformation of the protein upon binding (Glusker, 1991). Divalent cofactors are known to inhibit many enzymes. Among different cofactors zinc, magnesium were found to inhibit cellulase activity while copper and iron were found to stimulate the activity (James *et al*., 1999). Saxena *et al*., (1992) have shown that metal ions activate the CMCase activity from *Cellulomonas spp*. and cobalt was the greatest stimulator.

The influence of a number of bivalent metals on the activity of *Streptomyces atratus* glucose isomerase has been studied. Mg^{2+} ions are activators and Co^{2+} ions are stabilizers of the glucose isomerase activity (Tashpulatova and Davranova, 1991). Of a number of metal ions examined, Ca^{2+} and Co^{2+} were found to be slightly activating, while Pb^{2+} and Hg^{2+} were the most potent inhibitors of cellulase from *Thermomonospora fusca* (Ferchak and Pye, 1983). Some studies have shown that Zn^{2+} and Cu^{2+} metallic ions had an inhibitory effect, which is a common feature of cellulases and xylanases (Paradis *et al*., 1993), including plant cellulases (Ohmiya *et al*., 1995).

Here, an attempt is made to understand the mechanism of inactivation/activation of endoglucanase in presence of different cofactors, in order to understand the structure-function relationship of this enzyme. The role of cofactors (calcium, zinc, copper and iron) and EDTA on the activity and stability of the enzyme was investigated. The concentrations in the range

of 0 - 6 mM were taken for the activity measurements. The enzyme did not show any significant change in the activity in the presence of cationic salts like $CaCl_2$, $ZnCl_2$, $CuCl_2$ and $FeCl_2$ retaining most of its original catalytic activity (Fig. 9). In presence of $CaCl_2$, $CuCl_2$ and $FeCl_2$, the enzyme did not show any change in activity till 4 mM but, there was slight reduction of 2-5% activity at 5 mM concentration of these metal ions. Table 5 shows the activity of endoglucanase in presence of these salts. In presence of zinc there was no effect as the enzyme retained 100% activity up to 5 mM. The 2-5% loss in the activity at 5 mM concentrations of Ca^{2+}, Zn^{2+}, Cu^{2+} and Fe^{2+} was completely reversible on dialysis.

All the above results show that the metal ions did not have any effect on the activity of the isolated endoglucanase from *A. aculeatus*. Enzyme inhibition by cations usually suggest the presence of at least one suphydryl group of cysteine in the active site, whose oxidation by the cations destabilizes the conformational folding of the enzyme (Rouvinen *et al*., 1990) or leads to the formation of disulphide bonds at an irregular position of the protein (Ohmiya *et al*., 1995). Fe^{2+} and Mn^{2+} had no significant effect on EG II. Similar results were also observed in endoglucanases purified from other *Bacillus* strains (Mawadza *et al*., 2000). This shows that suphydryl groups are not involved in the activity or present at the active site of the isolated endoglucanase from *A. aculeatus*.

Effect of cofactor EDTA on the activity of endoglucanase was investigated. In presence of EDTA, the enzyme activity was 1.5 fold from 1 mM to 6 mM concentrations. The activity was measured after 2 h pre-incubation of endoglucanase in presence of different concentrations of EDTA in 20 mM sodium acetate buffer pH 5.0 at 40°C. The reversibility of the

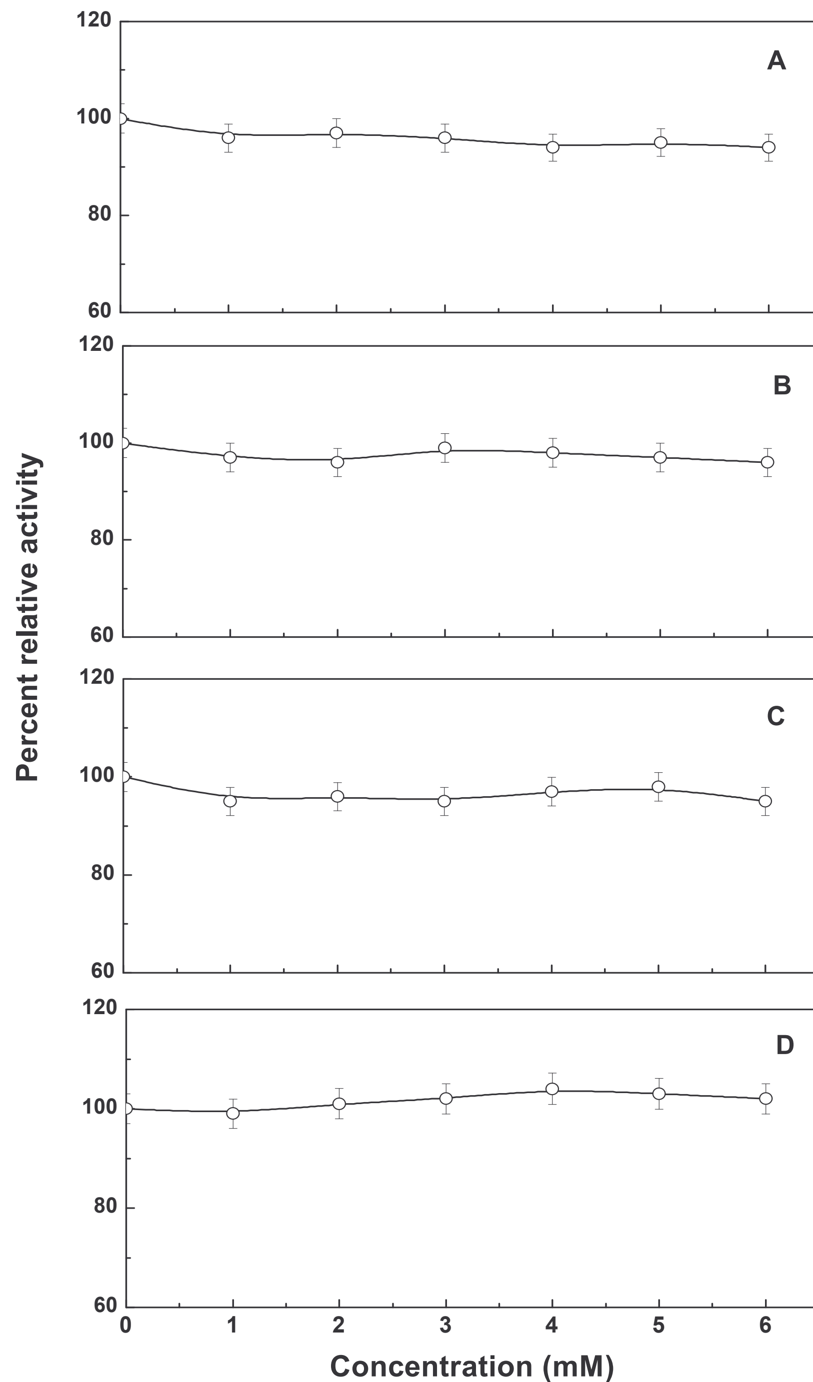


Fig. 9: The effect of cofactors on endoglucanase activity. The enzyme was pre-incubated with various cofactors and then activity measured with 0.5% CMC in 20 mM sodium acetate buffer, pH 5.0 at 40°C in presence of different concentrations (A) in presence of $FeCl_2$; (B) in presence of $CuCl_2$; (C) in presence of $CaCl_2$ and (D) in presence of $ZnCl_2$.

Table 5: Effect of different cofactors on endoglucanase activity

Treatment	Concentration (mM)	Relative activity (%)
Control	-	100
EDTA	2	150 ± 5
EGTA	2	136 ± 5
$CaCl_2$	5	96 ± 3
$CuCl_2$	5	98 ± 4
$ZnCl_2$	5	102 ± 3
$FeCl_2$	5	95 ± 4

enzyme activity in presence of EDTA was checked with proper controls. The activity profile of endoglucanase in presence of different concentrations of EDTA and after removal of EDTA by dialysis is shown in Fig. 10. Increased activity was retained even after the removal of EDTA by dialysis against buffer overnight with 3 to 4 changes. Treatment of endoglucanase with EDTA, followed by dialysis to remove EDTA completely from the enzyme solution and addition of the metal ions (Ca^{2+}, Zn^{2+} and Fe^{2+}) did not have any effect on the endoglucanase activity. The enzyme was able to maintain the increased activity observed in presence of EDTA alone and after its removal (Fig. 10). These results indicate that the presence of EDTA had irreversibly activated the endoglucanase.

The authors Loprete and Hill (2002) have shown that EDTA or EGTA had no inhibition on the activity of endoglucanase from *Achlya ambisexualis* indicating that no divalent cations are required. Apart from the thiol-inhibitor Hg^{2+}, the only other ions tested that had significant effects on activity were Mn^{2+} and Ca^{2+}, both of which showed mild inhibition. The patterns of ion inhibition and sensitivity to sulphydryl-binding agents vary greatly among plant and microbial glucanases and their significance.

The nature of activation of endoglucanase in presence of EDTA was further characterized by steady state kinetic analysis. Endoglucanase activity was measured in presence of different substrate concentrations. The increasing concentration of EDTA affected both K_m and V_{max} values, leading to a different intercept on the linear regression lines of the velocity axis of Lineweaver- Burk plot. The slope of this gives the K_m/V_{max} values which were plotted against the EDTA concentrations. The control had an apparent K_m of

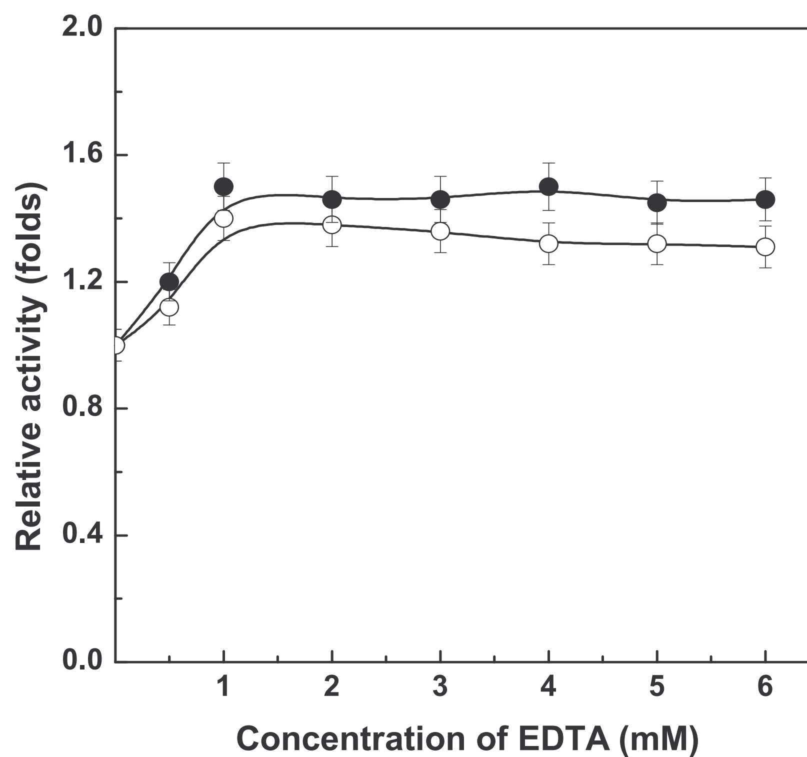


Fig. 10: The effect of EDTA on endoglucanase Activity. The enzyme was pre-incubated with EDTA and then dialyzed against buffer to remove EDTA and activity measured with 0.5% CMC in 20 mM sodium acetate buffer, pH 5.0 at 40 °C in presence of different concentrations of EDTA. (●) In presence of EDTA and (○) after removal of EDTA by dialysis.

0.060 ± 0.001% and K_{cat} was 1.3 ± 0.1 min^{-1}. In presence of EDTA, K_m value decreased to 0.040 ± 0.001% compared to control and had a K_{cat} of 1.9 ± 0.2 min^{-1} (Table 6). The decrease in the K_m and increase in the K_{cat} shows that the endoglucanase has attained the maximum catalytic efficiency in presence of EDTA. The authors Popa *et al*. (1977) in their work on viral neuraminidase have shown enhanced activity in presence of EDTA and the kinetic data obtained indicated that EDTA modifies the enzyme in such a manner that the affinity of the enzyme for the substrate is increased.

The stability of endoglucanase in presence of these cofactors and EDTA was probed through measurements of thermal denaturation temperature (T_m) in presence and absence of these salts. The thermal denaturation temperature was determined by monitoring the changes in the absorbance of endoglucanase at 287 nm as a function of temperature in the range of 25-90 °C. From the denaturation profile a plot of fraction unfolded versus temperature was obtained using the standard equation (Pace and Scholtz, 1997). The apparent thermal denaturation temperature (T_m) of endoglucanase was obtained by van't Hoff plot. The presence of cofactors (zinc, calcium, copper and iron) did not have any effect on the apparent thermal denaturation temperature (T_m) of endoglucanase, it was comparable to that of the control enzyme. Fig. 11 shows the apparent thermal denaturation curves of endoglucanase as a function of different concentrations of EDTA. The apparent thermal denaturation temperature of native endoglucanase was 57 ± 1 ° C, which increased in presence of 5 mM EDTA to 76 ± 1 °C. The increase in the apparent T_m was gradual and it increased with increase in concentration of EDTA, at 1, 2 and 3 mM concentrations the apparent T_m was found to be 66, 68 and 70°C, respectively.

Table 6. Kinetic parameters of endoglucanase in presence and absence of EDTA

Sample	**K_m (app) (%)**	**K_{cat} (min)**	**K_{cat}/K_m**
Control	0.060 ± 0.001	1.3 ± 0.1	22 ± 1
EDTA	0.040 ± 0.001	1.9 ± 0.2	43 ± 3

Activity measurements were done in sodium acetate buffer 20 mM, pH 5.0 using CMC as substrate.

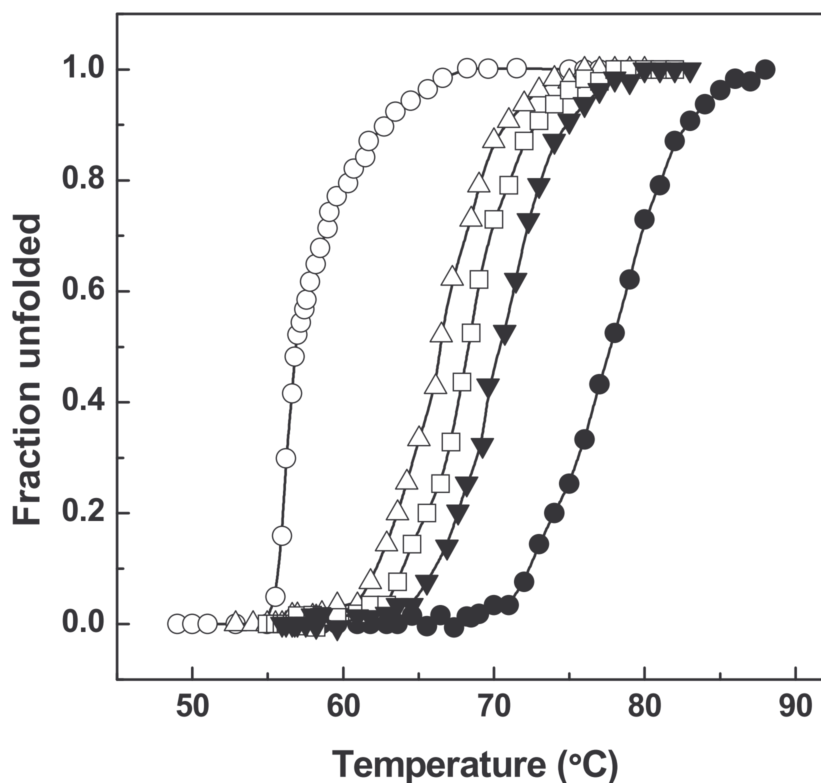


Fig. 11: Apparent thermal transition temperature profile of endoglucanase in presence of EDTA, in 20 mM sodium acetate buffer pH 5.0 as a function of temperature in the range of 25 - 90°C with 1°C /min increase in the temperature measured at 287 nm. (○) In absence of EDTA (Control) and in presence of (Δ) 1 mM, (□) 2 mM, (▼) 3 mM, and (●) 5 mM EDTA.

An increase of 19°C in apparent T_m was observed at 5 mM EDTA. Thus, thermal denaturation studies have clearly shown increased thermal stability of endoglucanase in presence of EDTA and there is no significant change in the apparent T_m in presence of other cofactors (Zn^{2+}, Ca^{2+}, Cu^{2+} and Fe^{2}+).

In order to probe the structural aspects of the endoglucanase, fluorescence emission spectral changes of the endoglucanase were investigated as a function of different concentrations of EDTA. No change in the relative fluorescence intensity of the endoglucanase in presence of EDTA was observed as shown in Fig. 12. This indicates that the environment around tryptophan is not affected in presence of EDTA and also there was no change in the tertiary structure of the endoglucanase. Presence of cofactors did not show any significant change in the fluorescence spectra, suggesting no change in the microenvironment of the endoglucanase in presence of these cofactors.

It is evident that in presence of EDTA, increase in the stabilization was observed and may bring about a change in the secondary structure of endoglucanase. The change in the secondary structure of endoglucanase as a function of EDTA and EGTA was followed by far-UV CD spectral studies. The far-UV CD spectra of endoglucanase in presence of EDTA and EGTA are shown in Fig. 13. The spectrum of native endoglucanase had a trough at 217 nm. There was an increase in the trough value at 217 nm from -3450 ± 30 to -4155 ± 50 and -3936 ± 45 deg.cm^2/dmol for native and in presence of EDTA (5 mM) and EGTA (2 mM), respectively. There is 20% and 11% increase in the ellipticity value at 217 nm in presence of EDTA and EGTA, respectively. The far-UV CD spectra of endoglucanase in presence of zinc, copper, calcium and iron did not show any change. In presence of EDTA,

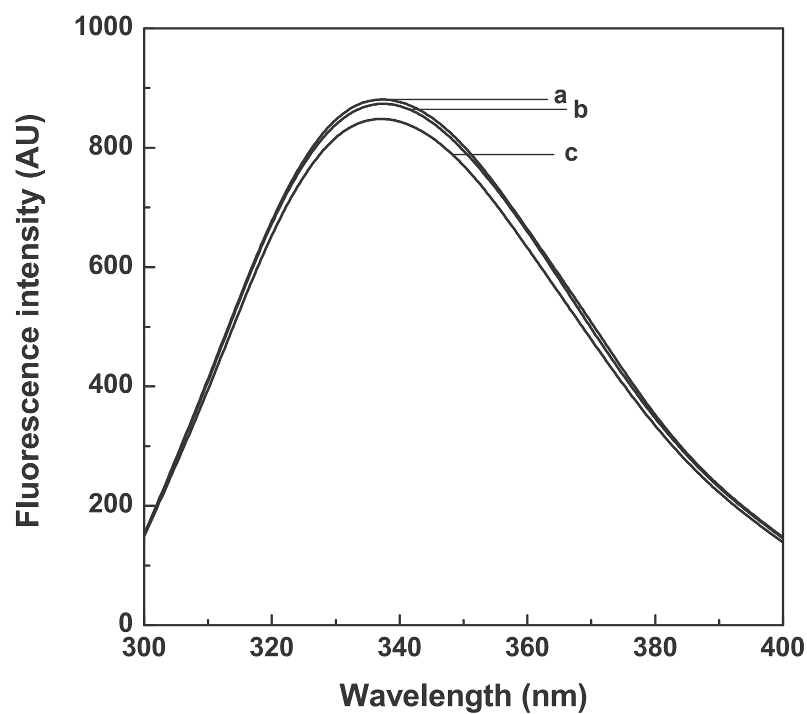


Fig. 12: The effect of EDTA on fluorescence emission spectra of endoglucanase. The spectra were measured in 20 mM sodium acetate buffer pH 5.0 at 25°C. The spectra were measured by excitation at 280 nm. (a) Control (in buffer only) and in presence of (b) 2 mM and (c) 5 mM EDTA.

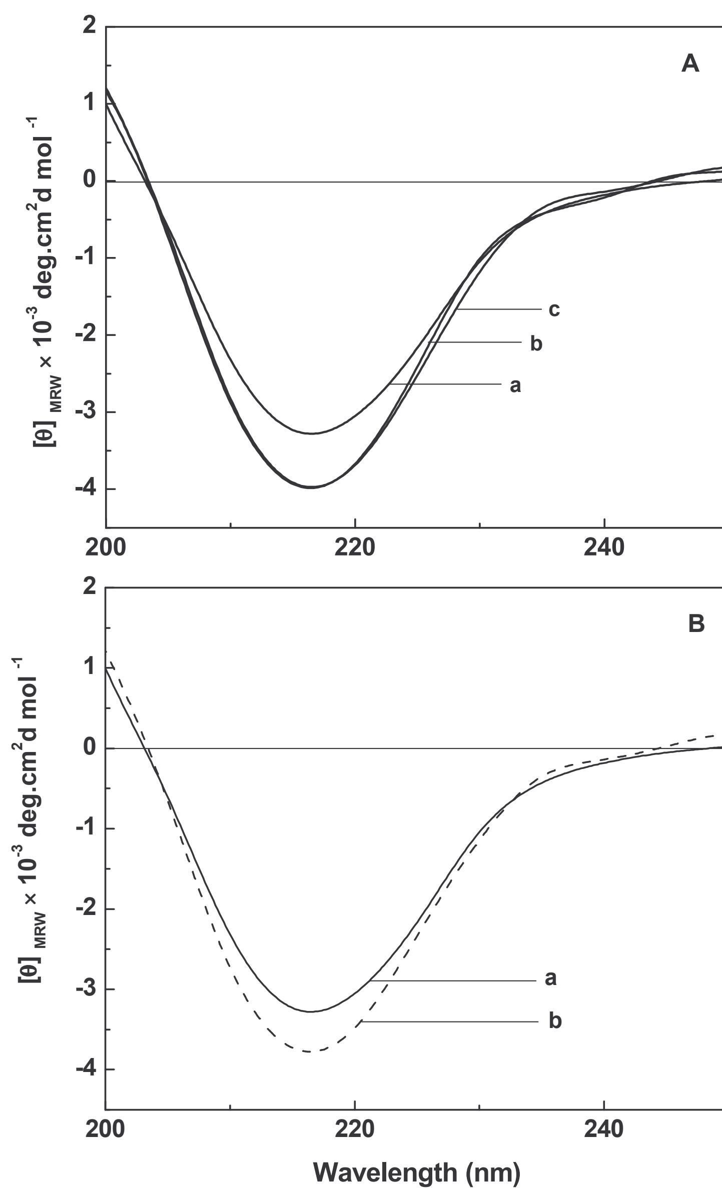


Fig. 13: Far-UV CD spectra of endoglucanase in presence and absence of cofactors. The spectra were measured in different concentrations of (A) EDTA and (B) EGTA after pre-incubation of the enzyme for 2 h (a) Control (in buffer only); (b) 2 mM and (c) 5 mM. The spectra were measured in 20 mM sodium acetate buffer pH 5.0 at 25 °C. The concentration of enzyme was 0.24 mg/mL.

there was a prominent change in the secondary structure of the endoglucanase which could be responsible for the increased activity and stabilization of the enzyme.

In order to further investigate the role of EDTA, we have monitored the exposure of hydrophobic surfaces in presence of different concentrations of EDTA using the hydrophobic dye ANS, which binds to the hydrophobic surfaces on the proteins, which is sensitive to the polarity of its microenvironment (Krishnan *et al.*, 1996). Fig. 14A shows the fluorescence of ANS-bound endoglucanase in the presence and absence of different concentrations of EDTA. The fluorescence of ANS alone in buffer shows emission maxima at 512 nm, ANS on binding to protein shows a shift in the emission maxima to 480 nm in the presence of EDTA with increase in the fluorescence intensity. This clearly shows that the enzyme exists in a flexible conformational state with exposed hydrophobic surfaces which become more accessible to the hydrophobic dye, ANS.

The profile of emission intensity versus EDTA concentration is shown in Fig. 14B. At 5 mM concentration of EDTA there is maximum change taking place, above 5 mM concentration of EDTA causes quenching leading to the decrease in fluorescence emission intensity. Prolonged incubation of endoglucansase in presence of 5 mM EDTA does not lead to any significant alteration in the profile. These results indicate that EDTA brings about change in the structure of the endoglucanase such that its hydrophobic surfaces are exposed to a greater extent compared to the native enzyme. Krishnan *et al.*, (1996) have shown using bovine carbonic anhydrase (BCA) that in the absence of EDTA, ANS reports a weak and broad transition at around 2 M GuHCl induced unfolding of BCA. However, in the presence of 2 mM EDTA, the transition becomes sharp suggesting the presence of EDTA influences the

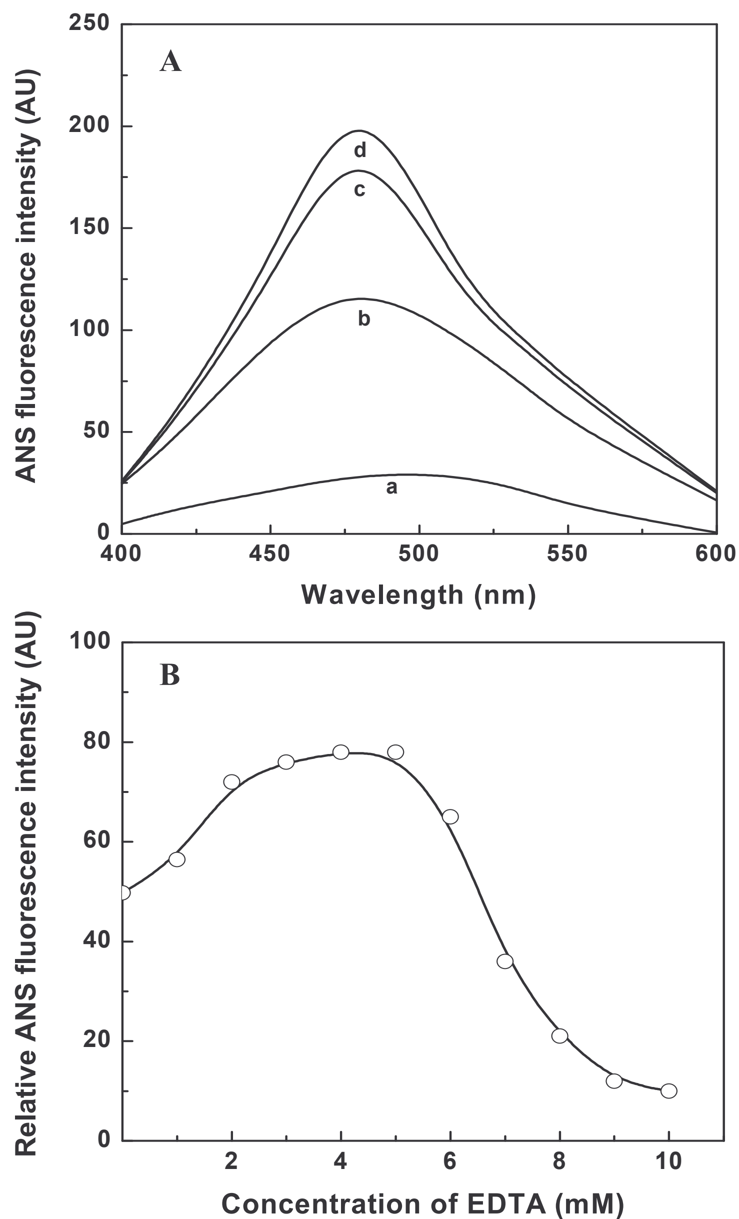


Fig. 14: (A) ANS fluorescence spectra of endoglucanase as a function of EDTA. The spectra were measured in 20 mM sodium acetate buffer pH 5.0 at 25 °C. The spectra were measured in different concentrations of EDTA after pre-incubation of the enzyme for 2 h at 25°C. (a) Buffer with ANS; (b) Protein with ANS; (c) in presence of 2 mM EDTA and (d) in presence of 5 mM EDTA. (B) ANS fluorescence emission intensity as a function of EDTA concentration.

unfolding behavior of BCA and leads to increased exposure of the hydrophobic surfaces. Thus, from the studies of this chapter it is clear that the cofactors, metal ions in particular did not have any significant effect on the activity of endoglucanase. EDTA activation of the endoglucanase suggests that EDTA may bind near the active site or in the region of the active site, bringing about a conformational change leading to a more open and flexible conformation resulting in the increased enzymatic activity.

