DECLARATION

I hereby declare that the thesis entitled **"Biochemical and Biophysical Properties of Ficin: Structure, Function and Stability"** which is submitted herewith for the degree of **Doctor of Philosophy** in **Biochemistry** to **University of Mysore, Mysore** is the result of research work done by me in the Department of Protein Chemistry and Technology, Central Food Technological Research Institute, Mysore, India under the guidance of **Dr. V. Prakash,** Director, Central Food Technological Research Institute, Mysore during the period of March 2004 - 2009.

I further declare that the results of this work have not been previously submitted either partially or fully for any other degree or fellowship.

Mr. Devaraj K. B.

Date: 20th August, 2009

Place: Mysore

CERTIFICATE

I hereby certify that the thesis entitled **"Biochemical and Biophysical Properties of Ficin: Structure, Function and Stability"** submitted by **Mr. Devaraj K. B.**, for the degree of **Doctor of Philosophy** in **Biochemistry** to **University of Mysore, Mysore** is the result of research work carried out by him in the Department of Protein Chemistry and Technology, Central Food Technological Research Institute, Mysore, India under my guidance and supervision during the period of March 2004 - 2009.

I further declare that the results of this work have not been previously submitted either partially or fully for any other degree or fellowship.

V. Prakash

Guide and Supervisor
&
DIRECTOR
Central Food Technological Research Institute,
Mysore - 570 020, India

Date: 20th August, 2009
Place: Mysore

ACKNOWLEDGEMENTS

*I express my deep sense of gratitude and heartfelt thanks to my guide and mentor Padmashree **Dr. V. Prakash**, Director, Central Food Technological Research Institute, Mysore, India for suggesting the research problem, excellent guidance, constant encouragement and constructive criticism throughout the course of this investigation and completion of thesis. The scientific way of approach, whether to be adapted in lab or life itself, that I have imbibed from him always guide me to progress in all walks of my life. I will cherish the memories of my association with such an elegant personality.*

It also gives me immense pleasure to express my sincere thanks to him as Director, Central Food Technological Research Institute, Mysore, for providing all the facilities to enable me to carry out my research work in the institute.

I extend my thanks to Dr. A. G. Appu Rao, Head, Department of Protein Chemistry and Technology at CFTRI and fellow colleagues for creating an inspiring working atmosphere to complete my research work.

I am grateful to Dr. (Mrs.) Lalitha R. Gowda for her help and valuable suggestions during the course of my work.

I wish to express my sincere thanks to Dr. Ramesh Kumar, Dr. Purnima Kaul, Dr. Radha and Dr. Jyothi Lakshmi for their constant support and encouragement during the course of my research work.

My special thanks to all my colleagues from the lab, both past and present, especially Sijo, Gangadhara, Guna Sekhar, Jay Kanth, Ashok, Srinivas, Anuradha, Arun and Roopesh for all the help and cooperation extended to me during the course of this study.

A number of colleagues and friends have always extended their helping hand at various stages during the course of this work. I am thankful to them for their support and cooperation.

I extend my special thanks to Mr. Venkatesh, Mr. Rao, Mr. Murthy and Mr. Suresha for their sincere support and cooperation extended to me during the course of this study.

I sincerely appreciate the tremendous support and encouragement rendered by my beloved parents, brother, sister, and family friends which has enabled me to successfully complete this work.

No words suffice my gratefulness and how indebted I am to my brother, Rajesh without whose support and constant encouragement, I would have never been able to reach this stage in my life.

The Senior Research Fellowship from Council of Scientific and Industrial Research, New Delhi is gratefully acknowledged.

Last but not the least, I am grateful to my friends, specially Manu, Manju, Jaypal, Panditha, Vijay, Gajendra, Puneeth, Rajashekara, Apurva and many others who stood as a tower of strength to achieve of my goal.

Date: 20th August, 2009 ***Devaraj K. B.***

Place: Mysore

CONTENTS

LIST OF ABBREVIATIONS

A	absorbance
Å	angstrom
ANS	8-anilino-naphthalene sulfonic acid
AU	arbitrary units
BAPNA	α-N-benzoyl-DL-arginine-*p*-nitroanilide
BSA	bovine serum albumin
CAPS	3-[cyclohexylamino]-1-propanesulfonic acid
[C]	concentrations of protein (mg/mL)
°C	degree Celsius
$CaCl_2$	calcium chloride
CD	circular dichroism
cm	centimeter
Cys	cysteine
Da	dalton
DEAE	diethylaminoethyl
DFP	di-isopropylfluorophosphate
DTNB	5, 5`-dithiobis-(2-nitrobenzoic acid)
E_a	activation energy
EDTA	ethylene diamine tetra acetic acid
Ficin	unless otherwise stated in results and discussion, from chapter 2 onwards ficin means from *Ficus carica*
Fig	figure
F_u	fraction unfolded
FPLC	fast protein liquid chromatography
g	gram
Gly	glycine
Glu	glutamate
GuHCl	guanidine hydrochloride
h	hour(s)

HCl	hydrochloric acid
His	histidine
HPLC	high pressure liquid chromatography
°K	degree Kelvin
K_{sv}	Stern-Volmer constant
KCl	potassium chloride
kcal	kilocalorie
L	liter
Leu	leucine
M	molar concentration
Met	methionine
MG	molten globule
mg	milligram
min	minute
mL	milliliter
mM	millimolar
mol	mole
MRE	mean residue ellipticity
MRW	mean residue weight
M_r	molecular markers
N	normality
NaCl	sodium chloride
NaOH	sodium hydroxide
nm	nanometer
P	pressure
PAGE	polyacrylamide gel electrophoresis
PCMB	*p*-chloromercuribenzoate
PITC	phenylisothiocyanate
Phe	phenylalanine
PMSF	phenyl-methanesulfonylfluoride
PVDF	polyvinyledine difluoride

R	universal gas constant
R_s	Stokes radius
rpm	rotations per minute
SD	standard deviation
SDS	sodium dodecyl sulfate
sec	second
SH	thiol group
Ser	serine
T	absolute temperature
TCA	trichloro acetic acid
TEMED	*N,N,N′,N′*,-tetramethyl ethylene diamine
TFA	trifluoroacetic acid
T_m	thermal melting temperature
Tris	tris (hydroxymethyl) amino methane
Trp	tryptophan
Tyr	tyrosine
UV	ultraviolet
v/v	volume by volume
$\overline{\nu}$	partial specific volume (mL/g) extrapolated to zero protein concentration
$\overline{\nu}_{app}$	apparent partial specific volume (mL/g) at a single protein concentration
V_e	elution volume
V_o	void volume
w/v	weight by volume
μ	chemical potential
μg	microgram
μmol	micromole
%	percentage
λ_{max}	wavelength maximum

$E^{1\%}_{1cm,\,\lambda max}$	absorption coefficient of 1% protein solution in 1 cm path length cell at its absorption maximum
ε	molar extinction coefficient ($M^{-1}cm^{-1}$)
ρ_o	density of solvent buffer
ρ_P	density of protein
ξ_3	preferential interaction parameter of protein
ϕ_2^o	isomolal partial specific volume of protein (mL/g) extrapolated to zero protein concentration
ϕ'°_2	isopotential partial specific volume of protein (mL/g) extrapolated to zero protein concentration
ΔV	volume change upon transfer of one mole of protein from one solvent system to another (mL/mol)
ΔG°	free energy change of the reaction when all of its reactants and products are in their standard states
$(\delta g_3/\delta g_2)$	preferential interaction parameter on g/g basis
$(\delta m_3/\delta m_2)$	preferential interaction parameter on mol/mol basis

LIST OF FIGURES

LIST OF TABLES

SYNOPSIS

OF

Ph. D. THESIS ENTITLED

BIOCHEMICAL AND BIOPHYSICAL PROPERTIES OF FICIN: STRUCTURE, FUNCTION AND STABILITY

SUBMITTED TO UNIVERSITY OF MYSORE, MYSORE, INDIA

FOR THE DEGREE

OF

DOCTOR OF PHILOSOPHY

IN

BIOCHEMISTRY

BY

DEVARAJ K. B.

DEPARTMENT OF PROTEIN CHEMISTRY AND TECHNOLOGY

CENTRAL FOOD TECHNOLOGICAL RESEARCH INSTITUTE

MYSORE – 570020, INDIA

AUGUST, 2009

Title of Ph. D. Thesis: BIOCHEMICAL AND BIOPHYSICAL PROPERTIES OF FICIN: STRUCTURE, FUNCTION AND STABILITY

The elucidation of physicochemical characteristics of enzymes helps to understand the structure-function and stability relationship. The name ficin has been used variously to describe the endoproteolytic enzyme activity in tree latex of the genus *Ficus* or the sulfhydryl protease or proteases prepared from the latex of the genus *Ficus*. Hence, the term ficin must be regarded as generic. The most extensively studied ficins are cysteine proteases isolated from the latex of *Ficus glabrata*. However, the proteases (ficins) from other *Ficus* species have not been investigated in detail. In the present study, an attempt has been made to purify and study the ficins from the latex of two different species *Ficus racemosa* and *Ficus carica*.

It is well known that the study of conformational changes induced by various treatments or denaturants would be very useful in understanding the structure-function and stability relationship of proteins and enzymes. The knowledge gained in these studies helps in better understanding the forces that determine the conformation of proteins and to optimize their stabilities. The effect of pH and denaturing agents such as urea and GuHCl on the conformation of ficin has been carried out to understand the structure-stability relationship and denaturation mechanism of ficin.

The problem of stability is of great concern when proteins and enzymes are used for various industrial applications. Hence, the stability with respect to structure and biological activity needs to be considered during production, isolation and purification, storage and ultimate application of the protein product. The introduction of cosolvents such as sugars and polyhydric alcohols into the solvent medium is found to stabilize biological macromolecules in solution. The cosolvents interact with protein in a diverse way, depending on the surface and physicochemical properties of the proteins.

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 "**Biochemical and Biophysical Properties of Ficin: Structure, Function and Stability**". 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 results of the investigation are divided into the following chapters:

Chapter 1: Isolation, Purification and Characterization of Ficins from the Latex of *Ficus racemosa* and *Ficus carica*

This chapter principally deals with the characterization of ficins from *Ficus racemosa* and *Ficus carica*. The purified ficin from the latex of *Ficus racemosa* exists as single isoform. The enzyme is a single polypeptide chain protein of molecular mass of 44500 ± 500 Da as determined by MALDI-TOF. The enzyme shows maximum activity at 60 ± 0.5° C and exhibits a broad spectrum of pH optima between pH 4.5–6.5. These results indicate that this protease is distinct from other known ficins with respect to its existence as a single isoform, molecular mass and pH optimum. The enzyme activity is completely inhibited by pepstatin-A indicating that the purified enzyme is an aspartic protease. Its enzymatic cleavage specificity studied using oxidized B-chain of insulin indicates that the protease preferably hydrolyzed peptide bonds C-terminal to glutamate, leucine and phenylalanine (at P_1 position). This aspartic protease shares a number of homology with several plant aspartic proteases with respect to pH optimum, molecular mass, inhibition by pepstatin, thermostability and its secondary structure and stability.

The ficin from *Ficus carica* was purified to homogeneity from the commercial crude proteinase mixture preparation. The purified ficin is a single polypeptide chain protein of molecular mass of 23100 ± 300 Da. The enzyme is active in the pH range of 6.5 - 8.5 and shows maximum activity at pH 7.0. The ficin activity is completely inhibited in presence of cysteine group specific

inhibitors which clearly confirms the participation of cysteine residue at the active site of the enzyme. The purified ficin contains three disulfide bonds and a single free cysteine residue. The N-terminal sequence has homology with plant proteinases of cysteine proteases family. The determined analytical observations reveal that the enzyme shares several homologies with many other plant cysteine proteases. The enzyme activity of ficin from *Ficus carica* is much higher as compared to the activity of ficin from *Ficus racemosa* and holds greater potential its study in depth. Therefore, the ficin isolated from *Ficus carica* 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 Structure and Stability of Ficin from *Ficus carica*

This chapter describes the effect of pH, urea and GuHCl on the activity, structure and stability of ficin. The pH-induced denaturation of ficin was followed over a range of pH 1.0 to 7.0. The pH induced transition of ficin is found to follow a simple cooperative two-state transition as studied by far-UV CD measurements at 222 nm. The denaturation process of ficin is reversible in the pH range of 2.4 – 7.0 and irreversible inactivation of the enzyme occurs when the pH is decreased further below 2.4. The maximum stability for structure as well as activity of ficin has been observed at pH 7.0. The results demonstrate that the pH induced denaturation of ficin leads to the formation of a partially folded conformation at low pH. The results show that ficin exists in a partially folded state at acidic pH with characteristic features of a molten globule.

Ficin molecule is resistant to unfolding by urea under neutral conditions as observed by florescence and far-UV CD studies. The enzyme is susceptible to urea unfolding at pH 3.0 and the changes are reversible. The urea unfolding transition of ficin is cooperative. The maximum conformational changes occur between 2 to 4 M urea with a transition mid point of 2.5 ± 0.1 M. The results indicate that urea-induced unfolding of ficin at pH 3.0 follows a simple two-

state transition. In order to further characterize such an unfolding process, the effect of GuHCl on the structure, function and stability of ficin has been followed under neutral conditions. The GuHCl-induced equilibrium unfolding of ficin as followed by changes in the fluorescence emission maximum and ellipticity values at 222 nm as probes indicates that the transition is sigmoidal in nature and follows simple two-state transition without formation of any intermediate state. The results obtained from folding and unfolding studies show a differential structural stability of ficin towards denaturation by urea and GuHCl.

Chapter 3: Effect of Cosolvents on Structure and Stability of Ficin from *Ficus carica*

In this chapter, the effect of cosolvents such as sorbitol, trehalose, sucrose and xylitol on the structure and stability of ficin was studied. Ficin looses 37% of its activity after 10 min incubation at 70° C and in presence of cosolvents the activity is protected up to different extent. The apparent melting temperature (T_m) of ficin is 72.0 ± 1.0° C. All the cosolvents are able to elevate the thermal melting temperature to different extents in a concentration dependent manner. The effect of cosolvents on the structural integrity of ficin was examined by fluorescence and circular dichroic spectral measurements. Fluorescence and far-UV CD spectra of ficin revealed that no gross conformational changes of the protein molecule occur in presence of cosolvents.

In order to understand the mechanism of cosolvent-mediated thermal stabilization of ficin, the partial specific volume and preferential interaction parameters were determined in presence of cosolvents. The apparent partial specific volume of ficin under isomolal and isopotential conditions is 0.726 ± 0.001 mL/g and 0.727 ± 0.001 mL/g respectively, in buffer only at pH 7.0. The determined preferential interaction parameters showed that, for all the cosolvents the preferential hydration parameter is found to be positive and the preferential interaction parameter $(\partial g_3/\partial g_2)_{T,\mu_1,\mu_3}$ (g/g) is found to be negative

indicating the deficiency of cosolvent molecules in the immediate domain of the protein. A higher value of preferential interaction parameter, being -0.104 ± 0.02 g/g in 40% sorbitol and a lower value of -0.034 ± 0.01 g/g is observed in presence of 10% xylitol. These results indicate that the principle driving force involved in stabilization of ficin is preferential hydration. Thus, the effect of cosolvent on stability of ficin depends on nature and concentration of cosolvents as well as the solution conditions under which these are added in protection to the enzyme.

The summary and conclusions in the thesis briefly highlights the salient features of the present investigation. The literature cited in the text is arranged in alphabetical order under reference section, which gives all the relevant details including title, journal name and year, volume and pagination.

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

Devaraj K. B.
(Candidate)

V. Prakash
Guide and Supervisor
&
DIRECTOR
Central Food Technological Research Institute,
Mysore - 570 020, India

Date: 20th August, 2009

Place: Mysore

INTRODUCTION

Proteins are the abundant bio-macromolecules and indispensable agents of biological functions. Chemically, proteins are polymers of amino acids with each amino acid residue joined to its neighbour by a specific type of covalent bond called peptide bond. Proteins occur in great variety: thousands of different kinds, ranging in size from relatively small peptides to large polymer with molecular weight in millions. This diversity and abundance reflect the central role of proteins in virtually all aspects of cellular structure and function. Proteins are perhaps the most versatile of all biological molecules.

Enzymes constitute a specialized and diverse group of proteins that have several roles in many physiological processes. They are the most remarkable and highly specialized biological catalysts, central to every biochemical processes. The enormous variety of biochemical reactions that comprise life are nearly all mediated by a series of enzymes. Hence cell maintains complete and balanced clusters of enzymes essential for all biological activities and functions.

Enzymes differ from ordinary chemical catalysts in several important aspects. They have extraordinary catalytic power, often far greater than that of synthetic or inorganic catalysts. They have a high degree of specificity for their substrates, they accelerate chemical reactions tremendously, and yet they function in aqueous solutions under very mild conditions of temperature and pH. These important special properties of enzymes are principally responsible for a variety of industrial applications. They have now become very important tools, not only in medicine but also in chemical industries, food processing industries and agriculture.

1. Proteases

Proteases or proteinases are the enzymes that hydrolyze peptide bonds in proteins. They are found in all living organisms (animals, plants and microorganisms) and execute a large variety of functions, extending from the cellular level to the organ and organism level. They are important for many physiological processes including digestion of food proteins, recycling of intracellular proteins, the blood coagulation cascade, antigen presentation and activation of variety of proteins including enzymes, peptide hormones and neurotransmitters (Neurath, 1984; Davie and Fujikawa, 1975; Rao, *et al.*, 1998). Some of the important physiological functions of proteases are: conversion of food proteins to peptides and amino acids, activation of zymogens to active enzymes, milk clotting in infant's stomach, fibrinolysis, collagen formation, virus assembly, protein translocation, immune response, germination of spores and plant seeds, extracellular protein transport, transfer of proteins across the membranes, complement activation etc.

Proteolytic enzymes are not only physiological necessity but also a potent hazard. They can disrupt the protein components of cells and tissues, when their activity is not controlled. Two principle regulatory mechanisms have been established, by which the activity of proteolytic enzymes themselves is regulated (Neurath, 1984). First one is the activation of inactive protease precursors (zymogens) by limited proteolysis and second is the inactivation of proteases by forming complexes with protein inhibitors.

Proteinases are selective for the position of peptide bond hydrolysis in the substrates. Based on the type of reaction they catalyze, proteinases are grossly subdivided into endopeptidases and exopeptidases (Hartley, 1960). An endopeptidase hydrolyzes the internal α-peptide bonds in polypeptide chain, tends to act away from the N-terminus or C-terminus end. The

exopeptidases require a free N-terminal amino group, C-terminal carboxyl group or both and hydrolyze a bond not more than three residues from the terminus end.

Proteases are classified in subgroup 4 of group 3 (hydrolases) in the Nomenclature Committee of the International Union of Biochemistry and Molecular Biology (Int. Union of Biochem., 1992). However, proteases do not comply easily with the general system of enzyme nomenclature due to their huge diversity of structure-function and activity profiles. The catalytic type of a protease relates to the chemical groups responsible for its catalysis of peptide bond hydrolysis. Six specific catalytic types that are recognized: serine, threonine, cysteine, aspartate, glutamate and metallopeptidases. They are sorted into four major classes (Hartley, 1960) based on the catalytic group of the enzyme involved in the neucleophilic attack at the carbonyl carbon of the susceptible peptide bond of the substrate: serine proteases (EC 3.4.21), cysteine (thiol) proteases (EC 3.4.22), aspartic proteases (EC 3.4.23) and metalloproteases (EC 3.4.24).

1.1. Serine proteases

Serine proteases are a diverse group of enzymes having different physical properties, but at the active site they are essentially identical. These are the enzymes so named because they have a common catalytic mechanism characterized by the possession of a particular reactive serine residue that is essential for their enzymatic activity. These proteases are well understood family of enzymes. They have a common reaction mechanism consisting of a common catalytic triad (Ser-Asp-His) of the three amino acids, serine, aspartate and histidine (Fastrez and Fersht 1973; Singer *et al.*, 1993).

Serine protease family includes trypsin, chymotrypsin, elastase, collagenase, subtilisin, the nine proteases involved in blood coagulation, and others (Barrett, 1994; Davie and Fujikawa, 1975; Kaneda *et al*., 1997). They are generally active at neutral and alkaline pH, with an optimum between pH 7.0 and 11.0, and show broad substrate specificities including esterolytic and amidase activity. The isoelectric point of serine proteases generally lies between pH 4.0-6.0. They are usually recognized by their irreversible inhibition by chemical inhibitors such as di-isopropylflurophosphate (DFP), phenylmethylsulfonyl fluoride (PMSF) and tosyl-L-lysine chloromethyl ketone (TLCK).

Serine alkaline proteases represent the largest subgroup of serine proteases and are active at highly alkaline pH. They are produced by several bacteria, molds, yeasts and fungi (Barrett, 1994). Subtilisins are the second largest subgroup of serine proteases isolated from *Bacillus* species. They have an optimum pH of 10.0 and exhibit broad substrate specificity (Phadatare *et al*., 1997). Cucumisin from sarcocarp of melon fruit (*Cucumis melo*) was the first characterized plant subtilisin class protease and subsequently more cucumisin-like proteases were isolated and characterized from other plants (Yamagata *et al*., 1994; Arima *et al*., 2000; Terp *et al*., 2000).

1.2. Cysteine proteases

Cysteine proteases have been found in all living organisms. More than twenty families of cysteine proteases have been described and many of which (e.g. papain, bromelain, ficain, animal cathepsins) are of industrial importance. Mammalian cysteine proteinases fall into two classes: caspases and the papain superfamily comprising the papain family, calpains and bleomycin hydrolases (Barrett *et al*., 1998; McGrath, 1999). Cysteine proteinases participate in various biological processes. The cathepsins alone are involved in protein breakdown in lysosomes, antigen presentation,

proteolytic processing of proenzymes and prohormones, fertilization, cell proliferation, differentiation and apoptosis (Chapman *et al*., 1997; Grzelakowska-Sztabert, 1998). Altered activity of endogenous cysteine proteinases may lead to numerous pathologies such as rheumatoid arthritis, multiple sclerosis, neurological disorders, tumours and osteoporosis. Thus cysteine proteases, in particular lysosomal cathepsins have attracted the interest of the pharmaceutical industry (Otto and Schirmeister, 1997; Leung-Toung *et al*., 2002).

Generally, cysteine proteases are active only in presence of reducing agents such as HCN or cysteine. They have neutral pH optima, although a few of them, e.g., lysosomal proteases are maximally active at acidic pH. Cysteine proteases are the proteins of molecular mass about 21-30 kDa, catalyze the hydrolysis of peptides, amide, ester and thiol ester bonds. They are susceptible to sulfhydryl agents such as *p*-chloromercurobenzoate (PCMB) and iodoacetamide but unaffected by di-isopropyl- fluorophosphate (DFP) (Otto and Schirmeister, 1997; McGrath, 1999). Papain is one of the best characterized typical cysteine protease isolated from the fruits of *Carica papaya*. The majority of the cysteine proteases that have been characterized are evolutionary related to papain and share a common fold.

The proteolytic activity of all cysteine proteases arises from the presence of catalytic Cys and His residues in active site centre. The general mechanism of cysteine protease action has been well studied with papain as the model enzyme. The mechanism of action of cysteine protease is very similar to that of serine proteases. The initial step in the catalytic process involves the non-covalent binding of free enzyme to substrate to form the complex. This is followed by the acylation of the enzyme with the formation and release of the first product. In the next deacylation step, the acyl-enzyme reacts with a water molecule to release the second product with the generation of free enzyme molecule (Storer and Menard, 1994).

1.3. Aspartic proteases

Aspartic proteases are commonly known as acidic proteases and depend on the aspartic residues in the catalytic locus of the active site, thus the name aspartic proteases. They are widely distributed in animals, microbes, viruses and plants. Most of these enzymes show maximum activity at low pH (2.0-4.0) and having isoelectric points in the range of pH 3.0-4.5. Their molecular masses are in the range of 30 to 45 kDa. The aspartic proteases are known to be inhibited by pepstatin. Calf chymosin, porcine pepsin, cathepsin D and penicillopepsin are extensively studied proteases of this class (Davies, 1990; Rawlings and Barrett, 1995).

Aspartic proteases are grouped into three families, namely, pepsin (A1), retropepsin (A2) and enzymes from pararetroviruses (A3) (Barrett, 1995). They are involved in a number of physiological and pathological processes such as digestion (pepsin), blood pressure homeostasis (renin), retroviral infection (HIV proteinase), hemoglobin degradation in malaria (plasmepsin) and intracellular proteolysis (cathepsin A) (Dunn, 1992). Pepsin and majority of the other family show cleavage specificity towards aromatic or bulky (hydrophobic) amino acid residues on both sides of the peptide bond (Barrett, 1995).

A general acid-base catalytic mechanism has been proposed for the hydrolysis of proteins by aspartic proteases (Pearl, 1987). Studies of crystallography have shown that the enzymes of the pepsin family are bi-lobed molecules with the active-site cleft located between the lobes and each lobe contributing one of the pairs of aspartic acid residues that is essential for the catalytic activity. In most of the enzymes of pepsin family, the aspartic residues are contained in an Asp-Thr-Gly motif in both the N- and C-terminal lobes of the enzyme molecule (Blundell *et al*., 1991; Rawlings and Barrett, 1995).

1.4. Metalloproteases

Metalloproteases are the most diverse of the catalytic type of proteases and most of them are exopeptidases. They are characterized by the requirement for a divalent metal ion for their catalytic activity (Barrett, 1995). This class includes the enzymes from variety of origins such as collagenases from higher organisms, hemorrhagic toxins from snake venoms, and thermolysin from bacteria. Some of the important enzymes of metalloproteases include carboxypeptidases A and B, carnosinase, glycyl-glycine dipeptidase, cytosol aminopeptidases. All these enzymes require Zn^{2+} as cofactor. Prolidase and imiodipeptidases require Mn^{2+} for their catalysis. The thermolysin is a metalloexopeptidases, with similar active site, specificity and mechanism as that of caroxypeptidase A. It is one of the first thermophilic enzymes reported and has been utilized for many industrial applications (Weaver *et al*., 1977; Hibbs *et al*., 1985; Okada *et al*., 1986; Shannon *et al*., 1989).

The mechanism of action of metalloproteases is slightly different from the other classes of proteases. The catalysis of these enzymes depends on the presence of bound divalent cations. Most of the enzymes containing the His-Glu-Xaa-Xaa-His (HEXXH) motif that has been shown by x-ray crystallography to form part of the site for binding the metal ion. All of them are inhibited by chelating agents such as EDTA. Based on the specificity of their action, metalloproteases can be divided into four groups such as neutral, alkaline, *Myxobacter*-I and *Myxobacter*-II. The neutral metalloproteases show specificity for hydrophobic amino acids, while the alkaline proteases possess a very broad specificity (Barrett, 1995). *Myxobacter* protease-I is specific for small amino acid residues on either sides of the cleavage bond, whereas protease-II is specific for lysine on the amino side of the peptide bond.

2. Proteases from plant origin

Plant genome encodes hundreds of proteases, but little is known about what role they play in the life of a plant. Proteases are biologically involved in a range of processes. In general, seed germination, proteolytic activation of proenzymes, senescence, scavenging of defective metabolites and protection against pests are accompanied by virtue of the presence of proteases (Brandy, 1985; Matlu and Gal, 1999; Berger and Altmann, 2000). Several proteases have been isolated from distinct parts of the plants ranging from seeds to latex and fruits. It has become apparent that most of the isolated plant endopeptidases have been identified as cysteine endopeptidases or more rarely as serine or aspartic proteases (Boller, 1986).

Serine proteases were once thought to be rare in plants but in recent years, several of these enzymes have been isolated (and duly purified) from various plant species. They are present in almost all parts of the plant, but seem to be more abundant in fruits. Serine proteases of plants are involved in many physiological processes such as microsporogenesis, protein degradation, signal transduction, differentiation, and in hypersensitive response (Antão and Malcata, 2005). As to their molecular mass, the plant serine proteases known at present vary from 19 to 110 kDa, but the majority lies between 60 and 80 kDa. The optimum pH for their activity is in the alkaline range (pH 7.0–11.0). The optimum temperature is rather variable among these enzymes, from 30 to 80° C (Antão, and Malcata, 2005). Many subtilisin-like proteases have been identified from different plant species. Cucumisin was the first serine protease isolated from a plant source and many other plant enzymes subsequently were purified and the roles of the vast majority of subtilisin-like proteases remain to be known (Kaneda and Tominaga, 1975; Antão and Malcata, 2005).

Only a small number of aspartic proteases have been isolated and studied from the plants. In plants, aspartic proteases are generally either secreted or targeted to the vascular/protein storage body compartments. These proteinases share a number of common characteristics with other aspartic proteinases: all have an acidic pH optimum, are inhibited by pepstatin and preferentially cleave peptide bonds between hydrophobic residues (Matlu and Gal, 1999). It has been suggested that plant aspartic proteases are involved in hydrolysis of storage and intracellular proteins (D'Hondt *et al*., 1993; Rodrigo *et al*., 1991; Elpidina *et al*., 1990; Runeberg-Roos and Saarma, 1998). Aspartic proteases play an important role in food industry, e.g., the cheese industry or in soy and cocoa processing (Verissimo *et al*., 1996).

The most widely utilized proteases are cysteine proteases of plant origin for many industrial applications. Generally, majority of the proteases isolated from lattices, fruits and seeds of plant belong to cysteine super family (Boller, 1986). Cysteine proteases of plants play a major role in intracellular and extra cellular processes such as development and ripening of fruits (Brandy, 1985), degradation of storage proteins in germinating seeds, activation of proenzymes and degradation of defective proteins (Kembhavi *et al*., 1993; Otto and Schirmeister, 1997). Besides, enzymes in the latex are also involved in protection of the plants against predator attack (Konno *et al*., 2004).

The important cysteine proteases of plants origin are papain (*Carica papaya*), bromelain (*Ananus comosus*), and ficin (*Ficus glabrata* and *Ficus carica*), and have been extensively used in food and pharmaceutical industry. The molecular mass of these enzymes lies between 21-30 kDa. They have an optimum pH in the neutral region and most of them are thermostable. Papain (EC 3.4.22.2) is the most well investigated plant cysteine protease from the latex of *Carica papaya* (Otto and Schirmeister, 1997).