Endoglucanase was isolated from Aspergillus aculeatus obtained commercially to homogeneity. The purified enzyme showed single band and had a molecular weight of 45,000 Da, with a specific activity of 1.4 U/mg. The purified enzyme showed high specific activity towards CMC and low activity towards avicel. The activity of the isolated enzyme was optimum at pH 5.0 and temperature 40°C respectively. The isoelectric point of the enzyme was 4.3. Apparent T_m *was found to be 57°C. In presence of DTT it maintained 60% activity. Treatment of the endoglucanase with DEPC resulted in the modification of the histidine residues present in the enzyme with a concomitant loss of 70% of the original enzymatic activity. EDAC completely inactivated the endoglucanase. NBS didn't have any effect on the activity. At higher temperature, the enzyme lost half of its activity. Inhibition studies using EDAC showed that the catalytic residues are two acidic amino acids. The catalytic activity of endoglucanase was 1.5 fold in presence of EDTA. Kinetic parameters showed decrease in the* K_m *and increase in the* K_{cat} *indicating that the endoglucanase has attained the maximum catalytic efficiency in presence of EDTA. There is increase in the ellipticity value at 217 nm by 20% in presence of EDTA. The apparent thermal transition temperature (*T_m*) of native endoglucanase increased from a control value of 57°C to 76°C in presence of EDTA.*

2. Effect of pH, Urea and GuHCl on the folding and unfolding of Endoglucanase

In this chapter, the effect of pH and denaturants such as urea and GuHCl on endoglucanase has been investigated. The denaturation of proteins/enzymes occurs due to environmental changes such as temperature, pH, pressure and addition of denaturants like urea and GuHCl (Tanford, 1968, 1970). Solvent additives can affect the macromolecular structure by direct interaction with the macromolecule or by indirect action through effects on the structure and properties of the solvent or by a combination of both these mechanisms (Gekko and Timasheff, 1981a). The interaction of urea with several proteins has been well documented using various biophysical techniques (Prakash *et al*., 1981; Robinson and Jencks, 1965; Kauzman, 1959; Zou *et al*., 1998).

Unfolding of many proteins that have been studied can be explained in terms of a two-state model (Privalov, 1979), where folded and unfolded states of the protein exist in rapid equilibrium with no observable intermediates. However, it has also been shown in case of many proteins that, folding involves discrete pathways with the formation of intermediate states between native and denatured states (Kim and Baldwin, 1990; Ptitsyn, 1995; Pande *et al*., 2007). Such intermediates are found to possess special characteristics.

The structural stability of a protein depends on its ability to acquire a unique three-dimensional structure. In most of the cases inactivation occurs at lower concentrations of denaturants than that required to bring about unfolding of the molecules (Yao *et al*., 1984; Tsou, 1986, 1993). The unfolding

of the protein in guanidine salts has two transitions, the first transition occurring between native to intermediate and the second transition occurring between intermediate state and unfolded state (Suryaprakash and Prakash, 2000). Interaction of denaturants with several proteins has been studied with respect to structure and functional relationships (Fan *et al*., 1996; Kumar *at al*., 2003; Shrivastava and Das, 2007).

A detailed study on the intermediates formed at lower concentrations of denaturants for human serum albumin and papain has been done (Muralidhara and Prakash 1997, 2002; Satish *et al*., 2002). They suggest the presence of two important structural features namely, molten globule state at lower concentrations of denaturants and secondly, unfolding process leading to complete denaturation or unfolded state at higher concentrations of denaturants. These intermediates have been found to accumulate under equilibrium conditions like low or high pH, high temperature and presence of denaturants such as urea and GuHCl. The medical, pharmaceutical and industrial applications of proteins depend on the present knowledge of their folding and unfolding patterns. The analysis of the intermediate states is crucial in understanding the mechanism of protein folding.

2.1. Effect of pH on the structure and stability of endoglucanase

Acid induced unfolding of proteins lead to formation of conformations that is different from that of the folded and unfolded proteins in presence of denaturants. Numerous studies have revealed the compactness and the amount of secondary structure of the intermediate states which is very much dependent on the type of the protein (Haq *et al*., 2002; Naseem *et al*., 2004; Kadi *et al*., 2006). The characteristic features of the molten globule state are

that it is less compact than the native state but more compact structure than unfolded state, contains extensive secondary structure and may or may not possess tertiary structures. Many studies have shown that secondary structure of protein in molten globule state is similar to that of the native state (Vassilenko *et al*., 2002).

Catalytic activity of an enzyme is dependent on various parameters like pH, temperature and denaturants. Stability of enzyme towards it decides its functionality and applications. Therefore, pH denaturation of endoglucanase was monitored by various spectroscopic techniques in order to probe the conformational changes in its structure, identification and characterization of the intermediates at extreme conditions of pH. The effect of pH on the endoglucanase activity is shown in Fig. 15. The optimum pH for the activity of the enzyme was 5.0 (Gajendra *et al*., 2007). Beyond pH 4-6, significant loss in the activity and stability has been observed. The endoglucanase showed 50% of its activity at pH 2.0.

The fluorescence emission spectra of endoglucanase in response to change in pH values are shown in Fig. 16. The native enzyme (pH 5.0) has fluorescence emission maximum (λ_{max}) at 336 nm, which shifted to 342 nm at pH 2.0, indicating the exposure of tryptophan residues to the solvent. The relative fluorescence intensity decreased with decrease in the pH. The fluorescence intensity of endoglucanase was measured to detect small changes in the emission maximum. There was red shift in the fluorescence emission maximum, indicating that at acidic pH, there is change in the tertiary structure of the protein due to exposure of the buried tryptophans to

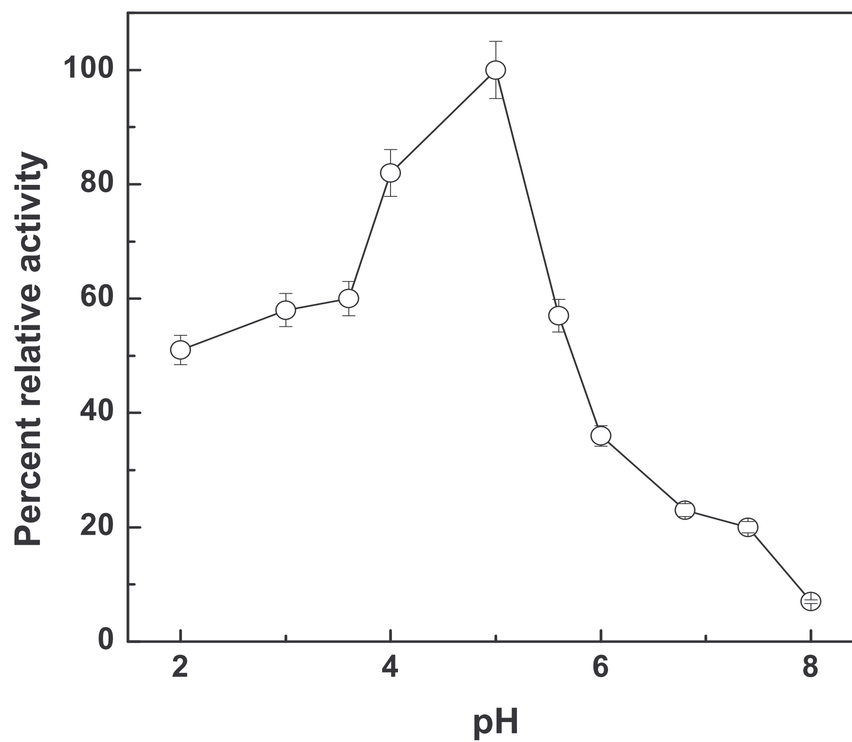


Fig. 15: Endoglucanase activity as a function of pH. Buffers used were at 20 mM concentration glycine-HCl (pH 1-2), sodium acetate (pH 3.0-5.6) and citrate phosphate (pH 6-8.0).

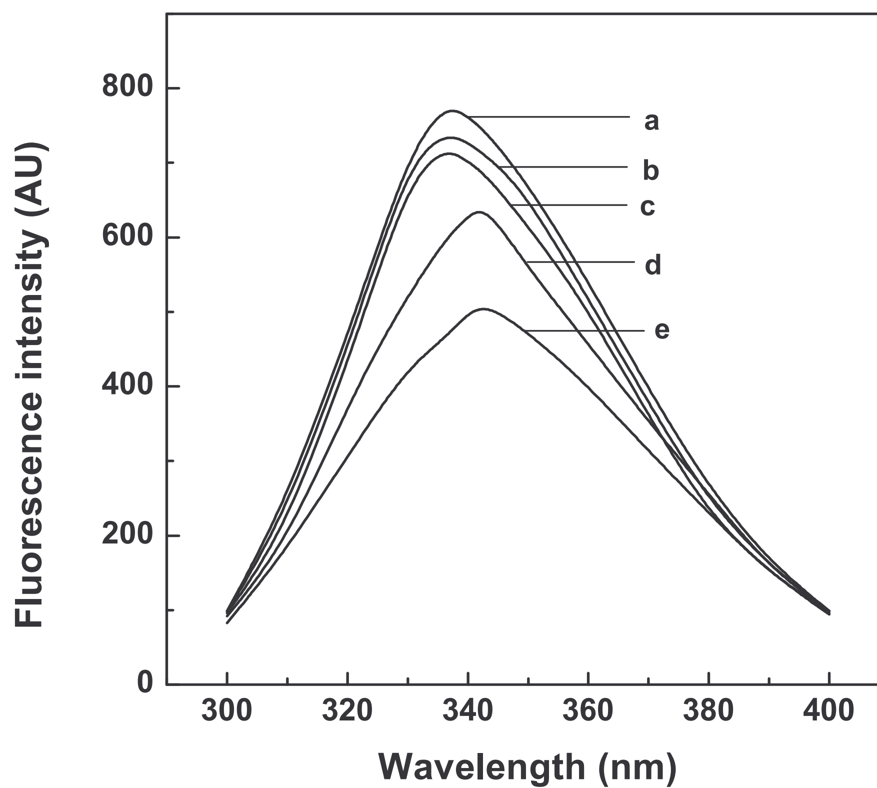


Fig. 16: Intrinsic fluorescence emission spectra of endoglucanase as a function of pH. The protein was excited at 280 nm and the emission was recorded in the wavelength range 300-400 nm (a) at pH 7.0, (b) pH 5.0, (c) pH 4.0, (d) pH 3.0 and (e) pH 2.0.

the polar solvent. Similar results were also reported for glucose/xylose isomerase (Jiang, 1990; Pawar and Deshpande, 2000).

Near-UV CD spectra showed a marked difference at pH 2.0 as shown in Fig. 17A. At pH 5.0, there is a strong ellipticity with positive peak, whereas at pH 2.0 it showed negative peak. These significant conformational changes are indicative of fixed orientations of aromatic residues in the protein solutions. Addition of acid (pH 2.0) induced a more flexible environment around the aromatic residues. Endoglucanase is a predominantly β-structured protein, the changes in the secondary structure of endoglucanase at pH 2.0 and pH 5.0 was monitored by far-UV CD measurements. The far-UV CD spectra showed no change in the secondary structural content at pH 2.0, indicating that a native like secondary structure persists even at low pH (Fig. 17B). The CD curves at pH 2.0 suggested the presence of native structure although there is loss of activity. This shows that endoglucanase at low pH adopts, like many other proteins, the features of the molten globule state having a substantial amount of secondary structure and tertiary structure is less organized than the native state. Recently, many reports have shown that molten globule state besides having secondary structure may or may not have a well defined tertiary structure (Boren *et al*., 1999; Dubey and Jagannadham, 2003).

The exposure of the hydrophobic surfaces of endoglucanase to solution was monitored by ANS binding studies. ANS is non-fluorescent in aqueous solution, whereas its fluorescence emission intensity increases in hydrophobic

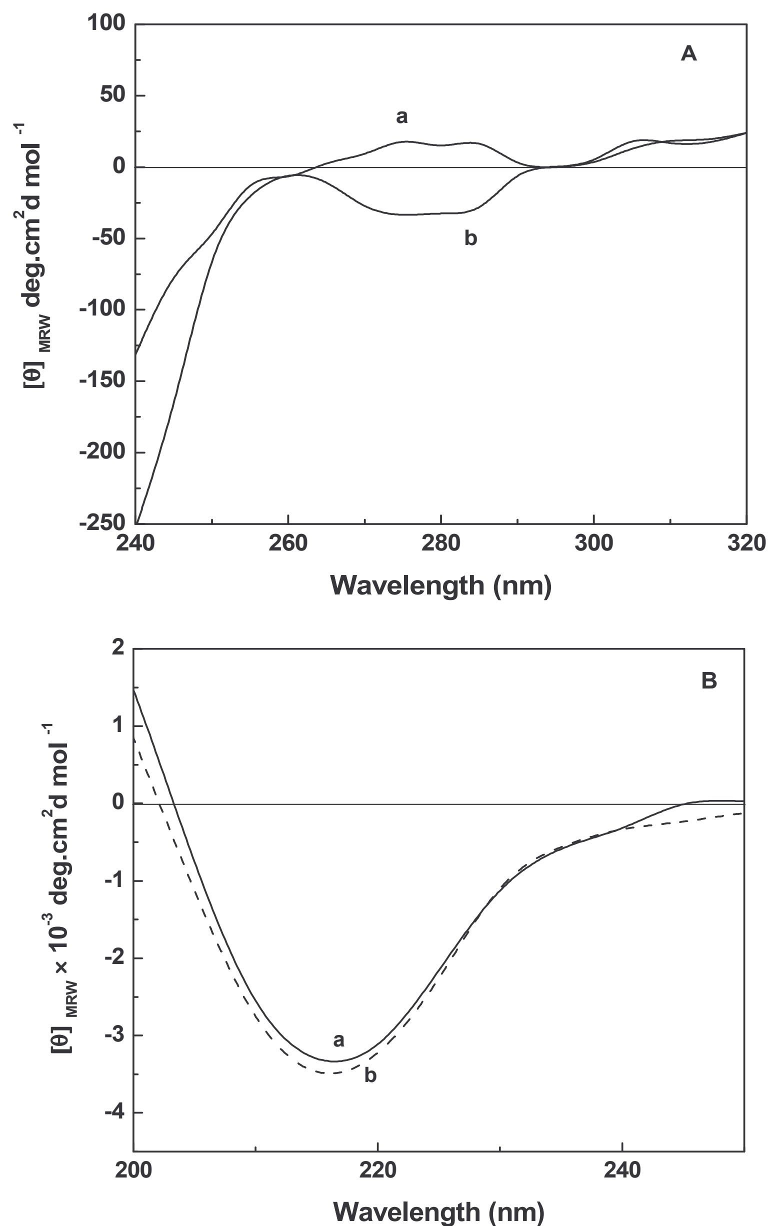


Fig. 17: Effect of pH on the CD spectra of endoglucanase. The enzyme was incubated at the test pH for 2 h. (A) Near-UV CD spectra of endoglucanase at (a) pH 5.0 and (b) pH 2.0. (B) Far-UV CD spectra of endoglucanase at (a) pH 5.0 and (b) pH 2.0 (Representative spectra are only shown for clarity).

environment. ANS binding experiment carried out with endoglucanase at pH ranging from 2.0 - 5.0 revealed an increase in the relative fluorescence intensity of the ANS bound protein. ANS fluorescence emission spectra of endoglucanase at different pH are shown in Fig. 18. ANS fluorescence intensity increased with decrease in the pH. This indicates the unfolding of the protein molecule at low pH, exposing the hydrophobic groups to which ANS binds. ANS emission maximum of the bound protein shifted to 495 nm at pH 2.0. This blue shift of ANS fluorescence is due to the increased hydrophobicity of the environment around the protein molecule. These results show the presence of a large number of solvent accessible non polar clusters in the protein at pH 2.0 and the existence of hydrophobic patches is a good indication for the conformational change of the protein molecule (Fan *et al*., 1996; Dubey and Jagannadham, 2003).

Quenching of intrinsic tryptophan fluorescence of endoglucanase in pH 2.0 and 5.0 was carried out using acrylamide to determine the changes in the microenvironment of protein. The Stern-Volmer plot for quenching of intrinsic tryptophan fluorescence by acrylamide at pH 5.0 and 2.0 are depicted in Fig. 19. The quenching constants (K_{sv}) calculated for pH 5.0 and 2.0 from the slopes of the Stern-Volmer plot were 5 and 11.2 M^{-1}, respectively. The pH 2.0 sample after dialysing against pH 5.0 had a K_{sv} value of 10 M^{-1}. The Stern-Volmer plot indicates that the aromatic amino acids at pH 2.0 are more exposed, compared to the native folded conformation at pH 5.0, hence tryptophan fluorescence is quenched more in the case of denatured protein. The results of quenching experiments give an idea about the relative solvent exposure of different types of fluorophores. More the fluorephore is exposed,

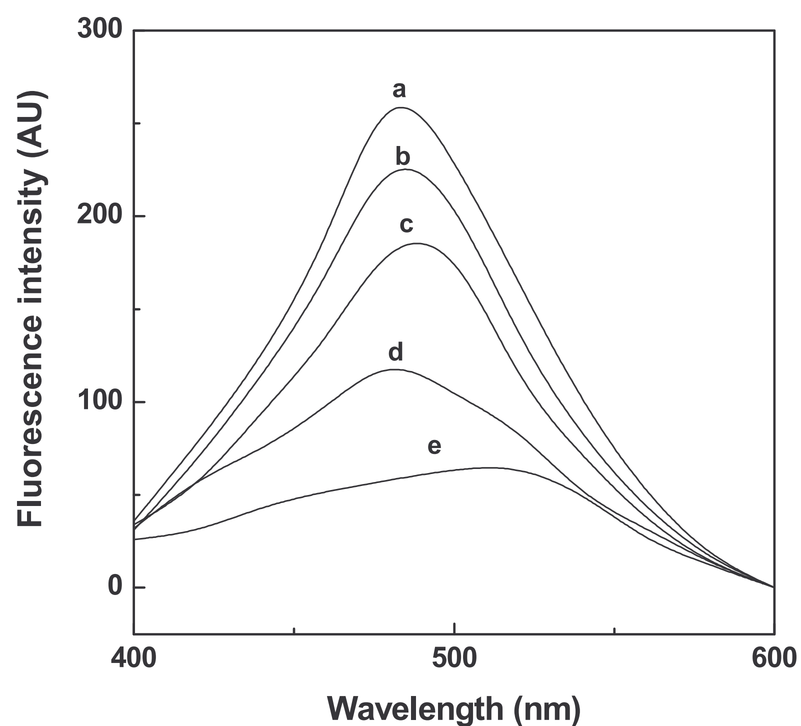


Fig. 18: ANS fluorescence spectra of endoglucanase as a function of pH. The spectra were recorded in the region of 400-600 nm after excitation at 380 nm at 25°C (a) in pH 2.0; (b) in pH 3.0; (c) in pH 4.0; (d) in pH 5.0 and (e) ANS in buffer.

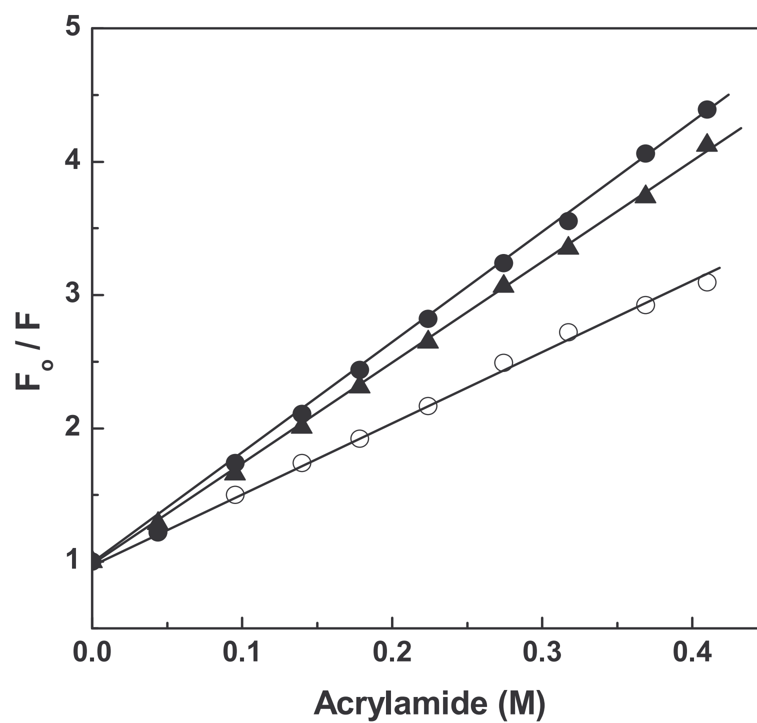


Fig. 19: Stern-Volmer plot for fluorescence quenching by acrylamide of endoglucanase at 25°C at pH 5.0 (○), pH 2.0 (●) and pH 2 sample after dialysis against pH 5 (▲).

more effective the quencher will be in reducing the fluorescence intensity exhibited by that molecule (Eftink and Ghiron, 1976; Verley and Pain, 1991; d'Amico *et al*., 2003).

Enzyme activity is very sensitive to unfolding by urea. The changes in the secondary structure of endoglucanase at pH 5.0 and 2.0 as a function of urea concentrations were monitored by far-UV CD measurements. The increase in urea concentration showed loss in enzyme activity due to unfolding of the endoglucanase by urea. The shift in emission wavelength at pH 2.0 in urea is shown in Fig. 20. Unfolding of the endoglucanase at pH 2.0, as a function of urea concentration showed that at 8 M urea, tryptophan residues were completely exposed to the solvent and the emission maximum shifted from 336 to 358 nm as shown in Fig. 20. The change in the secondary structure of endoglucanase at pH 2.0 is shown in Fig. 21A, indicated destabilization of the molecule as pH decreased. There was further loss of the structure at pH 2.0 as evidenced in the presence of 8 M urea by decrease in the molar ellipticity values at 217 nm (Fig. 21B). At pH 2.0 the endoglucanase had a completely aperiodic structure as shown in Fig. 21A. Folding or unfolding intermediates of proteins, including the molten globule intermediate tends to expose hydrophobic surfaces that become more accessible to the hydrophobic dye ANS. Molten globule state is characterized by the presence of hydrophobic core due to exposed tryptophan residues to solvent. Thus, endoglucanase at low pH shows structural characteristics of a molten globule state (Goto, 1991; Sivaraman *et al*., 1997). To characterize the intermediate state, the hydrodynamic properties of the intermediate state and the folded state has to be compared (Uversky *et al*., 1992; Uversky 1993). The effect of pH on the molecular size of endoglucanase was followed by gel filtration on

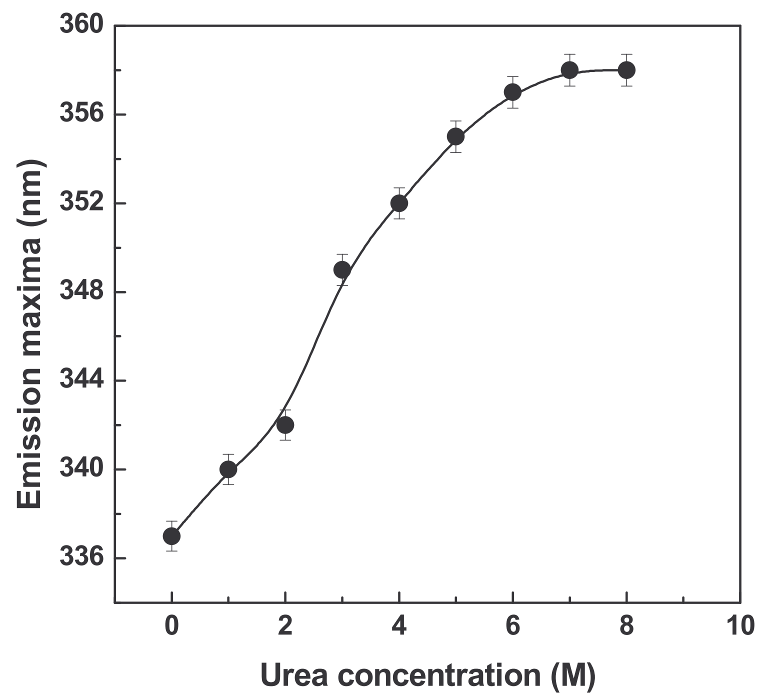


Fig. 20: Effect of urea denaturation on the fluorescence emission maxima of endoglucanase at pH 2.0 in presence of different concentration of urea.

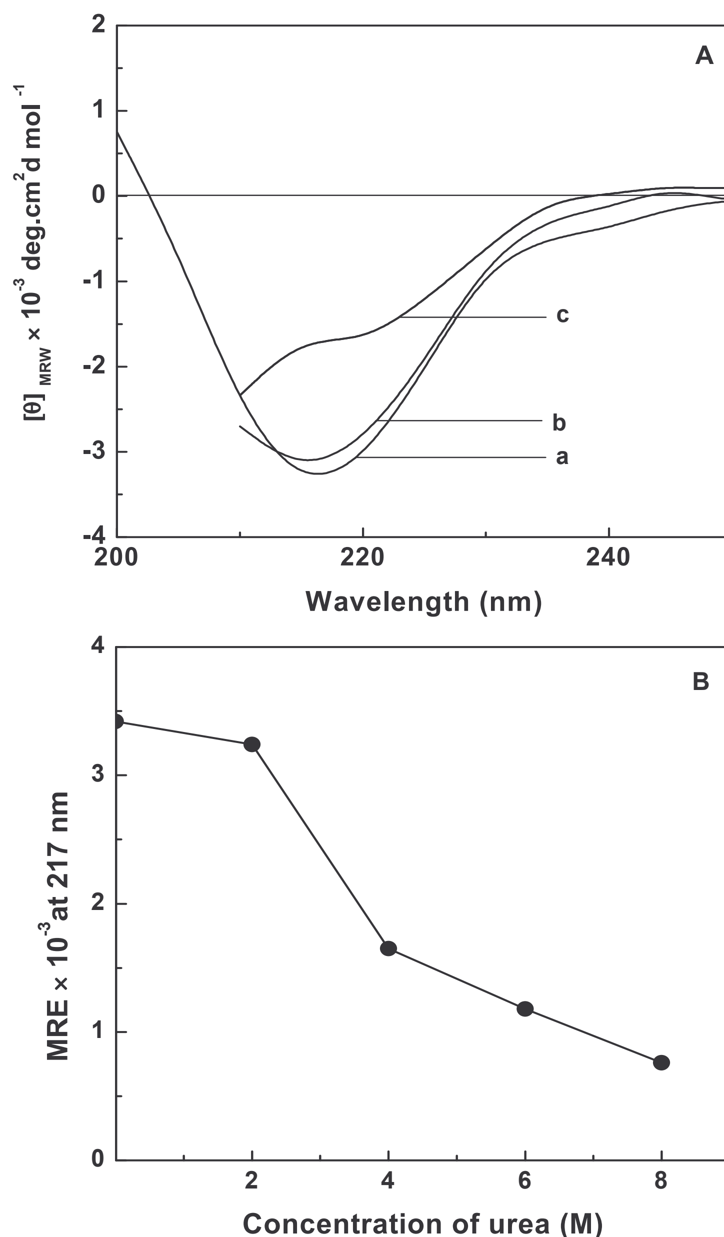


Fig. 21: Effect of urea denaturation on the structure of endoglucanase at pH 2.0. (A) As measured by far-UV CD spectra by addition of urea at pH 2.0. Appropriate blanks were used as reference for baseline corrections. (a) Control (only in buffer); (b) in 2 M and (c) in 4 M urea. (B) Relative changes in far-UV CD ellipticity values of endoglucanase at 217 nm at pH 2.0 in presence of urea.

FPLC, to determine the differences in the molecular properties of the intermediate state with that of the native and the unfolded states and also for determining their Stokes radii. In Fig. 22A is shown the elution profile of endoglucanase at the native and the intermediate state. The enzyme eluted at 16.1 mL as a single peak detected at 280 nm. The Stokes radii for the native and the unfolded states were found to be 31.0 Å and 43.7 Å, respectively. The acid induced state has Stokes radius of 34.4 Å (Fig.22B). These results show increase in the molecular size of the protein molecule at acidic pH (2.0) which could be due to opening up of the molecule or change in the state of association of the molecule. But in comparison to the unfolded state the intermediate state is more compact in nature.