3. Industrial applications of proteases

Proteases, which firmly maintain first place in the world enzyme market, play an important role in biotechnology. They represent one of the three largest groups of industrial enzymes and accounts for nearly 60% of the total world sale of enzymes. Besides their industrial applications, they play an important role in basic research. Their selective peptide bond cleavage is exploited in the elucidation of structure-function relationship, in the synthesis of peptides, and in the sequencing of proteins (Rao *et al*., 1998). Proteases are thus an exceptionally important group of enzymes not only in biology but also in biotechnology and biochemical industries.

There are many industrial applications for proteases, though often mixtures of these rather than purified enzymes are used. They are used in cheese making, tenderizing meet, clarifying beers and flavor enhancers of cheeses and pet foods. They are also used in the leather industry to dehair and make the leather more supple, in cleaning materials, such as eco-washing powders and contact lens cleaning fluids to site a few examples. In pharmaceutical industry, proteases are extensively used for the preparation of ointments (Rao *et al*., 1998; Uhlig, 1998; Leisola *et al*., 2001). Some of the important industrial relevant endopeptidases are listed in the Table 1.

Proteases from plant sources have received special attention in pharmaceutical industry and biotechnology due to their property of being active over wide ranges of temperatures and pH (Uhlig, 1998). Plant sources have yielded many useful cysteine endopeptidases, among them papain, bromelain, and ficin have been extensively utilized in food and medicine industry. The commercial importance of cysteine proteases of plants and animal source cathepsins is due to their strong proteolytic activity against a broad range of protein substrates (Polaina and MacCabe, 2007). In Table 2 is shown some of the major industrial applications of cysteine proteases.

Table 1: Industrially important endopeptidases

Enzyme	Source	EC number
Serine proteases		3.4.21
Chymotrypsin	Pancreas	3.4.21.1
Trypsin	Pancreas	3.4.21.4
Subtilisins	*Bacillus subtilus*	3.4.21.12
Cysteine proteases		3.4.22
Cathepsin B	Lysosomal	3.4.22.1
Papain	Latex of *Carica papaya*	3.4.22.2
Ficin	Latex of *Ficus* trees	3.4.22.3
Bromelain	Pineapple (*Ananas comosus*)	3.4.22.4
Aspartic proteases		3.4.23
Pepsin	Stomach	3.4.23.1
Chymosin	Stomach	3.4.23.4
Metalloproteases		3.4.24
Thermolysin	*B. thermoproteolyticus rokko*	3.4.24.27
Neutral proteinase	*Bacillus* species	3.4.24.28

(Ref: Rao et al., 1998; Uhlig, 1998; Leisola et al., 2001)

Table 2: Major industrial applications of cysteine proteases

Applications	Enzymes used	Reasons (uses)
Biological detergent	papain, bromelain	protein stain removing
Baking industry	bromelain, papain	dough relaxation, lowering the protein level of flour in biscuit manufacturing, better bread volume and browning uniformly
Brewing industry	bromelain, papain	removing cloudiness during storage of beers, splitting the proteins in malt
Dairy industry	bromelain, papain	whey hyrolysates, cheese ripening, sweetener,
Photography industry	ficin	dissolving gelatin of the scraped film allowing to recovery of silver present
Food industry	bromelain, papain, cathepsins	tenderization of meat, make high level nutriments, health foods, make soluble protein products, gelatin stabilization, dry fermented food ripening, sea foods: surimi production, protein hydrolysates
Cosmetics industry	bromelain, papain	peeling effect, tooth whitening, can help to dispel pimples, clean face
Pharmaceutical industry and medicine	Papain, bromelain	kill the lymphatic leukemia cells, probacteria, parasites, helping diminish inflammation, soft lens cleaning
Textile and leather industry	bromelain, papain	used to process wool, refining of silks, depilatory for tanning the leathers
Chemical industry	bromelain, papain	synthesis of aspartum, antitumor compounds, bioactive peptides

(Ref: Rao et al., 1998; Uhlig, 1998; Leisola et al., 2001; Polaina and MacCabe, 2007)

4. Ficin from *Ficus* species

The genus *Ficus* includes more than 1800 named species making them one of the largest genuses in the *Moraceae* family. They are widely scattered over the tropical and subtropical regions of the world. Such diverse form is as the common edible fig (*F. carica*), the wild fig (*F. glabrata*), the sycamore fig (*F. sycotmorus*), the banyan tree *(F.indica* and *F. benghalensis*), various strangling figs (several species which include *F. bonplandiana* and *F. padifolia*), the rubber tree (*F. elastica*) and the creeping fig (*F. repens*) belong to this genus (Williams *et al*., 1968). The important characteristics of this genus include vines, shrubs and trees, and the bearing of fruit at some period in the life of the plant.

It has been know for many years that milky latex flowing from cuts of stem, leaves and unripe fruits of many species in the genus *Ficus* contains proteolytic activity (Williams *et al*., 1968). This is another common characteristic feature of the genus *Ficus*. The importance of laticifers and the latex is not very clear. Experimental evidence has shown that the latex generally contributes to protecting the plant against predators in both mechanical (by wound coagulation) and chemical (by the presence of toxic substances) manner (El Moussaoui *et al*., 2001). Konno *et al*. (2004) provided the evidence that the cysteine proteinases (and not the proteinase inhibitors) stored in the laticifers of papaya are the active compounds in its defense against herbivorous insects.

The name "ficin" has been used variously to describe the endoproteolytic enzyme activity in tree latex of the genus *Ficus* (Jones and Glazer, 1970), or the sulfhydryl protease or proteases prepared from the latex (Buttle, 2004). Hence the term 'ficin' must be regarded as generic. The term ficin was first coined by Robbins (1930) for the purified white powder with antihelminthic activity that was obtained from members of the genus

Ficus. The name ficin was also called by Walti (1938) for the crystallized proteolytic enzyme from the clarified latex of unknown *Ficus* species.

Cysteine proteases isolated from the latex of plants have mostly been reported in multiples. The latex of papaya (*Carica papaya*) plant contains four structurally and functionally distinct cysteine proteases (Glazer and Smith, 1971; Lynn 1979). Likewise, the lattices of *Ficus glabrata* and *Ficus carica* contain number of cysteine proteases (Englund *et al*., 1968; Kramer and Whitaker, 1964). Similarly, the latex of *Calotropis gigantia* also been shown to contain four cysteine proteases (Pal and Sinha, 1980). However, these multiple forms of proteases would have arisen by autodigestion from common precursor or as artifact of the preparation procedure has been excluded (Jones and Glazer, 1970; Sengupta *et al*., 1984).

Most of the studies on the *Ficus* proteinases, known collectively as ficin have been carried out on enzymes prepared from the commercially available dried latex of *Ficus glabrata*. In comparison, there are few studies on ficin from the latex of *Ficus carica*. Proteolytic enzymes present in the latex of both species have long been recognized as sulfhydryl proteases. Majority of the studies are concerned with the isolation and enzymatic properties of ficins from the latex of *Ficus glabrata* (Liener and Friedenson, 1970). These isolated ficins are differing from each other chromatographically, electrophoretically, and compositionally but still all posses cysteine residue at their active sites.

Englund *et al*. (1968) have studied a major sulfhydryl ficin (EC 3.4.22.3) among several active components of *Ficus glabrata* latex. They purified ficin to homogeneity by subjecting *Ficus glabrata* latex to salt fractionation and chromatography on CM-cellulose. Their studies showed that ficin is a single polypeptide protein of molecular mass of 25,000 ± 750 Da. This ficin hydrolyses wide variety of peptide bonds, but more efficiently

the peptide bonds following aromatic residues. They also reported several additional points of similarity between ficin and papain. Both enzymes are single polypeptide proteins with similar amino acid composition, contain three disulfide bonds and have a single cysteine residue upon which activity depends. In 1992 IUBMB recommended the name "Ficain" for this major proteolytic component of the latex *Ficus glabrata* (Buttle, 2004).

Latex of *Ficus glabrata* has long been known to contain several proteolytic components when subjected to ion-exchange chromatography on CM-cellulose. Initially only a major ficin was studied by Englund *et al*. (1968). Later a comparative study of other components was carried out by Jones and Glazer (1970) and Kortt *et al*. (1974). All other proteolytic components of the latex purified to homogeneity consisted of single polypeptide chain proteins of 25000-26000 Da molecular mass with amino terminus leucine residue. They have different retention behaviour on cation exchangers, different motilities on polyacrylamide gel electrophoresis and difference in amino acid composition, but still they all belong to cysteine protease family exhibit similar specificity and kinetic properties towards the B-chain of oxidized insulin, casein and other synthetic ester substrates. They have also showed that these components are not arisen by autodigestion from common precursor or as artifact of the preparation procedure.

The early kinetic works on catalytic properties of ficin (EC 3.4.22.3) revealed a striking similarity with papain (EC 3.4.22.2), which is a well characterized thiol proteinase. The available reports show that ficin shares many common properties with papain (Liener and Friedenson, 1970). The amino acid sequence neighboring to essential cysteine residue has been determined and resemble closely with the corresponding one in papain (Liener and Friedenson, 1972). The essential thiol group of ficin is the part of its catalysis is transiently acylated during catalysis and evidences have also proved the participation of histidine residue at active site. It is therefore not

surprising that the mechanism of action of ficin is similar to that of papain (Lowe and Williams, 1965; Husain and Lowe, 1968).

Sgarbieri *et al*. (1964), Kramer and Whitaker (1964), Williams *et al*. (1968) and Jones and Glazer (1970) have reported the multiplicity of proteinases both in the latex of *Ficus glabrata* and *Ficus carica*. Similarly Marini-Bettolo *et al*. (1963) have also reported the multiplicity of proteases isolated from the latex of *Ficus anthelmintica*. All these proteases differ from each other chromatographically, compositionally and quantitatively but still belong to cysteine protease family. Contrast to these results, ficin-E isolated from the latex of *Ficus elastica* is a serine-centered protease, indicating for the first time that the latex of trees of the genus *Ficus* contains other classes of proteases (Lynn and Clevette-Radford, 1986).

5. Stability of proteins

Protein stability is one of the most important issues, which also has a role in commercial production of enzymes. Hence, the stability with respect to structure and biological activity needs to be considered during production, isolation and purification, storage and ultimate application of the protein product. Research on the enzyme stability has two main purposes: for a better understanding of the mechanisms of denaturation and stabilization of proteins from a fundamental point of view, and try to improve the stability of enzymes that are used industrially.

Protein stability is ultimately the delicate balance of stabilizing and destabilizing interactions (Jaenicke, 1991). A variety of different physico-chemical forces play a major role in stabilizing the unique three-dimensional structure of a protein. Both the strength and specificity of many of these forces strongly depend on environmental conditions in such way that changes in the environmental conditions can reduce or even eliminate part

of the conformational interactions, while remainder are unchanged or even intensified. The regular native conformation of protein molecule is stable at physiological pH values, temperature and salt concentration and may easily be destroyed by slight changes in the environmental conditions such as pH, temperature, pressure and ionic strength (Mozhaev and Martinek, 1982).

Upon extraction of proteins from their biological sources, they are exposed to different environments which may bring the conformational changes directly related to protein stability. Different non-covalent and covalent factors are associated with the protein stability in solution. The important intrinsic structural factors which play a major role in protein stability are: salt bridges, hydrogen bonding, hydrophobic interactions, disulfide bonds, amino acid composition and binding of ligands (Kristjansson and Kinsella, 1991). The knowledge of the mechanism of inactivation is fundamental to develop a strategy for obtaining protein and enzymes with increased stability. The ideal approach to stabilize the proteins always requires the identification of basic mechanisms involved in protein instability

The major limitation in utilization of enzymes for industrial applications is their relative instability under operational conditions. The resistance of the catalytically active protein structure towards high temperatures, pHs and other denaturing influences, is one of the most important criteria for commercial utilization of the enzymes. There are three general ways to obtain stable enzymes: (i) isolation from extremophilic microorganisms, (ii) production in genetically manipulated non-extremophilic hosts, and (iii) stabilization of unstable enzymes by modification (Janeček, 1993). The last approach employs different strategies to obtain stable enzymes such as immobilization, chemical modification, protein engineering and solvent engineering and protein design. In many instances the later one represents the simplest and most effective approach

to minimizing protein inactivation. Several experimental investigations carried out on the effect of nonspecific additives such as neutral salts, amino acids and polyhydric compounds showed their ability to increase thermal and storage stability (Arakawa and Timasheff, 1982a; Timasheff and Arakawa, 1988; Timasheff, and Arakawa, 1997).

6. Role of cosolvents in protein stability

The structural stability of proteins is extensively controlled by the interactions between the protein and surrounding solvent molecules. Inclusion of stabilizing additives in protein formulations is the most common means of increasing the stability and shelf-life of the product. The cosolvents also change the stability/instability of proteins in general. It is a widely recognized phenomenon that some biological macromolecules in a purified state are unstable and may lose their structural integrity and biological activity. The introduction of cosolvents into the solvent medium has been found to stabilize many biological macromolecules. These additives are known to prevent the loss of enzyme activities (Bradbury and Jakoby, 1972), inhibit irreversible aggregation (Gerlsma and Stuur, 1972; Frigon and Lee, 1972) and increase the thermal transition temperature of the macromolecules (Gekko and Morikawa, 1981b; Lee and Timasheff, 1981; Rajeshwara and Prakash, 1994; Rajendran *et al*., 1995).

Influence of cosolvents on protein stability has been explained by several ways. However, the mechanism of stabilization is an individual protein dependent phenomenon, though several mechanisms have been proposed from different viewpoints. One of these addresses regarding the specific binding sites for glycerol on protein and that the stabilization is the result of small conformational changes induced by glycerol (Meyers and Jakoby, 1975; DiPaola and Belleau, 1978). The second idea is based on the high viscosity of a mixed solvent medium. The denaturation rates of protein

seem to be too slow for the viscous medium and this may be the main cause of protein stabilization (Schmitz and Schurr, 1972). The third idea is a cavity formation theory based on the surface tension effect of solvent and most of the cosolvents are known to increase the surface tension of the medium (Timasheff *et al.*, 1976). Protein denaturation may be minimized in a medium of high surface tension since the denaturation is accompanied by an increase in the surface area of protein requiring additional surface energy (Tiao-Yin and Timasheff, 1996). The fourth idea is that the stabilization effects due to entropy effect, and that the free energy change of transfer of the denatured protein from water to aqueous medium containing cosolvents is larger than that of the native protein (Gekko and Morikawa, 1981b).

Various substances and additives in solution stabilize or destabilize the native structure of proteins when added to aqueous protein solution as a reflection of their effect on the water structure around the protein. Solvent additives such as sugars, polyhydric alcohols and amino acids can affect macromolecular structure by direct interaction with the macromolecule and by indirect action through effects on the structure and properties of solvent, or by a combination of both of these mechanisms (Lee and Timasheff, 1981).

The stabilization effect of cosolvents is mainly due to solvent ordering effect or the alteration of water structure in the solvent. For a large number of additives the phenomenon of "preferential hydration" has been claimed as responsible for protein stabilization (Lee and Timasheff, 1981; Gekko and Timasheff, 1981; Lee and Lee, 1981; Gekko and Morikawa, 1981a; Arakawa and Timasheff, 1983, 1985a, b; Rajeshwara and Prakash, 1994; Sathish *et al*., 2007). Preferential hydration is a thermodynamic phenomenon that reflects the inability of organic additives to interact with proteins, leads to an exclusion of additives from the protein surface, which in turn increases the chemical potential of protein. The degree of preferential exclusion and the increase in chemical potential are directly proportional to the surface area of

protein exposed to solvent. Because of its nature, the cosolvents that stabilize the protein structures are also referred as thermodynamic boosters (Lee and Timasheff, 1981).

The detailed and thorough studies of Timasheff and coworkers have proved that the ability of organic solutes and some of the additives to stabilize the structure of proteins is related to the preferential hydration of the macromolecule in an aqueous medium containing these additives. Among the several ways of enzyme thermal stability enhancement, the use of such additives is of much importance owing to their structure-stabilizing effect. The selection of the appropriate additives depends on the characteristics of protein. Several industrially important proteins and enzymes have been stabilized against thermal denaturation using different cosolvents based on the preferential hydration mechanism (Rajeshwara and Prakash, 1994; Rajendran *et al*., 1995; Sathish *et al*., 2007).

7. Denaturation of proteins – Denaturants, pH and temperature

One of the most important problems in physical biochemistry is to understand how the information resided in the linear amino acid sequence of a protein is translated into a unique three dimensional structure, which confers to a protein its specific function. It is well known that the studies of solvent induced-denaturation of protein molecules are very useful for the understanding of protein stability and folding pathways (Wong and Tanford, 1973; Privalov, 1979). Such studies involve the monitoring of conformational changes brought about by various agents such as pH, GuHCl and urea.

In vitro studies of protein folding reactions necessarily begin with the protein in solution conditions designed to disrupt the non-covalent interactions that stabilize the native conformation. The stability of native

protein is a function of external variables such as pH, temperature, ionic strength, and solvent composition as they alter the various forces that are responsible for the intrinsic stability of the protein (Privalov, 1979). Therefore, a quantitative analysis of the role of such variables in the formation of the structure of protein is fundamental in describing the forces that are responsible for the conformational stability. These studies help to provide a deeper insight into the molecular basis of stability of proteins, which in turn can be used to standardize the protocols especially for proteins with very sensitive properties and can be applied for biotechnological applications.

A natural criterion for the native protein is its ability to perform a specific function. It is also natural to assume that the denatured state is a form where protein has lost its biological function due to the disruption of unique tertiary structure. Protein molecule under particular conditions loses completely all its elements of tertiary and secondary structure and this form is referred as completely unfolded state. The transition of the polypeptide chain from a disordered non-native state to the ordered native state is called protein folding.

It is generally accepted that protein folding is a spontaneous process, which does not normally require the presence of any additional factors, and many proteins can be efficiently refolded *in vitro* from completely unfolded state (Anfinsen, 1973). Consequently, all the sufficient information necessary for the correct folding of protein is embedded in the amino acid sequence of the protein. On the other hand, it is known that homologous proteins from different sources, possessing a similar spatial structure, can have different amino acid sequences. Thus, the information about the unique tertiary structure is incorporated in some common physicochemical properties of polypeptide chains, and does not depend very strongly on the structural features of individual amino acid residues (Anfinsen and Scheraga, 1975).

The conformation of a native protein is stabilized by physical forces of different kinds. For example, hydrogen bonds, secondary structure, hydrophobic interactions are responsible for the compaction, whereas, the unique tertiary structure is stabilized via electrostatic and van der Waals interactions and other minor forces responsible for stabilization (Pace, 1975). Because of their unique nature and the complexities of conformational forces, they respond to the changes in protein environment in an unique way. The solution conditions in which protein exists will have direct or indirect effect on the conformational stability of the protein molecule. In some cases, their structure is stabilized or in some other cases there is a destabilized effect observed based on the several thermodynamic parameters of the solution conditions that determine such process (Tanford, 1968; Matthews, 1993). Therefore, the study of unfolding and refolding *in vitro* may represent an essential step towards understanding of the protein self-organization.

It has been experimentally demonstrated in many cases of urea or GuHCl-induced transitions in small globular proteins can be described in terms of two-state (native ↔ denatured) model, where only folded and unfolded states of the protein exist in rapid equilibrium with no observable intermediates (Tanford, 1970; Privalov, 1979). But, now it is generally accepted that unfolding and refolding of a number of proteins involves different steps i.e., under certain conditions different intermediate states or partially folded conformations have been observed those do not appear to be native or completely unfolded state (Kim and Baldwin, 1990; Ptitsyn 1995; Privalov, 1996; Juneja and Udgaonkar, 2003).

Partially folded conformations are found to possess some special characteristic features, which are combination of properties of typical native and completely unfolded states (Ohgushi and Wada, 1983; Ptitsyn 1995; Redfield *et al*., 1994; Fink, 2005). It is now known that many globular

proteins may exist in at least four different conformations: the native (ordered state), molten globule, pre-molten globule and unfolded states. These different partially folded states play crucial roles in protein self organization, function, and misfolding/deposition (Ptitsyn 1995; Arai and Kuwajima, 2000; Fink, 2005; Uversky, 2002). Moreover, the aggregation of partially folded proteins is responsible for a number of human diseases and is a significant problem in biotechnology (Ptitsyn 1995). Because of these reasons, attentions of different researchers have been focused towards the partially folded conformations for understanding the mechanism.

A rational approach to protein engineering and design requires a fundamental thermodynamic description of protein systems at molecular level. The kinetic and equilibrium free energies of reversible unfolding of proteins are used as a useful technique to analyze the thermodynamic measurements of transitions and intermediate states (Pace, 1990; Fersht *et al*., 1992). The use of urea and GuHCl to alter the free energies of unfolded proteins has become very important step in conformational studies. In comparison to either acid or thermal unfolding, chemical agents such as urea and GuHCl are more effective in disturbing the non-covalent interactions. These denaturants (unlike temperature and pH induced denaturation) can in many cases reversibly transform proteins to completely unfolded state (Tanford, 1968; Matthews, 1993; Zou *et al*., 1998).

The basic mechanism of interaction of urea and GuHCl with proteins has not been well understood. They have been identified to interact directly with proteins (Simpson and Kauzmann, 1953) and also shown that at high concentrations they can cause substantial changes to the behaviour of the solvent itself (Breslow and Guo, 1990). The relative importance of these two effects in protein unfolding is still ambiguous. Suggestions have been made that these chemical denaturants unfold proteins by migrating into the interior of the protein and forming hydrogen bonds with the backbone of

protein molecule (Hedwing *et al*., 1991). The denaturants tend to increase the solubility of the proteins by preferential interactions and exposing the more hydrophobic surfaces which are buried inside the native molecules. It was clearly shown by the increased denaturant binding to denatured state than native state of the proteins (Aune and Tanford, 1969; Arakawa and Timasheff, 1985a). The quantification of GuHCl and urea interactions with proteins has been well documented. In a more generalized mechanism and extent of binding show that two urea molecules will bind to a single peptide bond and one each to aromatic amino acids (Lee and Timaheff, 1974; Prakash *et al*., 1981; Prakash and Timasheff, 1981).

In view of the above literature, the studies are undertaken in the area of proteases especially initially to isolate enzymes from the plant sources such as ficins from two different species, Ficus racemosa and Ficus carica. The ficins from both the species are purified and characterized to evaluate their enzymatic and physical properties and their structure-function relationship. Further, ficin molecule is subjected to changing solution parameters and its evaluation, in order to understand the structure-function and stability relationship using different stabilizing and destabilizing agents. The effect of pH and denaturing agents such as urea and GuHCl on the structure and stability of ficin is being followed to obtain a deeper insight into the folding and unfolding behaviour of the protein molecule. These equilibrium folding and unfolding studies helps to assess the molecular basis of stability of ficin, which in turn can be used to design protocols for various biotechnological applications. The effect of cosolvents such as trehalose, sorbitol, sucrose and xylitol on structure and stability of ficin and their specific interaction with the enzyme molecule are also investigated. Such studies are particularly important for understanding the basic mechanism of thermal stabilization of ficin, which has both scientific and industrial importance. The main objectives of the present study are discussed in detail in the section scope and objectives.

SCOPE AND OBJECTIVES

The characterization of specific proteolytic enzymes helps in better understanding of their roles in physiological processes as well as in commercial applications. Proteolytic enzymes from the plant sources have received special attention because of their wide range of activity and stability. Ficin belongs to the group of proteolytic enzymes and is isolated from the latex of *Ficus* trees. The most extensively studied ficins are cysteine proteases isolated from the latex of *Ficus glabrata*. However, the proteases (ficins) from other *Ficus* species have not been investigated in detail. In the present study, an attempt has been made to isolate and purify the ficins from the latex of two different species such as *Ficus racemosa* and *Ficus carica*. The purified enzymes are characterized for their physical, chemical and enzymatic properties, which would help to understand their structure-function relationship and their utilization in biotechnological applications.

The understanding of structure-function and stability relationship of proteins and enzymes under different conditions is fundamentally important. The solvent-induced equilibrium denaturation studies are very useful in understanding the structure, stabilization, and folding of proteins. The knowledge gained in these studies helps in better understanding of the complexities of the forces that stabilize proteins. Further, the structural intermediates that are formed upon folding and unfolding of proteins will provide valuable information about pathways. Not much information is available concerning the structure-function and folding pathway of ficin. Hence, the current studies on the effect of pH and denaturing agents such as urea and GuHCl on conformation of ficin would be important from the viewpoint of its structure-function and stability relationship.

The stability of proteins and enzymes is an important area and plays a greater role in the development of potentially large number of bioprocesses based on enzymes. Hence, stabilization processes needs to be well understood, particularly the thermal denaturation, which is by far an important form of protein inactivation. Cosolvents such as polyhydric alcohols and sugars are used for a longtime to stabilize proteins. Their positive action on protein stability is very complex and remaining unclear though several mechanisms have been proposed from different viewpoints. The relationship between protein stability and surrounding environment is a subject for numerous studies, and it is necessary to study the behaviour of variety of proteins and enzymes in presence of various additives in order to establish a more general mechanism of stabilization. The present work is focused to study the effect of various cosolvents on structure and stability of ficin, and their preferential interaction with the enzyme, which would enhance better understanding of the mechanism of stabilization of ficin along with the study of the effect of pH and denaturants.

Followings are the specific objectives of the present study:

- Isolation, purification and characterization of ficins from *Ficus racemosa* and *Ficus carica*
- Effect of pH, urea and GuHCl on structure and stability of ficin
- Effect of cosolvents on structure and stability of ficin

The present investigation elucidates the enzymatic characteristics of the proteases isolated from the latex of *Ficus racemosa* and *Ficus carica*. The study involves purification of the enzymes using various conventional chromatographic and electrophoretic methods. The purified enzymes will be characterized for their physical and catalytic properties using different analytical techniques. The homology of the elucidated enzymatic characteristics is also explained in comparison with other important plant proteases.

The effect of pH and denaturing agents such as, urea and GuHCl on structure and stability of ficin from *Ficus carica* is investigated. Effect of pH and urea and GuHCl-induced equilibrium unfolding of ficin is followed to evaluate the folding mechanism and to understand the molecular basis of stability of the enzyme molecule. The conformational changes of the enzyme during folding and unfolding are monitored using several biophysical techniques to understand the same from various approaches.

Ficin purified from the latex of *Ficus carica* is studied for the mechanism of thermal inactivation and its stabilization using different cosolvents. The different cosolvents in this study are chosen based on the knowledge of their stabilizing effect on protein and after preliminary screenings. The stabilizing effect of cosolvents on ficin is also followed by various analytical techniques. Further, the mechanism of cosolvent-induced stabilization is being evaluated by partial specific volume measurements and related specific interaction parameters.

With these above objectives, studies are conducted to understand the ficin molecule in depth, particularly with respect to structure-function relationship of the enzyme. The data generated would help in understanding of the enzymatic properties of ficins from different *Ficus* species, and their potential in biotechnological applications. The folding and unfolding studies elucidate the mechanism of stability and define the conformational states during the folding and unfolding process of ficin molecule. The interaction of cosolvents with ficin gives deeper insight for evaluating the mechanism of inactivation and stabilization of the enzyme.

MATERIALS AND METHODS

1. CHEMICALS

Ficin (*Ficus carica*) Lot #031K76652, *p*-chloromercuribenzoate (PCMB), iodoacetamide, sodium tetrathionate, polyvinyledine difluoride membrane (PVDF), phenylmethyl-sulfonyl fluoride (PMSF), acrylamide, N-N'-methylene bis-acrylamide, sodium dodecyl sulfate (SDS), cysteine hydrochloride, 2-mercaptoethanol, N,N,N',N'-tetramethylethylenediamine (TEMED), urea, guanidine hydrochloride, ammonium persulfate, sucrose, D-sorbitol, trehalose, D-xylitol, carbonic anhydrase, bovine serum albumin (BSA), ribonuclease, ovalbumin, myoglobin, cytochrome c, aprotinin, casein, azocasein, gelatin, Tris (hydroxymethyl) amino methane (Trizma base), Coomassie brilliant blue R-250, 2, 4, 6-trinitrobenzenesulfonoic acid (TNBS), trifluoroacetic acid (TFA), α-N-benzoyl-DL-arginine-*p*-nitroanilide HCl (BAPNA), insulin B-chain, 3-[cyclohexylamino]-1-propanesulfonic acid (CAPS), 8-anilino-1-naphthalene-sulfonic acid (ANS), and DL-dithiothreitol (DTT) were purchased from Sigma-Aldrich Chemical Co., St. Louis, Mo, USA. DEAE Sephadex–A50, Sephadex G-25 and blue dextran 2000 were from Pharmacia LKB, Uppsala, Sweden. Spectrapor molecular membrane tubing was from Spectrum Medical Industries, Inc., Texas, USA. Coomassie brilliant blue G-250 was from Bio-Rad Laboratories, Richmond, USA. Ammonium sulfate, sodium chloride, potassium chloride, sodium dihydrogen orthophosphate, disodium hydrogen orthophosphate, sodium hydroxide, sodium carbonate, and ethylene diamine tetra acetic acid (EDTA) were from E. Merck (India) Ltd., Mumbai, India. Glycine, hydrochloric acid, acetic acid, trichloroacetic acid (TCA), Folin and Ciocalteu's phenol (FC) reagent and sodium acetate were from Qualigens Fine Chemicals, Mumbai, India. All other chemicals used were of analytical grade. Quartz triple distilled water was used in all the experiments.

2. METHODS

2.1. Purification of protease (ficin) from *Ficus racemosa*

2.1a. Collection of latex

Immature green fruits from *Ficus racemosa* trees growing locally were collected from the trees identified by their botanical characteristics. The fruits were freed of suckers and ants. Latex was collected by incising the stalk of the green fruit from the main branch. The collected latex was clarified by centrifugation at 10000 × g at 4° C for 60 min to remove the gum and other debris. The clarified extract was stored at –20° C until further use.