The endoglucanase at pH 2.0 is in acid induced intermediate state with native like secondary structure as shown by far-UV CD. The possible explanation for the maintenance of the secondary structure at pH 2.0 could be that addition of strong acid adds both protons and anions to the solution, these anions pair-up with positively charged groups thereby, shielding the columbic repulsion and permitting other forces to favor the folding. The positive charges do not have any effect due to lack of potential binding sites, as protein is maximally protonated in the acid unfolded state. Acrylamide quenching data also clearly shows that endoglucanase at pH 2.0 is in a partially unfolded form. The molten globule state by retaining or increasing the secondary structure attains the low energy compact protein (Shin *et al*., 1997). Goto *et al*., (1990) have proposed that upon acid-titration, intramolecular charge repulsions are the driving force for partial unfolding of the protein molecule. Similar results were shown for the intermediate states of glucose/xylose isomerase (Pawar and Deshpande, 2000), stem bromelain (Haq *et al*., 2002) and apomyoglobin (Nishi *et al*., 1995).

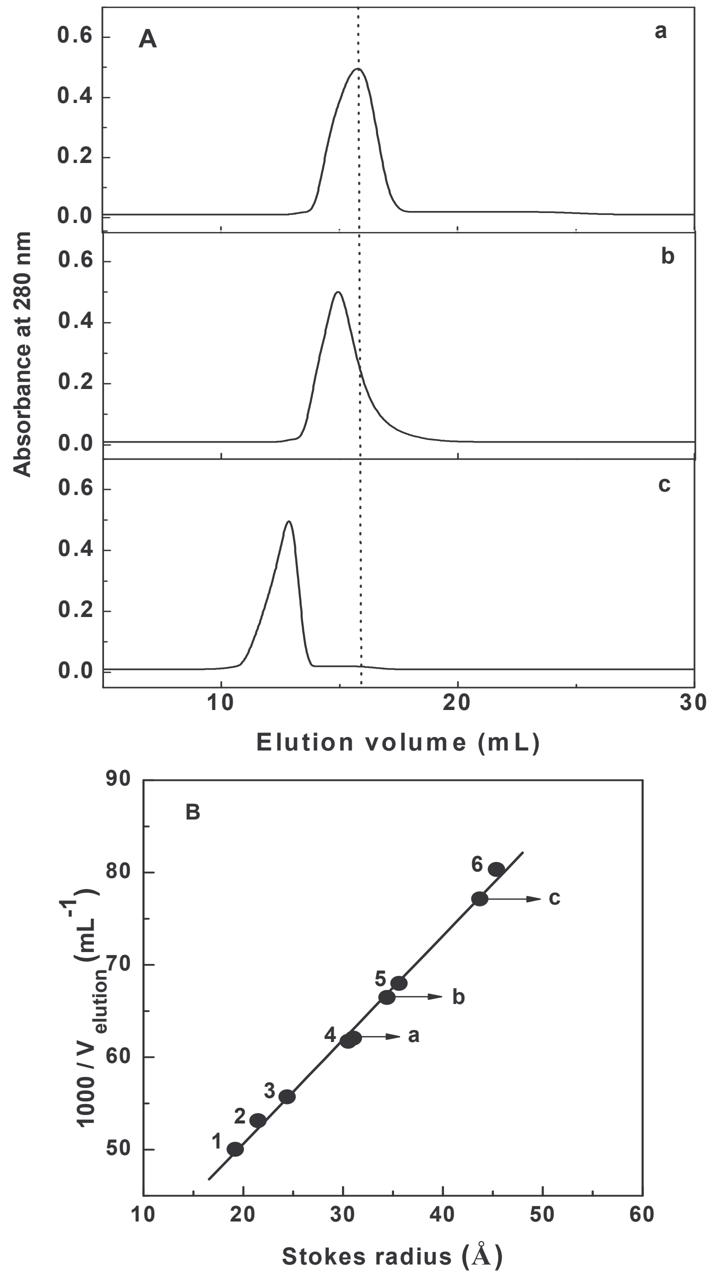


Fig. 22: The effect of pH on the elution profile of endoglucanase by size exclusion chromatography on FPLC:(A) (a) native state at pH 5.0, (b) acid-induce state at pH 2.0 and (c) urea-unfolded state. (B) Calibration curve obtained based on the elution profile of standard proteins of known Stokes radii: (1) ribonuclease (19.2 Å); (2) myoglobin (21.4 Å); (3) carbonic anhydrase (24.4 Å); (4) ovalbumin (30.5 Å); (5) BSA (35.5 Å) and (6) alcohol dehydrogenase, 45.35 Å (a) native state (pH 5.0); (b) acid induced state pH 2.0 and (c) urea-unfolded state.

Unfolding of the enzyme by urea at pH 2.0 indicates that endoglucanase is susceptible to unfolding. The decreased activity at pH 2.0 was not caused due to the loss in the secondary structure of the protein. Cellulases have similar catalytic mechanism to that of lysozyme (Rouvinen *et al*., 1990; Divne *et al*., 1994). The enzyme catalysis takes place by general acid-base catalysis mechanism, where two glutamic acid residues are involved in the catalysis. One of the glutamic acid residues is negatively charged and the other one positively charged. Negative charge is important to maintain or stabilize the exo-carbenium intermediate that is formed during the catalysis (McCarter and Withers, 1994). Since, the pK_a of glutamic acid may be affected, it is assumed that at this pH 2.0, it exists in the protonated state and hence can not interact with the substrate and doesn't stabilize the intermediate which is required for the protonation of the second glutamic acid residue. Therefore, an overall reduction in the enzyme activity was observed and also the H-bonding around the catalytic residues could also be affected at this pH and this in turn affects the rate of hydrolysis of the enzyme (Koivula *et al*., 1996).

These studies are important to understand the folding patterns of endoglucanases and further, it gives an insight to elucidate the general mechanism of folding of cellulases. The results presented here indicate the presence of a partially unfolded state caused by pH-induced denaturation. This partially unfolded state shows characteristic features of a molten globule state as indicated by various biophysical techniques. In the next chapter the effect of denaturants such as urea and GuHCl on the folding and unfolding pattern of endoglucanase is investigated in order to understand the mechanism of stability of cellulase.

2.2. Effect of urea and GuHCl on folding and unfolding of endoglucanase

The function of a protein depends on its ability to acquire a unique three-dimensional structure. Understanding how this process occurs is one of the greatest challenges for protein chemists. Proteins are known to acquire different conformational states during their unfolding by various denaturants. Urea and GuHCl, commonly used as protein denaturants, generally bring about unfolding of proteins by disrupting their secondary and tertiary structures. In spite of numerous reports on the use of these denaturants, the exact mechanism of action on the protein structure is not clearly understood.

The present investigation describes the biophysical characterization of endoglucanase to understand the structure-function relationship in presence of urea and GuHCl. In order to understand the unfolding process a number of parameters like enzymatic activity, intrinsic fluorescence and circular dichroic studies are carried out and results are analyzed.

Enzyme activity measurements with the increasing denaturant concentrations are a very sensitive probe to detect even small structural changes that an enzyme undergoes. The effect of urea and GuHCl on the structure and function of endoglucanase was studied by equilibrating the enzyme at pH 5.0 for 24 h in presence of different concentrations of denaturants. The activity profile of endoglucanase as function of urea and GuHCl concentrations is shown in Fig. 23. The denaturation process of endoglucanase in presence of increasing concentrations of urea and GuHCl is combination of two distinct processes. The first transition upto 2 M urea and GuHCl, where the enzymatic activities were 2.3 and 1.9 fold, respectively,

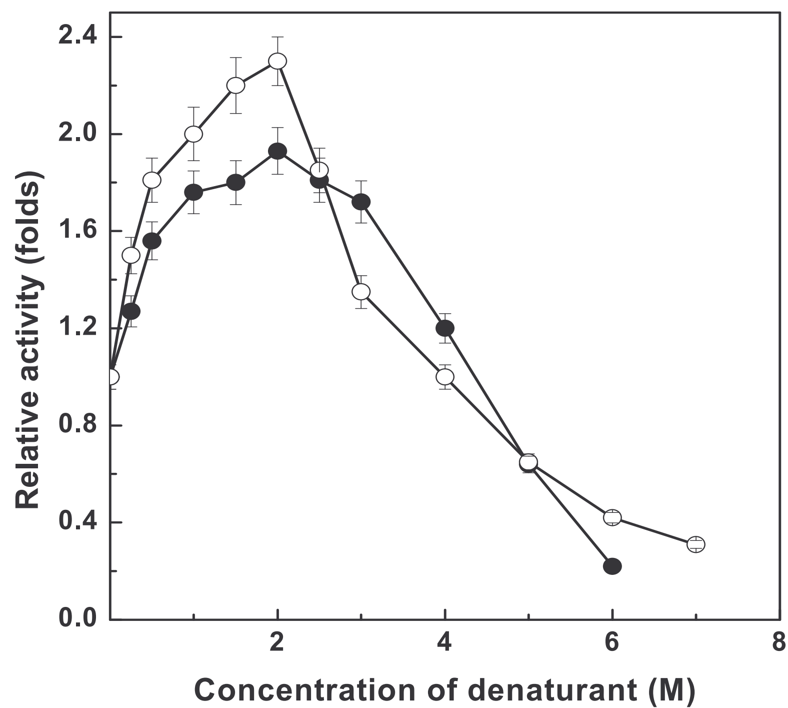


Fig. 23: The effect of denaturants on endoglucanase activity. The enzyme was pre-incubated with different concentrations of urea and GuHCl and activity measured with 0.5 % CMC in 20 mM sodium acetate buffer, pH 5.0 at 40 °C (○) urea and (●) GuHCl.

which gradually decreased with increasing concentrations of denaturants. The enzyme loses its total activity above 5 M urea and GuHCl in 20 mM sodium acetate buffer, pH 5.0. Denaturants at higher concentrations cause inactivation and unfolding by disruption of secondary and tertiary structures of the proteins. The enzyme was completely inactivated at 7 M urea and 6 M GuHCl, respectively.

To further characterize the activity-enhanced states (2 M urea and GuHCl), kinetic constants (K_m and K_{cat}) for the enzyme in presence and absence of denaturants were determined. Kinetic parameters of endoglucanase in low concentrations of the denaturants were determined using Lineweaver-Burk plot. In the absence of denaturants, the apparent K_m for the endoglucanase was 0.060 ± 0.001% and the catalytic constant was 1.3 ± 0.1 min^{-1}. In presence of 2 M urea, the K_m was 0.035 ± 0.001% and K_{cat} was 3.0 ± 0.3 min^{-1}, where as in the presence of GuHCl, the K_m was found to be 0.041 ± 0.001% and K_{cat} was 2.5 ± 0.2 min^{-1}. The decrease in the K_m and increase in the catalytic constant clearly suggests that endoglucanase is having more affinity towards the substrate due to the flexibility at the active site and is achieving a maximal catalytic efficiency in low concentrations of the denaturants. Similar results were shown for other enzymes where, the activity of the enzyme increased in presence of low concentrations of urea and GuHCl (He, 1993; Fan *et al*., 1996; Ahmad *et al*., 2007; Gangadhara *et al*., 2008a).

The fluorescence emission spectra of the endoglucanase in different concentrations of urea are shown in Fig. 24. As seen from the fluorescence emission spectra of native (0 M urea), intermediate (2 M urea) and denatured

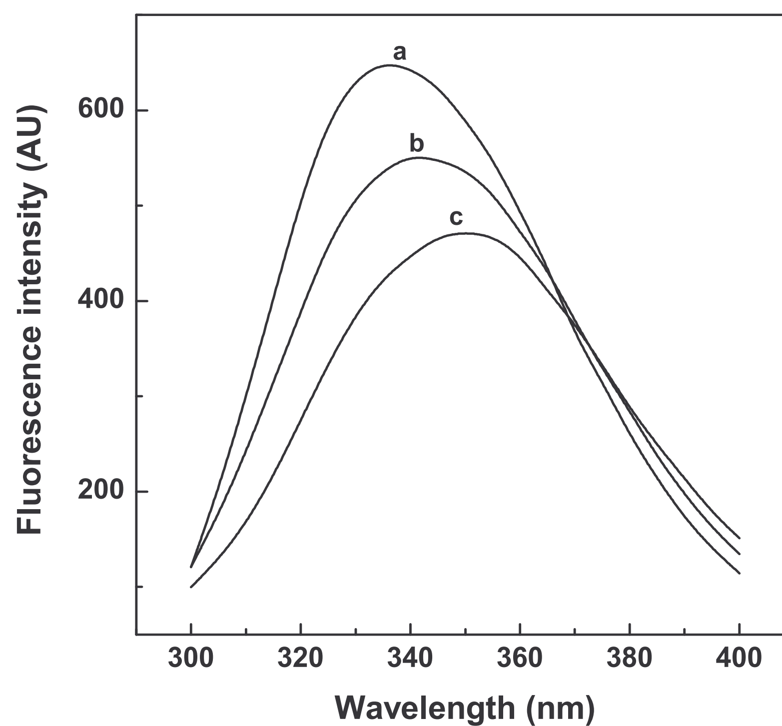


Fig. 24: Effect of urea on intrinsic fluorescence emission spectra of endoglucanase. (a) Control (in buffer only); (b) in 2 M and (c) in 8 M urea. The spectra was recorded in 20 mM sodium acetate buffer, pH 5.0 at 25°C and excitation at 280 nm.

endoglucanase (8 M urea) there is a gradual decrease in the fluorescence intensity with increasing concentration of urea. The fluorescence emission maximum was shifted from control value of 336 nm to 342 nm and 350 nm in case of the intermediate and denatured states, respectively. In Fig. 25 the fluorescence emission spectra of native endoglucanase, the intermediate state (2 M) and fully unfolded state (6 M) in presence of GuHCl is shown. The fluorescence emission maxima shifted under these conditions from a control value of 336 nm to a value of 342 nm in case of intermediate and finally to 353 nm in case of the unfolded state. These results show that the endoglucanase molecule is unfolding with increase in concentration of urea and GuHCl.

The effect of increasing concentration of urea and GuHCl on the secondary structure of endoglucanase was studied by measuring the far-UV CD spectrum of endoglucanase equilibrated with different concentrations of urea and GuHCl. The far-UV CD spectra of native endoglucanase showed a minimum at 217 nm. In Fig. 26 is shown the far-UV CD spectra of endoglucanase in presence of urea and GuHCl. The mean residual value at 217 nm for the native protein is -3450 ± 30 deg.cm^2 dmol^{-1} and in presence of 2 M urea and GuHCl were -3650 ± 40 and -4216 ± 50 deg.cm^2 dmol^{-1}, respectively. Thus, indicating that at 2 M urea and GuHCl, the enzyme possessed secondary structure similar to that of native circular dichroic spectral amplitude. The ellipticity values at 217 nm of endoglucanase in different concentration of urea are summarized in Table 7. As seen from the table, the ellipticity value of the enzyme increased by 5% in presence of 2 M urea compared to control. In Table 8 is summarized the ellipticity values at 217 nm of endoglucanase in presence of different concentrations of GuHCl. The ellipticity value of the enzyme at 217 nm increased by 22% in presence of

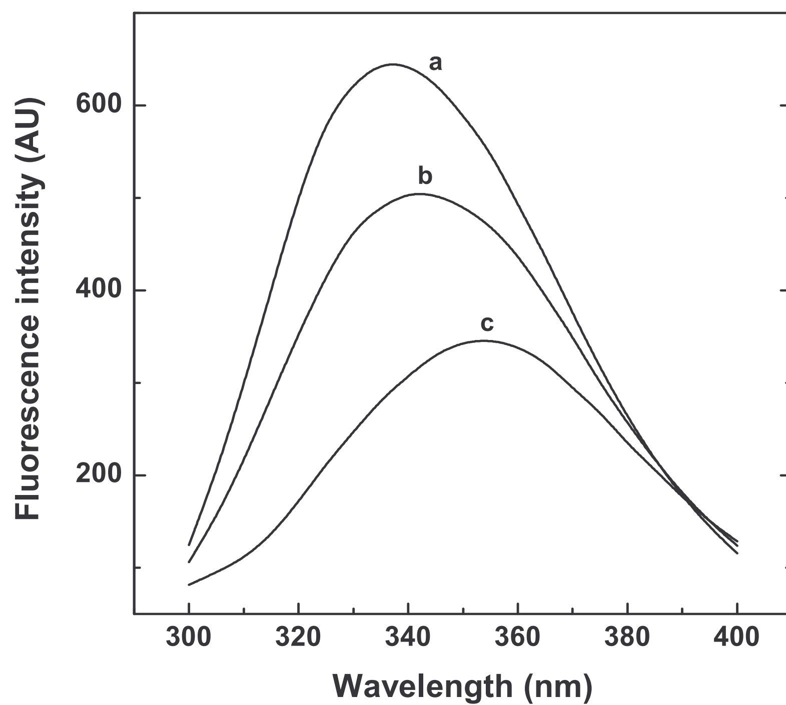


Fig. 25: Effect of GuHCl on intrinsic fluorescence emission spectra of endoglucanase. (a) Control (in buffer only); (b) in 2 M and (c) in 6 M GuHCl. The spectra was recorded in 20 mM sodium acetate buffer pH 5.0 at 25 °C and excitation at 280 nm.

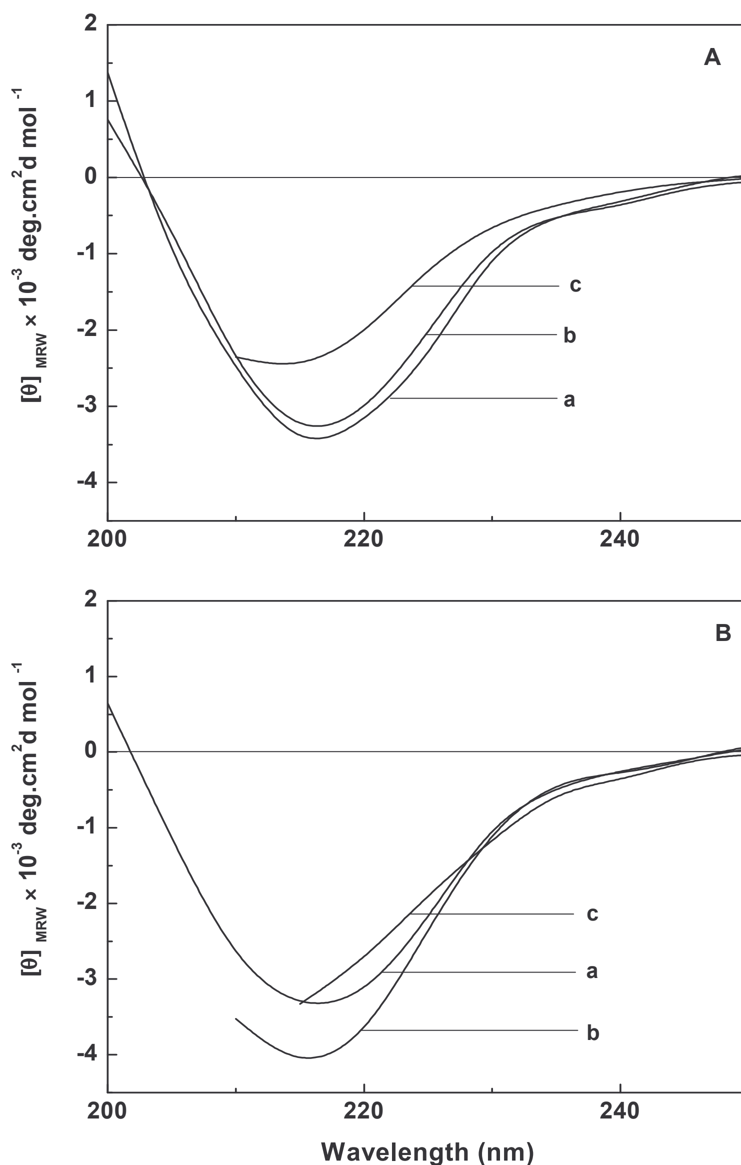


Fig. 26: Effect of different concentrations of denaturants on far-UV CD spectra of endoglucanase (A) urea and (B) GuHCl. (a) Control (in buffer only), (b) in 2 M and (c) in 4 M of respective denaturants in 20 mM sodium acetate buffer, pH 5.0. (Representative spectra are only shown for clarity).

Table 7: Changes in the ellipticity values of endoglucanase at 217 nm in different concentrations of urea in 20 mM sodium acetate buffer, pH 5.0.

Concentration of urea (M)	MRE values at 217 nm (deg.cm^2 dmol^{-1})
Control*	-3450 ± 30
1	-3500 ± 35
2	-3650 ± 40
4	-2475 ± 25
6	-1639 ± 15
8	-764 ± 15

** The enzyme in 20 mM sodium acetate buffer, pH 5.0*

Table 8: Changes in the ellipticity values of endoglucanase at 217 nm in different concentrations of GuHCl in 20 mM sodium acetate buffer, pH 5.0.

Concentration of GuHCl (M)	MRE values at 217 nm (deg.cm^2 dmol^{-1})
Control*	-3450 ± 30
1	-3500 ± 35
2	-4216 ± 50
4	-3325 ± 25
6	-1845 ± 15

** The enzyme in 20 mM sodium acetate buffer, pH 5.0*

2 M GuHCl. Further, high concentrations of urea and GuHCl did not allow measurement of spectrum between 200-210 nm. The loss of secondary structure occurs as the concentration of urea and GuHCl increases and maximum loss was observed at 8 M urea and 6 M GuHCl. The molecule acquires more random/aperiodic type of structure with concomitant loss in the secondary structures. Similar to our results, subtle conformational changes in secondary structure in presence of xylanase from *Chainia* at low urea concentrations were observed (Kumar *et al*., 2003). With further increase in the concentration of urea and GuHCl the enzyme loses its secondary structure and also the activity. Thus, from the far-UV CD spectral studies it is clear that the intermediate state observed at 2 M urea and GuHCl is in between the native and completely unfolded state.

These results indicate that the enhanced activity of endoglucanase in presence of (2 M) urea and GuHCl could be due to the conformational changes of the molecule leading to a more open and flexible structure of the molecule (Kumar *et al*., 2003; Fan *et al*., 1996). It has been shown that the substrate binding in *Chainia* xyalanases takes place due to hydrogen bonding and reorganization of the water molecules between the enzyme and the substrate. Urea can penetrate into the active site cleft of the enzyme leading to a more open conformation at the active site causing the increased activity of the enzyme. Endoglucanases being very similar to the xylanases in mode of action and structure, it can possibly be assumed that interaction with substrate proceeds in a similar manner. Similar increase in the activity of nuclease P1 in presence of low concentrations of urea and GuHCl was shown to be due to increase in catalytic constant as a result of more open and flexible conformation of the enzyme (Gangadhara *et al*., 2008a).

Fig. 27 shows the urea induced unfolding of endoglucanase as monitored by the changes in circular dichroic rotations at 217 nm and fluorescence emission measurements. It can be seen from the plot that urea unfolding transitions of endoglucanase monitored by both the methods are cooperative and overlapping as indicated by sigmoidal curves. The maximum changes occur between 2 to 6 M uea with a transition mid point of 4 ± 0.2 M as indicated by both CD and fluorescence measurements. The refolding was studied by dilution of the protein sample which has been completely equilibrium unfolded in 8 M urea. The transiton curves for the process of refolding was close to that of the unfolding curves. Thus, urea induced unfolding of endoglucanase can be described by a simple two-state model. It is known that protein denaturation is highly cooperative having a two-state model and no significant intermediates present during transition from native to denatured states (Privalov, 1979).

The equilibrium-unfolding curves of endoglucanase in presence of different concentrations of GuHCl obtained by different methods are shown in Fig. 28. In both, fluorescence and CD the changes are due to single transition. The fluorescence studies probe the changes in the tertiary structure of a protein and the far-UV CD signal measures the changes in the secondary structure. It is observed that unfolding is cooperative and coincidental as indicated by the sigmoidal curves. The maximum changes occur between 2 to 5 M GuHCl with a transition mid point of 3 ± 0.2 M as indicated by both CD and fluorescence measurements. The refolding transition curves were close to unfolding curves, indicating that the endoglucanase simply follows a two-state model for transition. This shows that the intermediate formed may

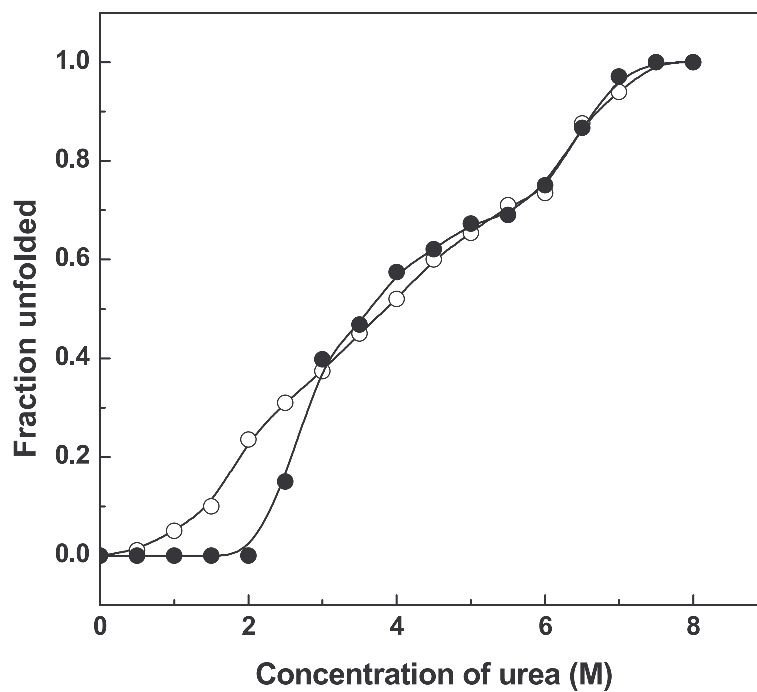


Fig. 27: Urea induced unfolding of endoglucanase in 20 mM sodium acetate buffer pH 5.0 as followed by different techniques (○) fluorescence spectroscopy and (●) far-UV circular dichroism.

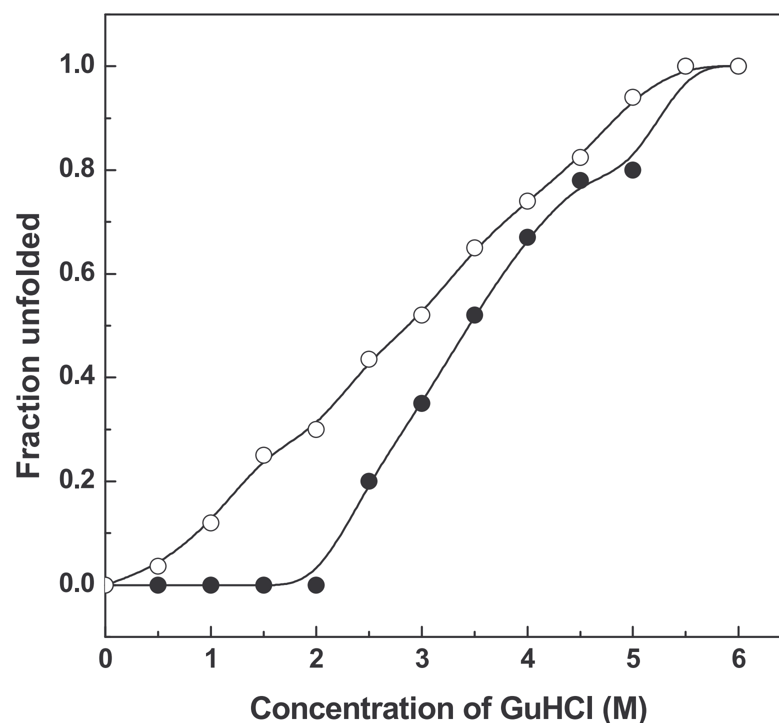


Fig. 28: GuHCl induced unfolding of endoglucanase in 20 mM sodium acetate buffer pH 5.0 as followed by different techniques (○) fluorescence spectroscopy and (●) far-UV circular dichroism.

be less stable and immediately gets transformed to the other state, hence cannot be detected. Similar results were obtained for endoglucanse III from *Trichoderma reesi* showing that it data from the equilibrium studies fit a simple two-state model (Arunachalam and Kellis, 1996).

The effect of lower concentrations of urea and GuHCl on the thermal stability of endoglucanase was monitored through the measurements of the apparent thermal transition temperature of the endoglucanase both in presence and absence of urea and GuHCl. The plot of fraction unfolded versus temperature was obtained using standard equation (Pace and Scholtz, 1997) from the denaturation profile. The thermal denaturation profile of urea and GuHCl are shown in Fig. 29 A and B. The thermal denaturation profile shows that there is a shift in the apparent T_m of endoglucanase in presence of low concentration of urea and GuHCl. The apparent thermal transition temperature of endoglucanase increased from a control value of 57 ± 1°C to a maximum value of 62 ± 1°C at 2 M urea. The apparent T_m in presence of GuHCl was 61 ± 1°C at 2 M concentration. These results indicate an increased thermal stability of the enzyme in presence of low concentration of urea and GuHCl. Similar results were shown in case of other proteins in presence of low concentrations of urea and GuHCl (Satish *et al*., 2002; Gangadhara *et al*., 2008a).