2.1b. Isolation and purification of protease from the latex

To the clarified extract, solid ammonium sulphate (51.6 g/100 mL) was added to obtain 80% saturation at 4° C. The precipitated protein was removed by centrifugation at 10000 × g for 30 min. The precipitate thus obtained was redissolved in 0.05 M Tris-HCl buffer pH 7.0. This protein fraction was loaded onto a BioSep-SEC-S2000 column (600 × 21.2 mm, Phenomenex, Torrance, USA) pre-equilibrated in the same buffer at a flow rate 5 mL/min at room temperature (25 ± 2° C). The eluted protease active fractions were pooled and concentrated using the 10000 Da cutoff Amicon Ultra (Millipore Corporation, USA) centrifugal filter devices. The retentate obtained was loaded onto a DEAE-Sephadex A-50 column (20 × 1.5 cm) pre-equilibrated with 0.05 M Tris-HCl buffer pH 7.0 at a flow rate of 25 mL/h at 4° C. The unbound fraction was eluted in the same buffer, which did not have any protease activity. Subsequently elution of bound protein, using a linear gradient of 0–0.5 M NaCl in the same buffer was carried out to obtain the active fractions. The active fractions were pooled and dialyzed against 0.05 M sodium acetate buffer pH 5.5.

2.2. Purification of ficin from *Ficus carica*

The enzyme was purified using SP-Sepharose cation-exchanger column on fast protein liquid chromatography (FPLC). The crude preparation of ficin obtained commercially from Sigma was dissolved in sodium acetate buffer pH 5.0 (0.05 M) and centrifuged. The clear supernatant ficin solution was directly applied to a Hiload 16/10 SP-Sepharose high performance column (Amersham Biosciences AB, Uppsala, Sweden) pre-equilibrated with the same buffer at 20° C. The column was washed with two column volumes (about 40 mL) of buffer to remove unbound fractions. The bound proteins were eluted with a linear gradient of 0-0.6 M NaCl in the same buffer at the flow rate of 1.0 mL/min. The total gradient elution volume was 100 mL that constitutes about six column volumes. The eluted proteins were separated into five active fractions. The major ficin fraction II was collected separately and pooled. The pooled fractions were dialyzed extensively against 0.05 M sodium phosphate buffer, pH 7.0 and this fraction was used in all further studies.

2.3. Protein estimation

The protein concentration of the ficins was quantified by the dye binding (Coomassie brilliant blue G-250) method of Bradford (1976) and by the method of Lowry's using Folin and Ciocalteu's phenol (FC) reagent (Lowry *et al*., 1951). Bovine serum albumin (BSA) was used as the standard protein.

2.4. Measurements of protease activity

2.4a. Using azocasein as substrate

Protease activity of the ficin from *Ficus racemosa* was assayed using azocasein as the substrate according to the reported method (Bendicho *et al*., 2002). The assay mixture consisting of 1 mL of 0.5% azocasein in 0.05 M sodium acetate buffer at pH 5.5 and 10–100 µg of enzyme was incubated at

60 ± 2° C for 30 min. The reaction was stopped by addition of ice cold TCA (10% w/v) and centrifuged at 10000 × g. In the control, enzyme was inactivated by TCA prior to incubation with substrates. The supernatant (0.5 mL) was mixed with an equal volume of 0.5 M NaOH and incubated for 15 min. The absorbance of the developed color was measured at 440 nm. All the assays were corrected for non enzyme hydrolysis using buffer in place of enzyme. One unit of enzyme activity is defined as the amount of enzyme that increases the absorbance of 1.0 at 440 nm per minute under the assay conditions.

2.4b. Using casein as substrate

The assay of the ficin from *Ficus carica* with casein as substrate was based on the method described by Kunitz (1947). Casein concentration of 1% (w/v) was prepared in sodium phosphate buffer pH 7.0 containing 5 mM cysteine hydrochloride. To one mL of substrate, one mL of purified enzyme containing 0-10 μg of ficin was added and the reaction mixture was incubated at 55° C for 20 min. The reaction was stopped by the addition of 2 mL of 10% TCA and incubated for 10 min at room temperature, and centrifuged. The absorbance of the supernatant was measured at 280 nm. In case of blank substrate was added after the enzyme was first inactivated by TCA. One unit of activity is defined as the amount of enzyme that increases the absorbance by 0.001 per min under given assay conditions.

2.4c. Using other food proteins as substrates

Proteolytic activity of ficin isolated from *Ficus racemosa* was also examined using natural food proteins that are commonly hydrolyzed or used in protease assay. The different substrates used were: albumin, gelatin, arachin (isolated according to Monteiro and Prakash, 1994) and glycinin (isolated as described by Tanh and Shibasaki, 1976). Substrate concentration of 1% (w/v) was prepared in acetate buffer pH 6.0. One milliliter of purified

enzyme (100 μg) was added to 1 mL substrate. The reaction mixture was incubated at 60° C for 30 min. The reaction was stopped by the addition of 2 mL of 10% TCA and incubated for 10 min at room temperature and centrifuged. The absorbance of soluble peptides in the supernatant was measured at 280 nm. In case of blank, substrate was added after the enzyme was first inactivated by TCA. One unit of activity is defined as the amount of enzyme that increases the absorbance by 0.001 per min under given assay conditions.

2.4d. Using BAPNA as synthetic substrate

Enzyme activity of ficin with α-N-benzoyl-DL-arginine-*p*-nitroanilide hydrochloride (BAPNA) was quantified by measuring its ability to cleave an amide bond in small molecular weight synthetic substrate (Englund *et al*., 1968). The extent of hydrolysis was determined by measuring the product (p-nitroaniline) formed at 410 nm (ε 8800 for p-nitroaniline). Activity of ficin is expressed as amount of p-nitroaniline formed by one mole of protein per min. In case of thermal inactivation and denaturation studies all the enzyme activity was measured both in presence and absence of third component in 0.05 M sodium phosphate buffer containing 5 mM cysteine hydrochloride. The residual activity was calculated as percentage of its original activity.

2.5. Size exclusion chromatography

2.5a. High performance liquid chromatography (HPLC)

The apparent molecular mass and homogeneity of the enzyme purified from the latex of *Ficus racemosa* was determined using a BioSep-SEC-S2000 (300 × 7.8 mm, Phenomenex, Torrance, USA.) analytical gelfiltration column, in a Waters HPLC system. The eluant used was 0.05 M sodium phosphate buffer, pH 7.4 and the column was equilibrated with the same buffer before loading the proteins. The protein samples were eluted at a flow rate of 1 mL/min. The column was calibrated using standard proteins

such as bovine serum albumin (66.3 kDa), ovalbumin (43.5 kDa), carbonic anhydrase (29 kDa), ribonuclease (13.7 kDa), cytochrome c (12.4 kDa) and aprotinin (6.5 kDa). The proteins fractions were detected at 280 nm.

2.5b. Fast protein liquid chromatography (FPLC)

The homogeneity and apparent molecular mass of the purified ficin was determined on fast protein liquid chromatography (FPLC) using Superdex-75 HR 10/30 gelfitration column (Amersham Biosciences AB, Uppsala, Sweden) calibrated with ovalbumin (43.5 kDa), carbonic anhydrase (29 kDa), myoglobin (17.6 kDa), ribonuclease (13.7 kDa) and aprotinin (6.5 kDa). The column was equilibrated with 0.02 M sodium phosphate buffer at pH 7.0. The proteins were eluted at a flow rate of 30 mL/h and the eluted protein fractions were checked for their absorbance at 280 nm.

2.5c. Determination of Stokes' radius

Determination of stokes radius of proteins is particularly important to define the different conformational states of proteins. Stokes' radii measurements of ficin at different conditions were performed by size-exclusion chromatography experiments on FPLC using Superdex-75 HR 10/30 gelfiltration column. The column was equilibrated with respective buffers specific for each state of ficin such as, 0.02 M sodium phosphate buffer pH 7.0, the buffer containing 6.0 M GuHCl and KCl-HCl buffer of pH 1.4. The elution was carried out at a flow rate of 30 mL/h and the absorbance of eluted fractions was read at 280 nm. A set of proteins with known molecular mass and Stokes' radii (R_S) (bovine serum albumin, 35.5 Å; ovalbumin, 30.2 Å; carbonic anhydrase, 24.4 Å; myoglobin, 21.4 Å; ribonuclease 13.7, 19.3 Å) were used as standards and a calibration curve of log R_S versus migration rate has generated (Uversky, 1993). Blue dextran was used to determine the void volume.

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 ± 2° C. From the stock solution of acrylamide-bisacrylamide (29.2:0.8%), a staking gel of 5% and separating gel of 10% was prepared. The samples were prepared in the Tris buffer pH 8.3 containing 10% glycerol and 0.02% bromophenol blue. After an initial prerun for 30 min, the samples were loaded and electrophoresed at a constant voltage (100 Volts) for 2-3 h. The gels were stained with Coomassie brilliant blue R-250 prepared in 50% methanol in 10% acetic acid for 2 h and destained in 50% methanol containing 10% acetic acid.

2.6b. Sodium dodecyl sulfate polyacrylamide gel electrophoresis

Sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) at pH 8.3 was carried out according to the method of Laemmli (1970) in a discontinuous buffer system. The polyacrylamide gel of 12% containing 0.1% SDS was cast in 1.5 mm slab gel apparatus. Electrophoresis was performed in 0.025 M Tris-0.3 M glycine buffer pH 8.3 using 12% acrylamide gel containing 0.1% SDS as electrode buffer at 100 Volts. The protein samples (20-40 µg) were mixed with sample buffer pH 6.8 containing 5% (v/v) 2-mecaptoethanol, 10% (v/v) glycerol, 2% (w/v) SDS and 0.1% (w/v) bromophenol. Samples were heated in a boiling water bath for 5 min and subjected to electrophoresis. After the electrophoresis, the gel was stained with 0.1% Coomassie brilliant blue R-250 and destained in 50% methanol containing 10% acetic acid.

2.6c. Gelatin-embedded PAGE for protease activity

Gelatin-PAGE (Felicioli *et al*., 1997) was performed by including gelatin (0.5% w/v final concentration) to the polyacrylamide (10%) gel as described above. Following electrophoresis at pH 8.3 the gel was washed

three times in distilled water and then incubated at 37° C in 0.05 M sodium acetate buffer pH 5.5 for 30 min. After incubation the gel was stained with Coomassie brilliant blue as described earlier. The presence of protease is detected as clear white band against a dark blue background.

2.6d. Electroblotting of proteins

Electroblotting of the purified ficins onto polyvinyledine difluoride (PVDF) membrane was carried out using the standard procedure described by Matsudaira (1987). Following electrophoresis, the gel was immediately rinsed in transfer buffer (10 mM CAPS containing 10% methanol and 0.1% SDS, pH 11.0) for 15 min. The PVDF membrane was cut to the required size was soaked in methanol for 5 min and equilibrated with transfer buffer for 15 min prior to transfer. Semi-dry electro-blotting was carried out using a semi-dry blotting apparatus (Towbin *et al.,* 1979). The transfer was carried out for 2 h using a current of 0.8 mA/cm^2 of the blotting paper. After transfer the membranes were stained with Coomassie blue R-250 and destained in 50% methanol. The band corresponding to ficins were excised, washed with methanol and dried.

2.7. Molecular mass determination

The molecular mass of the purified ficins was determined by MALDI-TOF. Matrix Assisted Laser Desorption Ionization Mass Spectroscopy (MALDI-MS) analysis were performed on a Bruker Daltonics Ultraflex MALDI TOF/TOF system (Bruker-Daltonics, Bremen, Germany) in the reflective positive ion mode. The purified enzymes were dialyzed extensively against water. The samples were prepared by mixing equal volumes of protein and matrix. Matrix was saturated α-cyano-4-hydroxycinnamic acid (Sigma Aldrich Chemie GmbH) prepared separately in CH_3CN/H_2O/TFA (80:20:0.1). The samples were then dried under atmospheric pressure at 25° C and loaded on to probe slide. The instrument was calibrated with the standards ranging in masses between 10-100 kDa.

2.8. Effect of pH on ficin activity and stability

The protease activity of ficins as a function of pH was determined using the following buffers of 50 mM each: citrate buffer (pH 4.0-6.0), sodium phosphate buffer (pH 6.5-7.5) and Tris-HCl buffer (pH 8.0-9.5). A plot of the relative activity versus pH was employed to obtain the pH optimum. To test the pH stability, the enzyme (0.5 mg/mL) in different specified buffers was incubated for different time intervals. At the end of the incubation period, aliquots of incubated enzyme were assayed for remaining activity. Enzymes were assayed against azocasein for ficin from *Ficus racemosa* and BAPNA as substrate for the ficin from *Ficus carica,* respectively as described in assay methods.

2.9. Determination of the protease group specificity

The residue involved in catalytic activity of the purified ficins was identified by using group specific chemical inhibitors of proteases. The enzyme was pre-incubated for 30 min at 37° C with different inhibitors specific for all classes of proteases: serine protease inhibitors (DFP and PMSF), cysteine protease inhibitors (PCMB, iodoacetamide and sodium tetrathionate), metalloprotease inhibitor (EDTA) and the aspartic protease inhibitor (pepstatin-A). The residual activity enzymes were determined as described previously. The assay performed without any of the inhibitors is taken as reference initial activity.

2.10. Amino acid analysis

Amino acid analysis of ficin 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 up 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 N_2 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.

2.11. Estimation of tryptophan

Tryptophan content of the purified ficin was estimated by spectrophotometric titration method as described by Spande and Witkop (1967) using N-bromosuccinimide (NBS) in Shimadzu UV 160A double beam recording spectrophotometer. To 3 mL of 0.5 mg/mL ficin solution in 0.05 M sodium acetate buffer at pH 4.0, NBS solution of 8.0×10^{-3} M was added in 10 µL aliquots through Hamilton syringe with constant stirring. 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 addition of NBS was continued until no further decrease in the absorbance with doe allowance for the small decrease expected from dilution. The number of tryptophan residues per mole of protein was calculated by the 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 ficin 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. Determination of thiol groups

The number of free cysteine and disulfide bridges of ficin were determined by using the sulfhydryl specific reagents 5, 5`-dithiobis-(2-nitrobenzoic acid) (DTNB). The assay was performed according to the method described by Ellman (1959) and Habeeb (1972). A molar extinction coefficient value of 13600 M^{-1} cm^{-1} was used for the calculation of sulfhydryl groups and in turn disulfide content of the enzyme.

2.13. Calculation of absorption coefficient

The extinction coefficient of purified ficin from the latex of *Ficus carica* was determined by Kjeldahl nitrogen estimation method (Jaenicke, 1974). The absorbance of different concentration solutions of purified ficin was recorded at 280 nm. These solutions were used for nitrogen estimation and a conversion factor of 6.25 was used to calculate the protein content of the samples. The specific extinction coefficient was calculated using Beer-Lambert`s law; $E^{1\%}_{280nm}=Acl$, where A, absorption at 280 nm; c, concentration of protein in mg/mL and l, path length of cuvette in centimeter (1 cm).

2.14. Determination of N-terminal sequence

Amino terminal sequences of purified ficins were determined by Edman degradation method. The purified ficins were transferred following SDS-PAGE to polyvinyledine difluoride (PVDF) membrane in 10 mM CAPS-10% methanol buffer by electroblotting at 0.8A/cm^2 at constant current for 2h and stained with Coomassie brilliant blue R-250. The band corresponding to the protease was excised and loaded directly to the gas phase protein sequencer (Applied Biosystems 447A) for automated Edman degradation. β-lactoglobulin (Applied Biosystems) was used as the standard to calibrate the instrument.

2.15. Determination of cleavage specificity of the enzyme

The cleavage specificity of the protease from the latex of *Ficus racemosa* was investigated by digesting the oxidized B-chain of bovine insulin and determining amino acid sequences of the peptides produced. The assay was carried out according to the method described elsewhere (Kim *et al*., 1995). The digestion was carried out in acetate buffer pH 5.5 (0.05 M) for 1 h at 60° C. The ratio of purified enzyme to substrate was 2% (w/w). The reaction was terminated by addition of TFA and clarified by centrifugation. The clear supernatant which contained the digested insulin

peptides were fractionated directly by RP-HPLC using a C-18 Symmetry Shield column (4.6 × 150 mm, 5 μm) on a Waters HPLC system equipped with a 1525 binary pump and Waters 2996 photodiode array detector set at 230 nm. The solvent used were 0.1% TFA (A) and 70% acetonitrile containing 0.05% TFA (B). A linear gradient traversing from 0 to 70% B in 60 min at a flow rate of 0.7 mL/min was employed. The peptide fractions were detected at 230 nm and collected. The peptides were identified by their sequence following Edman degradation in an automated gas phase protein sequencer as described earlier (Section 2.14).

2.16. Thermal inactivation studies of the enzyme

Thermal stability of the protease from the latex of *Ficus racemosa* at different temperatures was determined. Enzyme samples in 0.05 M acetate buffer pH 5.5 were incubated at the test temperatures for a reference time of 15 min, cooled rapidly and assayed for the remaining activity at 60° C. The kinetics of thermal inactivation of the purified protease was studied using a constant temperature bath, at the desired temperatures. Aliquots of enzyme removed at periodic intervals, were subjected to assay, after cooling to 4° C and the residual activity was measured as a percentage of initial activity. From the semi-logarithmic plot of residual activity as a function of time, the inactivation rate constants (k_r) were calculated.

Effect of cosolvents on thermal stability of purified ficin from the latex of *Ficus carica* was determined by incubating the enzyme solution at 70° C in the presence of different concentrations of cosolvents for 10 min in sodium phosphate buffer pH 7.0 using a water bath with shaker. The enzyme was immediately cooled in ice after incubation and the remaining activity was measured at 55° C using BAPNA as substrate. The enzyme activity in absence of cosolvent served as control and the percentage residual activity was calculated based on its original activity. The percentage residual activity was plotted against different concentrations of cosolvents.

2.17. Acid denaturation experiments

The samples of ficin for acid denaturation studies were prepared using following buffers of 20 mM each: KCl-HCl (pH 1.0-1.8), Gly-HCl (pH 2.0-3.5), sodium acetate (pH 3.5-5.0) and sodium phosphate (pH 6.0-7.0). The enzyme samples were incubated with buffers of desired pH at 25° C and allowed to equilibrate for 3 hours before taking all the spectroscopic measurements. In order to assess the reversibility of acid induced unfolding, ficin at various pH values was extensively dialyzed at 4° C against 20 mM sodium phosphate buffer, pH 7.0. This dialyzed preparation was compared to native ficin at pH 7.0 using fluorescence and CD measurements.

2.18. Folding and unfolding experiments

Unfolding of ficin was induced by incubating or dialyzing the enzyme with various concentrations of denaturants till the equilibrium is attained. Refolding was performed using the ficin that had been completely denatured by 8 M urea or 6 M GuHCl. The denatured ficin was either diluted with appropriate concentration of urea or GuHCl and the mixture was incubated till the reaction reached the equilibrium or alternatively the denatured protein was dialyzed against desired concentration of GuHCl/urea with several changes. The extent of unfolding and refolding of ficin was measured either by changes in emission maximum or change in the ellipticity values at 222 nm. The data are expressed in terms of the fraction unfolded (F_u) calculated from the standard equation (Pace and Scholtz, 1997).

2.19. Spectroscopic methods

2.19a. Absorption spectroscopy

Absorption spectra of protein in different conditions was recorded in Shimadzu UV 160A double beam recording spectrophotometer using the 1 cm quartz cell at 25° C in the rage of 230-360 nm. Respective buffer solutions were used as reference solution.

2.19b. Thermal denaturation studies

The thermal denaturation profile of ficin at different conditions was performed using a Cary Varian 100-bio UV-Visible spectrophotometer (Varian, Victoria, Australia). The spectrophotometer equipped with an electronically controlled thermal cuvette holder, which can accommodate six thermal quartz cuvettes. The thermal unfolding of ficin was monitored by recording the absorbance at 287 nm as a function of temperature in the range of 30-90° C at a scan rate of 1° C per min. The concentration of protein solution throughout the experiment was 1.05×10^{-5} M. The apparent T_m of ficin was obtained after normalizing the absorbance of native and denatured state. From the thermal denaturation profile, fraction of ficin unfolded was calculated using standard equation (Pace, and Scholtz, 1997).

$$F_u = (Y_F - Y)/(Y_F - Y_U) \qquad - - - - - - (2)$$

where Y_F is the absorbance of protein solution in the native state, Y_U the absorbance of protein solution in the unfolded state and Y is the absorbance of the protein solution at different temperatures. The apparent thermal denaturation temperature (T_m) is defined as the temperature at which the value of F_u is 0.5.

2.19c.Fluorescence spectroscopy

(i) Intrinsic fluorescence of protein

Fluorescence measurements of ficins under different conditions were carried out on Shimadzu (Model RF 5000) spectrofluorimeter. All the measurements were made at 25° C and appropriate blanks were used for baseline correction of fluorescence intensity. The intrinsic fluorescence was recorded in the wavelength ranging from 300 to 400 nm and the excitation of the protein solution was at 280 nm. The slit width for both excitation and emission was set at 5 nm.

(ii) ANS-binding studies of protein

For binding studies of ANS, ficin samples at different conditions were incubated with 100-fold molar excess of ANS for 30 min at 25° C in dark. The fluorescence of ANS was excited at 380 nm and emission was recorded between 400-600 nm. 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

Acrylamide quenching studies of the intrinsic fluorescence were performed by adding aliquots from the stock solution (5 M) of the quencher into a cuvette containing protein solution. The intrinsic fluorescence was excited at 280 nm, and the emission was recorded between 300-400 nm. Final fluorescence intensities were corrected for dilution effects. The decrease in fluorescence intensity at λ_{max} was analyzed by the Stern-Volmer equation (Eftink and Ghiron, 1981):

$$F_o/F = 1 + K_{sv}[Q] \qquad \text{– – – – – – (3)}$$

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.

2.19d. Circular dichroism spectroscopy

Circular dichroic (CD) spectral measurements of ficin under different conditions were carried out using an automatic recording Jasco J-810 spectropolarimeter (Japan Spectroscopic Co., Tokyo, Japan) fitted with a xenon lamp and calibrated with +d-10-camphor sulphonic acid. All the measurements were carried out at 25° C with a thermostatically controlled cell holder attached to water bath. Near-UV CD measurements in the region of 240-320 nm were determined with a 10 mm path length cell using the protein concentration of 47 μM. Far-UV measurements were recorded in the range of 190-260 nm using the protein concentration of 10 μM with 1mm

path length cell. The scan speed was set at 10 nm/min with a bandwidth of 1 nm and the spectra were taken as an average of three scans. The results were expressed as the mean residual ellipticity $[\theta]_{MRW}$ obtained from the relation

$$[\theta] = 100 \times \theta_{obs}/l \times c \qquad - - - - - - - - (4)$$

where θ_{obs} is the observed ellipticity in degrees. From the value of θ, the $[\theta]_{MRW}$ was calculated using a value of 115 for mean residue mass of the protease, c is the concentration in grams/liter and l is the length of the light path in cms. The values obtained were normalized by subtracting the base line recorded for the buffer under similar conditions. The analysis of the data for the secondary structure elements was done according to computer program of Yang *et al.,* (1986).

2.20. Measurements of partial specific volume

The partial specific volume measurements of ficin were carried out according to the standard procedure (Lee *et al*., 1979; Prakash, 1982; Prakash and Timasheff, 1981; 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)

$$\phi = 1/\rho_0[1-\{(\rho_p - \rho_0)/ C\}] \qquad - - - - - - - (5)$$

where ρ_p and ρ_0 are the densities of the protein solution and solvent respectively and C is the concentration of ficin in g/mL. The apparent partial specific volume of the protein ($\overline{\nabla}_{app}$) 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 (ϕ'^o_2) 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)

$$\xi_3 = (\delta g_3/\delta g_2)_{T, \mu_1, \mu_3} = 1/\rho_0 (\phi_2^o - \phi'^o_2) / (1 - \bar{\nabla}_3 \rho_0) \qquad - - - - - (6)$$

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° and ϕ'°_2 are the partial specific volume of the protein at isomolal and isopotential conditions, respectively and $\bar{\nabla}_3$ is the partial specific volume of the component three.

1. Isolation, purification and characterization of ficins from *Ficus racemosa* and *Ficus carica*

Proteolytic enzymes are multifunctional class of enzymes, which have many physiological functions ranging from generalized protein digestion to more specific regulated processes such as the activation of zymogens, blood coagulation, complement activation, inflammation process and liberation of physiological peptides from the precursor proteins. They are ubiquitous in nature (Neurath, 1984). Proteolytic enzymes constitute nearly half of the commercially utilized enzymes; frequently used in food processing, tenderization of meat, brewing, cheese elaboration, bread manufacturing, leather and textile industry (Rao *et al*., 1998). Thus, characterization of specific proteolytic enzymes helps in better understanding of their roles in physiological processes as well as in commercial applications.

Number of proteolytic enzymes have been identified and studied from the latex of several plant families such as *Caricaceae, Moraceae, Asclepiadaceae, Apocynaceae* and *Euphorbiaceae* (Caffini *et al*., 1988). Most of the plant derived proteases have been classified as cysteine proteases and more rarely belong to aspartic proteases (Boller, 1986). Proteolytic enzymes derived from the plant sources have received special attention because of their property of being active over a wide range of temperature and pH (Uhlig, 1998). The quest for valuable proteases with distinct specificity is always continuous for varied industrial applications.

The name 'ficin' has been used variously to describe the proteolytic enzyme activity in tree latex of the genus *Ficus* (Jones and Glazer, 1970), or the sulfhydryl protease or proteases prepared from the latex (Buttle, 2004). Hence the term 'ficin' must be regarded as generic. The genus *Ficus* contains more than 1800 named species making them one of the largest genuses in the *Moraceae* family. The ficins (EC 3.4.22.3) found in the latex of *Ficus*

glabrata and *Ficus carica* are cysteine proteases. These cysteine proteases are known to exist in a number of charged forms (Englund *et al*., 1968; Kramer and Whitaker, 1964). These proteases differ from each other chromatographically, compositionally and quantitatively with an essential cysteine at the active site (Liener and Friedenson, 1970).

The number of industrially used proteases of plant origin is small. Papain, bromelain and ficin are the most commercially important endopeptidases from the plant sources. Among these plant proteases, only papain and bromelain have been extensively utilized in medicine and many industrial applications as compared to ficin (Uhlig, 1998). There is perhaps very little information available on physical, chemical and enzymatic properties of ficins in general. In this study, we have purified and characterized the ficins from *Ficus racemosa* and *Ficus carica* for its enzymatic and chemical properties, which would help for understanding the structure and function of ficins from different *Ficus* species.

1.1. Purification and characterization of ficin from the latex of Ficus racemosa

The ficins isolated from the latex of different *Ficus* trees possess different characteristic properties. The most extensively studied ficins have been isolated from the latex of *Ficus glabrata* and *Ficus carica* and all belong to cysteine protease family. However the proteases (ficins) from other species are less known. Ficin-E isolated from *Ficus elastica* is a serine-centered protease, indicating for the first time that the latex of trees of the genus *Ficus* contains other classes of proteases (Lynn and Clevette-Radford, 1986). In this investigation, a protease from the latex of *Ficus racemosa* is purified and characterized for its biochemical characteristics. The characterized properties of the enzyme are further compared with that of other plant proteases.

Ficus racemosa is a moderate sized to large tree found in all parts of India in moist locality. Immature green fruits from *Ficus racemosa* trees growing locally were collected. The latex was collected by incising the stalk of the green fruit from the main branch. The collected latex was clarified by centrifugation at 10000 × g at 4° C for 60 min. The protease was purified to homogeneity from the latex by using conventional techniques that include the salt precipitation, molecular size-exclusion and anion-exchange chromatography as described in the methods and the steps are represented as a flow chart in Fig 1 (Devaraj *et al.,* 2008a). The total crude extract of the latex was used to detect the presence of proteases activity using gelatin-embedded PAGE. The gelatin-embedded PAGE of the crude extracts of latex indicated the presence of only a single protease (Fig 2 Inset). The presence of a single isoform in the *Ficus racemosa* latex is distinct as compared to the presence of several isoforms present in the latex of other *Ficus* trees. The lattices of *Ficus glabrata* and *Ficus carica* are known to contain a number of ficins (Liener and Friedenson, 1970).

The clarified latex was saturated to 80% ammonium sulfate at 4° C in the first step. This step afforded the removal of several non protein components including the polyphenols that absorb at 280 nm. The total activity recovered was 95%. This first step served in the separation and concentration of protein. Size exclusion chromatography of the concentrate by HPLC on BioSep-SEC-S2000 column was used as the second step of purification. The proteins resolved into three major fractions as shown in the Fig 2. Fraction two that exhibited maximum activity was pooled separately and concentrated by using a centrifugal device (10000 Da cutoff). This step resulted in 3.9 fold increase in the specific activity. A final step of anion exchange chromatography on DEAE-Sephadex A-50 was employed, to obtain the enzyme in a homogenous form. Several contaminating proteins were removed in the unbound fraction. A linear NaCl gradient (0–0.5 M)

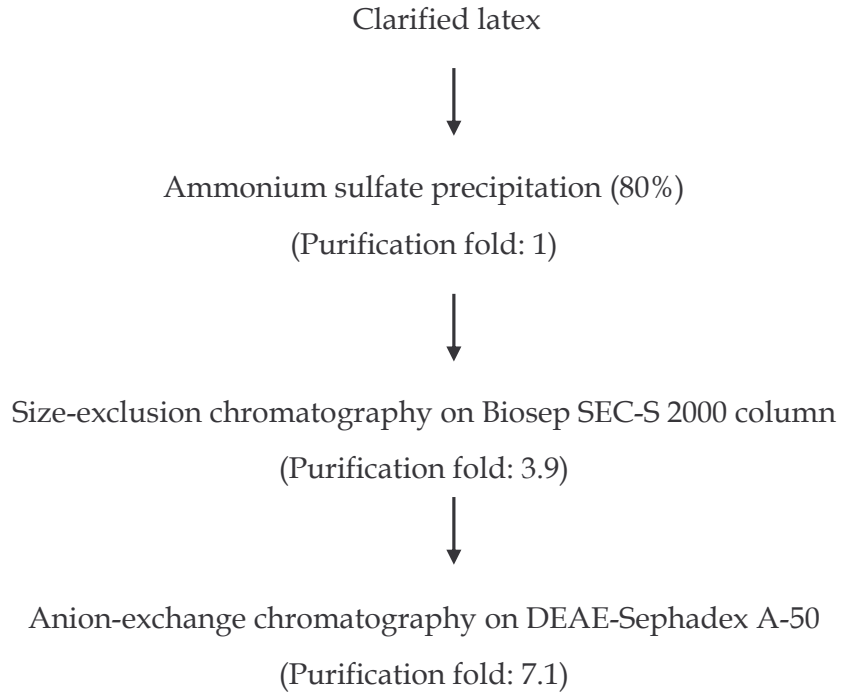

Fig 1: Flow chart for the purification of protease from the latex of *Ficus racemosa*.

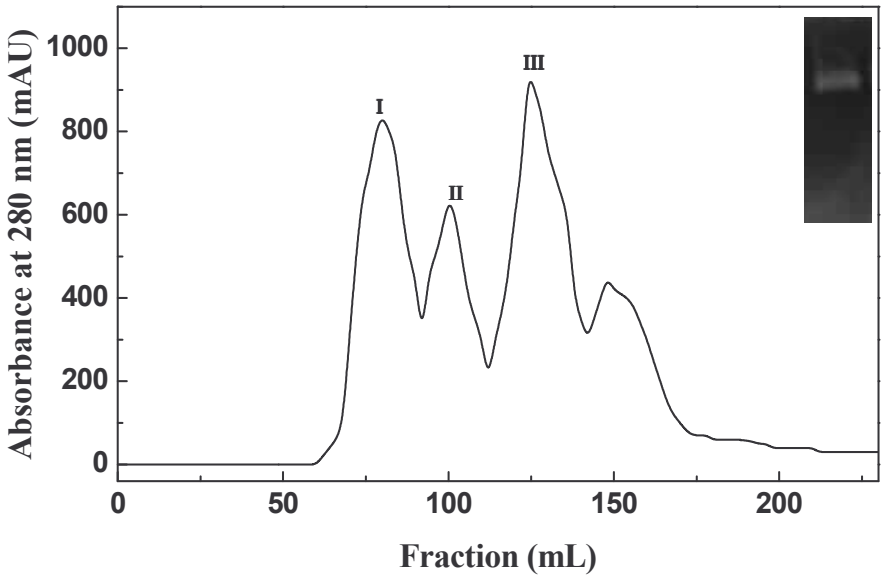

Fig 2: Size-exclusion chromatography profile of the latex proteins on BioSep-SEC-S2000. The column was pre-equilibrated with 0.05 M Tris-HCl buffer, pH 7.0. A flow rate of 5 mL/min was used and fractions were detected at 280 nm. Inset: Gelatin-embedded PAGE of crude extract of *Ficus racemosa* latex showing the protease activity.

was used to elute the bound protease (Fig 3). The fractions showing a high specific activity were recovered and pooled as indicated. The pooled fraction was dialyzed extensively (5 × 1L of 50 mM sodium acetate buffer pH 5.5). The results of the purification have been summarized in Table 3.

The specific activity of the purified protease was found to be 0.64 U/mg with a recovery of ~20% and existed as a single form with no isoenzymes. Among the several active components of commercial latex of *Ficus glabrata*, one major ficin was purified by CM-cellulose chromatography after reversible inhibition by sodium tetrathionate (Englund *et al*., 1968). Metrione *et al*. (1967) found evidence for three isoenzymes in a salt fractionated ficin. In contrast Sgarbieri *et al*. (1964) reported the separation of nine proteolytic components when salt fractionated *Ficus glabrata* latex was subjected to CM-cellulose chromatography.

The homogeneity of the purified enzyme was evaluated by native-PAGE, gelatin-embedded PAGE and gelfiltration chromatography. The purified enzyme was homogeneous, migrating as a single species on native-PAGE (Fig 4A). Gelatin-embedded PAGE, used specifically to detect proteases also shows a single activity band (Fig 4B). The single protein band visualized is indicative of a single isoform. Fig 5A shows the homogeneity of the enzyme on a BioSep-SEC-S2000 analytical gelfiltration column and the molecular mass estimated to be 44000 ± 1500 Da (Fig 5B). The apparent molecular mass of the purified enzyme estimated by SDS-PAGE is 43500 ± 500 Da indicated that the purified enzyme of *Ficus racemosa* is a single polypeptide chain protein (Fig 6 Inset). The molecular mass was also determined by MALDI-TOF was 44500 ± 500 Da (Fig 6). These results indicate that the purified protease is homogenous. The molecular mass of this protease differs from the other reported ficins. The molecular weight of the major ficin of *Ficus glabrata* determined by ultracentrifugation was 25500 ± 750 Da (Englund *et al*., 1968). These results taken together showed that

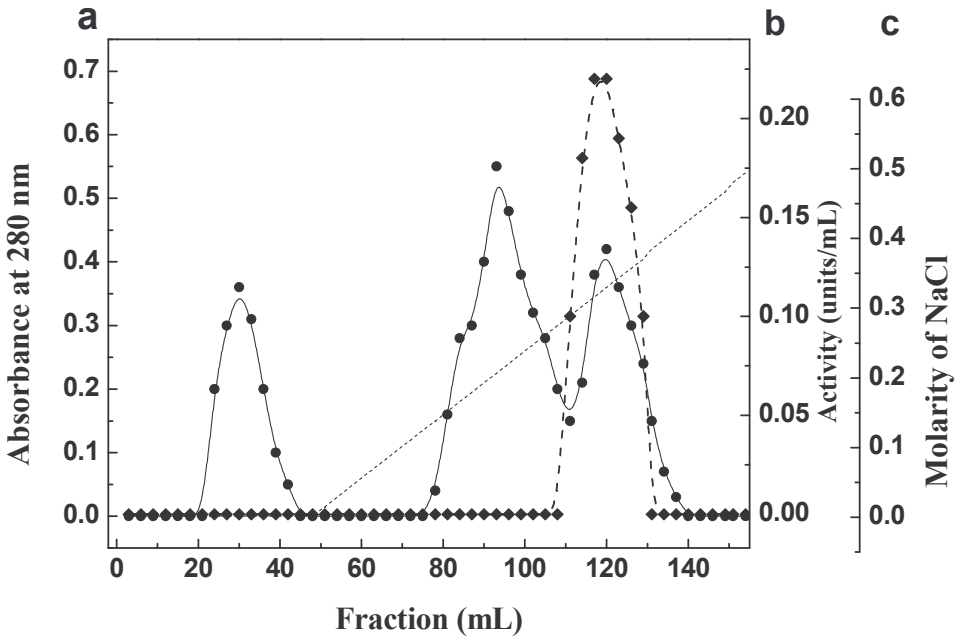

Fig 3: DEAE-Sephadex A-50 anion exchange chromatography profile of (Axis a) protein (—●—) and (Axis b) activity (---◆---). The column was washed with 0.05 M sodium acetate buffer pH 5.5 and protease eluted using a linear gradient (▪▪▪▪▪▪▪▪) of 0 – 0.5 M NaCl in the same buffer (Axis c).

Table 3: Purification steps of the protease from the latex of *Ficus racemosa

Steps	Total activity (Units)	Total protein (mg)	Specific activity (U/mg)	Purification (Fold)	Recovery (%)
Clarified latex	13.5 ± 2	150 ± 4	0.09 ± 0.01	-	100
80% $(NH_4)_2SO_4$ Precipitation	13.0 ± 2	143 ± 3	0.09 ± 0.01	1	95
Size Exclusion Chromatography (BioSep-SEC-S2000)	6.0 ± 1	17 ± 1	0.35 ± 0.05	3.9	45
DEAE-Sephadex A-50 Chromatography	2.6 ± 0.3	4 ± 0.5	0.64 ± 0.1	7.1	19

**These are the results of typical purification from 50 mL of the clarified latex of Ficus racemosa and are reproducible.*

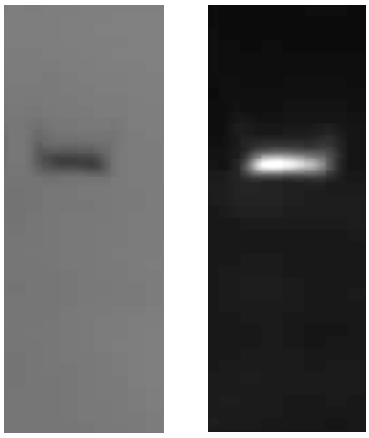

Fig 4: (A) Native-PAGE pattern of the purified protease in 10% gel to demonstrate the protease activity of the isolated enzyme. (B) Gelatin-embedded PAGE stained with Coomassie brilliant blue for detecting protease activity.

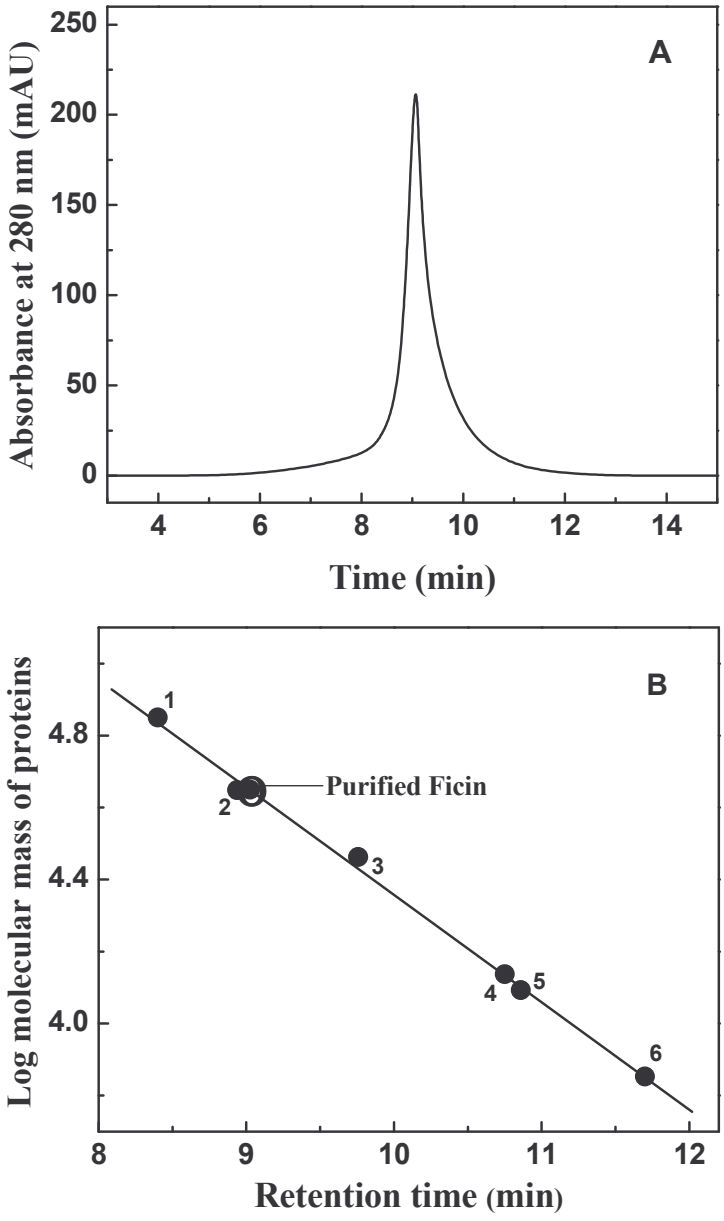

Fig 5: (A) The homogeneity of purified protease of *Ficus racemosa* by gelfitration chromatography using a BioSep-SEC-S2000 (300 × 7.8 mm, Phenomenex) analytical column, in a Waters HPLC system. The eluant used was 0.05 M sodium phosphate buffer, pH 7.4 at a flow rate of 1 mL/min. (B) The assessment of molecular mass of the protein. The column was calibrated with (1) bovine serum albumin, (2) ovalbumin, (3) carbonic anhydrase, (4) ribonuclease, (5) cytochrome c and (6) aprotinin.

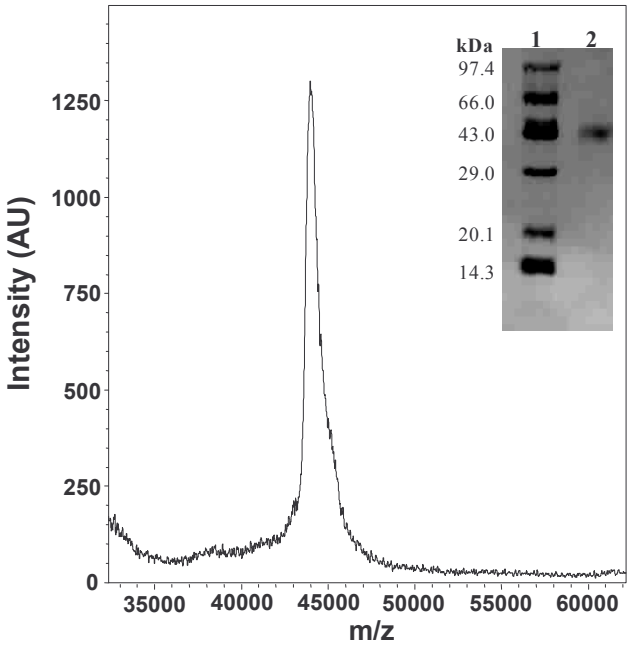

Fig 6: MALDI-TOF spectrum of the purified protease. Inset: SDS-PAGE (12% gel) of the purified protease. Lane 1 Molecular-mass markers: phosphorylase b (97.4 kDa), bovine serum albumin (66 kDa), ovalbumin (43 kDa), carbonic anhydrase (29 kDa), soy trypsin inhibitor (20.1 kDa) and lysozyme (14.3 kDa). Lane 2 Purified ficin from *Ficus racemosa*.

the purified protease of *Ficus racemosa* is distinct from other known ficins with respect to its existence as a single isoform and molecular mass.

The isolated protease had a pH activity profile as shown in Fig 7A. The maximum activity of the purified enzyme with azocasein was observed between pH 4.5–6.5. The enzyme is relatively active at low pH exhibiting 80% of its activity at pH 4.0. However, beyond pH 6.5 there is a sharp decline in the activity. Half the maximal activity is observed at pH 7.0. Varying the ionic strength from 0.1-0.6 M of the acetate buffer at pH 5.5 had no effect on the activity of the enzyme. Further increase in the ionic strength led to decrease in the activity. The enzyme also showed high stability over a wide pH range when incubated at 25 ± 2° C for 120 min. The residual activity was 95-80% when incubated at pH 4.0-7.0. However, the activity decreased rapidly when incubated at pH 8.0 (Fig 7B).

The relative activity of the purified protease in the presence of various inhibitors was carried out to establish the nature of the protease and its characteristics. The inhibitors evaluated include serine protease inhibitors (DFP and PMSF), cysteine protease inhibitors (PCMB, iodoacetamide and sodium tetrathionate), metalloprotease inhibitor (EDTA) and the aspartic protease inhibitor (pepstatin-A) (Table 4). Pepstatin, a high binding inhibitor specific for aspartic protease, inhibits the enzyme at relatively low concentrations. At pepstatin-A concentration as low as 80 μM, the inhibition of azocasein digestion was complete. This inhibition of the protease by pepstatin-A was further evaluated using oxidized insulin B-chain as the substrate. The results show that in the presence of pepstatin-A, the peptide was not digested (Fig 8). These results further show that the protease is an aspartic protease. The proteolytic activity of this enzyme was not inhibited by PCMB, sodium tetrathionate and iodoacetamide ruling out that this could be a thiol protease, as most proteases seen in the latex of other plants are thiol proteases in general. The ficin isolated from the latex of *Ficus glabrata*

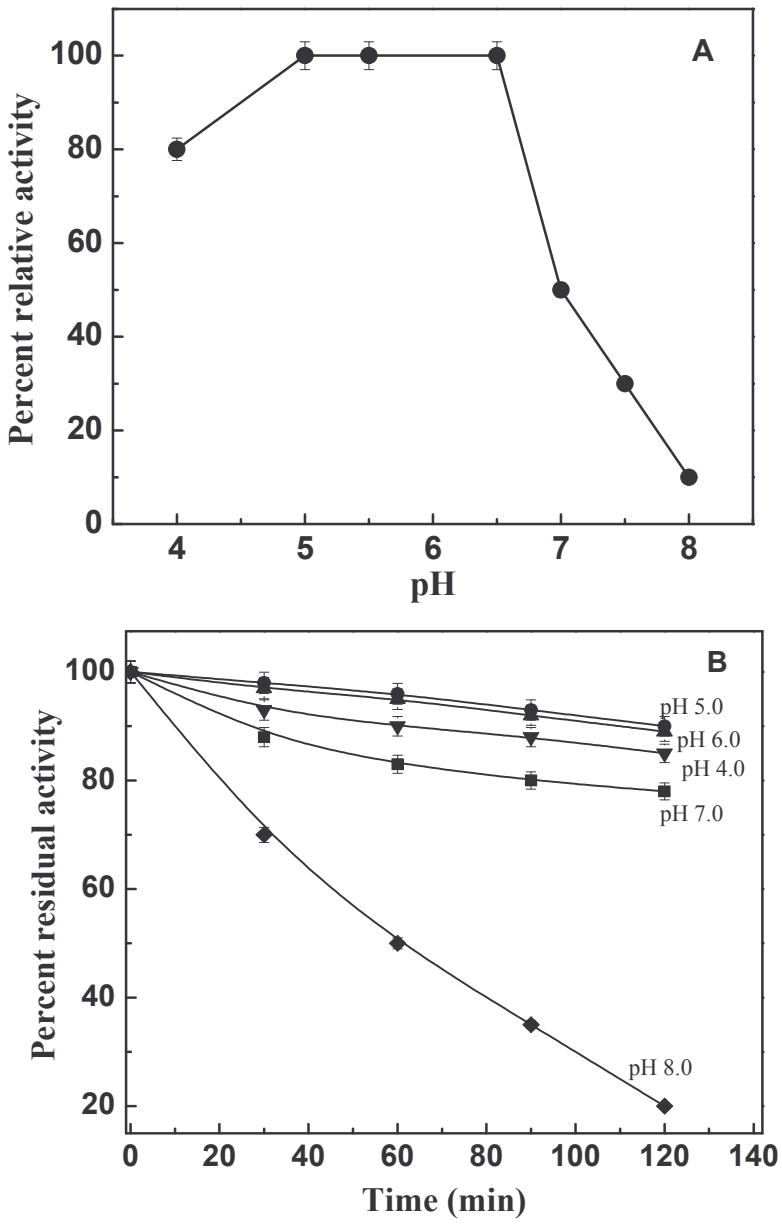

Fig 7: (A) Effect of pH on the activity of the purified protease and (B) pH stability. The buffers used were acetate (0.05 M) pH 4.0–6.0 and sodium phosphate (0.05 M) pH 6.5–8.0 and the enzyme were assayed as described in assay methods.

Table 4: Effect of inhibitors on the activity of the protease

Inhibitor	Concentration (mM)	Residual activity (%)
PCMB	1.0	98
Iodoacetamide	1.0	97
Sodium tetrathionate	1.0	99
DFP	1.0	97
PMSF	1.0	98
EDTA	5.0	99
Pepstatin-A	0.08	0

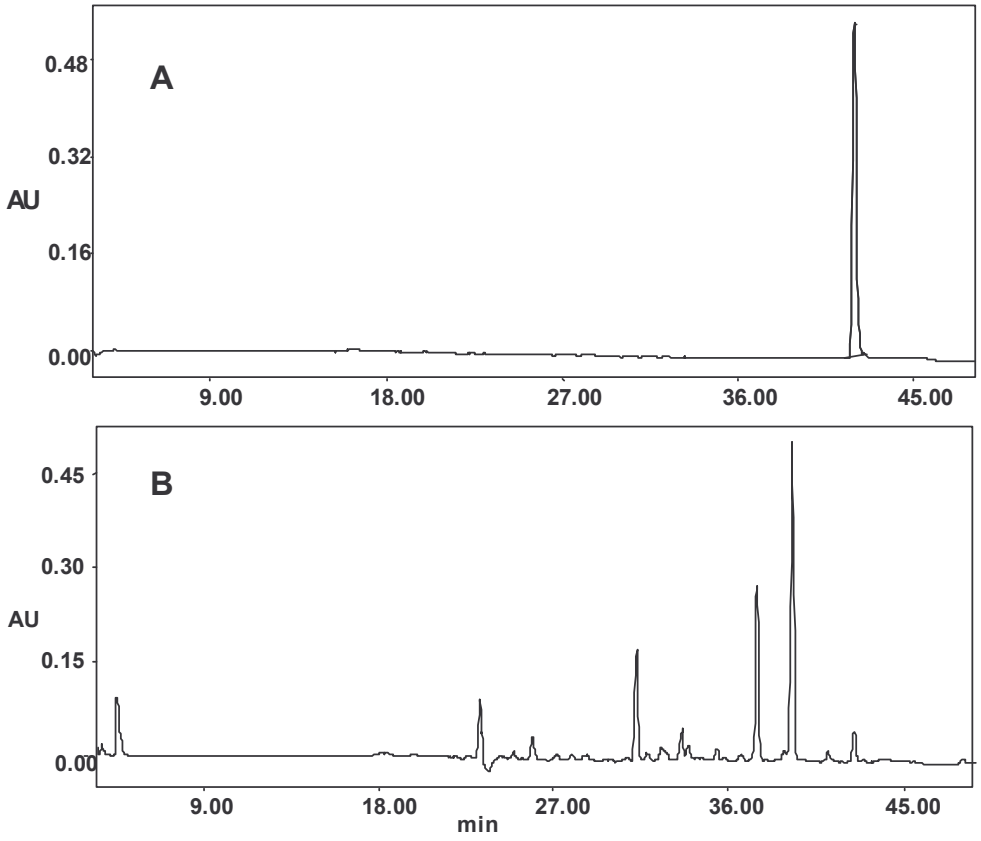

Fig 8: (A) RP-HPLC profile showing the pepstatin-A inhibition of the oxidized insulin B chain digestion by the protease of *Ficus racemosa*. (B) RP-HPLC profile of the *Ficus racemosa* protease cleaved peptides of oxidized B chain of insulin. The peptides were resolved using a Symetry Shield C-18 column (4.6 × 150 mm). The solvents used were 0.1% TFA and 70% acetonitrile containing 0.05% TFA.

and *Ficus carica,* as well as papain from latex of *Carica papaya* are all thiol proteases (Englund *et al*., 1968; Kortt *et al*., 1974). Different from these, a serine protease has been reported in the latex of *Euphorbia milii* (Yadav *et al*., 2006).

The optimum temperature for the proteolysis of azocasein was found to be 60 ± 0.5° C (Fig 9A). Even at higher temperatures (70° C) the enzyme exhibited 50% of its original activity indicating the thermal stability of the enzyme. The effect of temperature on the proteolytic activity was determined after incubating the enzyme for a reference time of 15 min at different temperatures (Fig 9B). The enzyme was incubated over a temperature range of 40–85° C and the residual activities measured at 60° C. The enzyme was stable up to 60° C with an apparent T_m of 70 ± 0.5° C. The semi-logarithmic plots of residual activity versus incubation time at different temperatures are characterized by straight lines of $r > 0.98$. Therefore the inactivation process can be attributed to a single exponential decay (Fig 10).

An investigation of specificity of the purified enzyme was carried out by determining the sites of the cleavage catalyzed by the enzyme using oxidized B-chain of insulin. The cleaved peptides were resolved by RP-HPLC as shown in Fig 11A and their amino acid sequence was determined by automated Edman degradation on an Applied Biosystems 447A protein sequencer. Analysis of the P_1-P_1` scissile bond specificity of the protease indicated that the $P_1$ residue was Phe, Leu or Glu and the preferred $P_1$` residue was Tyr (P_1-P_1` notations according to Schechter and Berger, 1967). The RP-HPLC peptide profile of oxidized B chain of insulin digested with ficin of *Ficus carica* (Fig 11B) is different, clearly indicating that the enzyme isolated from *Ficus racemosa* is different from that of *Ficus carica*. Earlier studies of Englund *et al* (1968) have shown that ficin obtained from the latex of *Ficus glabrata* preferably hydrolyses the peptide bonds following aromatic

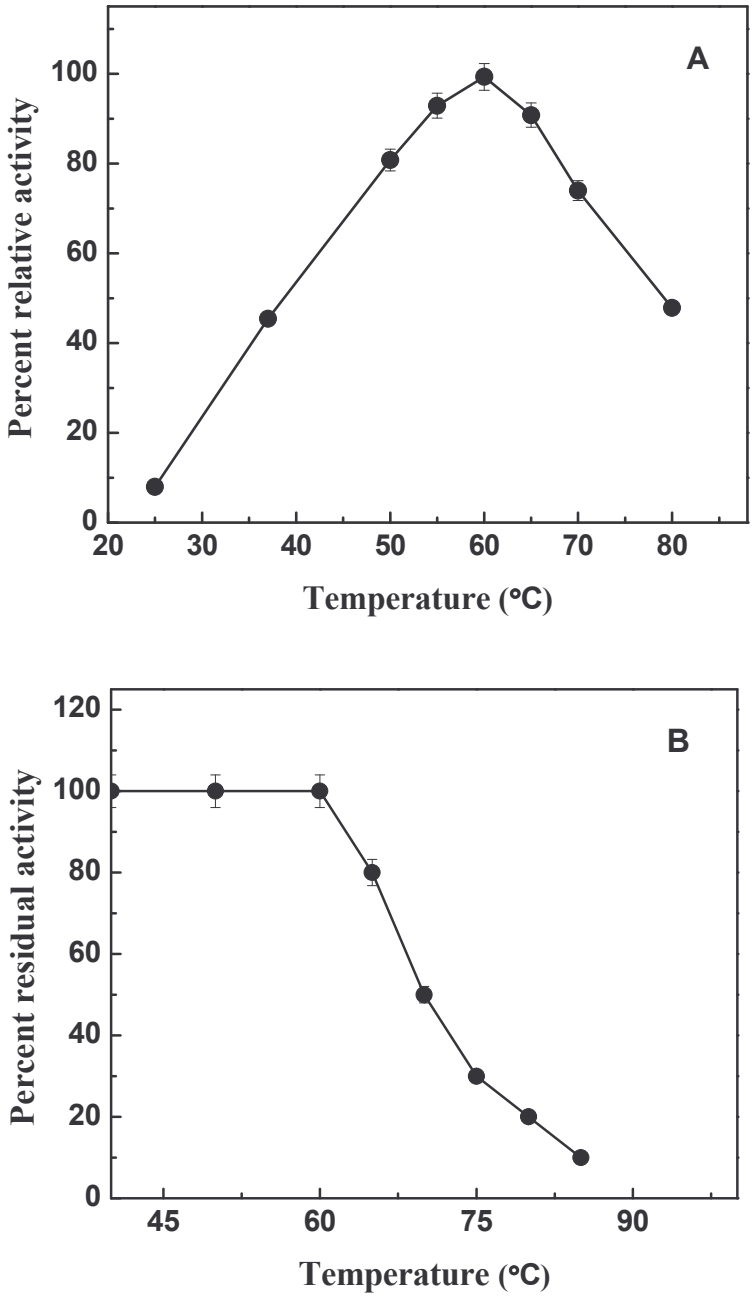

Fig 9: (A) Effect of temperature on the activity of the purified protease (temperature optimum). (B) Thermal stability of the protease at different temperatures. Enzyme samples in 0.05 M acetate buffer pH 5.5 were incubated at the test temperatures for a reference time of 15 min, cooled rapidly and assayed for the remaining activity at 60° C.

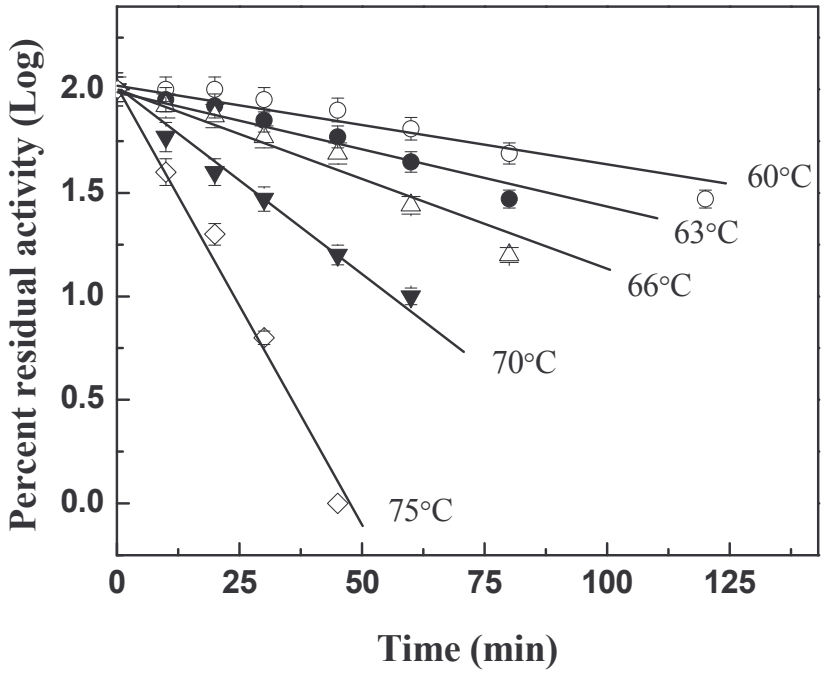

Fig 10: Thermal inactivation profile of the protease of *Ficus racemosa*. The enzyme was incubated at different test temperatures. Aliquots were drawn at different time intervals and cooled to 4° C and their residual activities were measured.