Acrylamide is a very sensitive quenching probe to monitor the accessibility of tryptophan residues. To assess the minor changes in the conformations of enzymes, the quenching of intrinsic fluorescence by acrylamide was measured and analyzed by Stern-Volmer plot as shown in Fig. 30A and B. The stern-Volmer plots for both native and intermediate states were nlinear nover the concentration range studied. But in intermediate state,

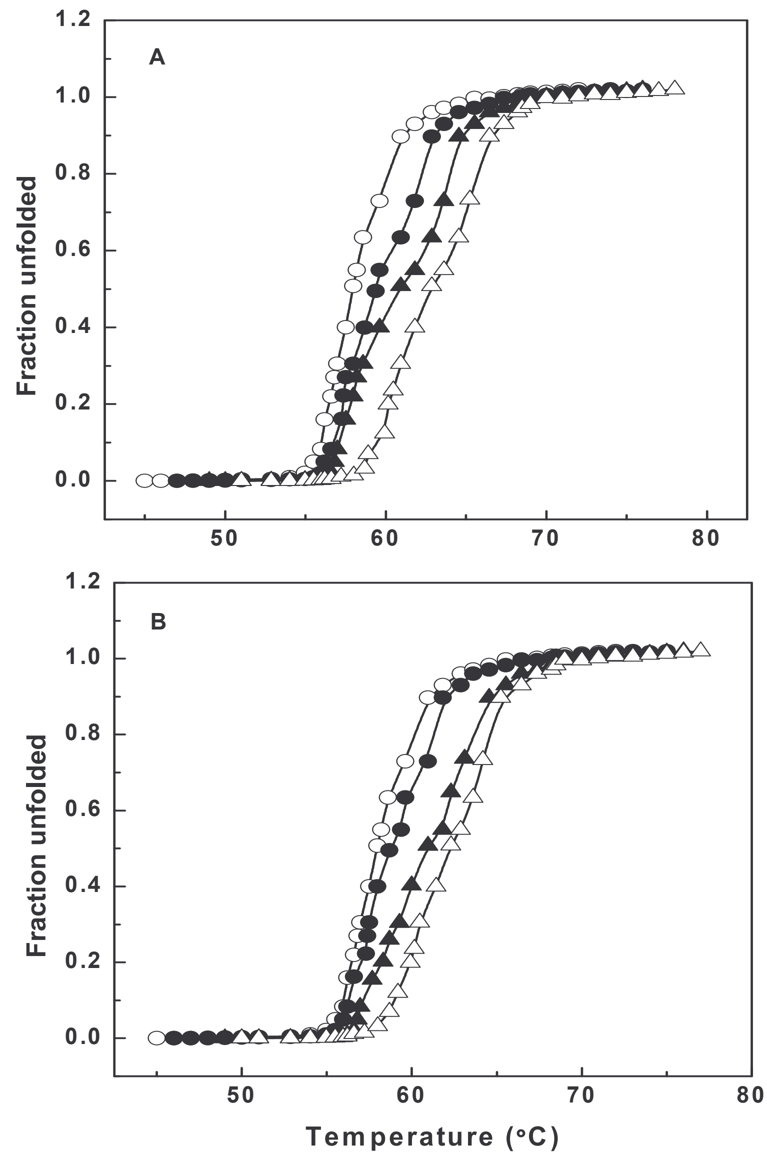


Fig. 29: Apparent thermal denaturation profile of endoglucanase in presence of different concentrations of denaturants as followed by monitoring the changes in absorbance at 287 nm as a function of temperature. (A) In presence of urea and (B) in presence of GuHCl. (○) control (in buffer only) and in presence of (●) 0.5 M , (▲) 1 M and (△) 2 M respective denaturants.

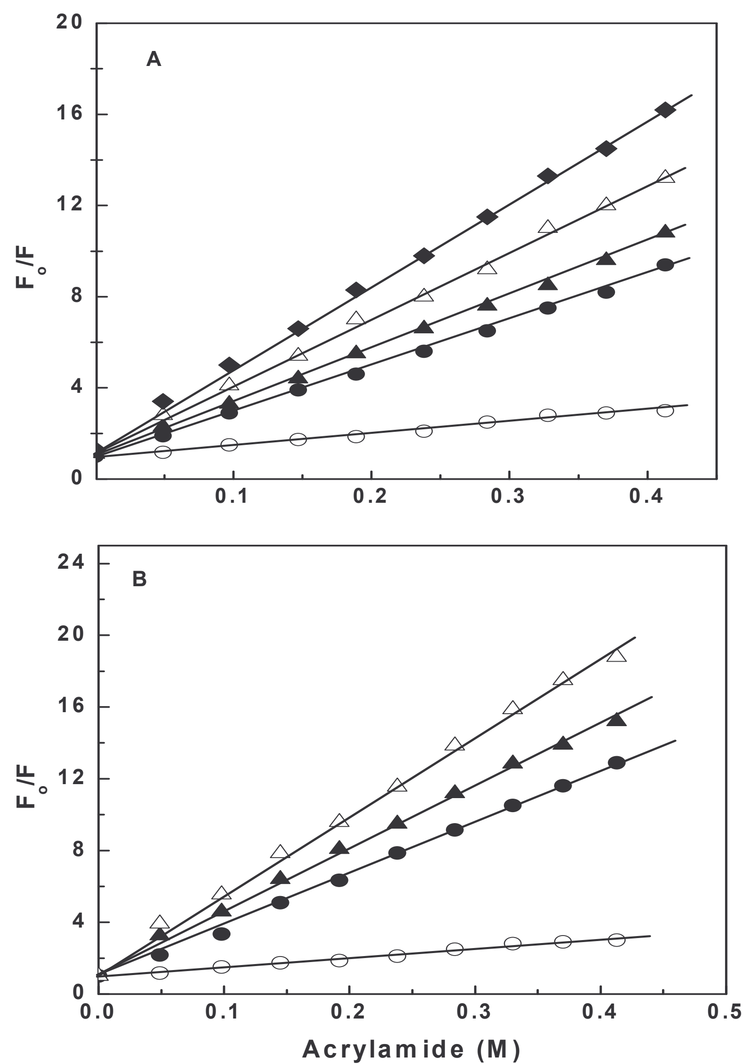


Fig. 30: Stern-Volmer plot of tryptophan quenching of endoglucanase by acrylamide in presence of different concentrations of denaturants. (A) in presence of urea and (B) in presence of GuHCl. (○) control (in buffer only) and in presence of (●) 2 M, (▲) 4 M, (△) 6 M and (■) 8 M respective denaturants.

the tryptophan residues are more accessible to acrylamide, with considerable increase in the K_{sv} values. The K_{sv} value for native enzyme is 5 M^{-1}, while it is 17.5 M^{-1} and 30 M^{-1} in case of intermediate states in presence of 2 M urea and GuHCl, respectively. In case of completely denatured state at 8 M urea and 6 M GuHCl, the Stern-Volmer plot showed an upward curvature which is characteristic state of quenching.

The exposure of the hydrophobic regions at lower concentrations of urea and GuHCl were studied by monitoring the ANS binding to the protein molecule. The hydrophobic dye ANS, binds to the hydrophobic surfaces on the proteins (Krishnan *et al*., 1996). Its fluorescence is sensitive to the polarity of its microenvironment, upon binding to the non-polar surfaces, depending on the extent of hydrophobicity of the surfaces, its emission maximum is shifted to the shorter wavelengths and the emission intensity is increased. This property of the dye has been demonstrated to identify the intermediates on the unfolding pathways of proteins (Semisotnov, 1991). Fig. 31 shows the fluorescence of ANS-bound endoglucanase in the presence and absence of urea and GuHCl. The protein bound to ANS shows increase in the fluorescence intensity and the emission maximum shifts from 512 nm to 480 nm, respectively. Three-fold increase in the fluorescence intensity is shown in presence of 2 M urea and GuHCl. ANS binds more strongly to the intermediate state than the protein in native or fully unfolded states (Ptitsyn, 1992). These results indicate that in presence of low concentrations (2 M) of urea and GuHCl there is exposure of the hydrophobic surface to the solvent due to conformational changes. This shows that there is flexibility in the enzyme molecule. Similar results were reported for other enzymes (Fan *et al*., 1996).

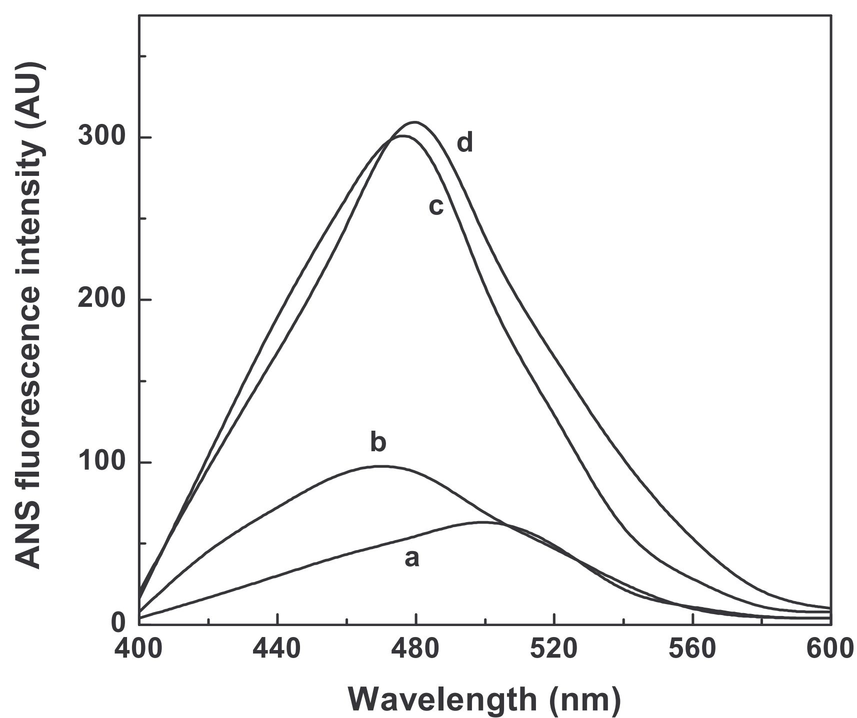


Fig. 31: ANS binding to endoglucanase as function of denaturants. The spectra were measured in 20 mM sodium acetate buffer pH 5.0 at 25 °C. The samples were incubated for 2 h in 2 M concentrations of urea and GuHCl before the measurements were taken. (a) ANS + Buffer; (b) Control (in buffer only); (c) 2 M GuHCl and (d) 2 M urea.

The present study shows that the enzyme is more stable in its activated form in 2 M urea and GuHCl. The conformational changes at low urea and GuHCl concentrations leads to activation followed by inactivation at higher concentrations of denaturants. Activation of cross-linked lactate dehydrogenase (Ma and Tsou, 1991) and glucan maltotetrahydrolase in presence of GuHCl (He, 1993), has suggested that flexibility at the active site is essential for the maximal expression of the catalytic activity. The activation of dihydrofolate reductase by urea and GuHCl is shown to be associated with the conformational change at the active site (Fan *et al.*, 1996), suggesting that the flexibility at its active site is essential for the full expression of the enzyme activity. A subtle conformational change at the active site has now been shown to accompany activation of dihydrofolate reductase before any global conformation change can be detected.

Understanding the structural changes in a protein molecule under various conditions would give an insight in understanding the structure and stability of proteins. In the present study the effect of pH and denaturing agents such as urea and GuHCl on structure-function and stability of endoglucanase has been investigated. The pH–induced denaturation of endoglucanase is irreversible at pH 2.0 with loss of 50% of the enzyme activity. The endoglucanase at pH 2.0 had an acid-induced intermediate state with native like secondary structure. This intermediate state showed high ANS fluorescence, one of the characteristic features of a molten globule state. The results indicate that pH-induced denaturation of endoglucanase leads to the formation of a partially folded conformation at low pH with characteristic features of a molten globule like intermediate state.

The results of folding and unfolding studies show the effect of denaturants such as urea and GuHCl on the structure and function of endoglucanase. Endoglucanase showed an increase in the activity at low concentrations of denaturants up to 2 M followed by gradual decrease in the activity with increasing concentrations of denaturants. Kinetic parameters show decrease in the K_m and increase in the catalytic constant suggesting that endoglucanase is having more affinity towards the substrate and attains maximal catalytic efficiency in low concentrations of the denaturants. Apparent T_m of endoglucanase showed increased stabilization of endoglucanase against thermal denaturation in presence of 2 M urea and GuHCl, respectively. Denaturants induced unfolding of endoglucanase is cooperative and follows a simple two-state model. The results obtained in this study help to gain insight into the structure-function relationship of the enzyme in presence of denaturants such as urea and GuHCl. In the subsequent chapter endoglucanase molecule is investigated for its structural stability using different structure stabilizing cosolvents. These studies give insight into the mechanism of stabilization and thermal denaturation of endoglucanase.

3. Effect of Cosolvents on Structure and Stability of Endoglucanase

The stability of the protein depends mainly on the interaction of amino acid side chains and secondary structural elements. The electrostatic forces, hydrophobic interactions, van der Waal's forces, hydrogen bonds and salt bridges contribute to the overall stability of a protein. The study of enzyme inactivation and thermal denaturation in order to stabilize protein molecules is important for the enzyme based applications in various fields. Proteins on extraction from their source are subjected to changes in the environment which leads to structural changes and loss of stability.

Polyhydric alcohols and sugars have been used for many years as stabilizing agents for the maintenance of the biological activity of macromolecules (Tanford *et al*., 1962; Gerlsma and Sturr, 1972; Frigon and Lee, 1972). The solvents tend to influence the catalytic properties and stability of the enzymes to a great extent (Gangadhara *et al*., 2008b). The use of enzymes in organic solvents has extended their practical applications. The major limitation to industrial use of the enzyme is their relative instability under operational conditions, which involves exposure to extreme conditions like temperature, pressure, pH, denaturants and organic solvents. Carrying the enzyme reactions at higher temperatures has several advantages like high conversion rates, decreased microbial contamination, increased solubility of substrate and also shelf life (Klibnow, 1983). From the application point of view of these enzymes in industries, the search for development of thermostable enzymes for use in the food and pharmaceutical industries is of considerable interest.

Protein molecules in aqueous solutions are surrounded by a hydration shell, which is composed of water molecules attached to the protein surface. If an organic solvent is present, the solvent molecules tend to displace the water molecules both in the hydration shell and in the interior of the protein, thereby distorting the interactions responsible for maintaining the native conformation of the enzymes (Kita *et al*., 1994; Timsheff, 1998). In the case of sugars, the stabilizing effect is due to surface tension of water. The major factor in preferential hydration is the free energy which is required to form a cavity in the solvent, to accommodate the protein molecule (Lee and Timasheff, 1981). Because such a cavity would be larger for the unfolded protein, the free energy would increase and thereby the stability of the folded state is higher than the unfolded state (Arakawa and Timasheff, 1982; Ruan *et al*., 2003).

It is well known that proteins are stabilized in presence of cosovlents by preferential hydration and the molecules exist in a stable form with the solvent functioning as thermodynamic boosters (Gekko and Timasheff, 1981a; Lee *et al*., 1975; Shimizu and Smith, 2004). The proteins are preferentially hydrated around the surface in the presence of these stabilizers, leading to an increase in free energy and consequently protection against denaturation (Timasheff, 1993). A number of studies have been carried out to elucidate the nature of thermal stability of proteins which can be increased by addition of sugars (Gekko, 1982; Fujita *et al*., 1982; Gekko and Ito, 1990; Wimmer *et al*., 1997; Timasheff and Arakawa, 1997; Romero *et al*., 2003; Miroliaei *et al*., 2007; Nasiripourdori *et al*., 2009). Hence, the cosolvents that stabilize proteins are also known as thermodynamic boosters.

In this chapter an attempt is made to elucidate the mechanism of stabilization of endoglucanase by different cosolvents. The cosolvents interact with proteins in different ways depending on the physico-chemical properties of the protein and therefore, the selection of cosolvents depends on the nature of the protein. The cosolvents were selected based on the knowledge of their stabilizing effect on the protein after preliminary screening. This was investigated by activity measurements, fluorescence spectroscopy, circular dichroic spectroscopy, thermal denaturation temperature and partial specific volume measurements.

The effect of different concentrations of cosolvents such as glycerol, sorbitol and sucrose on the activity of endoglucanase was monitored by activity measurements in 20 mM sodium acetate buffer pH 5.0 at its optimum conditions after 2 h pre-incubation. The Fig. 32 shows the activity profile of the endoglucanase in presence different concentrations of cosolvents (range of 0 - 40%). The activity of the enzyme increased with increasing concentration of cosolvents. At 40% concentration, the enzyme activity was highest showing a four-fold increase in the activity in presence of each cosolvent.

The effect of each cosolvent on the thermal stability of endoglucanase in 20 mM sodium acetate buffer pH 5.0 was measured through measurements of enzyme activity. Here, the enzyme with and without cosolvents was heated at 90°C for different time intervals and then cooled to room temperature and the activity was measured. The enzyme loses nearly 50% of its activity after heating at 90 °C for 1 h and it remains so even after heating for more than 3 h. In presence of different cosolvents, the enzyme activity was protected. The protection of the enzyme activity by cosolvents increased with increasing

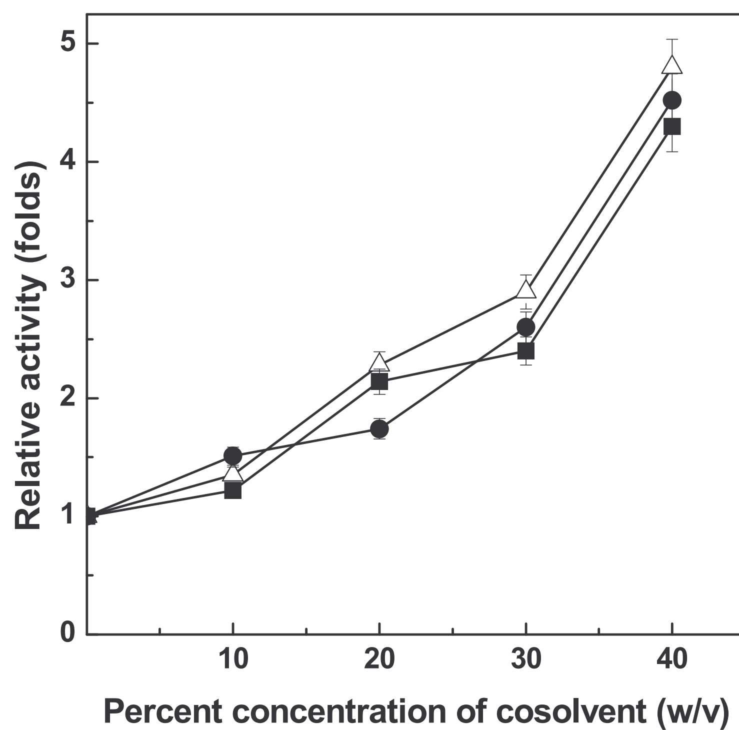


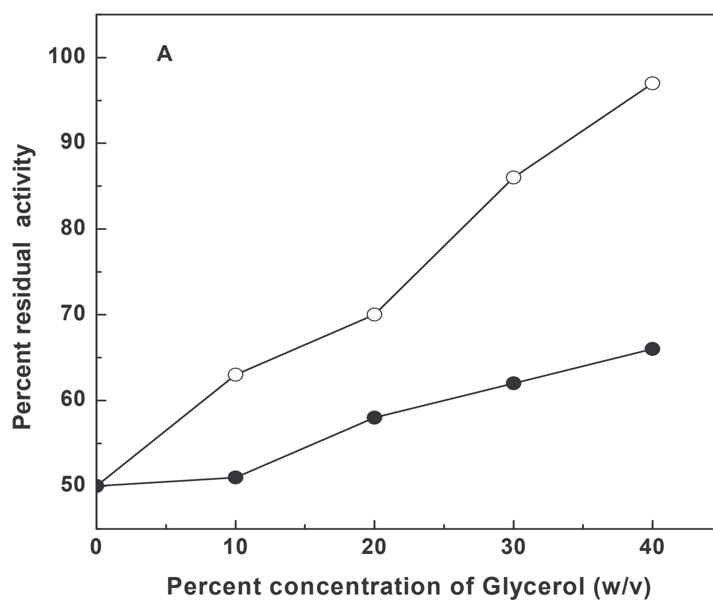
Fig. 32: Relative activity of endoglucanase at its optimum temperature as a function of cosolvent concentration in 20 mM sodium acetate buffer pH 5.0. (∆) Sucrose; (●) Sorbitol and (■) glycerol.

concentrations of each cosolvent. Increased thermal stability of proteins in presence of cosolvents has been reported (Rajendran *et al*., 1995; Satish *et al*., 2007).

The activity of the enzyme was also checked for reversibility of its original activity after exposure to 90 °C for 1 h in presence of cosolvents and after cooling, the cosolvent was removed by gel filtration on Sephadex G-25 column. The enzyme fraction eluted in the void volume was used for the reversibility studies. In Fig. 33 and Fig. 34 are shown the activity profile of the endoglucanase in presence of cosolvents over a range of 0 - 40%. From the figure, it is evident that the enzyme retains its activity to different extents in different cosolvents at different concentrations. In presence of 10% glycerol (Fig. 33A), the enzyme retains 63% of its original activity and it increases gradually to 97% at 40% glycerol concentration. Similarly in case of sucrose (Fig. 33B), the retention of the activity of 69% at 10% concentration was observed and it further increased to 145% at 40% concentration of sucrose. In case of sorbitol (Fig. 34), the enzyme retained 55% of its original activity at 10% concentration and at 40% concentration it retained approximately 92% of its original activity. This increase in the activity could be related with the formation of an activated complex in presence of a cosolvent to an activated state depending on the nature and the concentration of the cosolvent. Based on the above results the hierarchy of the effectiveness of different cosolvents on the stabilization of the endoglucanase is in the order:

Sucrose > Glycerol > Sorbitol

From Table 9, it is clear that sucrose and glycerol at 40% concentration offer maximum protection against the thermal inactivation as observed after



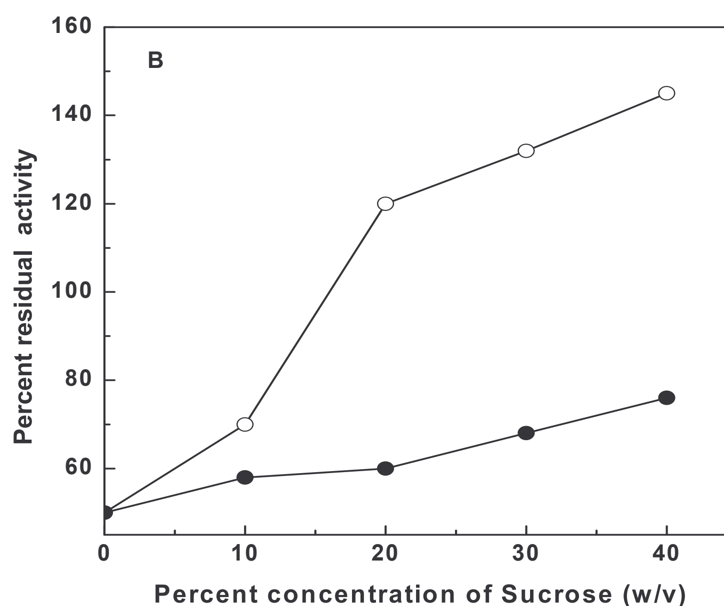


Fig. 33: Activity profile of endoglucanase in 20 mM sodium acetate buffer pH 5.0 as a function of cosolvent concentration at 90°C for 1 h. (A) in presence of glycerol and (B) in presence of sucrose. (○) in presence of cosolvent, (●) after removal of cosolvent by gel filtration.

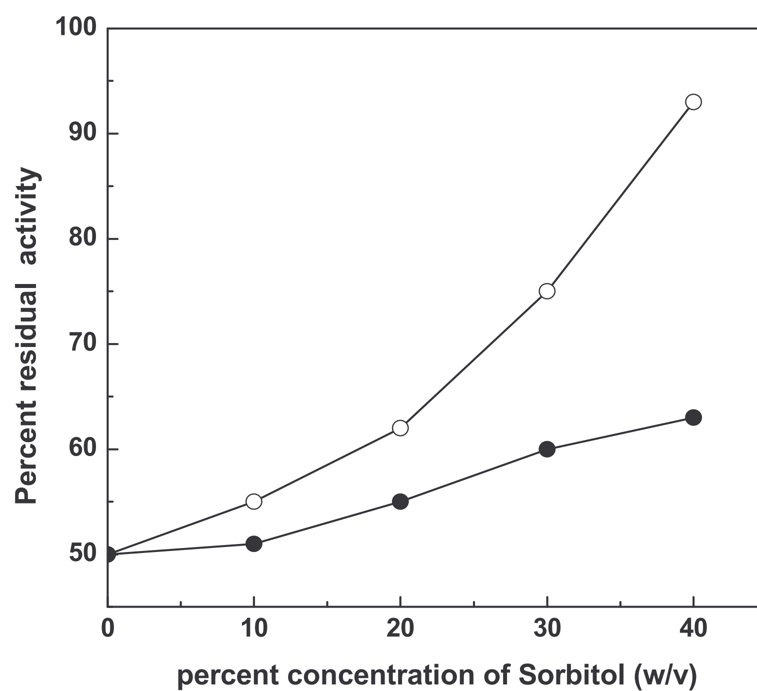


Fig. 34: Activity profile of endoglucanase in 20 mM sodium acetate buffer pH 5.0 as a function of sorbitol concentration at 90°C for 1 h. (○) in presence of cosolvent, (●) after removal of cosolvent by gel filtration.

Table 9: Reversibility of activity of endoglucanase as a function of cosolvent concentration after exposed to 90°C for 1 h in presence and after removal of cosolvents by gel filtration procedure

Cosolvent	Concentration (%) (w/v)	Residual specific activity* (%)	
		In presence of Cosolvent	After removal of cosolvent by gel filtration
Sorbitol	10	55 ± 2	51 ± 1
	20	62 ± 2	54 ± 1
	30	75 ± 2	60 ± 2
	40	92 ± 3	62 ± 2
Glycerol	10	63 ± 2	51 ± 2
	20	70 ± 2	58 ± 2
	30	86 ± 2	62 ± 2
	40	97 ± 3	66 ± 2
Sucrose	10	69 ± 2	57 ± 1
	20	119 ± 2	60 ± 2
	30	132 ± 3	68 ± 3
	40	145 ± 3	76 ± 3

** Residual specific activity of endoglucanase in 20 mM sodium acetate buffer, pH 5.0 is 50 ± 2 %. The cosolvent concentration shown is in the same buffer only.*

removal of cosolvent by gel filtration, the enzyme had the residual specific activity values of nearly 76 and 66%, respectively. Sorbitol was the least effective of all the cosolvents used having a recovery value of 62% of its original activity. The presence of cosolvents have been shown to prevent the thermal inactivation of many enzymes (Rajeshwara and Prakash, 1994; Rajendran *et al*., 1995; Satish *et al*., 2007)

The structural changes of the endoglucanase in presence and absence of cosolvents were monitored by fluorescence emission spectroscopic studies. The effect of cosolvents on the changes in the microenvironment of tryptophan residues have been monitored by intrinsic fluorescence measurements. The fluorescence emission spectra (Fig. 35) showed quenching of fluorescence intensity accompanied by red shift in the emission maximum above 60°C. The enzyme showed 70% quenching in the fluorescence intensity with a red shift in the fluorescence emission maxima from 336 nm to 348 nm at 60°C. The endoglucanase was exposed to 60°C for different time intervals in presence and absence of cosolvents and their fluorescence spectra were measured. Stabilization by cosolvents was studied as a function of their increasing concentration. In all the cases, the extent of stabilization was maximum at the highest concentrations of cosolvents (40%). Thus, from the enzyme activity measurements of endoglucanase in presence of these cosolvents it is clear that the endoglucanase molecule is stabilized against thermal inactivation.