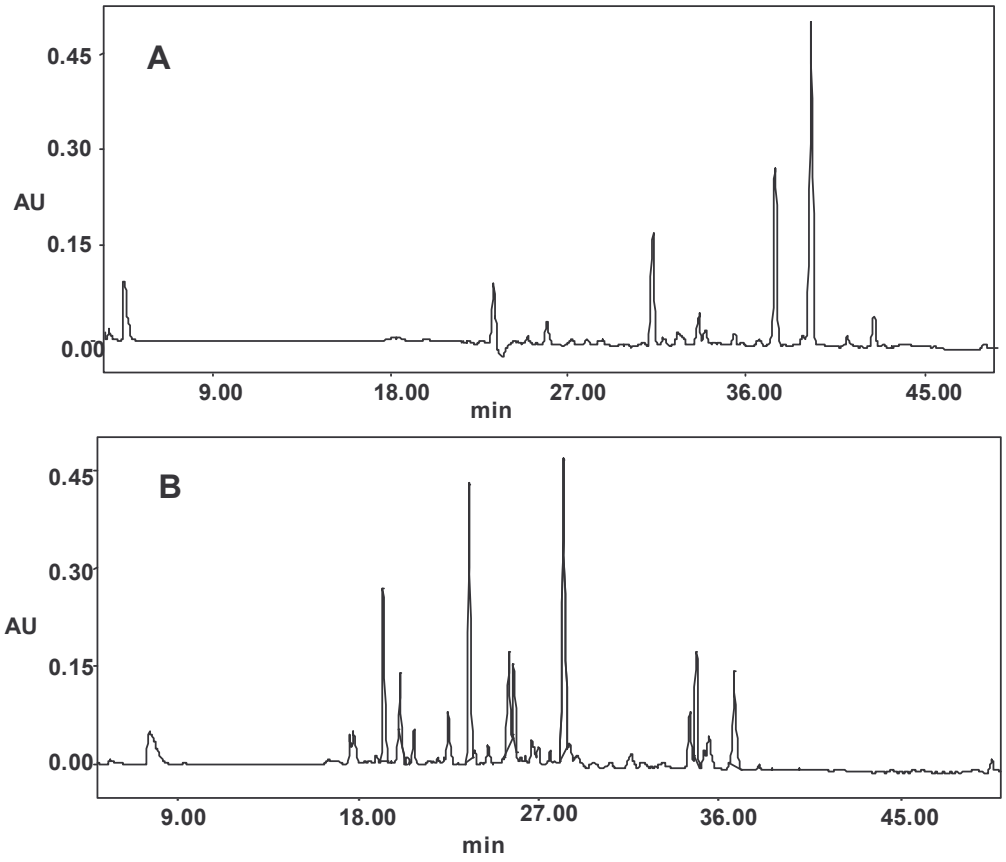

Fig 11: (A) RP-HPLC profile of the *Ficus racemosa* protease cleaved peptides of oxidized B chain of insulin. (B) Cleavage pattern of B chain of insulin hydrolyzed by ficin from the latex of *Ficus carica.* The peptides were resolved using a Symetry Shield C-18 column (4.6 × 150 mm). The solvents used were 0.1% TFA and 70% acetonitrile containing 0.05% TFA. The peptides were detected at 230 nm. These peptides were subjected to amino-terminal sequencing to deduce the cleavage specificity.

residues more efficiently as compared to others. A protease of musk melon fruit also showed broad specificity towards Leu, Phe, Glu, Ala, Val, Gly, Pro at P_1 position (Kaneda *et al*., 1997), but the preferential cleavage sites, as in the present study were hydrophobic and acidic amino acid residues at the P_1 position. Like the majority of the other aspartic proteases, the purified enzyme cleaves peptide bonds between residues with hydrophobic side chains (Fruton, 1976).

In order to study the possible food applications of the protease of *Ficus racemosa* the activity of the protease using different food proteins as substrates was studied. The highest activity was observed with casein (Table 5). Casein digestion constitutes the main process in the first phase of milk clotting in cheese making industry. The purified *Ficus racemosa* aspartic protease preferentially cleaves carboxy-terminus of Phe. It is well established that the first step of milk clotting starts with the cleavage of κ-casein at the Phe^{105}-Met^{106} bond (Sousa *et al*., 2001). Taking together the cleavage specificity of the purified protease and its ability to digest casein is suggestive of a milk clotting property. Nevertheless this will require a more detailed study. The purified enzyme also showed similar but lower activity towards arachin and glycinin digestion. Several endogenous seed storage proteins have been tested *in vitro* as substrates for plant aspartic proteases (Belozersky *et al*., 1989). The aspartic protease of *Ficus racemosa* hydrolyses arachin and glycinin, seed storage proteins of peanut and soy respectively. Therefore it may not be unreasonable to assume that the *Ficus racemosa* latex aspartic protease is physiologically associated with protein degradation for mobilization of nitrogen to the developing fruit. Runeberg-Roos and Saarma (1998) showed that the aspartic protease is localized at the developing tracheary elements of barley root tip cells.

Table 5: Hydrolyzing activity of the protease against different substrates

Substrate	Activity (Units/mg)
Casein	178 ± 5
Albumin	169 ± 5
Arachin	152 ± 6
Glycinin	135 ± 5
Gelatin	133 ± 6

The purified enzyme showed the following sequence from the amino-terminus, NH_2-EQELEQAGGYLA. This sequence is differed from the amino-terminus sequence NH_2-LPESVDWARFGAVN, determined for a ficin from *Ficus carica*. A BLAST search of the determined sequence did not show any matches with other plant proteases. Therefore the first 12 amino acid residues of the protease from *Ficus racemosa* were aligned and compared with amino-terminus sequences of other plant aspartic proteases (Table 6) like cardosin A, cardosin B (*C. cardunculus* L.), phytepsin *(H. vulgare*), At-Asp1 (*A. thaliana*), chlapsin (*Chlamydomonas reinhardtii)*, cyprosin B (*C. cardunculus)* and vigna AP (*V. unguiculata).* A similar amino-terminal Glu is observed in phytepsin. Val and Leu corresponding to Val^7 and Leu^9 are the first conserved amino acids in plant aspartic protease (Domingos *et al*., 2000). Leu^{11} of *Ficus racemosa* protease corresponding to Leu^9 of phytepsin is conserved. The amino-terminal sequences of plant aspartic proteases show heterogeneity and this could be the reason for the absence of any matches with BLAST search.

The far-UV CD spectrum for the protease was determined over the range of 190–260 nm in acetate buffer at pH 5.5 (0.05 M). Fig 12A shows the far-UV CD spectrum of the purified protease. The analysis of the spectrum indicated that the enzyme contains about 13% α-helix, 63% β-structures and aperiodic structures of 24%. The overall secondary structure of the purified aspartic protease of *Ficus racemosa* shows mainly the β-structures with very little α-helix. The three dimensional structure of cardosin A of *Cynara cardunculus* L. (PDB code: 1B5F) (Frazao *et al*., 1999) and prophytepsin of barley (PDB code: 1QDM) (Kervinen *et al*., 1999) show essentially the β-structures and very little α-helix. These results show that the purified aspartic protease of *Ficus racemosa* perhaps has secondary structural features similar to other plant aspartic proteases.

Table 6: Comparison of N-terminal amino acid sequence of *Ficus racemosa* protease with other aspartic proteases*

Enzyme	Amino-terminal sequence
Ficus racemosa protease**	**E Q E L E Q A G G Y L A**
Phytepsin (*H. vulgare*)	E E E G D I V A L K N Y
Vigna AP (*V. unguiculata*)	G T E T D I V A L K N Y
At-Asp 1 (*A. thaliana*)	G G D A D V V V L K N Y
Cardosin A (*C. cardunculus* L.)	D S G G A V V A L T N D
Cardosin B (*C. cardunculus* L.)	D S G G G I V A L T N D
Chlapsin (*C.reinhardtii*)	S S D Q G Q V T L K N G
Cyprosin B (*C. cardunculus*)	D S D G E L I A L K N Y

* *Domingos et al. (2000)*

** *The amino terminal sequence determined in this study*

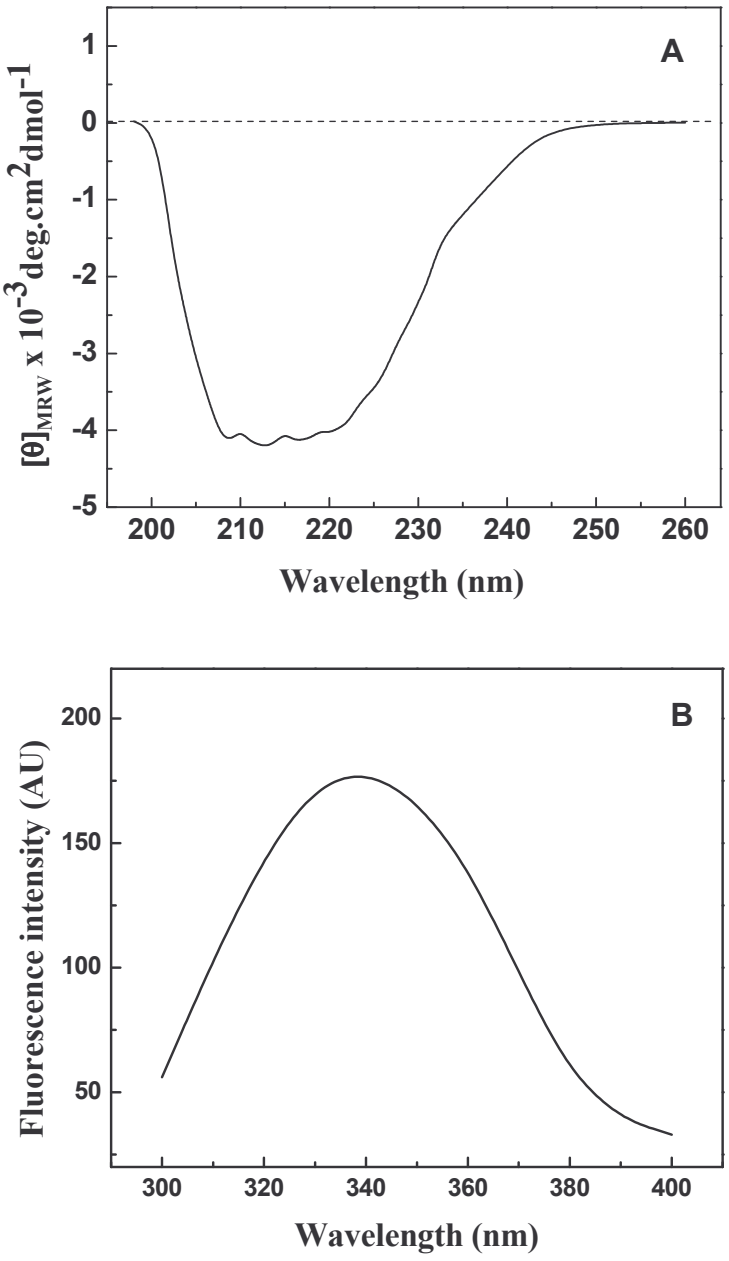

Fig 12: (A) Far-UV CD spectrum for the protease was determined over the range of 190–260 nm in acetate buffer at pH 5.5 (0.05 M). (B) The intrinsic fluorescence emission spectrum of the purified protease.

The intrinsic fluorescence emission spectrum of purified protease of *Ficus racemosa* was determined in acetate buffer at pH 5.5 (0.05 M). Tryptophans are known to display emission maximum at 330 nm or less in hydrophobic environment, whereas tryptophans localized in more hydrophilic surroundings like bulk water have an emission peak around 350 nm (Eftink and Ghiron, 1981). The purified enzyme exhibited an emission maximum at 338 ± 0.5 nm (Fig 12B), indicating that tryptophan residues were partially exposed to hydrophilic microenvironments.

The enzyme purified from the latex of *Ficus racemosa* unlike other multiple forms of ficin known to date exists as a single isoform. It is a monomer of molecular mass 43,000 ± 2000 Da, much larger than the ficins isolated from *Ficus glabrata* and *Ficus carica*. The purified protease shows marked thermal stability as evidenced by its stability with an apparent T_m of 70 ± 0.5° C. Unlike ficins of *Ficus carica* and *Ficus glabrata*, the reported protease of *Ficus racemosa* is an aspartic protease with a broad pH optimum (4.5–6.5). The protease from the plant lattices are noted for their broad specificity and their effectiveness at digesting protein but the preferred amino acid residues are different. The cleavage specificity of the *Ficus racemosa* protease is towards acidic and hydrophobic amino acids, the preferred P_1 residues being glutamate, leucine and phenylalanine. The characterized properties of this protease share several homologies with other plant aspartic proteases with respect to pH optimum, molecular mass, inhibition by pepstatin, thermal stability and its secondary structural features. All these results put together indicate that the thermostable protease purified from *Ficus racemosa* is unique differing from the known ficins.

1.2. Purification and characterization of ficin from the latex of Ficus carica

Ficin (EC 3.4.22.3), a proteolytic enzyme present in the latex of both *Ficus glabrata* and *Ficus carica* trees has been recognized as sulfhydryl enzyme, contains a cysteine residue at active site essential for its activity (Liener and Friedenson, 1970). Although crude ficin is of considerable commercial importance, ficin as such has not been fully characterized. Until now, most of the work has been carried out on the enzyme prepared from the latex of *Ficus glabrata* and a very few studies on the ficin isolated from the latex of *Ficus carica* species. The ficin isolated from both these species is known to occur naturally in multiple forms distinguishable by ion-exchange chromatography (Liener and Friedenson, 1970).

A major ficin among the several active components of latex of *Ficus glabrata* is a single polypeptide chain protein (Englund *et al*. 1968). Sgarbieri *et al*. (1964) reported the separation of several proteolytic components by CM-cellulose chromatography from the lattices of both *Ficus glabrata* and *Ficus carica*. The available information indicates that ficin apparently shares many common properties with papain with regard to substrate specificity, esterase activity, transpeptidase reactions and activation by reducing agents (Liener and Friedenson, 1970). The amino acid sequence determined for neighboring residues of active site cysteine found to resembles closely the corresponding one in papain (Liener and Friedenson, 1972). In this study, we have purified and characterized ficin from the commercial crude proteinase mixture from the latex of *Ficus carica* for its enzymatic and chemical properties, which would help for understanding the structure and function of ficins in general from different *Ficus* species and also other plant cysteine proteases.

The lattices of *Ficus glabrata* and *Ficus carica* are known to contain multiple forms of proteolytic components (Liener and Friedenson, 1970). These multiple forms of ficin can be distinguished by ion-exchange chromatography. Hence, the commercial crude preparation of ficin from the *Ficus carica* was subjected to cation-exchange chromatography using SP-Sepharose column on FPLC. The column was equilibrated with sodium acetate buffer pH 5.0 (0.05 M). Fig 13 shows the chromatographic separation of proteolytic components of crude preparation of ficin. These components have been designated as ficin I, II, III, IV and V in order of their elution from the column. Previous report of Sgarbieri *et al*. (1964) has shown the separation of nine proteolytic components when salt fractionated *Ficus glabrata* latex was subjected to CM-cellulose chromatography. Similar to the results in this study, the presence of six active fractions of ficin from the latex of *Ficus carica* was also reported by Richter *et al*. (2002), when the crude ficin was subjected to CM-cellulose column on FPLC.

The major ficin fraction II (which constituted about ~60% of total activity) was collected separately and subjected to further studies. Hence throughout this study ficin simply refers to fraction II. The purified ficin showed the activity of 6824 ± 26 units per mg of protein against casein. The activity of ficin was also studied using BAPNA as synthetic substrates showed the activity that one mol of enzyme hydrolyses 0.65 mol of BAPNA per min. These results indicated that relatively low level of activity with BAPNA when compared to casein hydrolysis. The exhibition of the greatest activity of ficin from *Ficus glabrata* with casein and a relatively low level of activity with BAPNA was also reported by Englund *et al*. (1968).

The homogeneity of the ficin fraction II was evaluated by SDS-PAGE and gelfiltration chromatography on FPLC using Superdex 75 column. In both the cases the purified enzyme was found to be homogenous. The purified ficin eluted as single peak on gel filtration chromatography and the

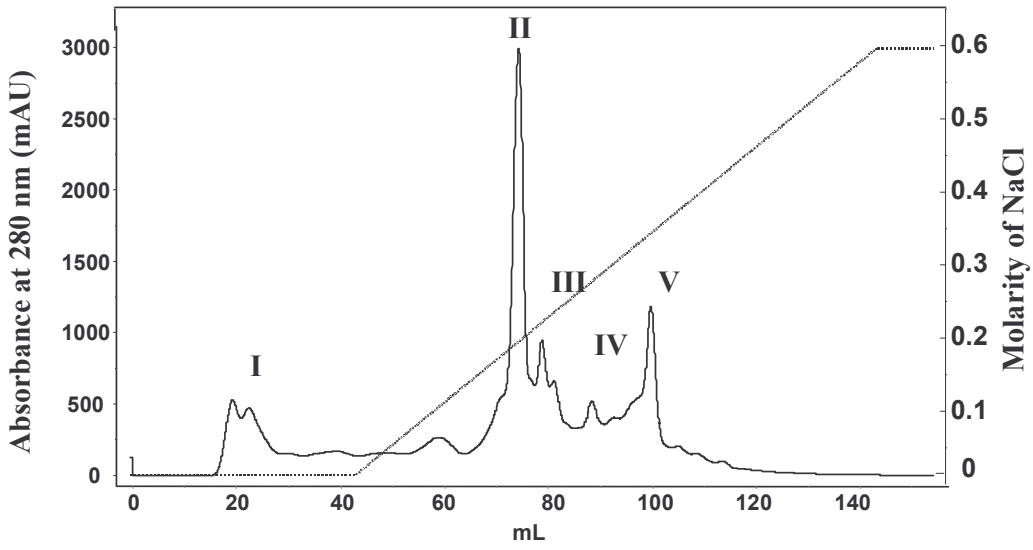

Fig 13: Chromatographic separation of crude preparation of ficin on FPLC using Hiload 16/10 SP-Sepharose high performance column (Amersham Biosciences AB, Uppsala, Sweden). The column was equilibrated with 0.05 M sodium acetate buffer at pH 5.0. The bound proteins were eluted using linear gradient (.....) of 0-0.6 M NaCl in the same buffer and the eluted fractions were read at 280 nm.

molecular mass was determined to be 23400 ± 500 Da (Fig 14). The homogeneity and molecular mass was further evaluated by SDS-PAGE. As shown in the Fig 15, the purified ficin migrated as a single band on SDS-PAGE. These results put together indicated that the purified ficin is homogenous and the enzyme is a single polypeptide chain protein. The molecular mass of ficin was also determined by MALDI-TOF and it was found to be 23100 ± 300 Da (Fig 16). These results are comparable with the earlier reports. The estimated molecular mass of purified ficin falls in the range of molecular mass 20000 - 35000 Da, which is reported for other cysteine proteases (Turk *et al.,* 1997). The molecular mass of ficin fractions isolated from *Ficus glabrata* were also in the range between 25000-26000 Da, which are very similar to our results (Englund *et al.,* 1968; Williams and Whitaker, 1969; Jones and Glazer, 1970*)*.

The purified ficin has pH activity profile as shown in the Fig 17A. The maximum activity was observed between pH 6.5-8.5 and showed optimum activity at pH 7.0. These results show that the enzyme is more active in the neutral range as compared to the acidic range. The lowest activity was observed in acidic range indicating the liable nature of ficin in acidic conditions. Similar results have been reported earlier by Kramer and Whitaker (1964) that the different ficin fractions from *Ficus carica* are more active in the neutral pH range. The acid liable nature of ficin from *Ficus glabrata* was also observed by Englund *et al*. (1968).

The effect of temperature on the activity of purified ficin was evaluated by measuring the activity at different temperatures. Fig 17B shows the activity profile of the enzyme at different temperatures. The enzyme was stable up to 60° C and showed maximum activity at 55° C. Even at higher temperatures the activity of the enzyme was not reduced rapidly. These results show a marked thermal stability of the enzyme at higher temperatures.

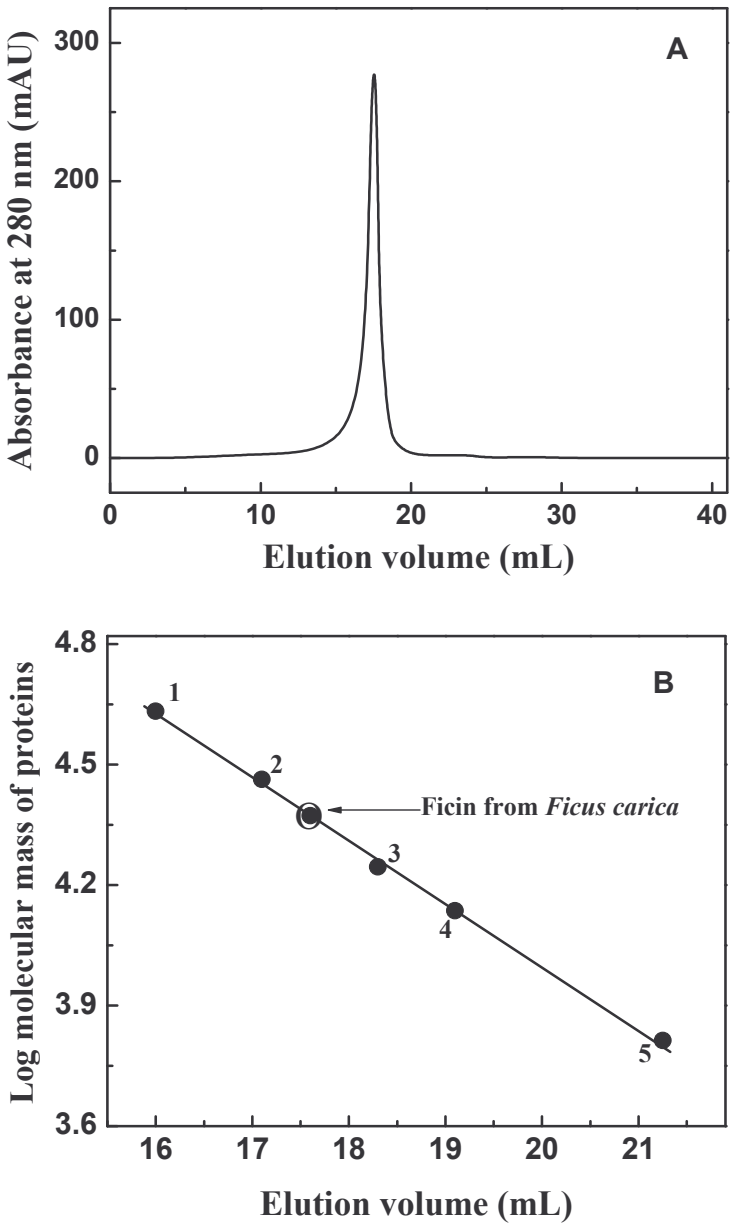

Fig 14: (A) Homogeneity of purified ficin by FPLC on Superdex 75 gelfiltration column (Amersham Biosciences AB, Uppsala, Sweden). The column was equilibrated using 0.02 M sodium phosphate buffer, pH 7.0 and the proteins were eluted at the flow rate of 30 mL/h. (B) Assessment of molecular mass of the protein. The column was calibrated with (1) ovalbumin, (2) carbonic anhydrase, (3) myoglobin, (4) ribonuclease and (5) aprotinin.

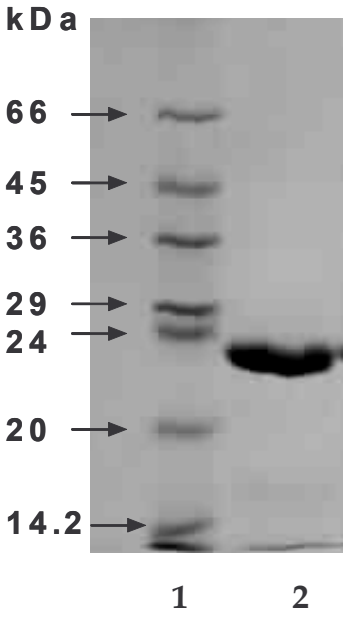

Fig 15: SDS-PAGE pattern of the purified ficin in 12% gel. Lane 1: Molecular-mass markers: bovine serum albumin (66 kDa), ovalbumin (45 kDa), glyceraldehyde 3-phosphate dehydrogenase (36 kDa), carbonic anhydrase (29 kDa), trypsinogen (24 kDa), soy trypsin inhibitor (20 kDa), and α-lactalbumin (14.2 kDa). Lane 2: Purified ficin.

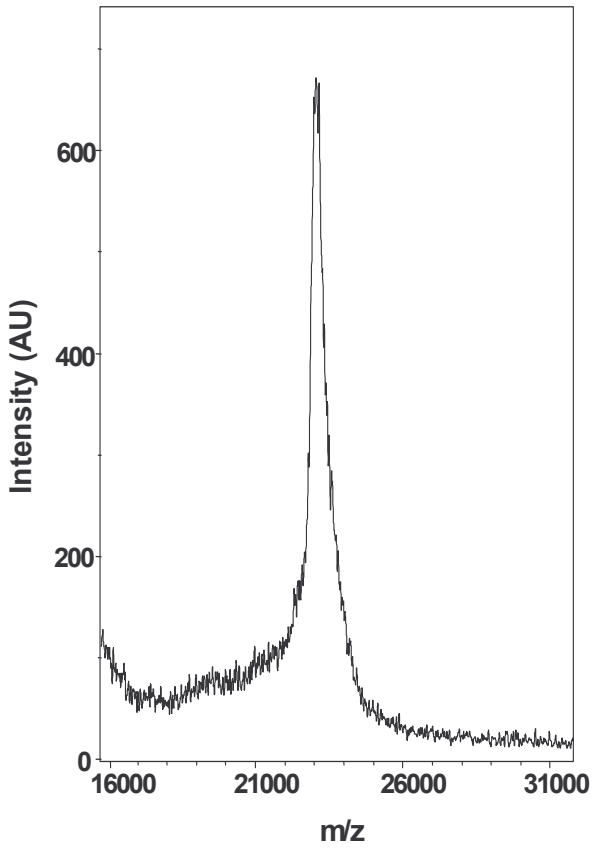

Fig 16: MALDI-TOF spectrum of purified ficin from *Ficus carica*. The enzyme sample was mixed in equal volumes with α-cyano-4-hydroxycinnamic acid, dried at atmospheric pressure and loaded onto probe slide. The measurements were carried out in the reflective positive ion mode.

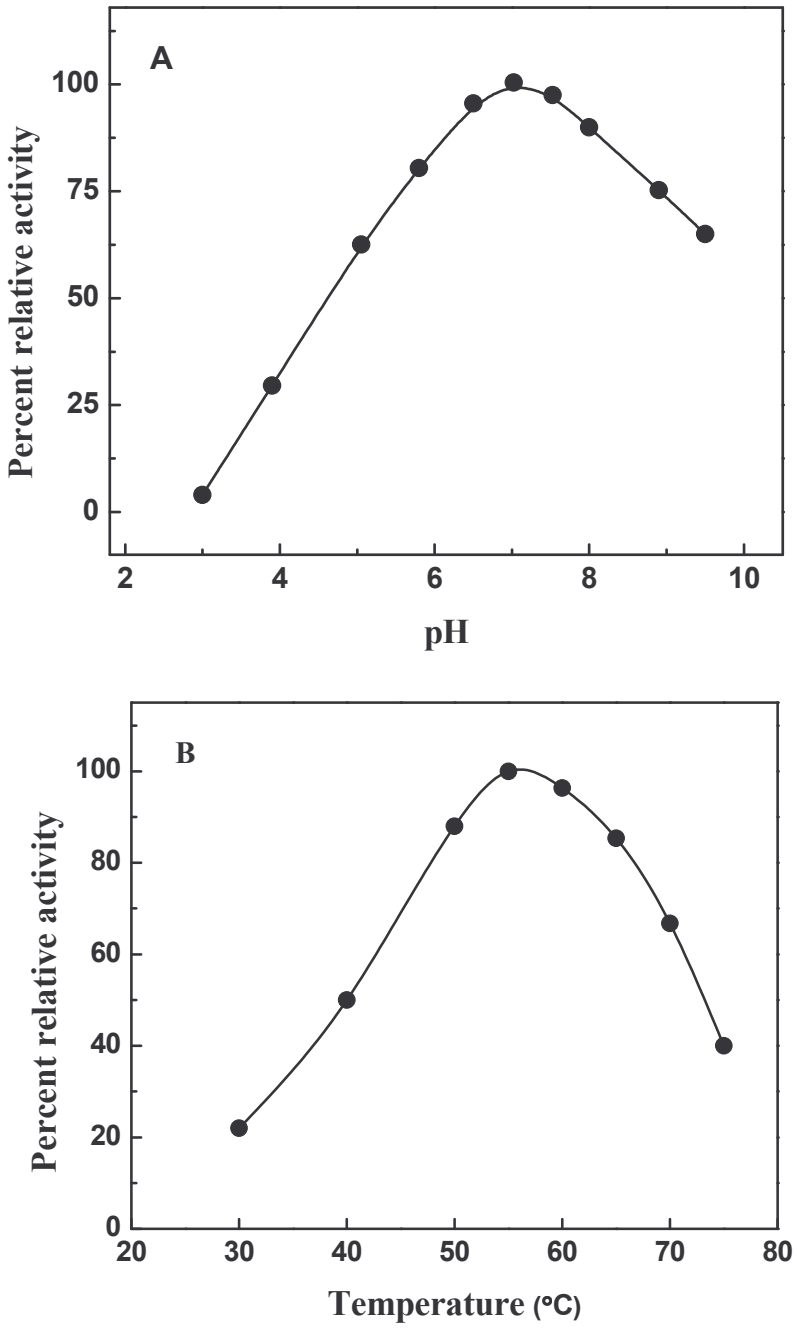

Fig 17: (A) Effect of pH on the activity of the purified ficin using the following buffers: 50 mM citrate buffer (pH 4.0-6.0), 50 mM sodium phosphate buffer (pH 6.5-7.5) and 50 mM Tris-HCl buffer (pH 8.0-9.5). (B) Effect of temperature on the activity of ficin. Activity of the ficin was determined using BAPNA as substrate at different temperatures.

The activity of the purified ficin was determined in presence of cysteine group specific inhibitors such as PCMB, iodoacetamide and sodium tetrathionate. The ficin activity was completely inhibited in presence of these inhibitors clearly confirms the participation of cysteine residue at the active site of the enzyme.

The first fourteen residues of the N-terminal sequence determined for the purified ficin showed the following sequence from amino-terminus end, NH_2-L P E S V D W A R F G A V N. The first amino-terminal residue of ficin is Leu and the previous investigations have also shown the presence of Leu at amino-terminus end of ficin (Richter *et al*., 2002; Jones and Glazer, 1970). Although there was some variation, the core of this sequence was common to proteases of 18 species we found using the blast network service search machine (http://www.ch.embnet.org/index.html), in the protein databases (www.expasy.org) hosted by Swiss Institute of Bioinformatics (SIB). The comparison of the N-terminal sequence of ficin with similar sequences of other plant cysteine proteases (Lee *et al*., 1997) is shown in Table 7. The core sequence has homology in all plant proteases of cysteine protease family, indicating the conservation of residues at amino-terminus end.

Amino acid composition of purified ficin is shown in the Table 8. With few exceptions, the amino acid composition of purified ficin is similar to that of ficin isolated from *Ficus glabrata* (Kortt *et al*., 1974). The free cysteine and disulfide content of the ficin was determined by Ellman method using DTNB. The purified ficin contains three disulfide bonds and a single free cysteine residue upon which the activity depends. The cysteine content and disulfide bridges of the purified ficin are similar to the ficin from *Ficus glabrata* (Englund *et al*., 1968) and other plant cysteine proteases (Turk *et al*., 1997). The above determined analytical observations reveal many similarities with respect to enzymatic activity, molecular mass, pH

Table 7: Comparison of N- terminal sequence of ficin to N-terminal sequences of other plant cysteine proteases*

Proteases	N-terminal sequences
Ficin (*Ficus carica*)**	**L P E S V D W A R F G A V N**
Papain (*Carica papaya*)	I P E Y V D W R S K G A V T
Chymopapain (*Carica papaya*)	Y P Q S I D W R A K G A V T
Bromelain (*Ananus comosus*)	A V P Q S I D W R D Y G A V
Papaya proteinase Ω (*Carica papaya*)	L P E N V D W R K K G A V T
Actinidin (*Actinidia chinensis*)	L P S Y V W R S A G A V V D
Ficus racemosa protease***	E Q E L E Q A G G Y L A

** Lee et al. (1997)*
*** The amino terminal sequence determined in this study*
**** The amino terminal sequence determined in this study (Page 68)*

Table 8: Amino acid composition of ficin from *Ficus carica*

Amino acids	**Gram percentage**	**No. of residues** (value to the nearest integer)
Asp	8.0 ± 0.15	15
Glu	12.2 ± 0.30	22
Ser	6.0 ± 0.05	16
Gly	7.4 ± 0.10	29
His	1.2 ± 0.10	2
Arg	8.8 ± 0.20	13
Thr	4.0 ± 0.20	9
Ala	6.8 ± 0.15	21
Pro	2.5 ± 0.10	6
Tyr	7.1 ± 0.10	10
Val	7.7 ± 0.10	16
Met	0.6 ± 0.05	1
Cys	3.1 ± 0.05	7
Ile	4.4 ± 0.10	9
Leu	8.3 ± 0.20	17
Phe	3.8 ± 0.10	6
Trp*	4.8 ± 0.10	6
Lys	3.3 ± 0.15	5

**Tryptophan content of the purified enzyme was determined by spectrophotometric method using NBS.*

optimum and stability, amino acid composition and role of disulfide bonds among the ficin fractions of *Ficus carica* and *Ficus glabrata*.

The extinction coefficient value ($E^{1\%}_{280}$) for the purified ficin was determined using nitrogen estimation method and it was found to be 20.9 ± 0.2. This value is consistent with the amino acid composition of ficin. The extinction coefficient value range of 15-25 has been reported for many plant cysteine proteases (Turk *et al*., 1997) and our value is found to occur in this range. The $E^{1\%}_{280}$ value of 20.2 ± 0.7 for different fractions of ficin was previously reported by Kramer and Whitaker (1964). Englund *et al*. (1968) have also shown the $E^{1\%}_{280}$ value of 21.0 for the major ficin isolated from the *Ficus glabrata*. These results further reveal the similarity of ficin fractions from *Ficus carica* and *Ficus glabrata*.

The far-UV CD spectrum for the purified ficin was determined in sodium phosphate buffer at pH 7.0 (0.02 M). Fig 18A shows the far-UV CD spectrum of the purified ficin. The analysis of the spectrum indicated that the enzyme contains about 21% α-helix, 43% β-structures and aperiodic structures of 36%. The intrinsic fluorescence emission spectrum of purified ficin is shown in Fig 18B. The purified ficin exhibited an emission maximum (λ_{max}) at 347 ± 1 nm, indicating that tryptophan residues are exposed to hydrophilic microenvironments.

The ficin isolated from the *Ficus carica* belongs to cysteine protease family. It is a single polypeptide chain protein of molecular mass 23100 ± 500 Da. The enzyme is active in the neutral pH range and shows maximum activity at pH 7.0. The purified ficin shares may similarities with ficin isolated from other *Ficus* species i.e. *Ficus glabrata*, with respect to enzymatic activity, molecular mass, pH optima and stability, amino acid composition and role of disulfide bonds. Ficin also shows a fair amount of homology with other plant cysteine proteases.

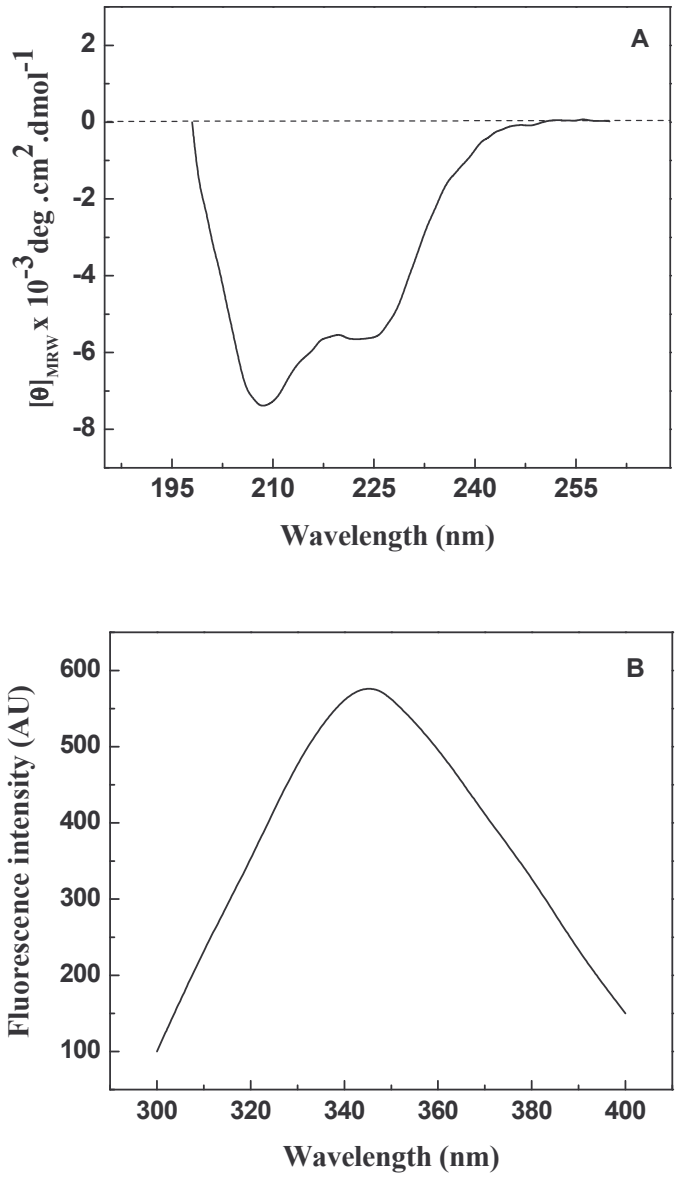

Fig 18: (A) The far-UV CD spectrum for the purified ficin determined in sodium phosphate buffer at pH 7.0 (0.05 M). (B) The intrinsic fluorescence emission spectrum of ficin.

The results presented in this chapter will help in understanding the enzymatic properties of ficin from two different Ficus species. Ficins isolated from different Ficus species exhibit different characteristic features. The ficin isolated from Ficus racemosa exists as single isoform with no multiple forms. This is a distinct characteristic feature as compared to the ficins isolated from Ficus carica and Ficus glabrata which are known to exist in several isoforms. It is shown in the present study that ficin from Ficus racemosa is an aspartic protease hitherto not known in the genus Ficus. This aspartic protease has broad cleavage specificity and shares a number of homology with several plant aspartic proteases with respect of pH optimum, molecular mass, inhibition by pepstatin, thermostability and its secondary structure and stability.

Ficin (EC 3.4.22.3), a cysteine protease isolated from the latex of Ficus carica exists as multiple isoforms. Although crude ficin (multiple forms) is of considerable commercial importance, ficin molecule as such has not been fully characterized. Therefore in the present study, the major fraction of ficin from Ficus carica, which constituted nearly about 60% of total activity was purified and characterized. The enzyme is a monomeric protein of molecular mass of 23 kDa and active in the neutral pH region. The characterized enzymatic properties of this ficin shares many similarities with ficin from Ficus glabrata and also shares several homologies with many other plant cysteine proteases. The enzyme activity of ficin from Ficus carica is much higher as compared to the activity of ficin from Ficus racemosa and holds greater potential for its study in depth. Therefore the ficin isolated from Ficus carica is subjected to further detailed studies in the next chapters to understand the structure-function and stability relationship of the enzyme. Hence, in the subsequent chapters, ficin refers to the ficin isolated from Ficus carica.

2. Effect of pH, urea and GuHCl on structure and stability of ficin from *Ficus carica*

Elucidation of the mechanism by which proteins fold from a denatured, structureless state, to their biologically active form is central to understanding the structure-function and stability relationship of proteins and enzymes. Such studies are fundamentally important for both theoretical and applicative aspects of the proteins and enzymes. The studies of solvent-induced denaturation of protein molecules are particularly useful for the understanding of protein stability and folding pathways (Pace, 1990; Matthews, 1993). The elucidation of folding pathways of globular proteins remains one of the major objectives of the protein chemistry and among the many theoretical and experimental approaches to this problem, denaturation studies continue to play a crucial role.

Protein folding is generally a highly cooperative process. The unique three-dimensional structure which confers to a protein its specific function is generally accepted as a thermodynamically most stable conformation (Anfinsen, 1973). Unfolding of many proteins that have been studied can be described in terms of two-state model where only folded and unfolded states of the protein exist in rapid equilibrium with no observable intermediates (Privalov, 1979; Schellman, 1987). However several studies have shown the existence of intermediate conformational states for several proteins (Kim and Baldwin, 1990; Ptitsyn 1995; Privalov, 1996).

The characterization of unfolded and partially folded state of protein is central to understand the protein folding and its stability. Therefore it is important and necessary to identify and define the partially folded conformations of proteins in the protein folding phenomenon. The molten globule state has been known to be an intermediate state between the native and unfolded states of several proteins. The molten globule state is

characterized by the presence of substantial secondary structure arranged in a native-like overall fold, a compact shape with slightly larger than the native protein, formation of a hydrophobic core exposed to solvent and lacking in the detectable tertiary structure (Ptitsyn, 1995).

The molten globule structure(s) have been observed for many proteins under different conditions such as low and high pH or in low concentrations of denaturants urea and guanidine hydrochloride (GuHCl) (Kuwajima and Arai, 2000). The molten globule state is important in the protein folding area, because it generally corresponds to late folding intermediate, which under normal folding conditions at neutral pH is difficult to study because of its transient life time (Colon and Roder, 1996; Mok *et al*., 2005). Hence for the structural studies, they are stabilized and populated at extremes of pH, typically at acidic pH. The molten globule conformations formed under acidic conditions have been reported for several proteins, and the role of such conformation in the mechanism and pathway of protein folding has been well discussed (Goto *et al*., 1990a and b; Arai and Kuwajima, 2000).

Solvent-induced equilibrium denaturation studies are very useful in understanding the structure, stability and folding of proteins. Such studies would give an insight in understanding the forces that determine the conformation of proteins and to optimize their stabilization at molecular level. *In vitro* studies of protein folding and unfolding reactions necessarily begin with the protein in solution conditions designed to disrupt the non-covalent interactions that stabilize the native conformation. The extremes of pH and temperature, and chaotropic agents such as urea and guanidine hydrochloride are commonly used in protein folding-unfolding studies (Tanford, 1970; Matthews, 1993). The present study is aimed to investigate and analyze the different effects of pH, urea and GuHCl on the structure, stability and folding and unfolding process of ficin.

2.1. Effect of pH on structure and stability of ficin

The use of pH changes to study the unfolding of a protein is very convenient approach because, in contrast to chemical denaturants such as guanidine or urea, the unfolding agent H^+ itself becomes part of the native system. The extensive studies on the folding pathways of papain as well as other related cysteine proteases have been well established (Edwin and Jagannadham, 1998; Haq *et al.*, 2002; Dubey and Jagannadham, 2003), however not much information is available on structural aspects of ficin till date. The investigations on conformational behaviours of similar proteases from different sources of cysteine proteases family would certainly help in understanding the folding pathway of these proteases. The denaturation of ficin was followed over a range of pH 1.0 to 7.0, and the structural perturbations of the protein molecule were monitored by various spectroscopic methods.

As studied in the first chapter, ficin isolated from *Ficus carica* showed the maximum activity between the pH 6.5 and 8.5 and optimum activity at pH 7.0. The lowest activity was observed at acidic pH range. Here, the effect of pH on the thermal stability of ficin was further investigated and Fig 19 shows the apparent thermal denaturation curves of ficin as function of pH. The apparent thermal melting temperature (T_m) of ficin decreased as the pH decrease or increase from the optimum pH 7.0. A decrease of 11° C in apparent T_m was observed at pH 4.0.

The CD spectra of a protein in far-UV region are essentially used to monitor the conformation of the polypeptide backbone. The pH induced transition of ficin was followed by far-UV CD and Fig 20 shows the effect of pH on the mean residue ellipticity (MRE) of the ficin at 222 nm. As evident from the ellipticity values, the pH induced transition of ficin was found to follow a cooperative single step two-state transition. Apparently no changes

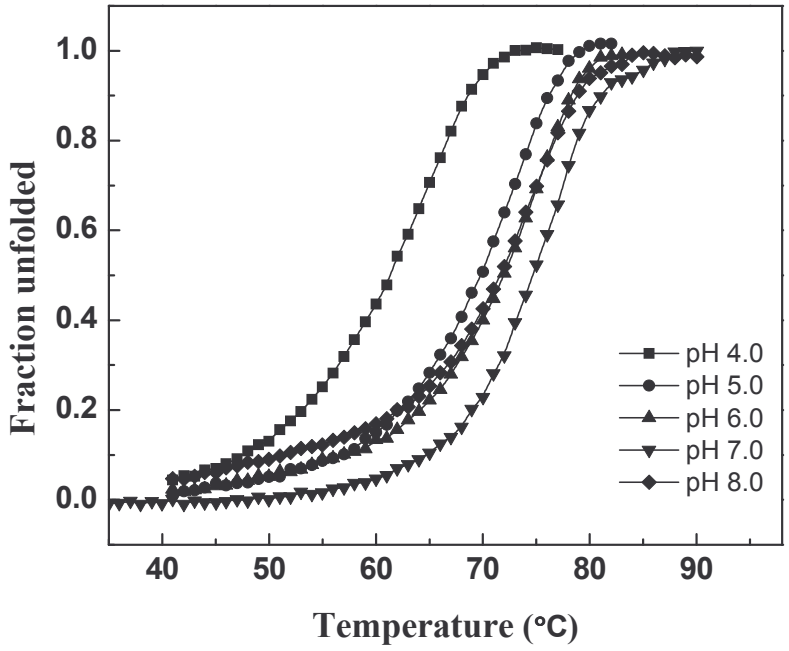

Fig 19: Apparent thermal denaturation curves of ficin as a function of pH. Buffers used were: 50 mM citrate buffer (pH 4.0-6.0), 50 mM sodium phosphate buffer (pH 6.5-7.5) and 50 mM Tris-HCl buffer (pH 8.0-9.5).

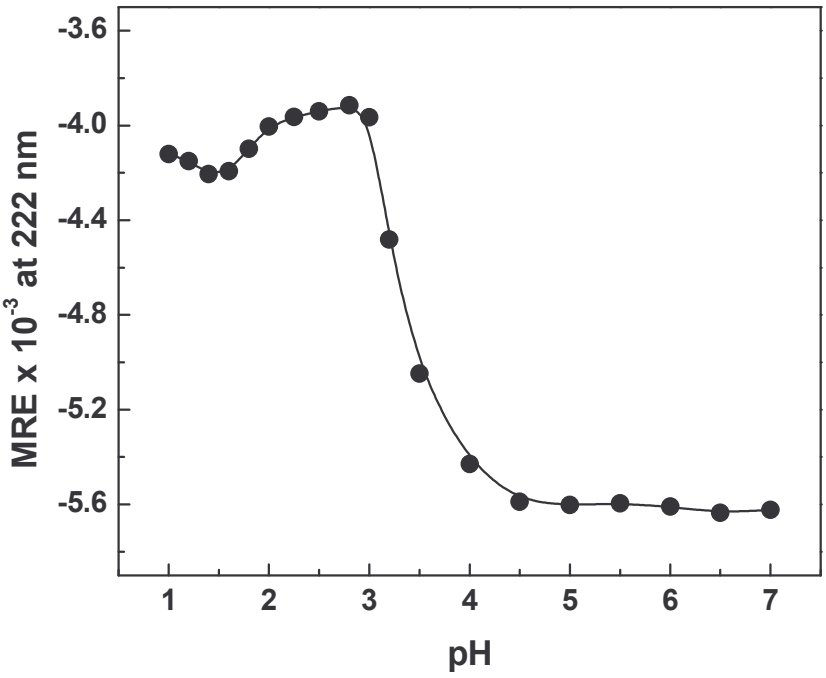

Fig 20: Relative changes in far-UV CD ellipticity values of ficin at 222 nm as a function of pH. The experiments were carried out at 25° C using the protein concentration of 10 μM.

in the ellipticity were observed between pH 7.0 and 4.0. However, when pH decreased below 4.0, the ellipticity decreased markedly to a minimum value at pH 3.0 and remains apparently unchanged down to pH 2.2. The decreased ellipticity at 222 nm within the pH range 4.0-2.0 suggests the loss of secondary structure. At pH 3.0 about 31% loss of MRE at 222 nm was observed (calculated on the basis of MRE value at 222 nm of native state taken as 100%). Reversibility studies showed that ficin was completely reversible in the pH range 2.4-7.0 while it was found to be completely irreversible from pH 1.0 to pH 7.0. Protein unfolding by a decrease in pH could be due to Coulombic repulsion from the net positive charges of the polypeptide chain (Goto *et al*., 1990a and b; Matthews, 1993; Fink *et al.,* 1994; Arai and Kuwajima, 2000). Generally acid-induced unfolding is incomplete and the conformational states formed are different from that of native and fully unfolded states.

The pH induced structural changes of ficin was further monitored and characterized by far-UV CD, intrinsic fluorescence spectral measurements and ANS binding studies. The circular dichroic spectra of ficin under different states in both near- and far-UV wavelength regions were determined. CD spectra of proteins in the far-UV region are particularly sensitive to protein secondary structure. In Fig 21 is shown the far-UV spectra of ficin at pH 7.0 (native state) and pH 1.4 (acid-induced state). The spectrum for native state has two minima, one at 208 nm and other at 222, which is characteristic feature of α-helical structure. The spectrum of acid-induced state (pH 1.4) of ficin exhibited considerably all secondary structural features although there was a decrease in MRE value at 222 nm. In contrast, a complete loss of the secondary structure of ficin was observed in 6 M GuHCl. The MRE at 222 nm are -5623 deg. cm^2 $dmol^{-1}$for native state, -4205 deg. cm^2 $dmol^{-1}$ for the acid induced state at pH 1.4 and

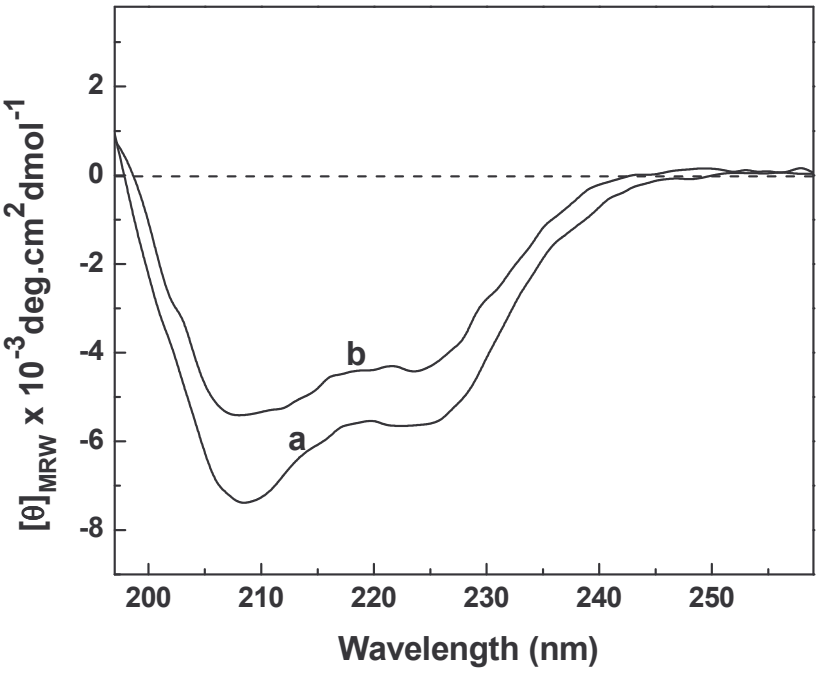

Fig 21: Far-UV circular dichroic spectra of ficin at different states. The spectra were recorded in the wavelength region 200-260 nm using protein concentration of 10 μM. (a) native state at pH 7.0, (b) acid-induced state at pH 1.4.

for the unfolded state in 6 M GuHCl, the MRE was -1121 deg. cm^2 $dmol^{-1}$. These observations suggest that the state of ficin molecule under acidic conditions is different from that of native or unfolded state.

In Fig 22 is shown the comparison of near-UV CD spectra of ficin at native state (pH 7.0), acid-induced state (pH 1.4) and unfolded state (in 6 M GuHCl). The near-UV CD spectra of proteins are used to monitor the asymmetry of the aromatic amino acid's environment. The near-UV CD spectrum for the native state (pH 7.0) exhibited two prominent positive peaks one centered at 283-285 nm and other at 272-275 nm and a negative band centered at 296-299 nm. The spectrum of acid-induced state (at pH 1.4, curve b) shows a substantial loss of these signals which results in the spectrum which is closely similar to that of unfolded state in GuHCl. The spectrum of acid-induced state has a prominent negative band approximately in the region of 270-280 nm. This suggests that a defined ordered structures in the vicinity of aromatic chromophores in the acid-induced state of ficin at pH 1.4. Recently many evidences supports the idea that the MG state besides having secondary structure may possess well defined tertiary structure as well (Kay and Baldwin, 1996; Song *et al.,* 1998; Shortle and Ackerman, 2001).

The exposure of hydrophobic surfaces of different states of ficin to solvents was monitored by changes in ANS fluorescence (Stryer, 1965), which is a commonly used method to detect the MG states of proteins Semisotnov, *et al.*, 1991,). The level of binding of ANS to ficin at different pH is shown in Fig 23. As evident from the figure, ANS binding to the protein is minimal in the pH range 7.0-2.5. A maximal binding of ANS was observed at pH 1.4. This marked increase in ANS fluorescence (about 7-fold) at pH 1.4 signifies the extensive solvent exposure of non-polar clusters. Further below pH 1.4, a slight decrease in ANS fluorescence was observed suggesting the reorganization of hydrophobic regions in the protein molecule.

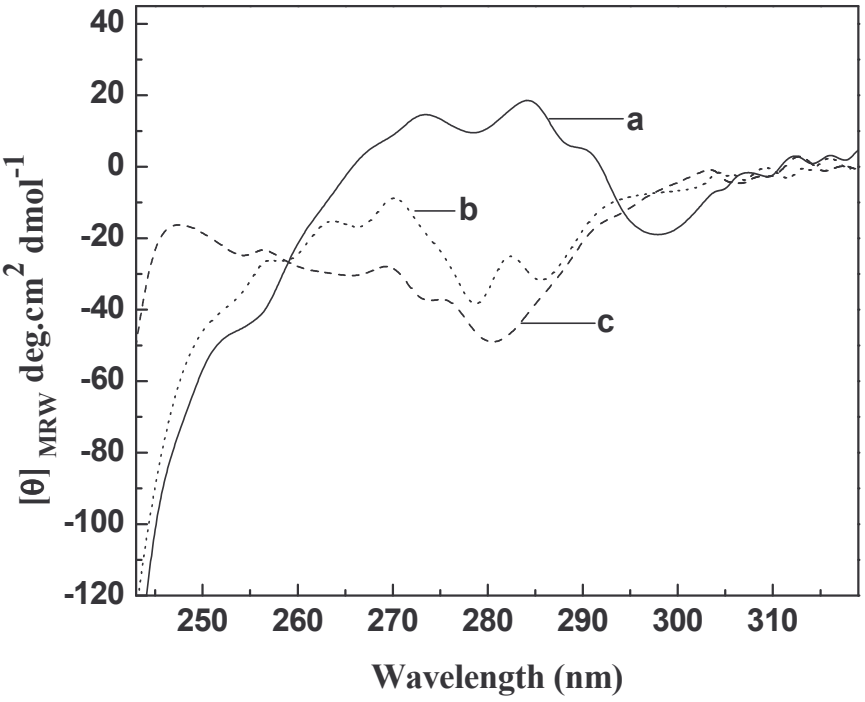

Fig 22: Near-UV circular dichroic spectra of ficin at different states. The spectra were recorded in region of 250-320 nm using the protein concentration of 47 μM (a) native state at pH 7.0, (b) in acid-induced state at pH 1.4 and (c) in 6M GuHCl-unfolded state.

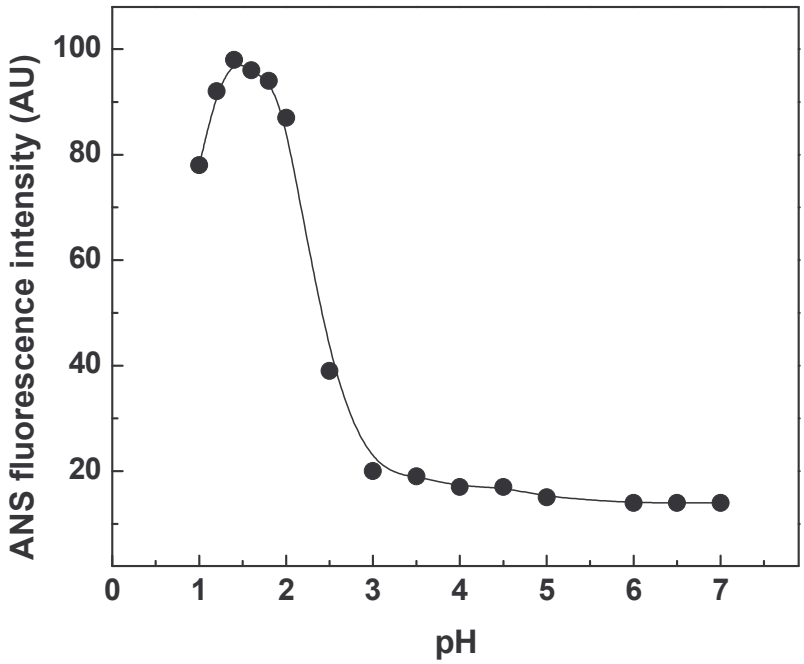

Fig 23: ANS fluorescence intensity as a function of pH at 478 nm. Ficin samples at different pH were incubated with 100-fold molar excess of ANS for 30 min at 25° C in dark. The fluorescence of ANS was excited at 380 nm and emission was recorded between 400-600 nm.

A comparison of ANS fluorescence emission spectra of ficin at native (pH 7.0), acid-induced (pH 1.4) and unfolded state (6M GuHCl) in the 400-600 nm range is shown in Fig 24A. As can be seen from the figure, the binding of ANS to the acid-induced state at pH 1.4 produced a large increase in fluorescence intensity along with a blue shift in emission maximum (λ_{max}), indicating the exposure of hydrophobic regions of the protein molecule. A very low binding of ANS to ficin at pH 7.0 is apparently related to non-accessibility of hydrophobic regions. These results clearly demonstrate that the spectrum of ficin at pH 1.4 has different conformation from that of native and unfolded state. The ANS fluorescence emission maximum (λ_{max}) of the enzyme at various pH values are shown in Fig 24B. These observations further suggest that acid induced state becomes a compact molecule with the exposure of hydrophobic clusters. All the above characteristics are typical of a MG state observed with several proteins (Goto *et al.*, 1990a; Ptitsyn, 1995, Arai and Kuwajima, 2000; Vassilenko and Uversky, 2002).

Intrinsic fluorescence spectra provide a sensitive means of characterizing proteins and their conformations. The spectrum is determined mainly by the polarity of the environment of tryptophan and tyrosine residues, and by their specific interactions. The emission maximum (λ_{max}) is an excellent parameter commonly used to monitor polarity of Trp, and is sensitive to conformational changes (Stryer, 1968). The intrinsic fluorescence spectra of ficin under native condition (pH 7.0), at pH 1.4 and in unfolded state (6 M GuHCl) are shown in Fig 25A. For native enzyme, the emission maximum was found to be at 347 nm. Where as in case of acid-induced state at pH 1.4 there is both a 20% decrease in maximum fluorescence intensity and a blue shift in emission maximum by 5 nm was observed. These results indicate the non-polar environment of tryptophan residues at MG state. The unfolded state (6 M GuHCl), however, exhibits a

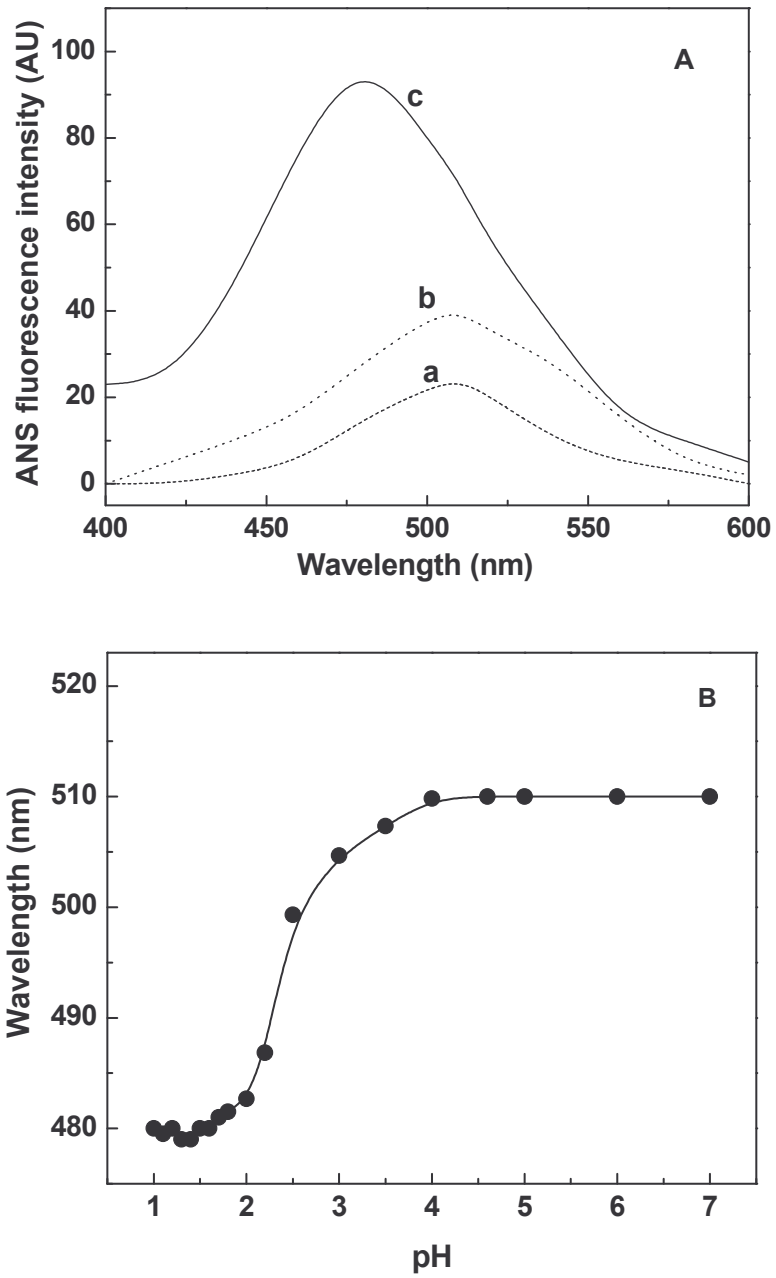

Fig 24: (A) ANS fluorescence emission spectra of ficin at (a) native, (b) GuHCl-unfolded states and (c) acid-induced states. Protein to ANS concentration was in the ratio 1:100 and the spectra were recorded in the region of 400-600 nm after exciting at 380 nm at 25° C. (B) pH dependence of ANS emission maxima (λ_{max}) of ficin.