The fluorescence emission spectra of endoglucanase in presence of different concentrations of glycerol are shown in Fig. 36. From the figure, it is clear that there was increase in the fluorescence intensity as a function of

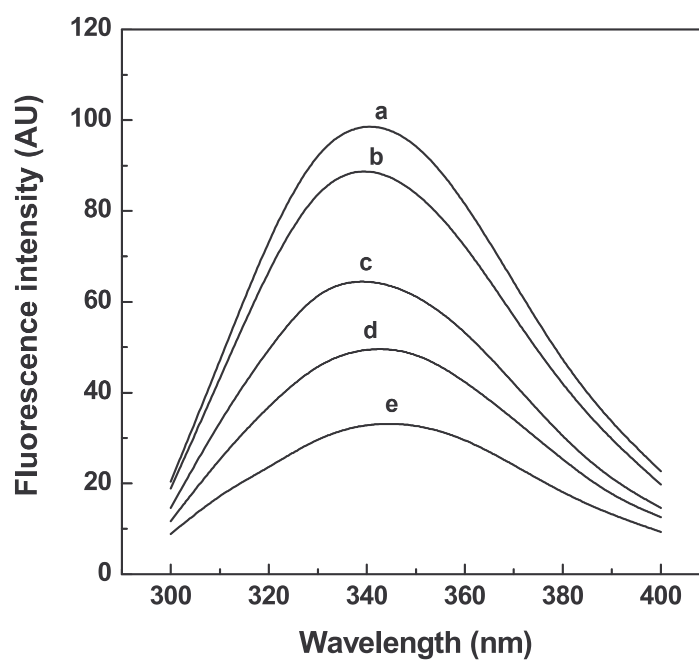


Fig. 35: Tryptophan fluorescence emission spectra of endoglucanase as a function of temperature. The temperatures (°C) used are (a) at 25 (control), (b) at 30, (c) at 40, (d) at 50 and (e) at 60, respectively in sodium acetate buffer, pH 5.0.

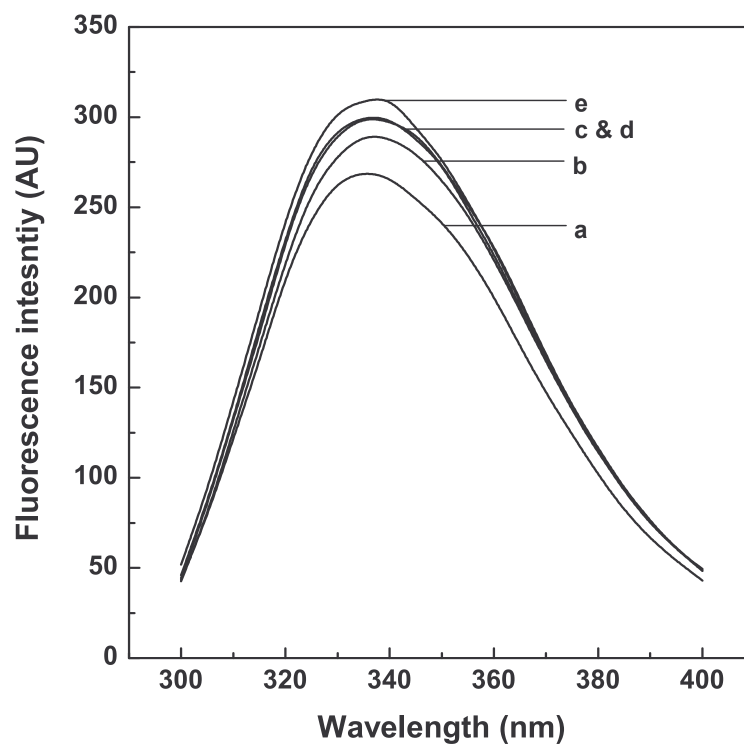


Fig. 36: Fluorescence emission spectra of endoglucanase in presence of different concentrations of glycerol in 20 mM sodium acetate buffer pH 5.0. (a) control (in buffer only), (b) in 10%, (c) in 20%, (d) in 30% and (e) in 40% glycerol.

glycerol concentrations. Apart from the increase in the fluorescence intensity, there was a shift in the wavelength of maximum emission at higher concentrations of glycerol. In Fig. 37 is shown the effect of sucrose on the fluorescence emission spectra of endoglucanase. There was an increase in the fluorescence intensity as the sucrose concentration increased. The fluorescence emission spectra of endoglucanase in presence of sorbitol are shown in Fig. 38. In case of sorbitol, the fluorescence intensity decreased as a function of different concentration. But here, there was no change in the wavelength of emission maximum even at higher concentrations. The wavelength maximum shifted from a control value of 336 nm to a value of 338 nm in presence of 40% glycerol and sucrose (Fig. 39). Similar reports on the stabilization of proteins by cosolvents without any major conformational changes in the protein structure are shown (Rajeshwara and Prakash, 1995; Satish *et al*., 2007).

The effect of temperature in the presence and absence of cosolvents on the structural conformation of endoglucanase was monitored by far-UV CD spectroscopy. In Fig. 40A, is shown, the far-UV CD spectra of native enzyme exposed to different temperatures (20 - 70 °C) for 15 min in absence of cosolvents. The native enzyme shows a minimum at 217 nm, where as the native enzyme exposed to higher temperature, shows a minimum at 205 nm. In presence of cosolvents, there were no major changes in the structure of endoglucanase and it resembles the pattern of the native enzyme as shown in Fig. 40B. The secondary structural elements of the native endoglucanase and the enzyme exposed to elevated temperatures in the absence and presence of cosolvents indicated that the β-sheet and α-helix content of the native enzyme was found to decrease from 62% and 14% to 54% and 6% in case of the

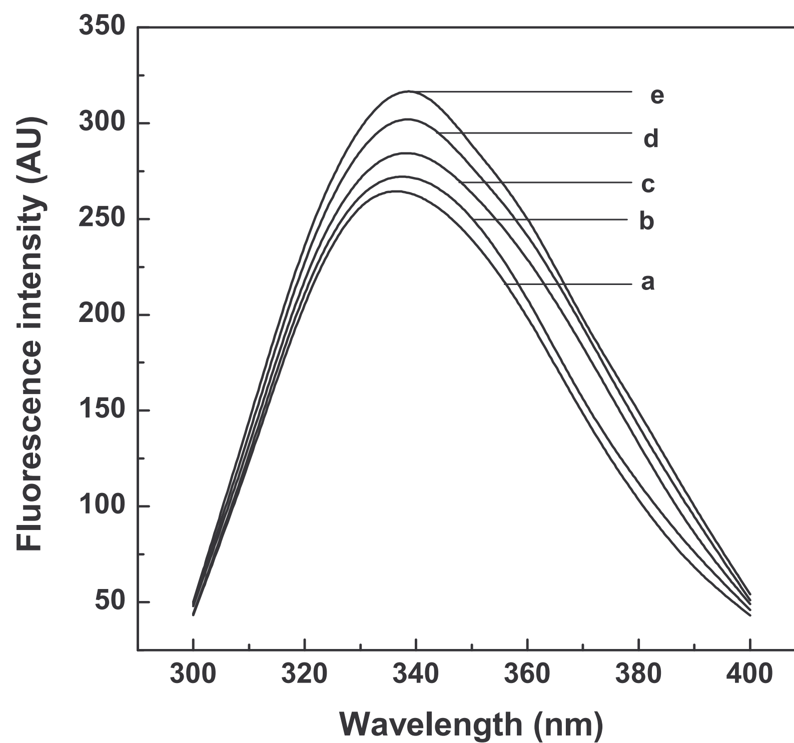


Fig. 37: Fluorescence emission spectra of endoglucanase in presence of different concentrations of sucrose in 20 mM sodium acetate buffer pH 5.0. (a) control (in buffer only), (b) in 10%, (c) in 20%, (d) in 30% and (e) in 40% sucrose.

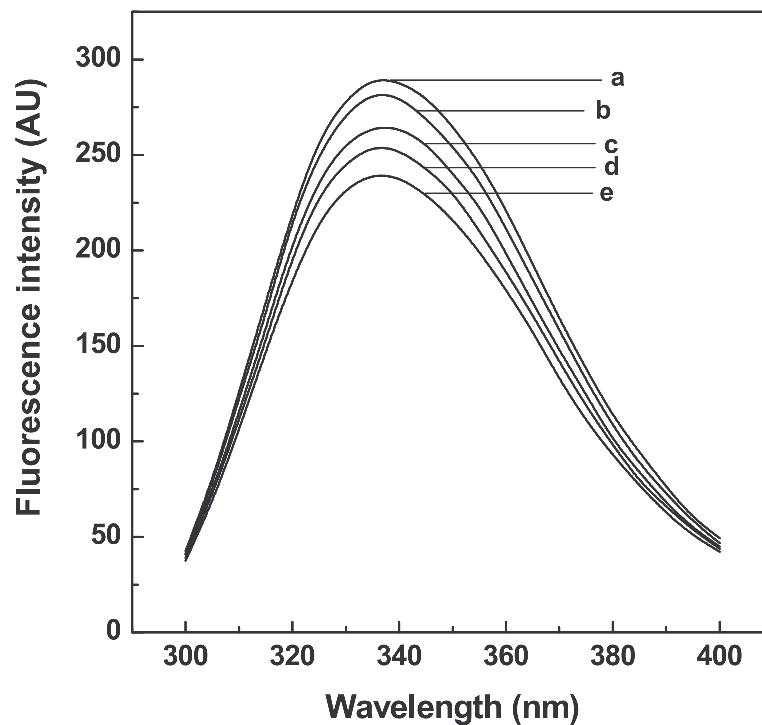


Fig. 38: Fluorescence emission spectra of endoglucanase in presence of concentrations of sorbitol in 20 mM sodium acetate buffer pH 5.0. (a) control (in buffer only), (b) in 10%, (c) in 20%, (d) in 30% and (e) in 40% sorbitol.

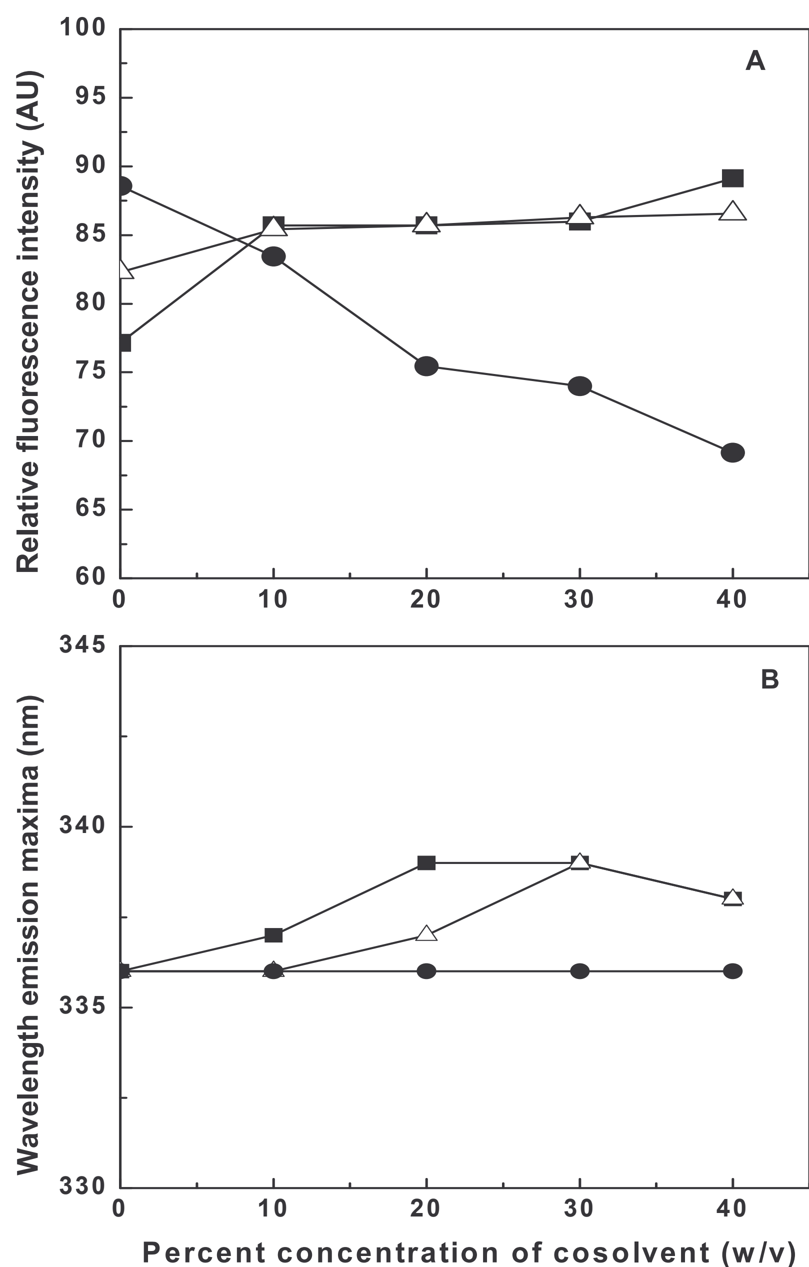


Fig. 39: (A) Changes in the fluorescence intensity of endoglucanase as a function of cosolvent. (B) Changes in fluorescence emission maxima of endoglucanase as a function of cosolvent concentrations. (■) glycerol (●) sorbitol and (Δ) sucrose.

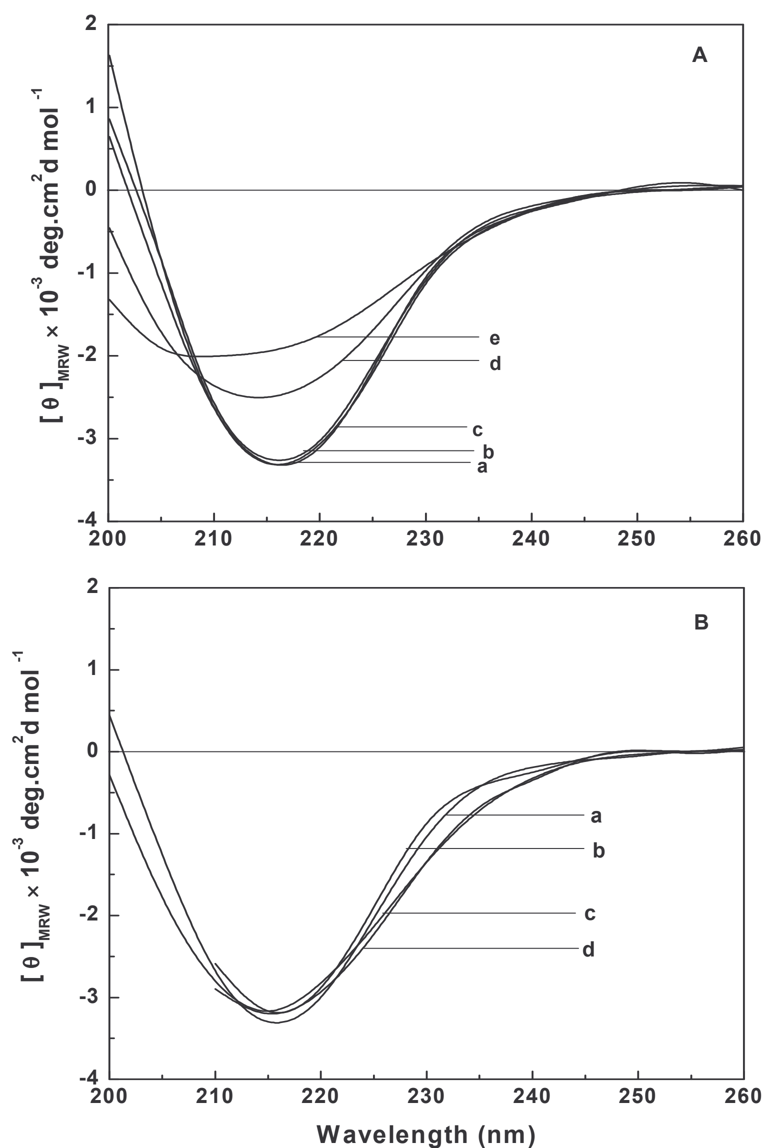


Fig. 40: (A) Far UV circular dichroic spectra of endoglucanase in the wavelength range 200-260 nm at the following temperatures for 15 min (°C)without cooling: (a) control (20); (b) 30; (c) 40 (d) 55 and (e) 70. (B) Far UV CD spectra of endoglucanase in presence and absence of cosolvents at 70°C in 20 mM sodium acetate buffer pH 5.0. (a) Native enzyme at 20°C; (b) Enzyme exposed to 70°C in presence of 40% glycerol; (c) Enzyme exposed to 70 °C in presence of 40 % sucrose and (d) Enzyme exposed to 70 °C in presence of 40% sorbitol.

enzyme exposed to higher temperature in absence of cosolvents (Table 10), where as in presence of cosolvents, the enzyme retains almost identical amount of β-sheet and α-helix contents as shown in Fig. 40B. The aperiodic structure increased to 34% for the enzyme exposed to higher temperature in comparison to the native enzyme. The enzyme exposed to high temperature in presence of cosolvents had a lower aperiodic structure compared to that in absence of cosolvents. This clearly shows that the cosolvents protect the enzyme to retain the native like conformation at higher temperatures. Similar observations have been shown for many proteins that cosovlents stabilize the protein molecules without affecting the secondary structure (Anuradha and Prakash, 2008; Gunasekhar and Prakash, 2008).

The stabilization of endoglucanase by different cosolvents to different extents as evident by the activity measurements, fluorescence and CD measurements has been further confirmed by apparent thermal denaturation temperature (T_m) measurements of the enzyme both in presence and absence of these cosolvents. The changes in the absorbance of the protein at 287 nm were monitored as a function of temperature in the range of 30 - 90 °C. From the changes observed, the apparent T_m of the endoglucanase was calculated from the plots of fraction unfolded versus temperature. In Fig. 41 is shown the thermal denaturation profile of endoglucanase in presence of different concentration of glycerol and sucrose. The thermal denaturation curve of endoglucanase indicated a shift in the apparent T_m as a function of glycerol concentrations. The apparent T_m shifted from a control value of 57 ± 1°C to a value of 64 ± 1°C in presence of 40% glycerol. The apparent T_m of endoglucanase in presence of 10, 20 and 30% glycerol was 58 ± 1, 61 ± 1 and 63 ± 1 °C respectively (Fig. 41A).

Table 10: Secondary structural content of endoglucanase as a function of temperature in 20 mM sodium acetate buffer pH 5.0.

Temperature	α- helix (%)	β- structure (%)	Random coil (%)
Control (20°C)	14 ± 1	62 ± 1	24 ± 1
55°C	9 ± 1	59 ± 1	32 ± 1
70°C	6 ± 1	54 ± 1	40 ± 1

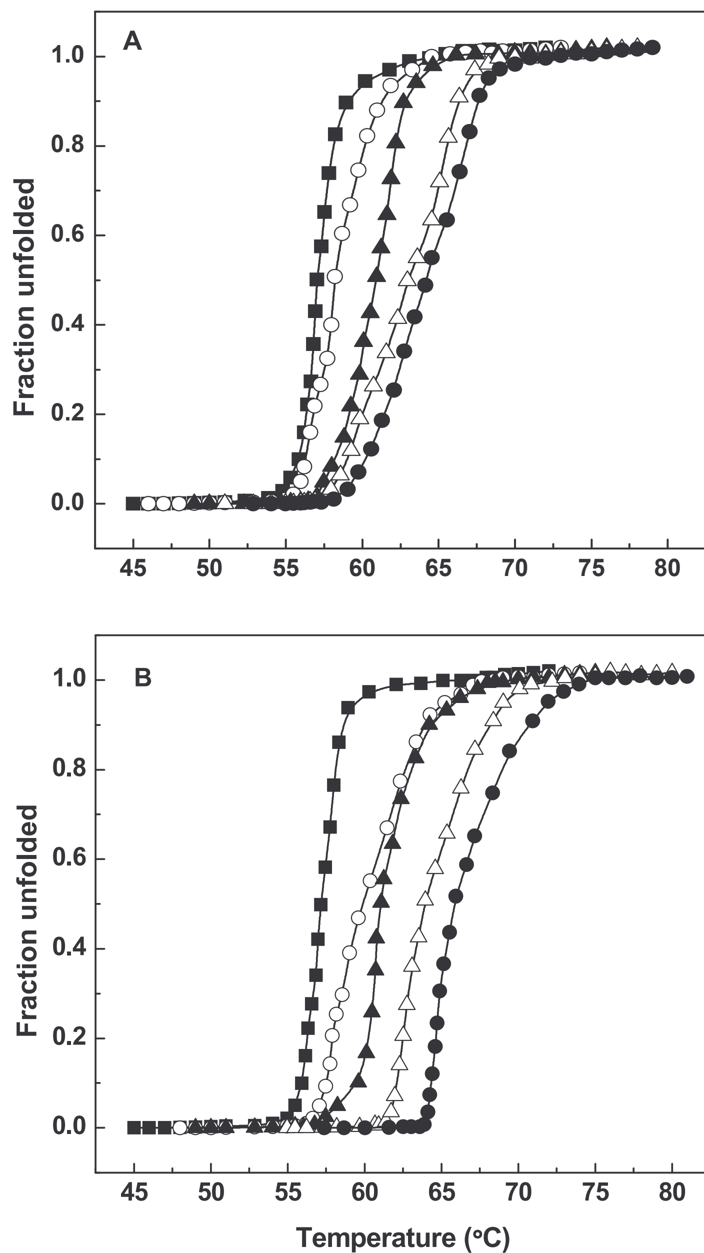


Fig. 41: Apparent thermal denaturation curves of endoglucanase in presence of different concentrations of (A) glycerol and (B) sucrose in 20 mM sodium acetate buffer pH 5.0. The change in absorbance at 287 nm was measured as a function of temperature (25-90 °C) with 1 °C /min. (■) Control, (○) in 10%, (▲) in 20%, (Δ) in 30% and (●) in 40% cosolvents of (A) and (B), respectively.

Fig. 41B and 42 shows the thermal denaturation profile of endoglucanase in presence of sucrose and sorbitol, respectively. The analysis of thermal denaturation profiles indicates a shift in the apparent thermal melting point temperature of endoglucanase as a function of cosolvent concentration. Here, the maximum shift in the T_m was seen in 40% sucrose and sorbitol with the apparent T_m values of 66 ± 1 and 64 ± 1°C, respectively compared to 57 ± 1°C of the control. The T_m values of 59 ± 1, 61 ± 1and 64 ± 1°C were determined from the thermal denaturation curves in presence of 10%, 20% and 30% sucrose, respectively. The apparent thermal denaturation temperature value of endoglucanase obtained for all the cosolvents used is summarized in Table 11. It is evident from the results that the cosovlents used in these studies were able to increase the apparent Tm of endoglucanase to different extents in a concentration dependent manner. This increase in the thermal denaturation temperature of endoglucanase in presence of cosolvents is a result of protection of enzyme by cosolvents against thermal inactivation. This increment in the thermal denaturation temperature of proteins and enzymes in presence of different cosolvents have been extensively studied (Gekko and Timasheff, 1981a; Zancan and Sola-Penna, 2005; Anuradha and Prakash, 2008). Thus, for all the cosolvents used the extent of thermal stabilization is dependent on the concentration of the cosolvents used.

The results of apparent T_m of the enzyme in presence of cosolvents indicate that endoglucanase is stabilized by cosolvents against thermal denaturation. The stabilization of proteins by cosolvents against the thermal denaturation has been studied (Timasheff, 1981; Rajendran *et al.*, 1995). During the thermal denaturation, the enzyme unfolds exposing the hydrophobic clusters of the protein to the solvent, increasing the surface area

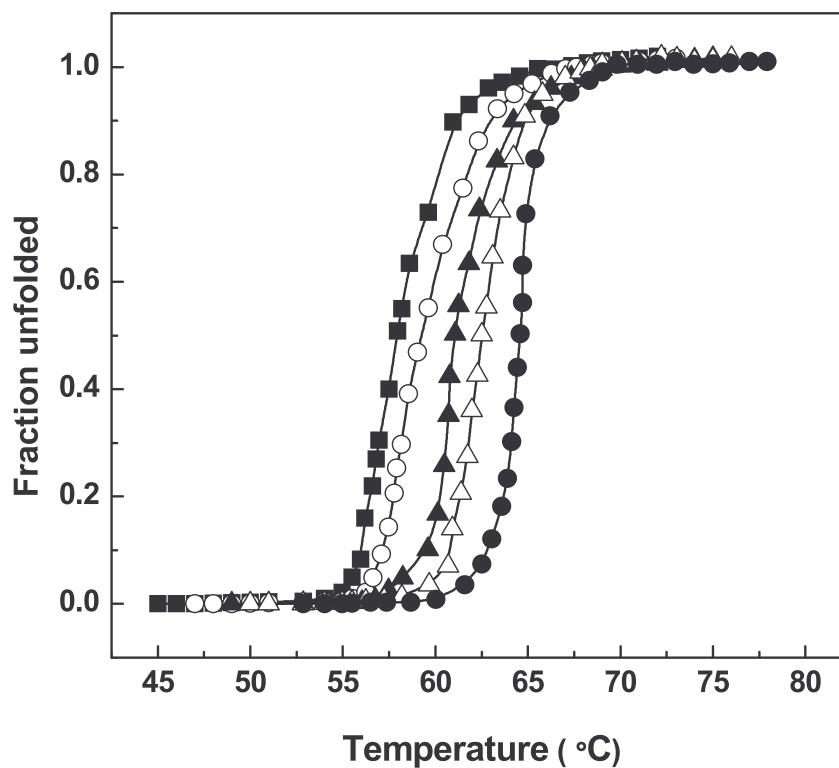


Fig. 42: Thermal denaturation profile of endoglucanase in the presence and absence of sorbitol in 20 mM sodium acetate buffer pH 5.0. The spectra were measured at 287 nm as a function of temperature in the range of 25 - 90 °C with 1°C/min increase. (■) Control, (○) 10%, (▲) 20%, (Δ) 30% and (●) 40%.

Table 11: Apparent thermal denaturation temperature of endoglucanase in presence of different cosolvents

Cosolvent concentration (%) (w/v)	Apparent T_m (°C)		
	Sorbitol	Sucrose	Glycerol
Control*	57 ± 1	57 ± 1	57 ± 1
10	59 ± 1	59 ± 1	58 ± 1
20	61 ± 1	61 ± 1	61 ± 1
30	62 ± 1	64 ± 1	63 ± 1
40	64 ± 1	66 ± 1	64 ± 1

**The endoglucanase in 20 mM sodium acetate buffer, pH 5.0 is control*

of contact between the protein and solvent. In the presence of cosolvents, the unfolding of the protein molecule needs more energy than the aqueous solution alone. Hence, higher temperatures are needed for the denaturation in presence of these cosolvent systems (Timasheff and Arakawa, 1997; Satish *et al*., 2007).

The above results indicate that the endoglucanase is stabilized by cosovlents against thermal denaturation and also maintains its structure. The stabilizing effect of these cosolvents depends on the physical properties of the protein and also the nature of cosolvent molecule. In order to understand the mechanism of interaction of endoglucanase with cosolvents, partial specific volume measurements were carried out in presence of these cosolvents using precision densitymetry method. In a three component system, water is considered as component one, protein as component two and cosolvent forms the third component according to notation of Stockmayer (1950). The presence of third component alters the solvent structure around the protein molecule which in turn affects the preferential interactions of cosolvent and partial specific volume of the protein (Satish *et al*., 2007).

The partial specific volume of endoglucanase was measured as a function of different protein concentrations in 20 mM sodium acetate buffer, pH 5.0, under both isomolal and isopotential conditions at 20°C. The apparent partial specific volume value of 0.723 ± 0.001 mL/g was obtained at isomolal and isopotential conditions, in buffer only. The apparent partial specific volume of endoglucanase was measured in different concentrations of cosolvents under isomolal and isopotential conditions. Fig. 43 shows the representative plot of apparent partial specific volume of endoglucanase in

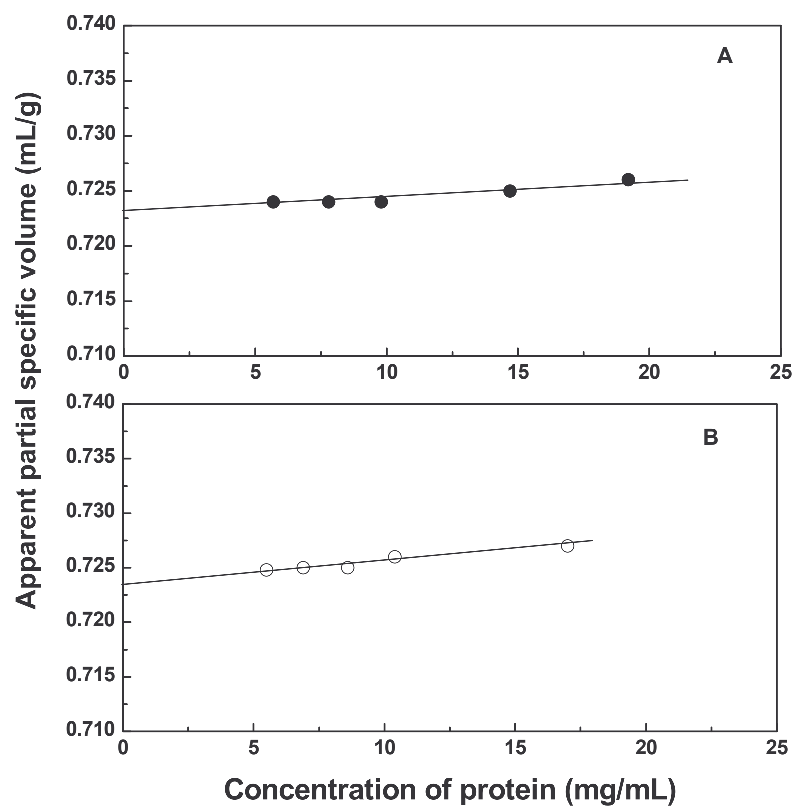


Fig. 43: Apparent partial specific volume of endoglucanase in 20 mM Sodium acetate buffer, pH 5.0 under isomolal (A) and isopotential (B) conditions at 20°C.

buffer alone. The isomolal and isopotential value of endoglucanase was almost constant as compared to the native value.

In Fig. 44, the representative plots apparent partial specific volume of endoglucanase as a function of different concentrations of glycerol is shown. In all the concentrations, there was little or no change in the apparent partialspecific volume of endoglucanase under isomolal condition. But, under isoptential condition, there was an increase in the apparent partial specific volume of endoglucanase as the concentration of glycerol increases. The apparent partial specific volume values of 0.731 ± 0.001, 0.735 ± 0.002, 0.743 ± 0.003 and 0.745 ± 0.003 mL/g were obtained in presence of 10, 20, 30 and 40% glycerol, respectively. From the isomolal and isopotential partial specific volume data, preferential interaction parameters of endoglucanase in presence of these cosolvents were calculated. Table 12 summarizes the apparent partial specific volumes and preferential interaction parameters of endoglucanase at different concentrations of glycerol. As seen from the table, the preferential interaction parameter $(\delta g_3/\delta g_2)_{T,\ \mu1,\ \mu3}$ was found to be negative, indicating the exclusion of glycerol from the domain of the protein.

The effect of different concentrations of sucrose on the apparent partial specific volume of endoglucanase has been shown in Fig. 45 under both isomolal and isopotential conditions. There was little or no change in the apparent partial specific volume of endoglucanase under isomolal condtion. But, under isopotential condtion, there was an increase in the apparent partial specific volume of endoglucanase as the concentration of sucrose increases. The apparent partial specific volume values of 0.744 ± 0.002, 0.746 ± 0.003, 0.751 ± 0.002 and 0.756 ± 0.003 mL/g were obtained in presence of 10, 20, 30 and 40% sucrose, respectively.

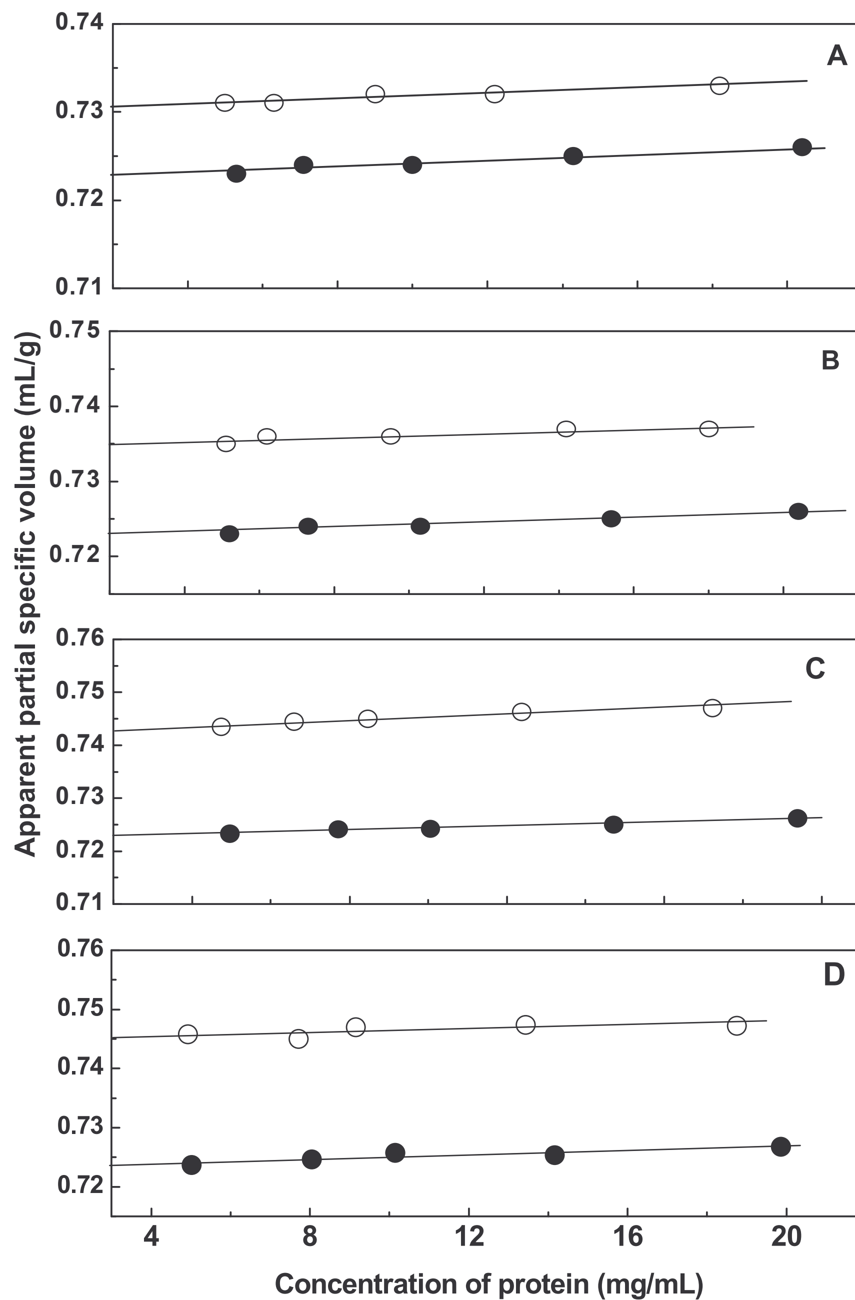


Fig. 44: Apparent partial specific volume of endoglucanase in 20 mM Sodium acetate buffer, pH 5.0 in presence of (A) 10%, (B) 20%, (C) 30% and (D) 40% glycerol under isomolal (●) and isopotential (○) conditions at 20 °C.

Table 12: Apparent partial specific volume and preferential interaction parameters of endoglucanase in glycerol

parameters	Concentration of glycerol (%) (w/v)			
	10	20	30	40
ϕ^0_2 (mL/g)	0.723 ± 0.001	0.724 ± 0.001	0.724 ± 0.001	0.723 ± 0.001
ϕ'^0_2 (mL/g)	0.731 ± 0.001	0.735 ± 0.002	0.743 ± 0.003	0.745 ± 0.003
g_3 (g/g)	0.108	0.237	0.391	0.580
m_3 (mol of solvent /1000g water)	1.18	2.57	4.25	6.30
$\delta g_3/\delta g_2)_{T, \mu1\ \mu3}$(g/g)	-0.033 ± 0.01	-0.051 ± 0.02	-0.099 ± 0.03	-0.126 ± 0.06
$(\delta g_1/\delta g_2)_{T, \mu1\ \mu3}$(g/g)	0.313 ± 0.04	0.219 ± 0.03	0.253 ± 0.03	0.218 ± 0.03
$(\delta m_3/\delta m_2)_{T, \mu1\ \mu3}$ (mol/mol)	-16.31 ± 1.40	-25.41 ± 4.40	-48.45 ± 1.40	-61.90 ± 8.40

The control app. partial specific volume of endoglucanase under isomolal and isopotential conditions is 0.723 mL/g in 20 mM sodium acetate buffer, pH 5.0.

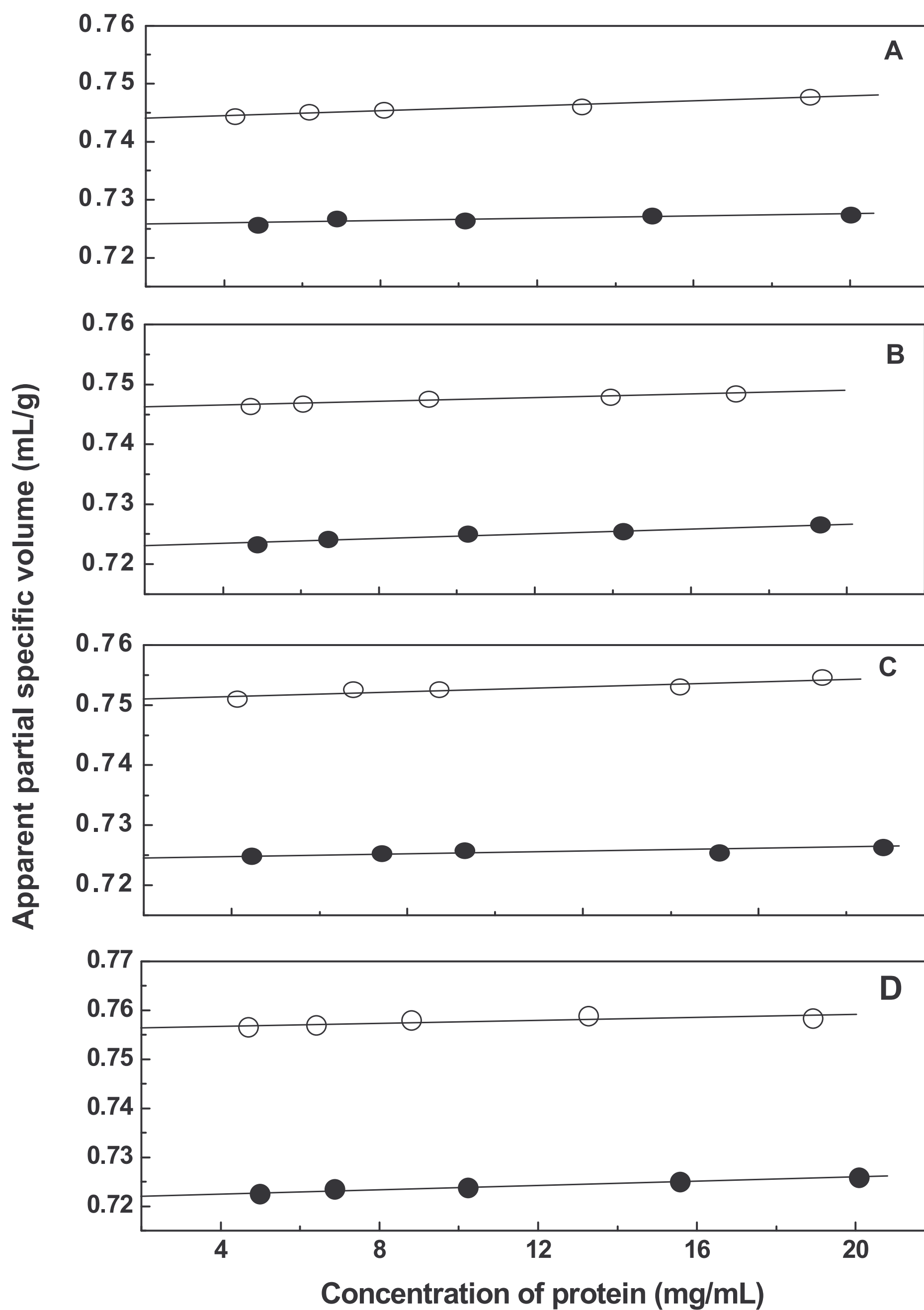


Fig. 45: Apparent partial specific volume of endoglucanase in 20 mM Sodium acetate buffer, pH 5.0 in presence of (A) 10%, (B) 20%, (C) 30% and (D) 40% sucrose under isomolal (●) and isopotential (○)conditions at 20 °C.

Table 13 summarizes the apparent partial specific volumes and preferential interaction parameters of endoglucanase at different concentrations of sucrose. The preferential interaction parameter was found to be negative, which indicates preferential exclusion of the sucrose molecules from the protein domain resulting preferential hydration. A negative interaction value increased from -0.051 ± 0.01 to -0.109 ± 0.04 on g/g basis for 10% to 40%, respectively.

In Fig. 46 is shown the partial specific volume of endoglucanase as a function of different concentrations of sorbitol in the range of 10-40%. The apparent partial specific volume of endoglucanase under isomolal condition did not show any change, while in isopotential condition, there was an increase in the apparent partial specific volume. The apparent partial specific volume values of 0.742 ± 0.002, 0.745 ± 0.003, 0.748 ± 0.002 and 0.751 ± 0.003 mL/g were obtained in presence of 10, 20, 30 and 40% sorbitol, respectively. The preferential interaction parameters are shown in Table 14. The preferential interaction parameter was found to be negative, with a value of -0.057 ± 0.01 to -0.104 ± 0.03 on g/g basis for 10% to 40%, respectively indicating preferential exclusion of the sorbitol molecules from the protein domain.

Thus, the results of partial specific volume indicated that the enzyme is stabilized in all the cosolvents used in a concentration dependent manner and the preferential interaction parameter indicates the extent of cosolvent exclusion. From Table 12, 13 and 14 it is clear that the preferential interaction parameter (ξ_3) is a negative value for all the cosolvents used, which indicate the phenomenon of preferential hydration or exclusion of the cosolvent

Table 13: Apparent partial specific volume and preferential interaction parameters of endoglucanase in sucrose

parameters	Concentration of sucrose (%) (w/v)			
	10	20	30	40
ϕ^0_2 (mL/g)	0.725 ± 0.001	0.723 ± 0.001	0.724 ± 0.001	0.722 ± 0.001
ϕ'^0_2 (mL/g)	0.744 ± 0.002	0.746 ± 0.003	0.751 ± 0.002	0.756 ± 0.003
g_3 (g/g)	0.106	0.229	0.370	0.537
m_3 (mol of solvent /1000g water)	0.31	0.67	1.08	1.57
$\delta g_3/\delta g_2)_{T, \mu1\, \mu3}$(g/g)	-0.051 ± 0.01	-0.068 ± 0.02	-0.079 ± 0.02	-0.109 ± 0.04
$(\delta g_1/\delta g_2)_{T, \mu1\, \mu3}$(g/g)	0.478 ± 0.02	0.298 ± 0.04	0.214 ± 0.03	0.204 ± 0.03
$(\delta m_3/\delta m_2)_{T, \mu1\, \mu3}$ (mol/mol)	-6.72 ± 0.90	-9.01 ± 1.20	-10.43 ± 1.40	-14.44 ± 2.10

The control app. partial specific volume of endoglucanase under isomolal and isopotential conditions is 0.723 mL/g in 20 mM sodium acetate buffer, pH 5.0.

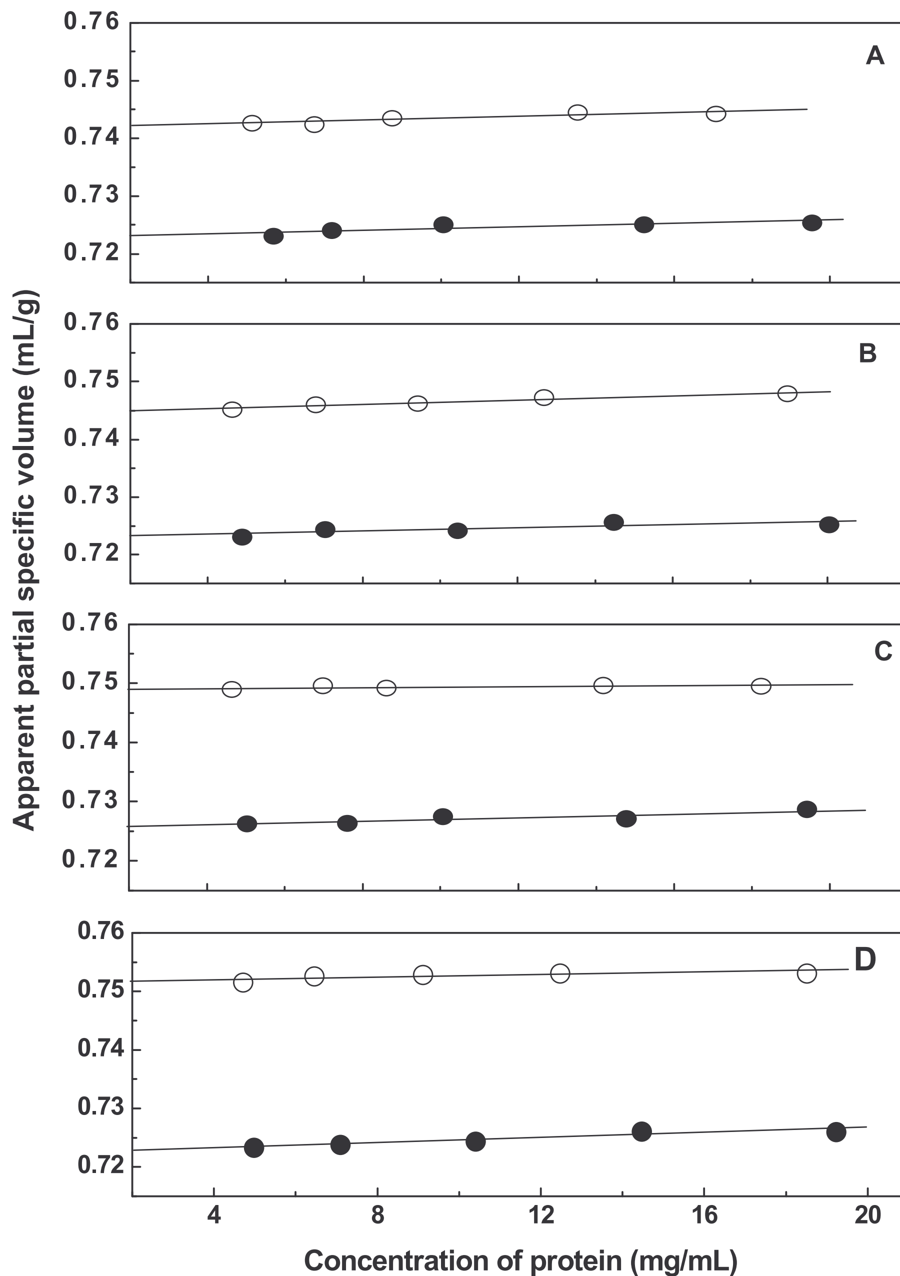


Fig. 46: Apparent partial specific volume of endoglucanase in 20 mM Sodium acetate buffer, pH 5.0 in presence of (A) 10%, (B) 20%, (C) 30% and (D) 40% sorbitol under isomolal (●) and isopotential (○) conditions at 20°C.

Table 14: Apparent partial specific volume and preferential interaction parameters of endoglucanase in sorbitol

parameters	Concentration of sorbitol (%) (w/v)			
	10	20	30	40
ϕ^0_2 (mL/g)	0.723 ± 0.001	0.723 ± 0.001	0.726 ± 0.001	0.723 ± 0.001
ϕ'^0_2 (mL/g)	0.742 ± 0.002	0.745 ± 0.003	0.748 ± 0.002	0.751 ± 0.003
g_3 (g/g)	0.107	0.231	0.375	0.548
m_3 (mol of solvent /1000g water)	0.59	1.27	2.07	3.03
$\delta g_3/\delta g_2)_{T, \mu 1\ \mu 3}$(g/g)	-0.057 ± 0.01	-0.072 ± 0.02	-0.074 ± 0.02	-0.104 ± 0.03
$(\delta g_1/\delta g_2)_{T, \mu 1\ \mu 3}$(g/g)	0.538 ± 0.02	0.313 ± 0.04	0.197 ± 0.03	0.190 ± 0.03
$(\delta m_3/\delta m_2)_{T, \mu 1\ \mu 3}$ (mol/mol)	-14.35 ± 1.50	-18.02 ± 2.10	-18.40 ± 2.40	-26.01 ± 3.40

The control app. partial specific volumes of endoglucanase under isomolal and isopotential conditions was 0.723 mL/g in 20 mM sodium acetate buffer, pH 5.0.

component from the domain of the protein molecule. The maximum hydration was observed in case of glycerol where the preferential interaction parameter was -0.126 ± 0.06 g/g and the lowest value was observed in case of 10% glycerol having a value of -0.033 ± 0.01 g/g (Fig.47). The preferential parameter calculated on a mol/mol basis is also summarized in the tables.

Polyols have been used to stabilize proteins (Gekko and Timasheff, 1981a; Gekko and Ito, 1990). Addition of sugars to protein in aqueous solution to increase the stability is shown by several studies (Back *et al*., 1979; Arakawa and Timasheff 1982) and polyhydric alcohols (Gekko and Morikawa, 1981, Graber and combes, 1989). The stabilization effect of these additives is attributed to their effect on water structure around the domain of the protein which inturn enhances the hydrophobic interactions between the non-polar groups of the protein molecules. The measurements of preferential interactions of cosolvents such as glycerol, sorbitol and sucrose have clearly indicated that endoglucanase is preferentially hydrated by all cosolvents.

In a three-component system, preferential hydration of a protein is a good indication that the third component is stabilizer of the structure of macromolecule (Pittz and Timasheff, 1978; Lee and Timasheff, 1981; Arakawa and Timasheff, 1982). A number of studies with a variety of proteins in different cosolvent systems have explained the phenomenon of preferential hydration (Rajeshwara and Prakash, 1994b; Rajendran *et al*., 1995; Radha *et al*., 1998). According to their hypothesis, sugars or polyols are preferentially excluded from the domain of the protein leading to unfavorable interactions between the protein and the solvent used as a result of ordering of the water

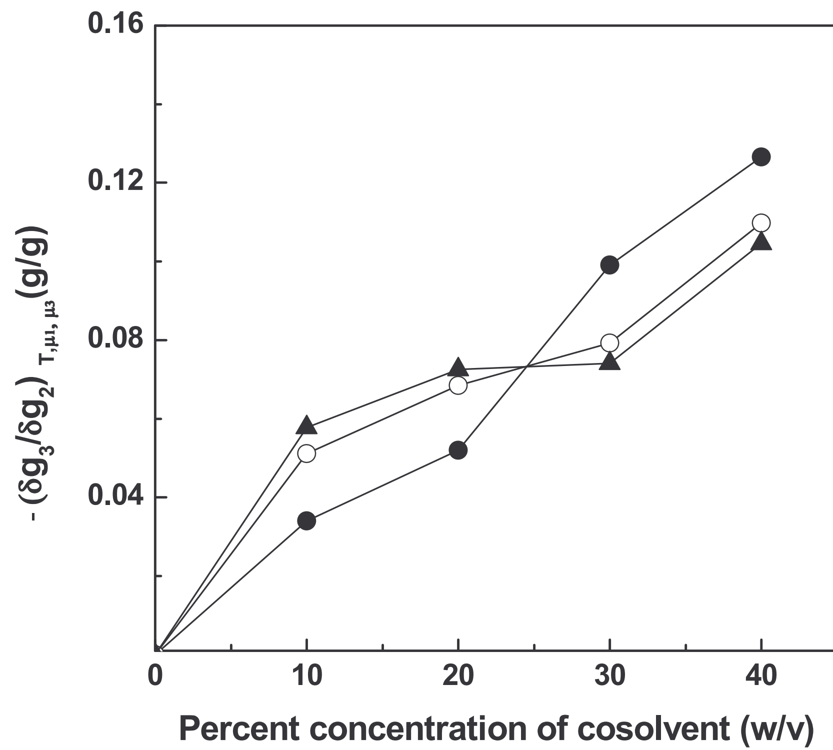


Fig. 47: Preferential interaction parameter (ξ_3) of endoglucanase as a function of cosolvent concentrations in 20 mM sodium acetate buffer pH 5.0. In presence of (●) Glycerol, (○) sucrose and (▲) sorbitol.

structure around the protein molecules. This in turn increases the free energy of the system, but more for the unfolded state of the protein due to large surface area, leading to increased unfavorable protein-water interactions. The degree of exclusion and increase in the chemical potential are directly proportional to the surface area of the protein. The native structure of the protein is stabilized thermodynamically, as the folded state is favored energetically in the equilibrium between the folded and the unfolded states of the protein.

The results showed that endoglucanase had four-fold activity in presence of 40% concentration of the cosolvents. The activity measurements at higher temperature showed that presence of cosolvents prevent loss of activity against thermal denaturation of endoglucanase. The thermal denaturation studies showed an increase in the apparent T_m of endoglucanase in a concentration dependent manner in presence of cosolvents. The results show that endoglucanase is stabilized by addition of cosolvents such as glycerol, sorbitol and sucrose. Addition of cosolvents does not induce any significant structural changes in the protein molecule as evidenced by results of fluorescence and CD spectroscopic measurements. The results obtained from the studies of preferential interaction of cosolvents with endoglucanase show that cosolvents are preferentially excluded from the domain of protein molecule. The preferential hydration parameter was found to be positive and the preferential interaction parameter was found to be negative in presence of all cosolvents, clearly indicating the phenomenon of preferential hydration or exclusion of cosolvents from the protein domain. These results indicate that the stabilization of endoglucanase is brought about by preferential hydration as indicated by preferential interaction measurements.

SUMMARY AND CONCLUSIONS

The knowledge of protein structure-function and stability relationship is very important from both academic and commercial point of view. Enzymes despite their potential role as catalysts, find wide applications in various industrial processes which are limited due to instability in solution. The limitation for their application is the instability under operational conditions due to exposure to extreme temperatures, pressure, pH, denaturants and organic solvents. Thus, in order to utilize these enzymes to its full potential, the structure-function stability relationship of enzymes needs to be understood. Thus, a detailed elucidation of the mechanism of its action and factors affecting stabilization and destabilization is important. A well defined three-dimensional structure is essential for an enzyme to perform its biological function, for which the enzyme structural stability and catalytic activity are interlinked.

The polymers cellulose and hemicellulose constitute the major portion of the organic matter on our planet. They form the cell walls of plants and are consequently the principal components of wood, cotton and grass. Cellulose, as a renewable raw material, is an ideal resource for the industrial production of paper, textiles and synthetic products. Cellulases from fungal origin are known to be most powerful in cellulose hydrolysis. These microorganisms produce a multi-component enzyme system consisting of endoglucanase, exoglucanase and β-glucosidase. A combination of these three types of enzymes is necessary for the complete hydrolysis of crystalline cellulose. Endoglucanase and exoglucanase are known to act synergistically in cellulose hydrolysis, while β-glucosidase is needed for removal of cellobiose, a strong

inhibitor of both endoglucanase and exoglucanase. The diversity of endoglucanase, exoglucanases and β-glucosidases from fungal origin is emphasized by many investigators. In the present study, an attempt is made to purify and characterize the enzyme from crude cellulases produced from *Aspergillus aculeatus* obtained commercially. *A. aculeatus* being large producer of enzyme endoglucanase, which was taken up for further isolation and characterization.

The structural stability of proteins is affected by a number of factors like water and salts. Cofactors play an important role in the catalytic activity and structural stability of proteins. They serve a variety of functions in proteins like enhancing the catalytic activity and some of them have the ability to inhibit. These influence the structural stability by altering the conformation of the proteins. The mode of action of these is very specific and depends on the nature of each cofactor. The pH and denaturants like urea and GuHCl are known to unfold the protein molecules and alter the hydrophobic interactions leading to the significant conformational changes with modified functionality. Cosolvents play an important role in the structural stability of the proteins which is very specific and dependent on the nature of the cosolvent used. The effect of cofactors, pH, denaturants and cosolvents on the structure, function and stability of the cellulase has been carried out. The mechanism of stabilization was studied by measuring the catalytic activity, fluorescence spectroscopy, circular dichroic spectroscopy and partial specific volume measurements.

A brief outline of the important results obtained in the present investigation is summarized as below.