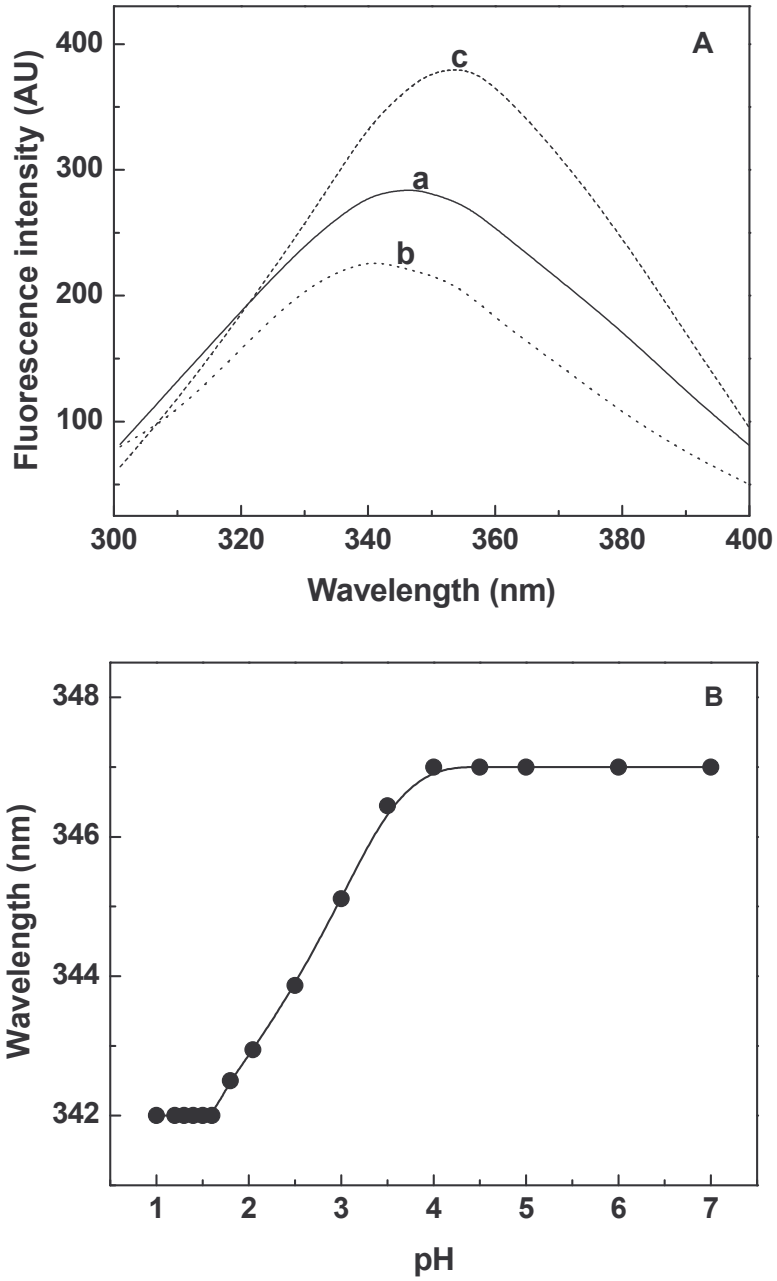

Fig 25: (A) Intrinsic fluorescence emission spectra of ficin at (a) native state, (b) acid-induced state and (c) 6M GuHCl-unfolded state. The excited wavelength was 280 nm and emission was recorded between 300 and 400 nm. (B) Intrinsic fluorescence emission maxima (λ_{max}) of ficin at different pH.

larger red shift with maximum emission occurring at 354 nm, which indicates that tryptophan residues are maximally exposed to the solvent. The pH dependence of intrinsic fluorescence emission maximum of ficin is shown in Fig 25B. The emission maximum was unchanged between the pH 7.0 to 4.0 but when the pH decreased further emission maximum suffers a blue shift indicating the internalization of tryptophan residues in a non-polar environment.

Quenching of Trp fluorescence by an external quencher is a common method to determine the solvent accessibility and microenvironment of Trp residues in MG state of proteins. Since MG state has been characterized by the formation of hydrophobic core exposed to solvent, it is useful to investigate the degree of exposure of Trp residues. In the present study, quenching of Trp fluorescence was determined based on the method of Eftink and Ghiron using the uncharged molecules of acrylamide (Eftink and Ghiron, 1981). Fig 26 depicts the Stern-Volmer plot for the quenching of fluorescence by acrylamide at native, acid-induced and GuHCl denatured states of ficin. The quenching of ficin is characterized by an upward curvature of Stern-Volmer plot. This is probably arising due to heterogeneous population of tryptophan. Proteins containing multiple tryptophans have shown both linear and curved Stern-Volmer plot in presence of acrylamide (Eftink and Ghiron, 1981) and ficin is known to contain six tryptophan residues (Devaraj *et al*., 2008b). In Table 9 is shown the Stern-Volmer constants (K_{sv}) fitted to the linear part of the curves. As seen from the table, K_{sv} value for acid-induced state is 7.2 M^{-1}, which is lower than the K_{sv} value obtained at native state (8.6 M^{-1}). These results are in good agreement with intrinsic fluorescence emission results. A blue shift of 5 nm in the emission maximum and a low K_{sv} value suggest the hydrophobic environment of tryptophan residues in the acid-induced state compared to native state at pH 7.0. On the other hand the K_{sv} value for the GuHCl-denatured state was significantly higher than those values for native and

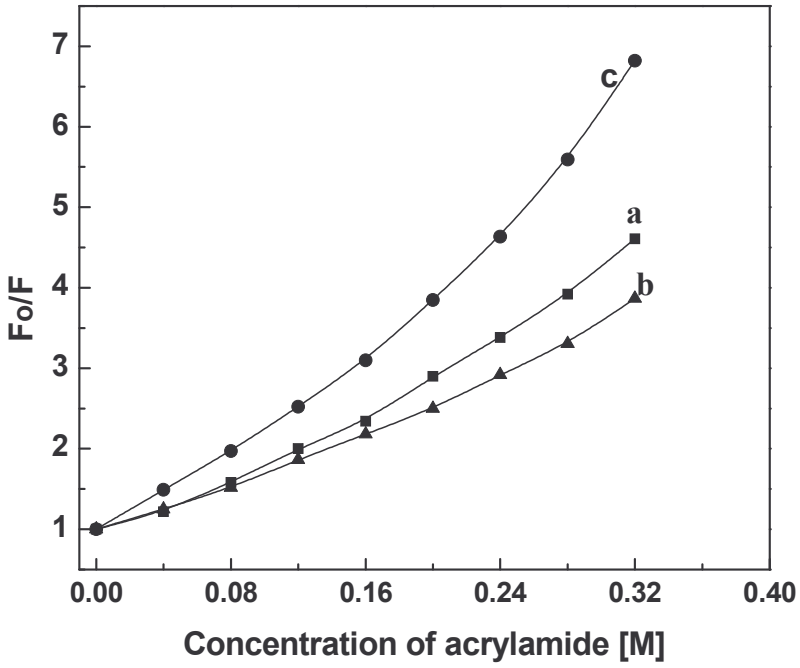

Fig 26: Stern-Volmer plot for the analysis of the acrylamide fluorescence quenching data of ficin at (a) native state, (b) acid-induced state and (c) unfolded state. F_o and F are the fluorescence intensities in the absence and presence of acrylamide, respectively.

Table 9: Stern-Volmer constants for acrylamide fluorescence quenching of ficin in different conditions

Condition of experiment	K_{sv} (M^{-1})
Native (at pH 7.0)	8.6
Acid-induced (at pH 1.4)	7.2
GuHCl-unfolded (at pH 7.0)	12.5

acid-induced states, suggesting that the tryptophans are more exposed to solvent, and consequently the fluorescence was quenched more in completely unfolded state in GuHCl.

To study the thermal unfolding of intermediate state relative to the native state, changes in fluorescence intensity at their emission maximum was measured as a function of temperature. Protein samples were incubated at desired temperature for 5 min before the measurements at a particular temperature. Reversibility was checked by rapidly cooling the heated sample at 85° C to the initial lower temperature (25° C). As seen from the Fig 27, the thermal unfolding of native ficin at pH 7.0 is a cooperative process. On the other hand, thermal transition of acid-induced state at pH 1.4 is a weak cooperative process in the temperature range used. In both the cases the thermal unfolding was found to be irreversible. The results show lack of cooperativity in the thermal unfolding of MG state of ficin. The lack of cooperativity in the thermal transition is associated with MG state that has been observed with many other globular proteins (Carra *et al.*, 1994; Colón and Roder, 1996; Uversky *et al.*, 1998; Naseem *et al.*, 2004)

A comparison of the hydrodynamic properties of the partially folded state with that of the folded native state and the unfolded one is particularly important in characterizing the intermediate state of proteins (Uversky *et al.*, 1992; Uversky, 1993). Size exclusion chromatography on FPLC was used to determine stokes radius of native, molten globule and unfolded states of ficin. In Fig 28 is shown the elusion profile of ficin at three different states and the elution volumes are 17.6 mL, 16.6 mL and 14.7 mL, respectively. The Stokes radii of the native and unfolded states were determined to be 23 Å and 35 Å, respectively. For the acid-induced state (at pH 1.4) the Stokes radius was 27 Å (Fig 29). These results indicate an increase in the hydrodynamic dimensions of the protein at pH 1.4, which could be due to the opening up of the tertiary structure as compared to the native state but is still much more compact than the unfolded state.

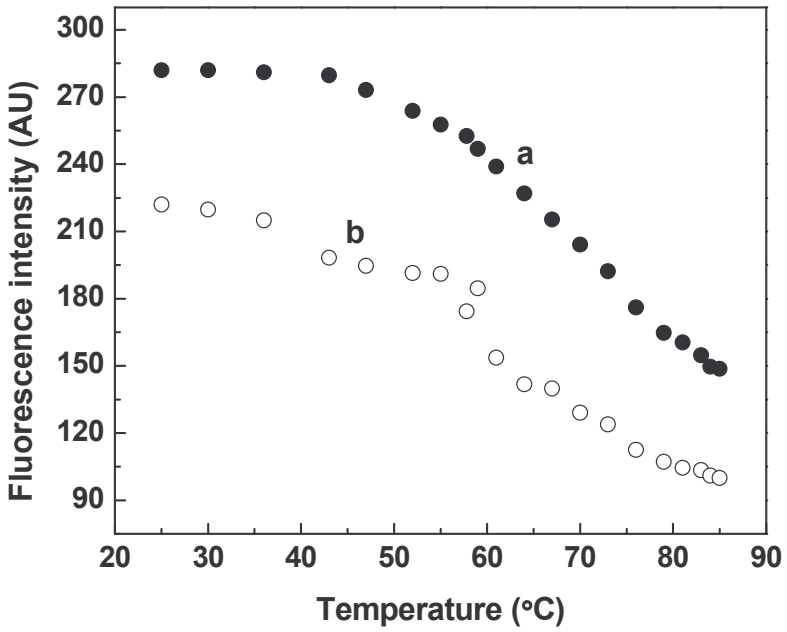

Fig 27: Thermal denaturation profile of ficin as measured by the changes in fluorescence intensity at 347 nm for native state and 342 nm for acid-induced state. (a) Native state at pH 7.0 in 20 mM sodium phosphate buffer; (b) acid-induced state at pH 1.4 in 20 mM KCl-HCl buffer.

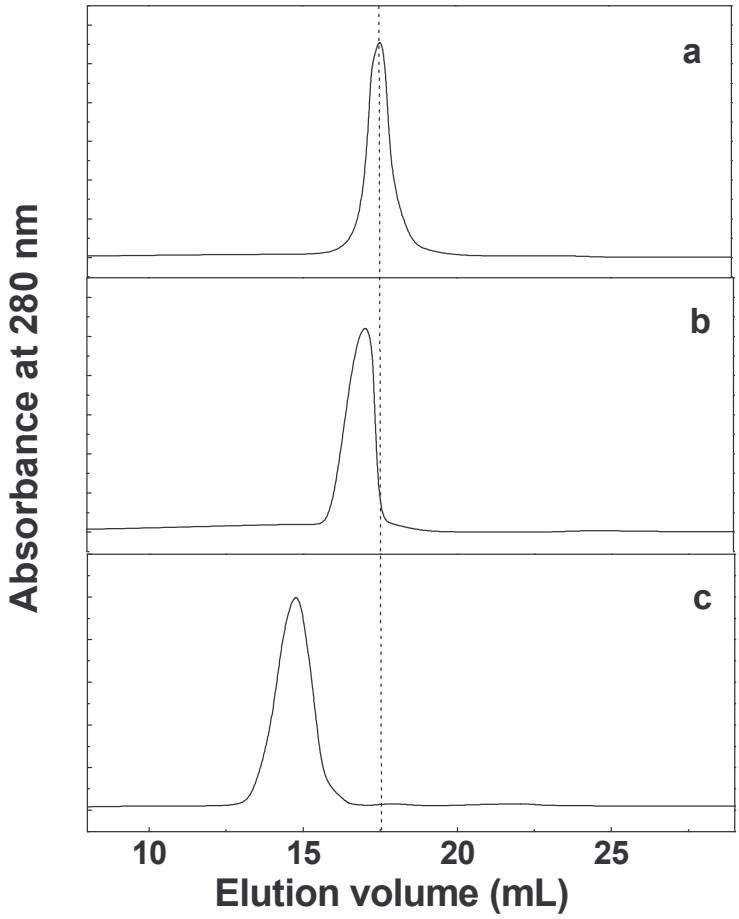

Fig 28: Size-exclusion chromatographic profile of ficin (a) native state at pH 7.0, (b) acid-induce state at pH 1.4 and (c) GuHCl-unfolded state, on FPLC using Superdex-75 HR 10/30 column (Amersham Bioscience AB, Uppsala, Sweden).

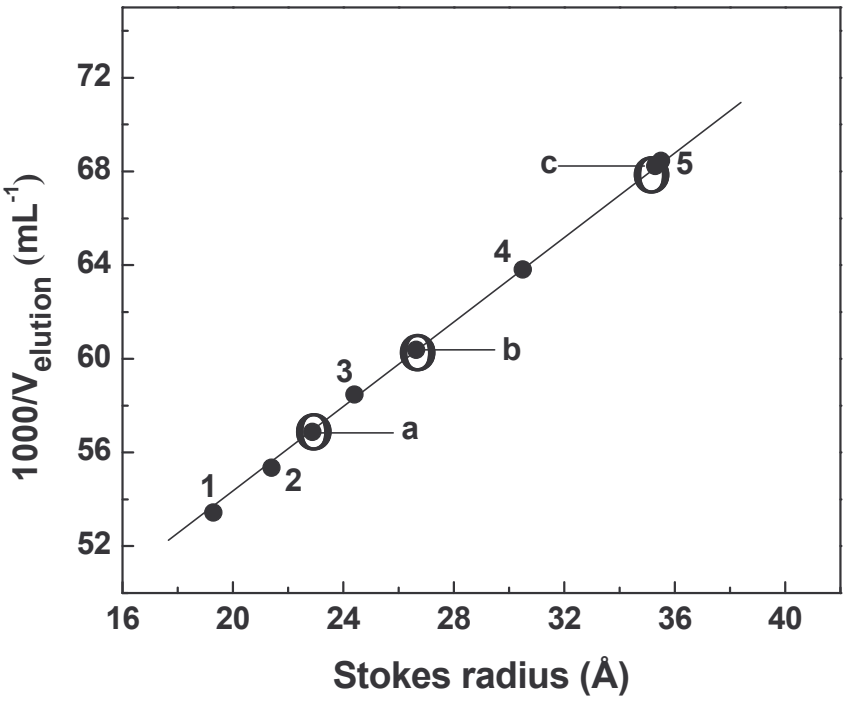

Fig 29: Assessment of Stokes radius of ficin at three different states: (a) native state at pH 7.0, (b) acid-induce state at pH 1.4 and (c) GuHCl-unfolded state ficin. Calibration curve drawn based on the elution rate of standard proteins of known Stokes radii: (1) ribonuclease (19.3 Å), (2) myoglobin (21.4 Å), (3) carbonic anhydrase (24.4 Å), (4) ovalbumin (30.5 Å) and (5) BSA (35.5 Å).

The present study demonstrates that ficin exists as a partially folded state at acidic pH with characteristic features of molten globule. The observed structural properties of ficin at pH 1.4 agrees with the definition of the molten globule state as it contains disordered tertiary structure but retain substantial secondary structure with strong ANS binding. A comparison of some of the conformational properties of ficin under native, molten globule and unfolded states are summarized in Table 10. Refolding process was found to be completely reversible up to pH 2.4 - 7.0 as judged by fluorescence and CD measurements as it regains all the spectral characteristics, while further decrease in pH up to 1.0 makes the denaturation process irreversible (Devaraj *et al.*, 2009).

Acids are known to denature proteins by affecting the electrostatic interactions. Acid-induced unfolding of proteins is often incomplete and acid-unfolded states assume the conformations that are located between native state and completely unfolded state (Matthew, 1993; Fink *et al.*, 1994; Arai and Kuwajima, 2000; Fink, 2005). The major driving force involved during acid denaturation is intra-molecular charge repulsion (Fink *et al.*, 1994), which may or may not overcome the interactions favouring the folded states such as hydrophobic forces, salt bridges and the metal ion-protein interactions. The mechanism of denaturation of a given protein at low pH is proposed to be complex and may involve intricate interplay between a variety of stabilizing and destabilizing forces leading to a compact structure, characteristic features of the molten globule or partially unfolded intermediate (Fink *et al.*, 1994; Arai and Kuwajima, 2000). The studies on the acid-induced unfolding of ficin reveal that ficin exhibits unfolding behaviour characteristics of Type I proteins as classified by Fink *et al,* (1994). In this class, when proteins are titrated with HCl in absence of any additional factors, shows two transitions, initially in the first phase the unfolding of protein molecule occurs in the vicinity o f pH 3 - 4 and then refolding to a

Table 10: Comparison of some physical parameters of ficin at three different states

Variables	Native state	Intermediate state	GuHCl-unfolded state
Fluorescence emission maximum (λ_{max}) (nm)	347 ± 1	342 ± 1	354 ± 2
ANS fluorescence (relative intensity) (AU)	23 ± 3	93 ± 8	39 ± 6
Stokes radius (Å)	23 ± 1	27 ± 1	35 ± 2

molten globule like conformation occurs in the second phase at lower pH. A similar kind of folding behaviour has also been observed with stem bromelaine (Haq *et al*., 2002).

The results obtained in the present study are in good agreement with the existing results. It has been demonstrated in many studies that the existence of a transient intermediate state during unfolding of papain as well as other related plant cysteine proteases. Papain exhibits the characteristics of MG state at acidic conditions as it contains substantial amount of secondary structure and significantly disordered tertiary structure with exposed hydrophobic regions (Edwin and Jagannadham, 1998). Detailed studies of the folding aspects of stem bromelain from *Ananus comosus* (Haq *et al*., 2002) and procerain from *Calotropis procera* (Dubey and Jagannadham, 2003) have also reported the existence of MG state at acidic conditions. These results are very informative to understand the folding aspects of plant cysteine proteases and further give an insight to establish more generalized mechanism for the folding behavior of plant cysteine proteases.

The denatured states of proteins are equal in importance to the native state in determining the stability and folding pathway of proteins. The results presented here indicate that the pH-induced denaturation of ficin leads to a partially unfolded state at acidic pH. The pH-induced denaturation of proteins is known to result in denatured states that are often less unfolded. The partial unfolded structure of ficin at low pH shows the characteristics of molten globule like intermediate state as studied by various biophysical techniques. In the next chapter the effect of chemical denaturants such as urea and GuHCl on structure and stability of ficin is investigated. These studies further help to elucidate the mechanism of folding pathway and the stability of ficin.

2.2. Effect of urea and GuHCl on structure and stability of ficin

The stability of proteins in solutions is a major concern of biologists and pharmacologists. The more in depth knowledge of protein denaturation and refolding process is central to understanding the protein stability. The mechanism by which proteins fold from a denatured structureless state to their biological active form is not very well understood, though many mechanisms have been proposed. Further, studying of the denaturation process of proteins provides a basis for the protein designing and helps to establish a molecular description of protein folding.

It is well known that urea and GuHCl are the most frequently used classical denaturants to study the protein stability and folding pathways. In comparison to either acid or thermal unfolding, chemical agents such as urea and GuHCl are more effective in disturbing the non-covalent interactions. These denaturants (unlike temperature and pH induced denaturation) can in many cases reversibly transform proteins to completely unfolded state. The extent of unfolding is generally greater than that of any other means of denaturation (Tanford, 1968; Matthews, 1993; Zou *et al*., 1998). Despite their widespread use, the mode of action of these agents on protein conformation is not clearly known. They may exert their effect directly, by binding to the protein, or indirectly, by altering the solvent environment (Schellman, 1987; Vanzi *et al*., 1998).

2.2a. Effect of urea

Chemical denaturants are frequently used to unfold proteins and to characterize the mechanisms and transition states of protein folding reactions. Urea is a widely used chemical denaturant in the protein structural studies. Despite its widespread use, the molecular mechanism underlying urea-induced denaturation is not well understood. Two classes

of interaction models are distinguished in the literature. In the first, direct interactions between urea and the protein are considered the main denaturation driving force (Tirado-Rives *et al.,* 1997; Mountain and Thirumalai, 2003). In the second, urea-induced changes in the water structure are suggested as indirect interactions that drive unfolding (Finer *et al.,* 1972; Hoccart and Turrell, 1993). The many recent studies support the direct interaction model (Klimov *et al.,* 2004; O'Brien *et al.,* 2007) and it is still unclear whether polar or apolar residues or the peptide backbone constitute the main interaction sites for urea. That the peptide backbone is an important interaction site for urea is now widely accepted and some studies also stress the importance of urea-protein hydrogen bonds to polar residues (Auton and Bolen, 2005; Moqlich *et al.,* 2005). However, other studies show the importance of apolar urea-protein contacts weakening the hydrophobic effect (Prakash *et al.,* 1981; Timasheff and Xie, 2003).

The effect of urea on structural and functional properties of ficin was studied by equilibrating the enzyme at neutral conditions (pH 7.0) in presence of different concentrations. The enzyme was equilibrated for 36 h before taking all spectral measurements. Changes in the fluorescence emission spectra (representative) of ficin after equilibration in presence of increasing concentrations urea are shown in the Fig 30. As seen from the spectra, a slight increment in the fluorescence intensity is observed with increasing concentration of urea without any shift in the emission maximum. Only about 13% of intensity increment was observed event at 8 M urea. This increase in the intensity is probably due to the loss of specific interaction of tryptophan residues with vicinal quenching groups, which consequently resulted in the increment in the fluorescence intensity (Sommers and Kronman, 1980). These results suggest that ficin molecule at neutral conditions is resistant to unfold even in presence of high concentration of urea. The stability or conformational rigidity of ficin at neutral conditions was further evaluated by far-UV CD spectral studies.

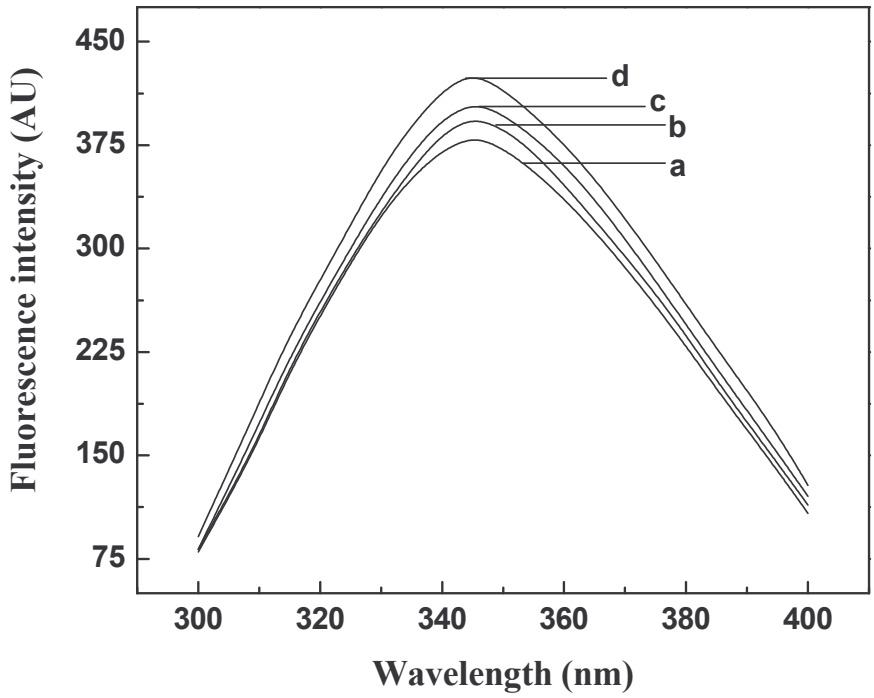

Fig 30: Intrinsic fluorescence emission spectra (representative) of ficin in presence of different concentrations of urea at neutral condition (pH 7.0). (a) Control in pH 7.0 sodium phosphate buffer, (b) in 2 M (c) in 4 M and (d) in 6 M, urea.

In Fig 31 is the shown the far-UV CD spectra (representative) of ficin in presence of 3M urea. The spectra of ficin in presence of different concentrations of urea were not affected significantly except some marginal changes in the ellipticity values at 222 nm. The mean residual ellipticity value at 222 nm for native enzyme is -5150 deg.cm^2 dmol^{-1} and in presence of 8 M urea is -4504 deg. cm^2 dmol^{-1}. These results clearly suggest the structural stability of ficin molecule. The structural stability of ficin, isolated from *Ficus glabrata* towards urea denaturation has been reported earlier by Englund *et al*. (1968). In several studies, it has already been reported that the urea does not always lead to complete unfolding. Superoxide dismutase and lysozyme for example are not completely unfolded in 10 M urea at neutral pH (Pace, 1975). Similar to our results, procerain, also a cysteine protease from *Calotropis procera* is resistant to denature by urea (Dubey and Jagannadham, 2003).

Activity measurements of enzymes in presence of different concentrations of denaturants are also an excellent probe to monitor the small changes induced by the denaturants. In Fig 32 is shown the effect of urea on the activity of ficin at neutral conditions. The enzyme looses 50% of its activity at 2 M urea and is completely inactivated at 6 M urea. In order to check the reversibility of the activity, the denatured protein sample diluted to desired concentrations of urea. The reversibility studies showed that the inactivation of enzyme was reversible. A recovery of about nearly 90% of the activity was observed after complete removal of urea. The activity loss and structural resistant nature of ficin towards urea denaturation at neutral conditions can not be compared. Because, the inactivation of the enzyme could be due to the changes in the vicinity of the active site and several studies have shown that the inactivation of many enzymes occurs before significant conformational changes of the molecule as a whole can be detected during denaturation process by urea or GuHCl (Ma and Tsou, 1991;

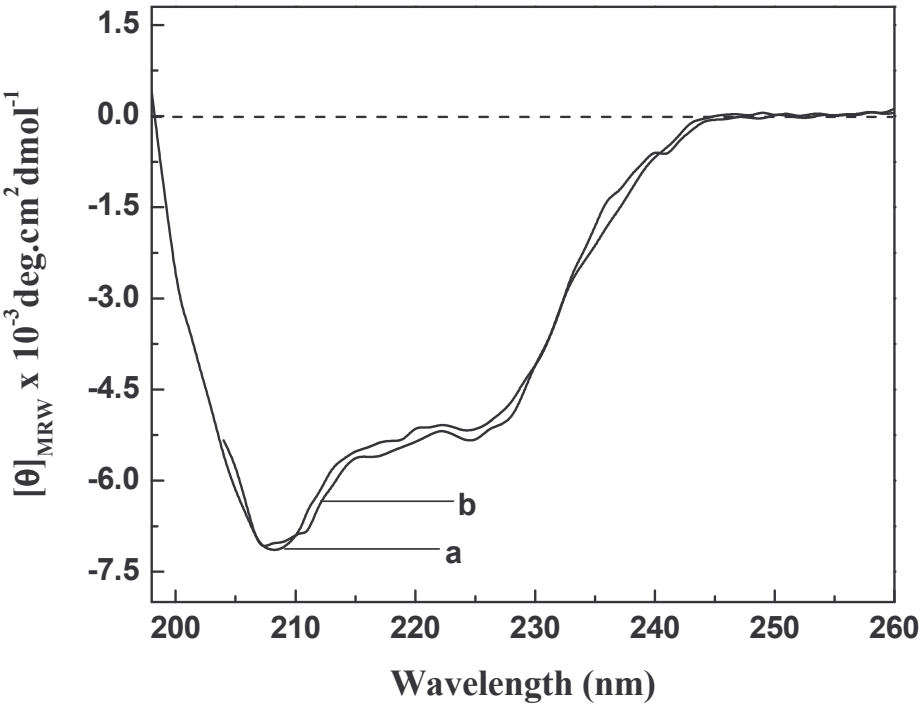

Fig 31: Effect of concentrations of urea on far-UV CD spectra (representative) of ficin at neutral condition (pH 7.0). (a) In buffer only and (b) in 3 M urea (Representative spectra are only shown for clarity).