Chapter 1: 1.1 Purification and Characterization of Endoglucanase from commercial source
1.2. Effect of Cofactors on Structure and function of endoglucanase

This chapter describes the isolation, purification and characterization of endoglucanase produced from *Aspergillus aculeatus* obtained commercially and effect of cofactors on the optimization of catalytic activity has been worked in greater detail. Endoglucanase was purified to homogeneity and the purity was checked by SDS-PAGE and FPLC. The amino acid composition of the isolated endoglucanase was in correlation with the literature values of other related endoglucanases. The optimum temperature and pH were found to be 40°C and 5.0, respectively. The isoelctric point was found to be 4.3. Lineweaver-Burk analysis showed that K_m was 0.060 ± 0.001% and V_{max} was 0.08 units (mg of protein)$^{-1}$ min^{-1}. The end product analysis was carried out by HPLC. The release of glucose and cellobiose as the end products during incubation with carboxymethyl cellulose as substrate confirmed it as endoglucanase. The secondary structure measurements carried out by far-UV CD spectra, showed that endoglucanase has 64% beta-sheets and 14% α-helical structures. The purified endoglucanase lost 50% of its activity at 90°C.

In the presence of dithiotreitol, endoglucanase activity was reduced to 60% compared to the control, indicating that disulfide bonds play an important role at the catalytic site. N-bromosuccinimide did not show any effect on the endoglucanase activity, showing that tryptophan is not involved in the catalytic activity. Carboxymethylation of the histidyl residues of the enzyme with diethylpyrocarbonate showed a 70% loss in the activity suggesting that these residues participated mechanistically and also in the

maintenance of the conformation necessary for the active enzyme. It may be playing a role in the binding of the substrate to the enzyme and proper orientation of the catalytic site of the enzyme for its hydrolysis. The participation of specific carboxyl groups in the active site of the enzyme were indicated by N- (3-Dimethylaminopropyl)-N-ethylcarbodiimide modification of endoglucanase where it was completely inhibited.

The catalytic activity of endoglucanase was not affected by the presence of metal ions (calcium, zinc, iron and copper). The fluorescence emission spectra of endoglucanase in presence of cofactors did not show any change, indicating that there was no change in the microenvironment of the aromatic chromophores. The far-UV CD spectra of endoglucanase in presence of cofactors did not show any change in the secondary structures. The enzyme activity was found to increase with increase in the concentration of ethylenediamine tetra acetic acid. The catalytic activity of endoglucanase was 1.5 fold in presence of 5 mM ethylenediamine tetra acetic acid. In presence of ethylenediamine tetra acetic acid, K_m decreased to 0.040 ± 0.001 % compared to control and had a K_{cat} of 1.9 ± 0.2 min^{-1}. The decrease in the K_m and increase in the K_{cat} shows that the endoglucanase has attained the maximum catalytic efficiency in presence of ethylenediamine tetra acetic acid. The fluorescence emission spectra of endoglucanase in presence of ethylenediamine tetra acetic acid showed no changes. The endoglucanase showed 20% increase in the ellipticity value at 217 nm in presence of ethylenediamine tetra acetic acid. The apparent thermal transition temperature (T_m) of native endoglucanase increased from a control value of 57°C to 76°C in presence 5 mM ethylenediamine tetra acetic acid. The endoglucanase isolated from *A.*

aculeatus is subjected to further detailed investigations in the next chapters to understand the structure-function and stability relationship of the enzyme.

Chapter 2: Effect of pH, urea and GuHCl on the folding and unfolding of endoglucanase

This describes the effect of pH, urea and guanidine hydrochloride on the folding and unfolding of endoglucanase. The pH induced denaturation of endoglucanase was followed over a pH range of 2.0 - 5.0. The pH denaturation is irreversible and the enzyme loses 50% of its activity at pH 2.0. Far-UV CD spectra of endoglucanase did not show any change in the secondary structure at pH 2.0. The 8-anilino-1-naphthalene sulphonic acid binding studies showed increased fluorescence intensity at pH 2.0, indicating the exposure of hydrophobic amino acids to the solvent. The pH induced denaturation leads to the formation of a partially folded intermediate at low pH with characteristic features of a molten globule.

In order to further characterize unfolding process, the effect of urea and guanidine hydrochloride on the structure and function were followed at pH 5.0. In presence of 2 M urea, the K_m was 0.035 ± 0.001% and K_{cat} was 3.0 ± 0.3 min^{-1}, where as in the presence of guanidine hydrochloride, the K_m was found to be 0.041 ± 0.001% and K_{cat} was 2.5 ± 0.2 min^{-1}. The decrease in the K_m and increase in the catalytic constant suggests that endoglucanase is having more affinity towards the substrate and has maximal catalytic efficiency in low concentrations of the denaturants. Further, the activity decreased with increasing concentrations of these denaturants. The T_m value increased to 60 and 61°C in presence of 2 M urea and guanidine hydrochloride, respectively compared to control value of 57°C, which indicates stabilization of

endoglucanase at low concentrations. The urea and GuHCl induced equilibrium unfolding of endoglucanase was followed by changes in the fluorescence emission maximum and ellipticity values at 217 nm as probes indicating that the transition is sigmoidal and follows a simple two-state transition. The results obtained from folding and unfolding studies showed differences in stability towards denaturation by urea and guanidine hydrochloride.

Chapter 3: Effect of cosolvents on structure and stability of endoglucanase

In this chapter, the structural stabilization of endoglucanase in presence of cosolvents such as glycerol, sorbitol and sucrose has been described. The endoglucanase showed four-fold increase in the activity at 40% concentration of the cosolvents. In presence of these cosolvents, the endoglucanase was able to maintain its original activity at 90°C. The apparent T_m value of the endoglucanase was found to be 57°C. All the cosolvents are able to elevate the apparent T_m to different extents in a concentration dependent manner. The structural integrity of endoglucanase was monitored by fluorescence and circular dichroic spectral measurements. Fluorescence spectra did not show any changes in presence of cosolvents. Far-UV CD spectra of endoglucanase showed no significant conformational changes in the protein molecule in presence of these cosolvents.

In order to understand the mechanism of cosolvent-induced stabilization of endoglucanase, the partial specific volume and preferential interaction parameters were determined in presence of cosolvents. The apparent partial specific volume of endoglucanase under isomolal and

isopotential condition is 0.723 ± 0.001mL/g in sodium acetate buffer at pH 5.0. The preferential interaction parameters showed that for all the cosolvents the preferential hydration parameter is positive and the preferential interaction parameter is negative indicating preferential hydration or exclusion of cosolvents from the protein domain. The preferential interaction parameter was found to be maximum with -0.126 ± 0.06 g/g in 40% glycerol and minimum value of -0.033 ± 0.01 g/g in 10% glycerol. These results indicate that stabilization of endoglucanase is due to preferential hydration. Thus, these cosolvents stabilize the enzyme against thermal denaturation which is dependent on the concentration and nature of the cosolvent used.

REFERENECES

Abuja, P. M., Pilz, I., Claeyssens, M. and Tomme, P. (1988a) Domain structure of cellobiohydrolase II as studied by small X-ray scattering, close resemblance to cellobiohydrolase I. *Biochem. Biophys. Res. Commun*. **156**, 180-185.

Abuja, P. M., Schmuck, M., Pilz, I., Tomme, P., Claeyssens, M. and Ester-Bauer, H. (1988b) Structural and functional domains of cellobiohydrolase I from *Trichoderma reesei*. *Eur. Biophys. J*. **15**, 339-342.

Ahmad, B., Shamim, T. A., Haq, S. K. and Khan, R. H. (2007) Identification and characterization of functional intermediates of stem bromelain during urea and guanidine hydrochloride unfolding. *J. Biochem*. **141**, 251-259.

Almin, K. E. and Eriksson, K. E. (1967) Enzymic degradation of polymers, I. Viscometric method for the determination of enzymic activity. *Biochim. Biophys. Acta*. **139**, 238-247.

Altman, J., Lipka, J. J., Kuntz, I. and Waskell, L. (1989) Identification of proton nuclear magnetic resonance of histidines in cytochrome b5 modified by diethylpyrocarbonate. *Biochemistry* **28**, 7516-7523.

Anfinsen, C. B. and Scheraga, H. A. (1975) Experimental and theoretical aspects of protein folding. *Adv. Protein Chem*. **29**, 205-300.

Antony, T., Kumar, S., Chauhan, M., Atreyi, M. and Khatri, G. S. (1996) Effect of Mg^{2+} and denaturants on the unfolding pattern of DNA T, A replication protein of *E. coli*. *Int. J. Biol. Macromol*. **19**, 91-97.

Anuradha, S. N. and Prakash, V. (2008) Structural stabilization of bovine β-lactoglobulin in presence of polyhydric alcohols. *Ind. J. Biotechnol*. **17**, 437-447.

AOAC. (1984) Official Methods of Analysis of the Association of Official Analytical Chemist's (William, S., Ed.), 14th Edn. Association of Official Analytical Chemist's Inc., Virginia, USA, pp. 988.

Arai, M. and Kuwajima, K. (2000) Role of the molten globule state in protein folding. *Adv. Protein Chem*. **53**, 209-271.

Arakawa, T. and Timasheff, S. N. (1982) Stabilization of protein structure by sugars. *Biochemistry* **21**, 6536-6544.

Arakawa, T. and Timasheff, S. N. (1983) Preferential interaction of proteins with solvent components in aqueous amino acid solutions. *Arch. Biochem. Biophys*. **224**, 169-177.

Arunachalam, U. and Kellis, J. T. Jr. (1993) Folding and stability of endoglucanase III, a single domain cellulase from *Trichoderma reesei*. *Biochemistry* **35**, 11379-11385.

Araujo, A. and D'Souza, J. (1986) Characterization of cellulolytic enzyme components from *Aspergillus terreus* and its mutants. *J. Ferment. Technol*. **64**, 463-468.

Ariki, M. and Fukui, T. (1978) Modification of rabbit muscle phosphorylase b by a water-soluble carbidiimide. *J. Biochem. (Tokyo)*, **83**, 183-190.

Back, J. F., Oakenfull, D. and Smith, M. B. (1979) Increased thermal stability of proteins in the presence of sugars and polyols. *Biochemistr*y **18**, 5191-5196.

Bakalova, N., Petrova, S. and Kolev, D. (1996) Separation of enzymes from polyenzyme mixtures used in medicine and pharmacy. I. Isolation and characterization of xylanase from *Aspergillus oryzae*. *Pharmazie* **51**, 761-764.

Bayer, E., Morag, E. and Lamed, R. (1994) The cellulosome- a treasure-trove for biotechnology. *TIBTECH* **12**, 379-386.

Bayer, E., Morag, E., Wilchek, M., Lamed, R., Yaron, S. and Shoham, Y. (1995) Cellulosome domains for novel biotechnological application. In: Petersen, S. B., Svensson, B. and Pedersen, S. (Eds) Carbohydrate bioengineering. Progress in biotechnology. Elsevier, *The Netherlands*. Vol. 10, 251-259.

Beguin, P. and Aubert, J. P. (1994) The biological degradation of cellulose. *FEMS Microbiol. Rev.* **13**, 25-58.

Beguin, P. (1990) Molecular biology of cellulose degradation. *Annu. Rev. Microbiol*. **44**, 219-248.

Beldman, G., Searle-VanLeeuwen, M. F., Rombouts, F. M. and Voragen, F.G. J. (1985) The cellulase of *Trichoderma viride*, purification, characterization and comparison of all detectable endoglucanases, exoglucanases and beta glucosidase. *Eur. J. Biochem*. **146**, 301-308.

Belghith, H., Ellouz-Chaabouni, S. and Gargouri, A. (2001) Biostoning of denims by *Penicillium occitanis* (Po16) cellulases. *J. Biotechnol*. **89**, 257-262.

Berghem, E. R., Pettersson, L. G. and Axio-Fredrikson, U. B. (1976) The mechanism of enzymatic cellulose degradation, purification and some properties of two different 1, 4-β-D-glucan glucanohydrolases from *Trichoderma* V*iride. Eur. J. Biochem*. **61**, 621.

Bhat, K. M. and Bhat, S. (1997) Cellulose degrading enzymes and their potential industrial applications. *Biotechnol. Adv*. **15,** 583-620.

Bhat, M. K. and Bhat, S. (1998) *Closttridium thermocellum* cellulosome: dissociation, isolation and characeterization of subunits and the potential biotechnological implications. In: Pandalai, S. G. (Ed.) Recent Research Developments in Biotechnology and Bioengineering, 1, Part I. Research Signpost, Trivandrum, India, 59-84.

Bhat, M. K. (2000) Cellulases and related enzymes in biotechnology. *Biotechnol. Adv*. **18**, 355-383.

Bidlingmeyer. B. A., Cohen. S. A. and Travin. T. L. (1984) Rapid analysis of the amino acids using pre-column derivartization. *J. Chromatogr*. **336**, 93-104.

Biely, P., Vrsanska, M. and Claeyssens, M. (1991) The endo-1, 4-β-glucanase I from *Trichoderma reesei*. Action on β-1, 4-oligomers and polymers derived from D-glucose and D-xylose. *Eur. J. Biochem*.**200**, 157-163.

Boisset, C., Borsali, R., Schulein, M. and Henrissat, B. (1995) Dynamic light scattering of the two domain structure of *Humicola insolens* endoglucanase V. *FEBS Lett*. **376**, 49-52.

Boren, K., Andersson, P., Larsson, M. and Carlsson, U. (1999) Characterization of a molten globule state of bovine carbonic anhydrase III: loss of asymmetrical environment of the aromatic residues has a profound effect on both the near- and far-UV CD spectrum. *Biochim. Biophys. Acta*. **1430**, 111-118.

Breslow, R. and Guo, T. (1990) Surface tension measurements show that chaotropic salting-in denaturants are not just water-structure breakers. *Proc. Nat. Acad. Sci. USA*. **87**, 167-169.

Casassa, E. F. and Eisenberg, H. (1961) Partial specific volumes and refractive index increments in multicomponent systems. *J. Phys. Chem*. **65**, 427-433.

Casassa, E. F. and Eisenberg, H. (1964) Thermodynamic analysis of multi-component solutions. *Adv. Protein Chem*. **19**, 287-395.

Clarke, A. J. (1987) Essential tryptophan residues in the function of cellulase from *Schizophyllum commune*. *Biochim. Biophys. Acta* **912**, 424-431.

Clarke, A. J. and Adams, L. S. (1987) Irreversible inhibition of *Schizophyllum commune* cellulase by divalent transition metal ions. *Biochim. Biophys. Acta* **916**, 213-219.

Clarke, A. J. and Yaguchi, M. (1985) The role of carboxyl groups in the function of endo-β-1, 4-glucanase from *Schizophyllum commune*. *Eur. J. Biochem*. **149**, 233-238.

Colman, R. F. (1989) In: Protein function: A practical approach (Creighton, T.E., Ed.) IRL Press, Oxford, UK. pp.1442-1478.

Covert, S., Bolduc, J. and Cullen, D. (1992) Genomic organization of a cellulase gene family in *Phanerochaete chrysosporium*. *Curr. Genet*. **22**, 407-413.

Cupo, J. F. and Pace, C. N. (1983) Conformational stability of mixed disulphide derivatives of β-lactoglobulin 'B'. *Biochemistry* **22**, 2654-2658.

d`Amico, S., Marx, J. C., Gerday, C. and Feller, G. (2003) Activity-stability relationships in extremophilic enzymes. *J. Biol. Chem*. **278**, 7891-7896.

Daveis, G. J., Wilson, K. S. and Henrsissat, B. (1997) Nomenclature for sugar-binding subsites in glycoside hydrolases. *Biochem. J*. **321**, 557-559.

Din, N., Forsythe, I. J., Burtnick, L. D., Gilkes, N. R., Miller, R. C. Jr., Warren, R. A. J. and Kilburn, D. G. (1994) The cellulose-binding domain of endoglucanase A CenA from *Cellulomonas fimi*: Evidence for involvement of tryptophan residues in binding. *Mol. Microbiol*. **11**, 747-755.

Din, N., Coutinho, J. B., Gilkes, N. R., Jervis, E., Kilburn, D. G., Miller, R. C. Jr., Ong, E., Tomme, P. and Warren, R. A. J. (1995) In: Interactions of cellulases from *Cellulomonas fimi* with cellulose. (Petersen, S. B., Svensson, B. and Pedersen, S., Eds.) Carbohydrate bioengineering. Progress in biotechnology. Elsevier, The Netherlands. Vol, 10, pp. 261-270.

Divne, C., Stahlberg, J., Reinikainen, T., Ruohonen, L., Pettersson, G., Knowles, J. K. C., Teeri, T. T. and Jones, T. A. (1994) The three-dimentional structure of cellobiohydrolase I from *Trichoderma* reveals a lysozyme like active site on a lectin like framework. *Science* **265**, 524-528.

Divne, C., Stahlberg, J., Teeri, T. T. and Jones, T. A. (1998) High-resolution crystal structures reveal how a cellulose chain is bound in the 50Å long tunnel of cellobiohydrolase I from *Trichoderma reesei*. *J. Mol. Biol*. **275**, 309-325.

Dubey, V. K. and Jagannadham. M. V. (2003) Differences in the unfolding of procerain induced by pH, guanidine hydrochloride, urea and temperature. *Biochemistry* **42**, 12287-12297.

Dubois, M., Gilles, K. A., Hamilton, J. K., Rebers, P. A. and Smith, F. (1956) Colorimetric method for determination of sugars and related substances. *Anal. chem*. **28**, 350-356.

Eftink, M. R. and Ghiron, C. A. (1981) Fluorescence quenching studies with proteins. *Anal. Biochem*. **114**, 199-277.

Eftink, M. R. and Ghiron, C. A., (1976) Exposure of tryptophanyl residues and protein dynamics. *Biochemistry* **16**, 5546-5551.

Ellman, G. L. (1959) Tissue sulfhydryl groups. *Arch. Biochem. Biophys*. **82**, 70-77.

Eriksson, K. E. and Pettersson, G. (1968) Studies on cellulolytic enzymes. V. Some structural properties of the cellulase from *Penicillium notatum*. *Arch. Biochem. Biophys*. **124**, 160-166.

Fan, Y. X., Ju, M., Zhou, J. M. and Tsou, C. L. (1996) Activation of chicken liver dihydrofolate reductase by urea and guanidine hydrochloride is accompanied by conformational change at the active site. *Biochem. J*. **315**, 97-102.

Ferschak, J. D. and Pye, E. K. (1983) Effect of cellobiose, glucose, ethanol and metal ions on the cellulase enzyme complex of *Thermomonospora fusca*. *Biotechnol. Bioeng*. **25**, 2865-2872.

Frigon, R. P. and Lee, J. C. (1972) The stabilization of calf-brain microtubule protein by sucrose. *Arch. Biochem. Biophys*. **153**, 587-589.

Fujita, Y., Yasuchika, I. and Yukinao, N. (1982) The effect of ployhydric alcohols on the thermal denaturation of lysozyme as measured by differential scanning calorimetry. *Bull. Chem. Soc. Jpn*. **55**, 1896-1900.

Gajendra S. N., Purnima Kaul and Prakash, V. (2007) Purification and characterization of a new endoglucanase from *Aspergillus aculeatus*. *J. Agric. Food Chem*. **55**, 7566-7572.

Gallagher, T., Snell, E. E. and Hackett, M. L. (1989) Pyruvuyl-dependent histidine decarboxylase: active site structure and mechanistic analysis. *J. Biol. Chem*. **264**, 12737-12743.

Gangadhara, B. N., Ramesh Kumar, P. and Prakash, V. (2008a) Enhancement of nuclease P1 activity in low concentration of denaturants. *Enzyme Microb. Technol*. **43**, 343-348.

Gangadhara, Ramesh Kumar, P. and Prakash, V. (2008b) Influence of polyols on the stability and kinetic parameters of invertase from *Candida utilis*: correlation with the conformational stability and activity. *Protein J*. **27**, 440-449.

Garrison, K. C. and Himes, R. H. (1975) The reaction between diethylpyrocarbonate and sulfhydryl groups in carboxylate buffers. *Biochem. Biophys.Res. Commun*. **67**, 1251-1255.

Gekko, K. and Ito, H. (1990) Completing solvent effects of polyols and guanidine hydrochloride on protein stability. *J. Biochem*. **109**, 572-577.

Gekko, K. and Timasheff, S. N. (1981a) Mechanism of protein stabilization by glycerol: preferential hydration in glycerol-water mixtures. *Biochemistry* **20**, 4667-4676.

Gekko, K. and Timasheff, S. N. (1981b). Thermodynamic and kinetic examination of protein stabilization by glycerol. *Biochemistry* **20**, 4677-4686.

Gekko, K. (1982) Calorimetric study on thermal denaturation of lysozyme in polyol-water mixtures. *J. Biochem. (Tokyo),* **91**, 1197-1204.

George, A. L. and Borders, C. L. (1979) Essential carboxyl residues in yeast enolase. *Biochem. Biophys. Res. Commun*. **87**, 59-65.

Gerlsma, S. Y. and Sturr, E. R. (1972) Effect of polyhydric and monohydric alcohols on the heat-induced reversible denaturation of lysozyme and ribonuclease. *Int. J. Pept. Protein Res*. **4**, 377-383.

Gilkes, N. R., Henrissat, D. G., Kilburn, R. C., Miller, Jr. and Warren, R. A. J. (1991) Domains in microbial β-1, 4-glycanases: sequence conservation, function and enzyme families. *Microbiol. Rev*. **55**, 305-315.

Glazer, A. N., DeLange, R. J. and Sigman, D. S. (1976) In: chemical modification of protein (Laboratory Techniques in Biochemistry and Molecular Biology,) (Work, T. S. and Work, E. Eds.) North Holland publishing company, Amsterdam, Netherlands Vol. 4, part I.

Glusker, J. P. (1991) Structural aspects of metal liganding to functional groups in proteins. *Adv. Protein Chem*. **42**, 1-76.

Goto, Y. (1991) Role of electrostatic repulsion in the acidic molten globule of cytochrome *c*. *J. Mol. Biol*. **222**, 679-686.

Goto, Y., Calciano, L. J. and Fink, A. J. (1990) Acid induced unfolding of proteins. *Proc. Nat. Acad. Sci. USA.* **87**, 573-577.

Graber, M. and Combes, D. (1989) Effect of polyols on fungal α-amylase thermostability. *Enzyme Microb. Technol*. **11**, 673-677.

Gunasekhar, P.M. and Prakash, V. (2008) Interaction of selected cosolvents with bovine α-lactalbumin. *Int. J. Biol. Macromol*. **42**, 348-355.

Haq, S. K., Rasheedi, S. and Khan, R. H., (2002) Characterization of a partially folded intermediate of stem bromelain at low pH. *Eur. J. Biochem*. **269**, 47-52.

He, R. Q. (1993) Conformation and activity change of D-glyceraldehyde-3-phosphate-dehydrogenase and 1, 4-α-D-glucan maltotetrahydrolase during denaturation by guanidine hydrochloride at low temperature. *Acta Biochem. Biophys. Sin*. **25**, 347-353.

Hedwig, G. R, Lilley, T. H. and Linsdell, H. (1991) Calorimetric and volumetric studies of the interactions of some amide in water and in 6molar dm-3 aqueous guanidine hydrochloride. *J. Chem. Soc., Faraday Trans*. **87**, 2975-2982.

Helmut Uhlig. (1998) Industrial enzymes and their applications, John wiley & Sons, Inc., USA, pp. 89 – 113.

Henrissat, B. and Bairoch, A. (1993) New families in the classification of glycosyl hydrolases based on amino acid sequence similarities. *Biochem. J*. **293**, 781-788.

Henrissat, B. and Bairoch, A. (1996) Updating the sequence-based classification of glycosyl hydrolases. *Biochem. J*. **316**, 695-696.

Henrissat, B. and Davies, G. (1997) Structural and sequence-based classification of glycoside hydrolases. *Curr. Opin. Struct. Biol*. **7**, 637-644.

Henrissat, B. (1991) A classification of glycosyl hydrolases based on amino acid sequence similarities. *Biochem. J*. **280**, 309-316.

Highley, T. L. (1975) USDA Forest Service Research paper FPL 247.

Hoare, D. G. and koshland, D. E. (1967) A method for the quantitative modification and estimation of carboxylic acid groups in proteins. *J. Biol. Chem*. **242**, 2447-2453.

Hoffren A. M., Teeri, T. T. and Teleman, O. (1995) Molecular dynamic simulation of fungal cellulose-binidng domains: differences in molecular rigidity but preserved cellulose-binding surface. *Protein Eng*. **8**, 443-450.

Horiike, K., Tojo, H., Yamano, T. and Nozaki, M. (1983) Interpretation of the Stokes radius of macromolecules determined by gel filtration chromatography. *J. Biochem*. **93** 99-106.

Hrmova, M., Biely, P. and Vrsanska, M. (1989) Cellulose and xylan-degrading enzymes of *Aspergillus terreus* and *Aspergillus niger*. *Enzyme Microb. Technol*. **11**, 610-615.

Imoto, T. and Yamada, H. (1997) Chemical modification. In: protein function: A practical approach. (Creighton, T. E., Ed.), IRL Press, New York, USA. pp. 279-316.

Irwin, D. C., Spezio, M., Walker, L. P. and Wison, D. B. (1993) Activity studies of eight purified cellulases: specificity, synergism and binding domain effects. *Biotechnol. Bioeng*. **42**, 1002-1013.

Iwama, M., Ohtsuki, R., Takaheshi, T. and Irie, M. (1984) Modification of glucosylases from *Rhizopus sp*. With 1-cyclohexyl-3-(-2-marpholinyl-4-ethyl) carbodiimide. *J. Biochem. (Tokyo)*, **96**, 329-336.

Iwasaki, T., Ikeda, R., Hayashi, K. and Funatsu, M. (1965) Biochemical studies on cellulase. II. Properties of two types of cellulase from *Trichoderma koningi*. *J. Biochem*. **57**, 478-487.

Iyer, P. V. and Ananthanarayan, L. (2008) Enzyme stability and stabilization-aqueous and non-aqueous environment. *Process Biochem*. **43**, 1019-1032.

James, J. A., Dixon, G. and Lamikanra, O. (1999) Characterization of partially purified cellulase from muscadine grapes (*Vitis rotundifolia Michx*). *Am. J. Enol. Vitic*, **50**, 19-24.

Jiang, J. X. and London, E. R. (1990) Involvement of denaturation-like changes in *Pseudomonas* exotoxin A hydrophobicity and membrane penetration determined by characterization of pH and thermal transitions. Role of two distinct conformationally altered states. *J. Biol. Chem*. **265**, 8636-8641.

Kadi, E. N., Taulier, N., Huerou, L. J. Y., Ginder, M., Urbach, W., Nwigwe, I., Kahn, P. C. and Waks, M. (2006) Unfolding and refolding of bovine serum albumin at acidic pH: ultrasound and structural studies. *Biophys. J*. **91**, 3397-3404.

Kauzmann, W. (1959) Some factors in the interpretation of protein denaturation. *Adv. Protein Chem*. **14**, 1-63.

Khademi, S., Zhang, D., Swanson, S. M., Wartenberg, A., Witte, K. and Meyer, E. F. (2002) Determination of the structure of an endoglucanase from *Aspergillus niger* and its mode of inhibition by palladium chloride. *Acta Crystallogr. D Biol. Crystallogr*. **58**, 660-667.

Kim, P. S. and Baldwin, R. L. (1990) Intermediates in folding reactions of small proteins. *Annu. Rev. Biochem*. **59**, 631-652.

Kim, Y. S., Jones, L. S., Dong, B. S., Kendrick, B. S., Chang, M. C., Manning, T. W., Randolph, J. F. and Carpenter. (2003) Effect of sucrose on conformational equilibria and fluctuations within the native-state ensemble of proteins. *Protein Sci*. **12**, 1252-1261.

Kita, Y., Arakawa, T., Lin, T.Y. and Timasheff, S. N. (1994) Contribution of the surface free energy perturbation to protein-solvent interactions. *Biochemistry* **33**, 15178-15189.

Kleywegt, G. J., Zov, J. Y. and Divne, C. (1997) Crystal structure of catalytic core domain of endoglucanase I from T. *reesei* at 3.6 Å resolution and a comparison with related enzyme. *J. Mol. Biol*. **272**, 383-95.

Klibnov, A. M. (1983) Stabilization of enzymes against thermal inactivation. *Adv. Appl. Microbiol.* **29**, 1-28.

Koivula, A., Reinikainen, T., Ruohonen, L., Valkeajarvi, A., Claeyssens, M., Teleman, O., Kleywegt, G. J., Szardenings, M., Rouvinen, J., Jones, T. A. and Teeri, T. T. (1996) The active site of *Trichoderma reesei* cellobiohydrolase II: the role of tyrosine 169. *Protein Eng*. **9**, 691-699.

Koshland, D. E. (1953) Stereochemistry and the mechanism of enzymatic reactions. *Biol. Rev*. **28**, 416-436.

Kraulis, P. J., Clore, G. M., Nilges, M., Jones, T. A., Pettersson, G., Knowles J and Gronenborn, A. M. (1989) Determination of the three-dimensional solution structure of the C-terminal domain of the cellobiohydrolase I from *Trichoderma reesei*. A study using nuclear magnetic resonance and hybrid distance geometry dynamical simulated annealing. *Biochemistry* **28**, 7241-7257.

Krishnan, R., Bakthisaran, R. and Ch. Rao, M. (1996) Molten-globule state of carbonic anhydrase binds to the chaperone-like α-crystallin. *J. Biol. Chem*. **271**, 27595-27600.

Kumar, A. R., Hegde, S. S., Ganesh, K. N. and Khan, M. I. (2003) Structural changes enhance the activity of *Chainia* xylanase in low urea concentrations. *Biochim. Biophys. Acta* **1645**, 164-171.

Ladish, M. R., Lin, K. W., Voloch, M. and Tsao, G. T. (1983) Process considerations in the enzymatic hydrolysis of biomass. *Enzyme Microb. Technol*. **5**, 82-102.

LaemmLi, U. K. (1970) Cleavage of structural proteins during the assembly of the head of bacteriophage T4. *Nature* **227**, 680-685.

Lange, N. K. (1993) In: *Trichoderma reesei* cellulases and other hydrolases. (Suominen, P. and Reinikainen, T., Eds.), Foundations for Biotechnical and Industrial Fermentation Research, Helisiniki, pp. 263-272.

Lee, J. C. and Timasheff, S. N. (1981) The stabilization of proteins by sucrose. *J. Biol. Chem*. **256**, 7193-7201.

Lee, J. C., Gekko, K. and Timasheff, S. N. (1979) Measurements of preferential solvent interactions by densimetric techniques. *Methods Enzymol*. **61**, 26-49.

Lee, J. C, Frignon, R. P. and Timasheff, S. N. (1975) Structural stability of calf brain microtubule protein. *Ann. N. Y. Acad. Sci*. **253**, 284-291.

Lenting, H. and Warmoeskerken, M. (2001) Guidelines to come to minimized tensile strength loss upon cellulase application. *J. Biotechnol*. **89**, 227-232.

Lewendon, A., Murray, I. A., Shaw, W. V., Gibbs, M. R. and Leslie, A. G. W. (1994) Replacement of catalytic histidine-195 of chloramphenicol acetyl transferase: evidence for a general base role of glutamate. *Biochemistry* **33**, 1944-1950.

Linder, M., Linderberg, G., Reinikainen, T., Teeri, T. T. and Pettersson, G. (1995b) The difference in affinity between two fungal cellulose-binding domains is dominated by a single amino acid substitution. *FEBS Lett.* **372**, 96-98.

Linder, M., Mattinen, M. L., Kontteli, M., Linderberg, G., Stahlberg, J., Drakenberg, T., Reinikainen, T., Pettersson, G. and Annila, A. (1995a) Indentification of functionally important amino acids in the cellulose-binding domain of *Trichoderma reesei* cellobiohydrolase I. *Protein Sci*. **4**, 1056-1064.

Loprete D. M and Hill, T. W. (2002) Isolation and characterization of an endo-(1, 4) - β-glucanase secreted by *Achlya ambisexualis*. *Mycologia* **94**, 903-911.

Lowry. O. H., Rosebrough, N. J., Farr, A. L. and Randall, R. J. (1951) Protein measurement with the Folin phenol reagent. *J. Biol. Chem*. **193**, 265-275.

Lundbald, R. L. and Noyes, C. M. (1984) In: Chemical reagents for protein modification CRC Press, Vols I and II. Boca Raton, Florida, USA.

Ma, Y. Z. and Tsou, C. L. (1991) Comparison of the activity and conformation changes of lactate dehydrogenase H4 during denaturation by guanidinium chloride. *Biochem. J*. **227**, 201-211.

Mansfield, S. D., Mooney, C. and Saddler, J. N. (1999) Substrate and enzyme characteristics that limit cellulose hydrolysis. *Biotechnol. Prog*. **15**, 804-816.

Mawadza, C., Kaul, R. H., Zvauya, R. and Mattiasson, B. (2000) Purification and characterization of cellulase produced by two *Bacillus* strains. *J. Biotechnol*. **83**, 177-187.

McCarter, J. D. and Withers, G. S. (1994) Mechanisms of enzymatic glycoside hydrolysis. *Curr. Opin. Struct. Biol*. **4**, 885-892.

McGinnis, K. and Wilson, D. B. (1993) Disulphide arrangement and functional domains of β-1, 4-endoglucanase E5 from *Thermomonospora fusca*. *Biochemistry* **32**, 8157-8161.

Medve, J., Stahlberg, J. and Tjerneld, F. (1994) Adsorption and synergism of cellobiohydrolase I and II from *Trichoderma reesei* during hydrolysis of microcrystalline cellulose. *Biotechnol. Bioeng*. **44**, 1064-1073.

Miles, E. W. (1977) Modification of histidyl residues in proteins by diethylpyrocarbonate. *Methods Enzymol*. **47**, 431-442.

Millett, F., Darley-Usmar, V. and Capaldi, R. A. (1982a) Cytochrome *c* is cross-linked to subunit II of cytochrome *c* oxidase by water soluble carbodiimide. *Biochemistry* **21**, 3857-3862.

Millett, F., de Jong, C., Paulson, I. and Capaldi, R. A. (1982b) Identification of specific carboxylate groups on cytochrome *c* oxidases that are involved in binding cytochrome *c*. *Biochemistry* **22**, 546- 552.

Miroliaei, M., Ranjbar, B., Manesh, H. N. and Gorgani, M.N. (2007) Thermal denaturation of yeast alcohol dehydrogenase and protection of secondary and tertiary structural changes by sugars: CD and fluorescence studies. *Enzyme Microb. Technol*. **40**, 896-901.

Mohlin, U. and Pettersson, B. (2002) Improved paper-making by cellulase treatment before refining. *Prog. Biotechnol*. **21**, 291-299.

Monsellier, E. and Bedoulle, H. (2005) Quantitative measurement of protein stability from unfolding equilibrium monitored with the fluorescence maximum wavelength. *Protein Eng*. **18**, 445-456.

Muhlard, A., Hegyi, G. and Toth, G. (1967) Effect of pyrocarbonate on proteins. Reaction of diethylpyrocarbonate with amino acids. *Acta Biochim. Biophys*. **2**, 19-29.

Muralidhara, B. K. and Prakash, V. (2002) Molten globule intermediates of human serum albumin in low concentrations of urea. *Ind. J. Biochem. Biophys*. **39**, 318-324.

Muralidhara, B. K. and Prakash, V. (1997) Molten globule state of human serum albumin in urea. *J. Protein Sci*., **72**, 831-834.

Murao, S., Kanomoto, J. and Arai, M. (1979) Isolation and identification of a cellulolytic enzyme producing microorganisms. *J. Ferment. Technol*. **57**, 151-156.

Murao, S., Sakamoto, R. and Arai, M. (1988). Cellulases of *Aspergillus aculeatus*. *Methods Enzymol*. **160**, 274-299.

Naseem, F., Ahmad, B., Asraf, M. T. and Khan, R. H. (2004) Molten globule-like folding intermediate of asialofetuin at acidic pH. *Biochim. Biophys. Acta* **1699**, 191-199.

Nasiripourdori, A., Manesh, H.N., Ranjbar, B. and Khajeh, K. (2009) Co-solvent effects on the structure and function properties of savinase: solvent-induced thermal stabilization. *Int. J. Biol. Macromol*. **44**, 311-315.

Nidetzky, B., Hayn, M., Macarron, R. and Steiner, W. (1993) Synergism of *Trichoderma reesei* cellulases while degrading celluloses. *Biotechnol. Lett*. **15**, 71-76.

Nidetzky, B., Steiner, W., Hayn, M. and Claeyssens, M. (1994) Cellulose hydrolysis by the cellulses from *Trichoderma reesei*: a new model for synergistic interaction. *Biochem. J*. **298**, 705-710.

Nishi, I., Kataoka, M. and Goto, Y. (1995) Thermodynamic stability of the molten globule state of apomyoglobin. *J. Mol. Biol*. **250**, 223-238.

Ohmiya, Y., Takeda, T., Nakamura, S., Sakai, F. and Hayashi, T. (1995) Purification and properties of a wall-bound endo-1, 4-β-glucanase from suspension-cultured poplar cells. *Plant Cell Physiol*. **36**, 607-614.

Ohnishi, A., Ooi, T., Kinoshita, S., Tomatsuri, H., Umeda, K., Ueda, S., Hata, Y. and Arai, M. (1999) Analysis of a catalytic acidic pair in the active centre of cellulase from *Aspergillus aculeatus*. *Biosci. Biotechnol. Biochem.* **63**, 2157-2162.

Olama, Z. A., Hamza, M. A., El-Sayed, M. M. and Abdel-Fattah, M. (1993) Purification, properties and factors affecting the activity of *Trichoderma viride* cellulase. *Food chem*. **47**, 221-226.

Ooi, T., Minamiguchi, K., Kawaguchi, T., Okada, H., Murao, S. and Arai, M. (1994) Expression of the cellulase (FI-CMCase) gene of *Aspergillus aculeatus* in *Saccharomyces cervisiae*. *Biosci. Biotechnol. Biochem*. **58**, 954-956.

Pace, C. N. and Scholtz, J. M. (1997) Measuring the conformational stability of a protein. In: Protein structure: A practical Approach (Creighton, T. E., Ed.), IRL Press, Oxford, U.K. pp. 299-321.

Pace, C. N., Shirley, B. A. and Thomson, J. A. (1989) In: Protein Structure: A Practical Approach (Creighton, T.E., Ed.), IRL Press, Oxford, UK. pp. 311-330.

Pace, C. N. (1975) The stability of globular proteins. *Crit. Rev. Biochem*. **3**, 1-16.

Paci, E. and Karplus, M. (2000) Unfolding proteins by external forces and temperature: the importance of topology and energitics. *Proc. Nat. Acad. Sci. USA*. **97**, 6521-6526.

Pande, M., Dubey, V. K., Sahu, V. and Jagannadham, M. V. (2007) Conformational plasticity of cryptolepain: accumulation of partially unfolded states in denaturants induced equilibrium unfolding. *J. Biotechnol*. **131**, 404-417.

Paradis, F. W., Zhu, H., Krell, P. J., Phillips, J. P. and Forsberg, C. W. (1993) The *xyn*Cgene from *Fibrobacter succinogens* S85 codes for a xylanase with two similar catalytic domains. *J. Bacteriol*. **175**, 7666-7672.

Parsiegla, G., Belaich, A., Belaich, J. P. and Haser, R. (2002) Crystal structure of the cellulase Ce19M enlightens structure/function relationships of the variable catalytic modules in glycoside hydrolases. *Biochemistry* **41**, 11134-11142.

Pawar, S. A. and Deshpande, V. V. (2000) Characterization of acid-induced unfolding intermediates of glucose/xylose isomerase. *Eur. J. Biochem*. **267**, 6331-6338.

Perkins, S. J., Johnson, L. N., Machin, P. A. and Phillips, D. C. (1979) Crystal structures of hen egg-white lysozyme complexes with gadolinium (III) and gadolinium (III)-N-acetyl-D-glucosamine. *Biochem. J*. **181**, 21-36.

Pittz, E. P. and Timasheff, S. N. (1978) Interaction of ribonucease A with aqueous 2-methyl-2, 4-pentanediol. *Biochemistry* **17**, 615-623.

Popa, L. M. and Repanovici, R. (1977) Enhancement and stabilization effect of EDTA on sendai virus neuraminidase activity. *Acta. Virol.* **21**, 280-287.

Prakash, V. and Timasheff, S. N. (1985) Calculation of partial specifc volumes of proteins in 8M urea solution. *Methods Enzymol*. **117**, 53-60.

Prakash, V. (1982) Partial specific volumes and interactions with solvent components of α-globulin from *Sesamum indicum L.* in urea and guanidine hydrochloride. *J. Biosci.* **4**, 347-359.

Prakash, V., Loucheux, C., Scheufele, S., Gorbunoff, M. J. and Timasheff, S. N. (1981) Interaction of proteins with solvent components in 8M urea. *Arch. Biochem. Biophys*. **210**, 455-464.

Privalov, P. L. (1979) Stability of proteins. Small globular proteins. *Adv. Protein Chem*. **33**, 167-204.

Ptitsyn, O. B. (1995) Molten globule and protein folding. *Adv. Protein Chem*. **47**, 83-229.

Ptitsyn, O. B. (1992) In: Protein Folding. (Creighton, T. E., Ed.), Freeman, W. H. and Company, New York, USA. pp. 243-300.

Radha, C., Muralidhara, B. K., Ramesh Kumar, P., Tasneem, R. and Prakash, V. (1998) Thermal stabilization of multimeric proteins: A case study with α-globulin. *Ind. J. Biochem. Biophys*. **35**, 76-85.

Rajendran, S., Radha, C. and Prakash, V. (1995) Mechanism of solvent-induced thermal stabilization of alpha-amylase from *Bacillus amyloliquifaciens*. *Int. J. Pept. Protein Res*. **45**, 122-128.

Rajeshwara, A. N. and Prakash, V. (1996) Effect of denaturant and cosolvent on the stability of wheat germ lipase. *J. Agric. Food Chem*. **44**, 736-740.

Rajeshwara, A. N. and Prakash, V. (1994) Structural stability of lipase from wheat germ. *Int. J. Pept. Protein Res*. **44**, 435-440.

Reese, E. T., Siu, R. G. H., and Levinson, H. S. (1950) Biological degradation of soluble cellulose derivatives. *J. Bacteriol*. **59**, 485−497.

Reinikainen, T., Ruohonen, L., Nevanen, T., Laaksonen, L., Kraulis, P., Jones, T. A., Knowles, J. K. C and Teeri, T. T. (1992) Investigation of the function of mutated cellulose binding domains of *Trichoderma reesei* cellobiohydrolase I. *Protein Struc. Funct. Genet.* **14**, 475-482.

Reinikainen, T., Teleman, O. and Teeri, T. T. (1995) Effects of pH and high ionic strength on the adsorption and activity of native and mutated cellobiohydrolase I from *Trichoderma reesei*. *Protein Struc. Funct. Genet*. **22**, 392-403.

Riehm and scheraga, H. A. (1996) Structural studies of ribonulcease XXI. The reaction between ribonuclease and a water soluble carbodiimide. *Biochemistry* **5**, 99-115.

Robinson, D. R. and Jencks, W. P. (1965) The effect of compounds of the urea-guanidium class on the activity of acetyl tetra-glycine ethyl ester and related compounds. *J. Am. Chem. Soc*. **87**, 2462-2470.

Romero, C. M., Lozano, J. M., Sancho, J. and Giraldo, G. I. (2007). Thermal stability of β-lactoglobulin in the presence of aqueous solution of alcohols and polyols. *Int. J. Biol. Macromol*. **40**, 423-428.

Roseman, M. and Jencks, W. P. (1975) Interaction of urea and other polar compounds in water. *J. Am. Chem. Soc.* **97**, 631-640.

Rouvinen, J., Bergfors, T., Teeri, T., Knowles, J. K. and Jones, T. A. (1990) Three-dimensional structure of cellobiohydrolase II from *Trichoderma reesei*. *Science* **249**, 380-386.

Ruan, K., Xu, C., Li, T., Li, J., Lange, R. and Balny, C. (2003) The thermodynamic analysis of protein stabilization by sucrose and glycerol against pressure induced unfolding. *Eur. J. Biochem*. **270**, 1654-1661.

Ryu, D. D. Y. and Mendels, M. (1980) Cellulases: Biosynthesis and applications. *Enzyme Microb. Technol*. **2**, 91-102.

Sakamoto, R., Kanamoto, J., Arai, M. and Murao, S. (1985) Purification and physicochemical properties of three β-glucosidases from *Aspergillus aculeatus* No. F-50. *Agric. Biol. Chem*. **49**, 1275–1281.

Sami, A. J. and Akhtar, M. W. (1993) Purification and characterization of two low-molecular weight endoglucanases of *Cellulomonas flavigena*. *Enzyme Microb. Technol*. **15**, 586-592.

Sathish, H. A., Ramesh Kumar, P. and Prakash, V. (2002) Effect of urea at lower concentration on the structure of papain-formation of stable molten globule and its characterization. *Ind. J. Biochem. Biophys*. **39**, 155-162.

Sathish, H. A., Ramesh Kumar, P. and Prakash, V. (2007). Mechanism of solvent induced thermal stabilization of papain. *Int. J. Biol. Macromol*. **41**, 383-390.

Saxena, S., Bahadur, J. and Varma, A. (1992) Effect of cobalt and nickel on growth and carboxymethyl cellulase activity of *Cellulomonas spp*. *Biometals* **5**, 209-212.

Scatchard, G. (1946) Physical chemistry of protein solutions. I. Derivation of the equations for the osmotic pressure. *J. Am. Chem. Soc*. **68**, 2315-2319.

Schlierf, M. and Rief, M. (2005) Temperature softening of a protein in single-molecule experiments. *J. Mol. Biol*. **354**, 497-503.

Schoemaker, S. P. and Brown, R. D. (1978a) Characterization of endo-1, 4- β-D glucanases purified from *Trichoderma viride*. *Biochim. Biophys. Acta* **523**, 147-161.

Schoemaker, S. P. and Brown, R. D. (1978b) Enzymic activites of endo- 1, 4-β-D-glucanases purified from *Trichoderma viride*. *Biochim. Biophys. Acta* **523**, 33-146.

Schulein, M., Tikhomirov, D. F. and Schou, C. (1993) *Humicola insolens* alkaline cellulases. In: Suominen, P and Reinikainen, T. (Eds). *Trichoderma reesei* cellulases and other hydrolases. Foundation for biotechnical and industrial fermentation research. Helsinki. Vol. 8, pp. 109-116.

Schulein, M. (1998) Kinetics of fungal cellulases. *Biochem. Soc. Trans*. **26**, 164-167.

Semisotnov, G. V., Rodionova, N. A., Razgulyaev, O. I., Uversky, V. N., Gripas, A. F. and Gilmanshin, R. I. (1991) Study of the molten globule intermediate state in protein folding by a hydrophobic fluorescent probe. *Biopolymers* **31**, 119-128.

Shewale, J. G. (1982) β-glucosidases: its role in cellulase synthesis and hydrolysis of cellulose. *Int. J. Biochem*. **14**, 435-443.

Shimizu, S. and Smith, D. J. (2004) Preferential hydration and exclusion of cosolvents from protein surfaces. *J. Chem. Phys*. **121**, 1148-1154.

Shin, I., Kreimer, D., Silman, I. and Weiner, L. (1997) Membrane-promoted unfolding of acetylcholinesterase: a possible mechanism for insertion into the lipid bilayer. *Proc. Nat. Acad. Sci. USA*. **94**, 2848-2852.

Shrivastava, R. and Das, A. K. (2007) Temperature and urea induced conformational changes of histidine kinases from *Mycobacterium tuberculosis*. *Int. J. Biol. Macromol*. **41**, 154-161.

Sivaraman, T., Kumar, T. K. S., Jayaraman, G. and Yu, C. (1997) The mechanism of 2, 2, 2-trichloroacetic acid-induced protein precipitation. *J. Protein Chem*. **16**, 291-297.

Somogyi, M. (1952) Notes on sugar determination. *J. Biol. Chem*. **195**, 19-23.

Spande, T. F and Witkop. B. (1967) Determination of the tryptophan content of proteins with *N*-bromosuccinimide. *Methods Enzymol.* **11**, 498-506.

Spezio, M., Wilson, D. B. and Karplus, P. A. (1993) Crystal structure of the catalytic domain of a thermophilic endocellulase. *Biochemistry* **32**, 9906-9916.

Sprey, B. and Bochem, H. P. (1993) Formation of cross-fractures in cellulose microfibril structure by an endoglucanases-cellobiohydrolase complex from *Trichoderma reesei*. *FEMS Microbiol. Lett*. **106**, 239-244.

Srisodsuk, M., Reinikainen, T. and Teeri, T. T. (1993) Role of the interdomain linker peptide of *Trichoderma reesei* cellobiohydrolase I in its interaction with crystalline cellulose. *J. Biol. Chem*. **268**, 20756-20761.

Stockmayer, W. H. (1950) Light scattering in multicomponent systems. *J. Chem. Phys*. **18**, 58-61.

Suryaprakash and Prakash, V. (2000) Unfolding of multimeric proteins in presence of denaturants. A case study of helianthinin from *Helianthus annuus L. Nahrung* **44**, 178-183.

Takada, G., Kawaguchi, T., Sumitani, J. and Arai, M. (1998) Expression of *Apergillus aculeatus* no. F-50 cellobiohydrolase I (*cbhI*) and β-glucosidase I (*bglI*) genes by *Saccharomyces cervisiae*. *Biosci. Biotechnol. Biochem*. **62**, 1615-1618.

Takada, G., Kawasaki, M., Kitawaki, M., Kawaguchi, T., Sumitani, J., Izumori, K. and Arai, M. (2002) Cloning and transcription analysis of the *Aspergillus aculeatus* no. F-50 endoglucanase 2 (*cmc2*) gene. *J. Biosci. Bioeng*. **94**, 482-485.

Takagi, S., Kobayashi, M. and Matsuda, K. (1989) Modification of essential carboxyl groups in rabbit muscle phosphorylase by water soluble carbodiimide. *J. Biochem. (Tokyo)*, **105**, 933-938.

Tanford, C. (1968) Protein denaturation. *Adv. Protein Chem*. **23**, 121-282.

Tanford, C. (1970) Protein denaturation. *Adv. Protein Chem*. **24**, 1-95.

Tanford, C. Buckely, C. E., De, P. K. and Lively, E. P. (1962) Effect of ethylene glycol on the conformation of γ-globulin and β-lactoglobulin *J. Biol. Chem*. **237**, 1168-1171.

Tashpulatova, B. A. and Davranova, K. D. (1991) Influence of metal ions on the activity and stability of glucose isomerase from *Streptomyces atratus*. *Chem. Nat. Cmpd*. **27**, 739-742.

Teeri, T. T., Koivula, A., Linder, M., Wohlfahrt, G., Divne, C. and Jones T. A. (1998) *Trichoderma reesei* cellobiohydrolases: why so efficient on crystalline cellulose? *Biochem. Soc. Trans*. **26**, 173-178.

Timasheff, S. N. (1993) The control of protein stability and association by week interactions with water: How do solvents affect these processes? *Annu. Rev. Biophys. Biomol. Struct*. **22**, 67-97.

Timasheff, S. N. (1998) Control of protein stability and reactions by weakly interacting cosolvents: the simplicity of the complicated. *Adv. Protein Chem*. **54**, 355-431.

Timasheff, S. N. and Arakawa, T. (1997) In: protein structure- a practical approach (Crieghton, T. E. Ed.), IRL Press, Oxford, UK. pp. 340-375.

Timasheff, S. N., Lee, J. C., Pittz, E. P. and Tweedy, N. (1976) The interaction of tubulin and other proteins with structure-stabilizing solvents. *J. Colloid Interface Sci*. **55**, 658-663.

Tomme, P., Warren, A. J. and Gilkes, N. R. (1995a) Cellulose hydrolysis by bacteria and fungi. *Adv. Microb. Physiol*. **37**, 1-81.

Tomme, P., Warren, R. A. Jr., Miller, R. C. Jr., Kilburn, D. G. and Gilkes, N. R. (1995b) In: Cellulose-binidng domains: classification and properties. (Saddler, J. N and Penner, M. H. Eds.) Enzymatic degradation of insoluble carbohydrates, American chemical Society. Washington, USA. 618, 142-163.

Tsou, C. L. (1986) Location of the active sites of some enzymes in limited and flexible molecular regions. *Trends Biochem. Sci*. **11**, 427-429.

Tsou, C. L. (1993) Conformational flexibility of enzyme active sites. *Science* **262**, 380-381.

Uversky, V. N. (1993) Use of fast protein size-exclusion liquid chromatography to study the unfolding of proteins which denature through the molten globule. *Biochemistry* **32**, 13288-13289.

Uversky, V. N., Semisotnov, G.V., Pain, R.H. and Ptitsyn, O. B. (1992) 'All-or-none' mechanism of the molten globule unfolding. *FEBS Lett*. **314**, 89-92.

Varley, P. G. and Pain, R. H. (1991) Relation between stability dynamics and enzyme activity in 3-phosphoglycerate kinases from yeast and *Thermus thermophilus*. *J. Mol. Biol*. **220**, 531-538.

Vassilenko, K. S. and Uversky, V. N. (2002) Native-like secondary structure of molten globules. *Biochim. Biophys. Acta* **1594**, 168-177.

Ward, M., Wu, Shan, Dauberman, J., Weiss, G., Larenas, E., Bower, B., Rey, M., Clarkson, K. and Bott, R. (1993) In: Cloning, sequence and preliminary analysis of small, high pI endoglucanase EGIII from *Trichoderma reesei*. (Suominen, P. and Reinikainen, T. Eds.). *Trichoderma reesei* cellulases and other hydrolases Foundation for biotechnical and industrial fermentation research, Helsinki. 8, pp. 153-158.

Wetzel, R. (1987) Harnessing disulfide bonds using protein engineering. *Trends Biochem. Sci*. **12**, 478-82.

White, A., Withers, S., Gilkes, N. and Rose, D. R. (1994) Crystal structure of the catalytic domain of the β-1, 4-glycanase Cex from *Cellulomonas fimi*. *Biochemistry* **33**, 12546-12552.

Wimmer, H., Olsson, M., Peterson, M. T., Hatti-Kaul, R., Peterson S. B. and Muller, N. (1997) Towards a molecular level understanding of protein stabilization: the interaction between lysozyme and sorbitol. *J. Biotechnol*. **55**, 85-100.

Withers, S. G. (2000) Mechanisms of glycosyl transferases and hydrolases. Carbohydr. *Polym.* **44**, 325-337.

Wood, T. M. (1992). Fungal cellulases. *Biochem. Soc. Trans*. **20**, 46-53.

Wood, T. M. and Gracio-Campayo, V. (1990) Enzymology of cellulose degradation. *Biodegradation* **1**, 147-161.

Wood, T. M. and McCrae, S.I. (1978) The cellulase of *Trichoderma koningii*. Purification and properties of some endoglucanase components with special reference to their action on cellulose when acting alone and in synergism with the cellobiohydrolase. *Biochem. J*. **171**, 61–72.

Wood, T. M. and McCrae, S. I. (1979) Synergism between enzymes involved in the solubilization of native cellulose. *Adv. Chem.* Series **181**, 181-209.

Wood, T. M., McCrae, S. I. and Bhat, K. M. (1989) The mechanism of fungal cellulase action. Synergism between enzyme components of *Penicillium pinophilum* cellulase in solubilizing hydrogen bond-ordered cellulose. *Biochem. J*. **260**, 37-43.

Woodward, J. and Wiseman, A. (1982) Fungal and other β-d-glucosidases- their properties and applications. *Enzyme Microb. Technol*. **4**, 73 - 79.

Xu, G. Y., Ong, E., Gilkes, N. R., Kilburn, D. G., Muhandiran, D. R., Brandeis, M., Carver, J. P., Kay, L. E. and Harvey, T. S. (1995) Solution structure of a cellulose-binding domain from *Cellulomonas fimi* by nuclear magnetic resonance spectroscopy. *Biochemistry* **34**, 6993-7009.

Yang, J. J., Wu, C-S. C. and Martinez, H. M. (1986) Calculation of protein conformation from circular dichroism. *Methods Enzymol*. **130**, 208-269.

Yao, Q. Z., Tian, M. and Tsou, C. L. (1984) Comparison of the rates of inactivation and conformational changes of creatine kinase during urea denaturation. *Biochemistry* **23**, 2740-2744.

Yoshida, K., Maeda, K., Kobayashi, M. and Ichishima, E. (1994) Chemical modification of a *Penicillium* 1, 2-α-D-mannosidase by water-soluble carbodiimide: identification of a catalytically important aspartic acid residue. *Biochem. J*. **303**, 97-103.

Zancan, P. and Sola-Penna, M. (2005) Trehalose and glycerol stabilize and renature yeast inorganic pyrophosphatase inactivated by very high temperatures. *Arch. Biochem. Biophys*. **444**, 52-60.

Zou, Q., Haberman-Rotinghaus, S. M. and Murohy, K. P. (1998) Urea effects on protein stability: Hydrogen bonding and the hydrophobic effect. *Protein Struc. Funct. Genet*. **31**, 107-115.

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