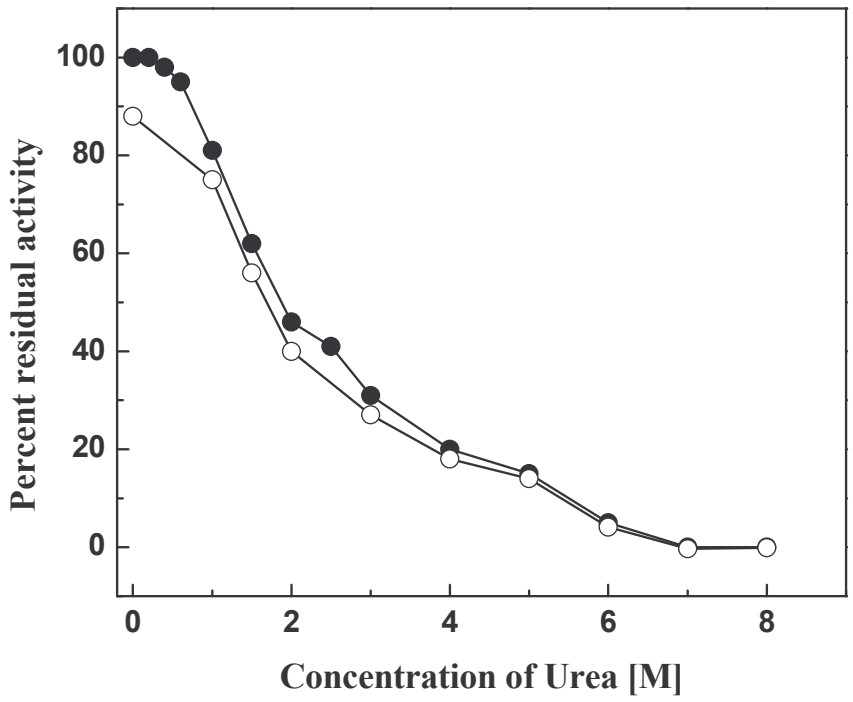

Fig 32: Activity profile of ficin as a function of urea at pH 7.0. Activity of ficin was carried out in presence of various concentrations urea using BAPNA as substrate: unfolding (●) and refolding (○).

Tsou, 1993; Zhou *et al*., 1993). The second reason for inactivation could also be the interaction between the urea and substrate itself (Hill *et al*, 1959; Nakamura and Soejima, 1970).

Since ficin is resistant to unfolding by urea under neutral pH, the unfolding of the enzyme was carried out at lower pH. The enzyme was susceptible to urea unfolding at pH 3.0 and the changes were reversible. The maximum incubation time sufficient for achieving equilibrium was standardized and the changes occurred within a maximum of 3 h with no further changes upto 12 h.

The modifications of microenvironments of aromatic residues of ficin due to denaturation by urea were studied by monitoring the changes in the fluorescence spectra as a function of urea concentration. In Fig 33A is shown the changes in fluorescence emission spectra of ficin in presence of increasing concentrations of urea and Fig 33B shows the fluorescence emission maxima as function of urea concentration. For native ficin, the emission maximum is at 347 nm as studied under neutral pH and the emission maximum shifts to 345 nm at pH 3.0, suggesting the aromatic residues are in the hydrophobic core of the protein at acidic condition. A red shift in the fluorescence emission maximum of the intrinsic fluorescence spectra, as well as an increase in intensities was observed upon urea induced denaturation of protein (Fig 33A). At 4.5 M urea, the maximum red shift of about 8 nm occurred in the emission maximum and further increase in the urea concentration up to 8.0 M, the emission maximum remains constant.

The fluorescence quenching experiments allow us to assess the relative solvent exposure of fluorophores (Eftink and Ghiron, 1981). The exposure of the protein interior and the red shift in the fluorescence emission maximum of ficin upon urea denaturation at pH 3.0 was further evaluated by acrylamide quenching of tryptophan fluorescence. Fig 34 presents the Stern-Volmer plot for analyzing the acrylamide fluorescence

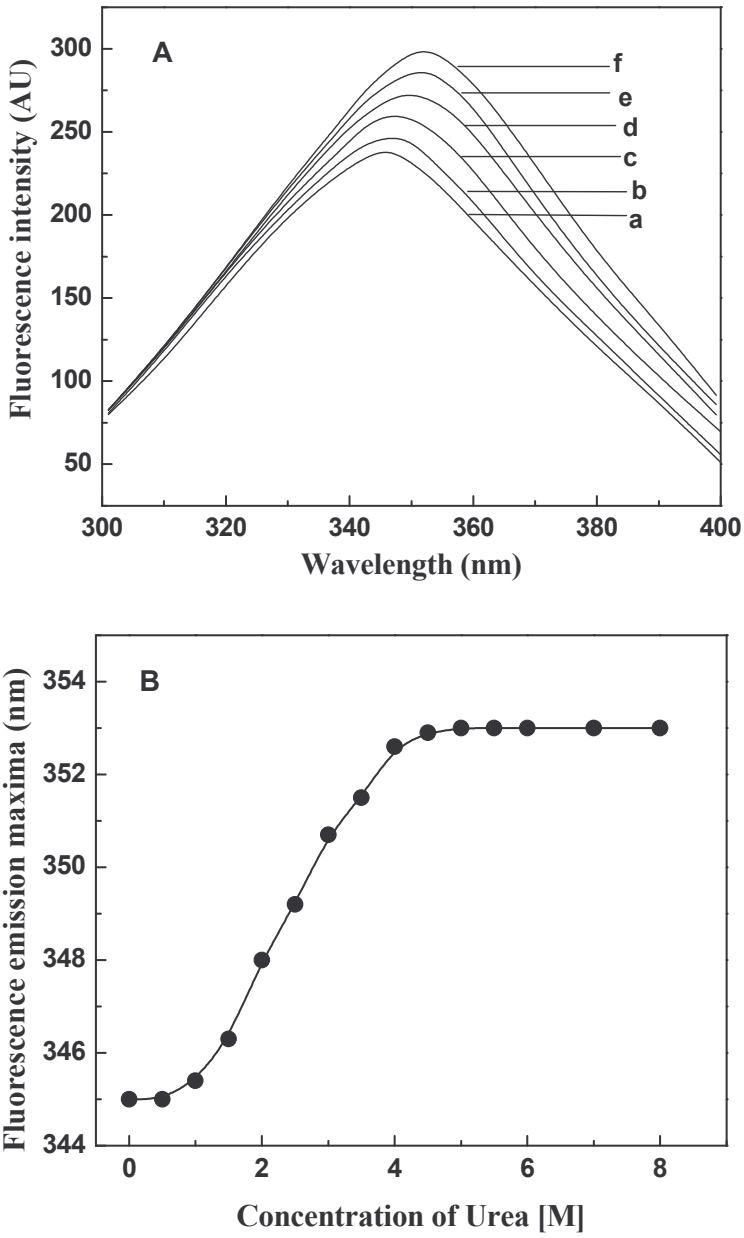

Fig 33: (A) Intrinsic fluorescence emission spectra of ficin in presence of various concentrations of urea at pH 3.0. (a) Control in Gly/HCl pH 3.0 buffer, (b) in 1.5 M (c) in 2.5 M, (d) in 3 M, (e) in 4 M and f) in 4.5 M urea. (B) Fluorescence emission maximum (λ_{max}) as a function of urea concentration at pH 3.0.

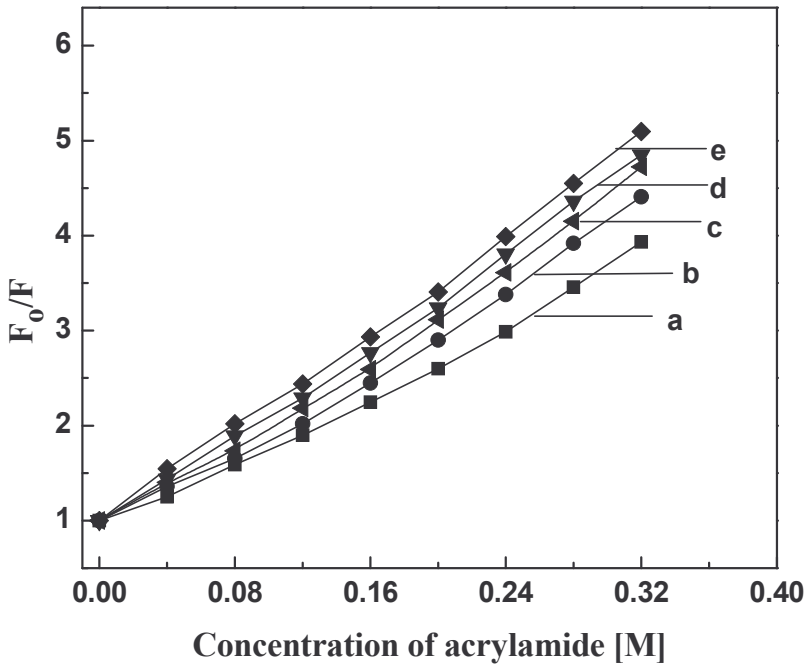

Fig 34: Stern-Volmer plot for the acrylamide fluorescence quenching of ficin in presence of different concentrations of urea at pH 3.0. a) In absence (only in buffer) and in presence of b) 2 M; c) 3 M; d) 4 M and e) 4.5 M urea.

quenching data of ficin in presence of different concentrations of urea. It is evident from the plot that the quenching is more effective by acrylamide with increase in the concentration of urea and maximum quenching occurred at 4.5 M. The results of Stern-Volmer plot thus indicate that aromatic amino acids of ficin are more exposed to solvent as the unfolding induced by urea. These results are consistent with the intrinsic fluorescence emission results. The red shift in the fluorescence emission maximum and high quenching of tryptophan fluorescence are indicative of the unfolding of protein molecule.

The exposure of any hydrophobic regions, which are buried inside the enzyme, on urea unfolding was monitored by ANS binding studies. The level of ANS binding to the protein in presence of different concentrations of urea is shown in Fig 35. From the figure, it can be observed that there was a minimal ANS binding to ficin in absence of urea at pH 3.0. ANS binding to the enzyme is maximal at 0.5-2.0 M urea concentration, which is suggestive of exposure of hydrophobic patches in this region of urea-induced unfolding pathway. The minimal binding of ANS was observed above the 2.0 M urea concentration.

Far-UV CD studies on urea-induced unfolding of ficin were carried out to investigate the effect of urea on the secondary structure of the protein molecule. In Fig 36 is shown the effect of urea concentration on the far-UV CD spectra of ficin. The spectrum of the ficin at pH 3.0 is similar to that of ficin at neutral pH except some loss in the ellipticity values. The enzyme contains nearly about 70% of secondary structures at pH 3.0 calculated based on the ellipticity values at 222 nm. As seen from the figure, the loss of secondary structure occurs as the concentration of urea increases and the maximum loss was observed at 4.5 M urea. Further, high concentration of urea will not allow the measurement of spectrum between 200-210 nm. Therefore, the ellipticity values at 222 nm are tabulated in the Table 11. The

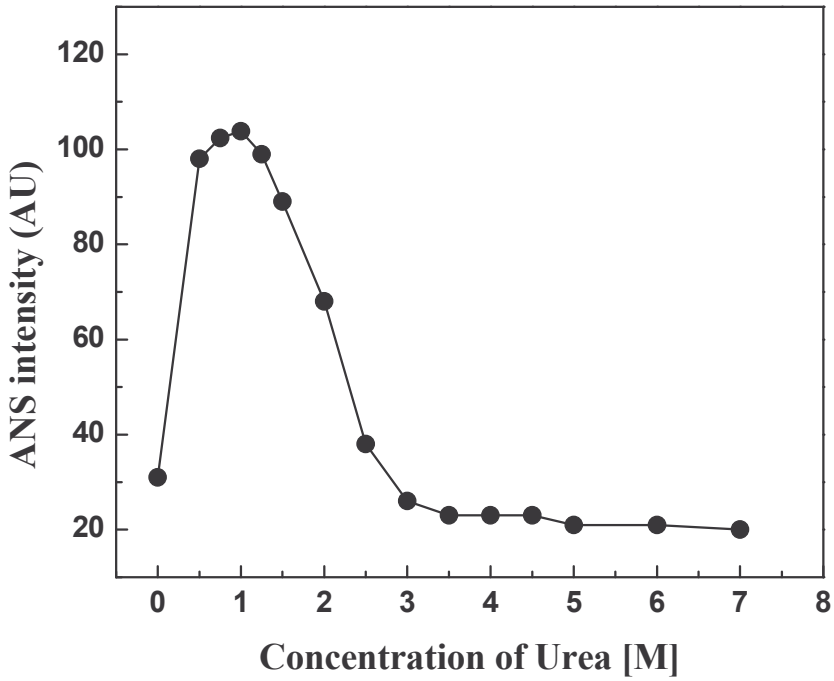

Fig 35: Effect of urea-induced unfolding (at pH 3.0) on ANS fluorescence. Protein to ANS concentration was in the ratio 1:100 and the spectra were recorded in the region of 400-600 nm after exciting at 380 nm at 25° C.

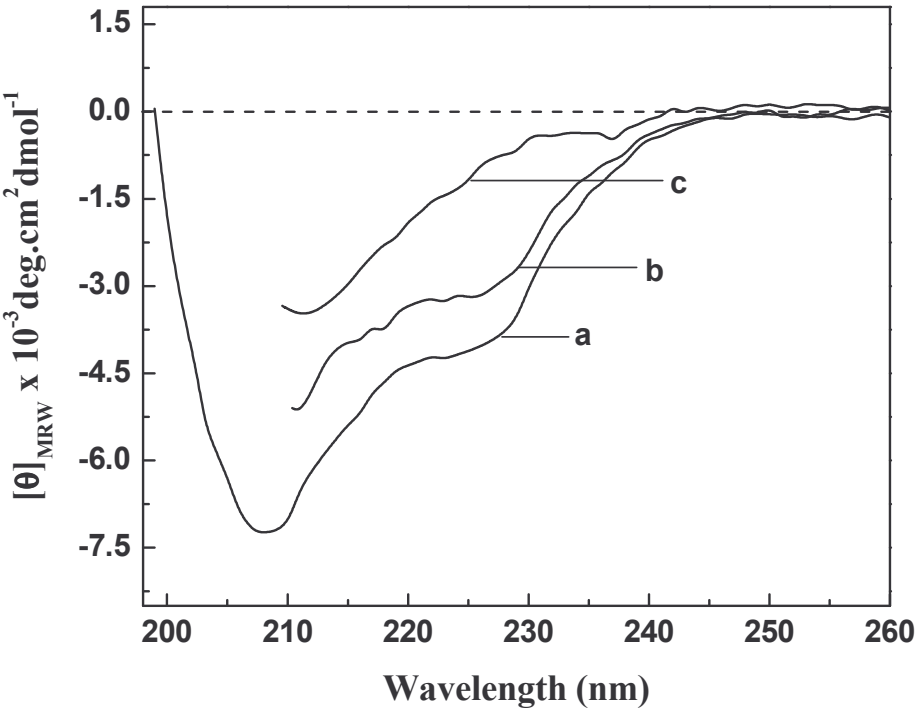

Fig 36: Effect of different concentrations of urea on far-UV CD spectra (representative) of ficin in acidic conditions (pH 3.0). a) At pH 3.0 in absence of urea and in presence of b) 2 M and c) 3 M urea (Representative spectra are only shown for clarity).

Table 11: Changes in the ellipticity values at 222 nm as a function of urea concentration at pH 3.0

Concentration of urea [M]	MRE values at 222 nm ($deg.cm^2dmol^{-1}$)
Control*	-4215 ± 45
1.0	-3833 ± 35
2.0	-3224 ± 30
3.0	-1549 ± 25
4.0	-1093 ± 15
5.0	-865 ± 15

** The enzyme is in sodium Gly-HCl buffer, pH 3.0 as control*

decrease in the ellipticity values at 222 nm with increasing concentration of urea reflects the disruption of secondary structures by urea. A marked decrease in ellipticity at 2 to 4 M concentration of urea indicates that, most of the secondary structures were disrupted in this transition region. There was no further major change in the ellipticity observed as the concentration of urea increased up to 8 M indicating that the maximum loss of structure occurred at 4 M urea.

The equilibrium unfolding transitions of ficin at pH 3.0 as a function of urea concentration was monitored by changes in the fluorescence emission maximum and ellipticity values at 222 nm as probes. The plots of urea concentration versus fraction unfolded are shown in Fig 37. It can be seen from the plot that, the urea unfolding transitions of ficin monitored by both methods are cooperative and coincidental as indicated by sigmoidal curves. The maximum changes occur between 2 to 4 M urea with a transition mid point of 2.5 ± 0.1 M as indicated by both CD and fluorescence measurements. The refolding was studied by dilution of the protein sample which had been completely equilibrium unfolded in 8 M urea. The transition curves for the process of refolding was close to that of unfolding curves. Thus, urea-induced unfolding of ficin can be described by a simple two-state model. It is generally recognized that protein denaturation is a highly cooperative process, for which small globular proteins may be approximated by a two-state model and no significant intermediates are present during the transitions between native ↔ denatured states (Greene and Pace, 1974; Privalov, 1979). All the above results indicate that urea-induced unfolding of ficin at pH 3.0 follows a simple two-state transition. To establish the complete folding pathway and stability, it is necessary to study the conformational stability of a protein molecule towards various treatments. In the next studies the effect of GuHCl, which is known to be a more potent protein destabilizer than urea, on ficin is investigated.

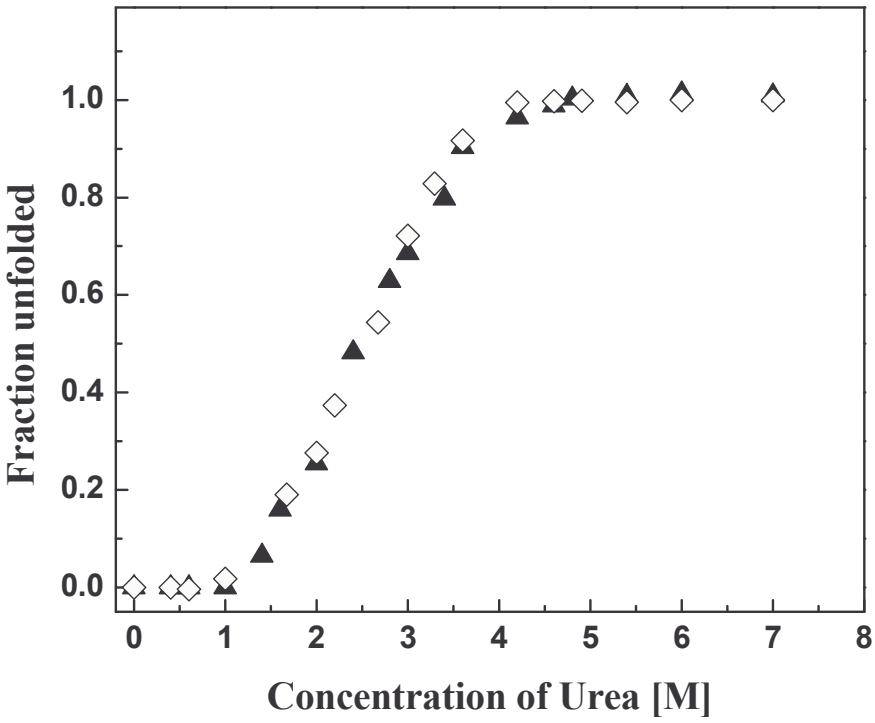

Fig 37: The urea-induced equilibrium unfolding transition curves of ficin at pH 3.0 measured by changes in the CD ellipticities at 222 nm (▲) and fluorescence emission maximum (◇).

2.2b. Effect of GuHCl

In majority of denaturation studies, chemical denaturants, generally urea or GuHCl are utilized to induce protein unfolding. However, GuHCl has been found to be more potent denaturant than urea. Despite the routine use of GuHCl in denaturation studies, the mechanisms by which it affects the protein stability are still not fully understood (Schellman 2002). GuHCl, a salt and also a bi-functional reagent, and its strong denaturing activity usually associated with the guanidinium ion. Thus, the GuHCl-induced modulation of protein stability through mechanisms is related to both its denaturant and ionic properties. GuHCl being bi-functional reagent favors interaction with peptide bonds of protein through hydrogen bonding. It has been shown that one molecule of GuHCl can hydrogen bonding with two peptide bonds to form a cyclic structure (Robinson and Jencks, 1965; Collins, 2004).

In the previous study, the effect of urea, a non electrolyte denaturing reagent on the structure, function and stability of ficin was followed. The obtained results indicated that ficin molecule, under neutral conditions, is resistant to denaturation by urea was susceptible to unfolding under acidic condition. In order to further characterize, an attempt was made to understand the effect of GuHCl on the structure, function and stability of ficin under neutral conditions. The enzyme was incubated with desired concentrations of GuHCl under neutral condition for 12 h to attain the equilibration before taking all the measurements.

The fluorescence emission spectral properties such as position, shape and intensity are mainly determined by the polarity and specific interactions of fluorophores and are very sensitive to any small changes in fluorophore's environment. Changes in the fluorescence emission spectra of ficin after denaturation in GuHCl solutions of increasing concentrations are shown in

Fig 38. The spectrum of ficin was not affected by the presence of GuHCl up to 1.8 M concentration. As seen from the spectra, with the increasing concentration above 1.8 M of GuHCl, the fluorescence emission intensity increased with a red shift in the emission maximum. In Fig 39 is shown the fluorescence emission maximum as function of GuHCl concentration. It can be seen from the figure, the emission maximum suffers a red shift as the concentration of GuHCl increase. The red shift in the wavelength maximum indicates the more exposure of fluorophores of the protein to the solvent environment, which is the characteristic of unfolding. The increase in fluorescence intensity may be the result of an increased distance between fluorophores and specific quenching groups of the protein (Halfman and Nishida, 1971; Sommers and Kronman, 1980).

Acrylamide quenching studies provide insights into the conformational changes of the protein by probing the solvent accessibility of fluorescent moieties (Eftink and Ghiron, 1981; Eftink and Selvidge, 1982). Quenching titrations were performed with sequentially added aliquots of acrylamide solutions to ficin samples equilibrated at different concentrations of GuHCl solutions. In Fig 40 is the shown Stern-Volmer plot for the tryptophans fluorescence quenching of ficin by acrylamide at various concentrations of GuHCl. As seen from the plot, the solvent accessibility of tryptophan residues was increased with increase in concentration of GuHCl. These results are consistent with the data obtained from the intrinsic fluorescence experiments.

The effect of GuHCl on the structural changes of ficin was further characterized by using far-UV CD measurements. Conformational changes in the secondary structure of the protein molecule were monitored in the region between 200 and 260 nm. The secondary structure of ficin was stable till 1.8 M GuHCl. The disorganization of secondary structure of ficin by

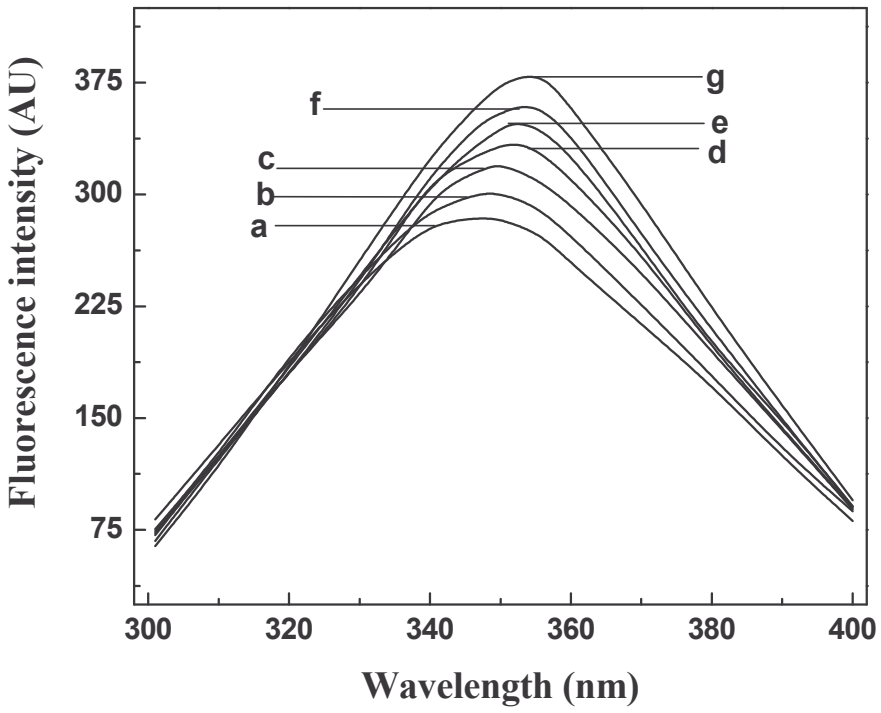

Fig 38: Intrinsic fluorescence emission spectra of ficin in presence of various concentrations of GuHCl at pH 7.0 a) in absence (only in buffer) and in presence of b) 2 M, c) 2.5 M, d) 3 M, e) 3.5 M, f) 4 M and g) 6 M GuHCl.

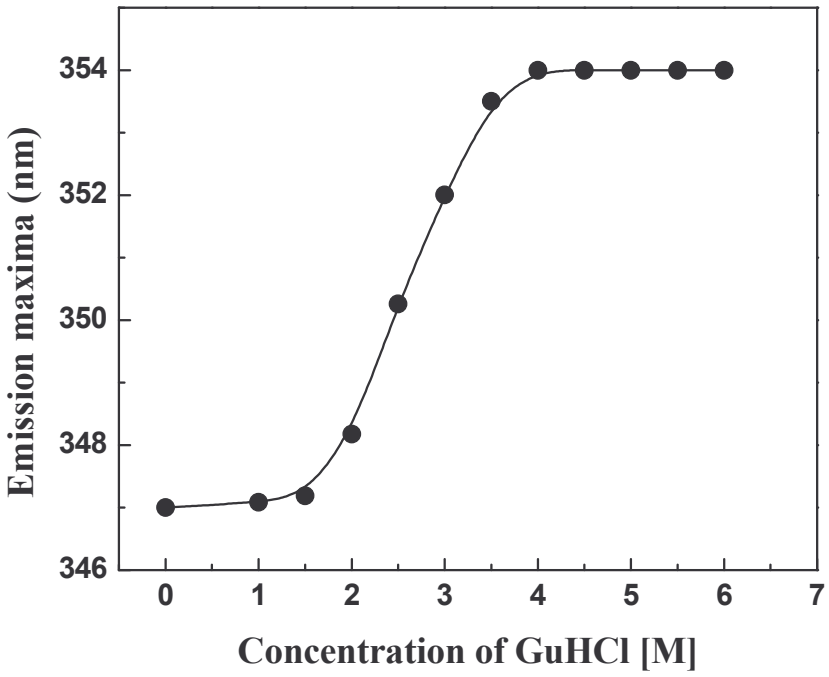

Fig 39: Intrinsic fluorescence emission maximum (λ_{max}) of ficin as a function of GuHCl concentration at pH 7.0.

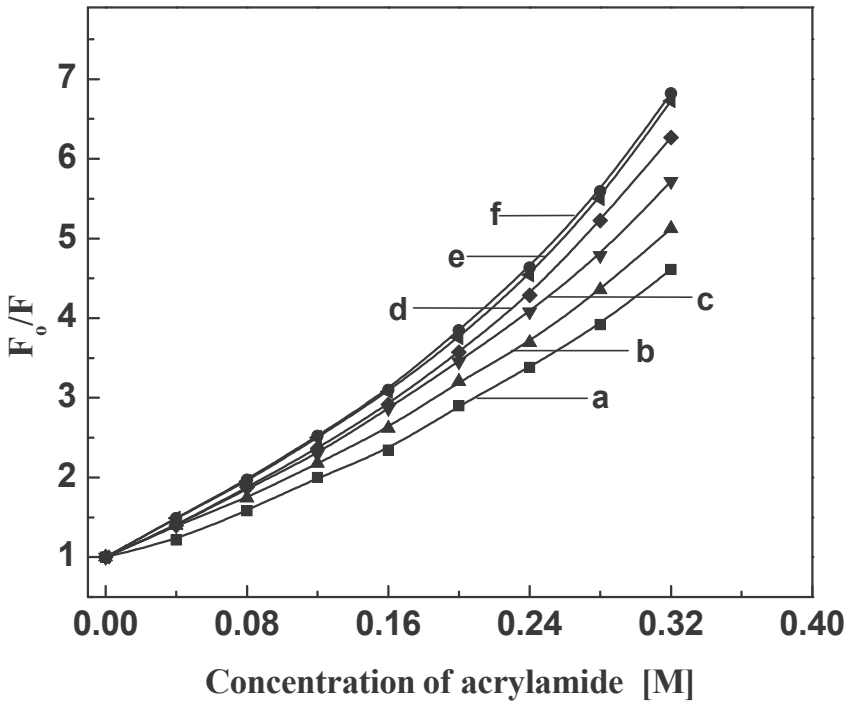

Fig 40: Stern-Volmer plot for the acrylamide fluorescence quenching of ficin in presence of different concentrations of GuHCl at pH 7.0: a) in absence (only in buffer) and in presence of b) 2.5 M, c) 3 M, d) 3.5 M, e) 4 M and f) 5 M GuHCl.

increasing concentrations of GuHCl as monitored by far-UV CD. The measurements of spectra could not be completed between 200-210 nm because of the presence of high concentration of GuHCl (Fig 41). Hence the ellipticity values at 222 nm are tabulated in Table 12. It can be seen from the spectra and ellipticity values, the loss in the secondary structure of the protein was observed with increase in the concentration of GuHCl. Most of the secondary structure was disturbed in the region between 2 and 3.5 M GuHCl. Further increase in concentration of GuHCl above 4 M did not show any major changes in the spectra indicating the complete loss of structure occurs at 4 M GuHCl concentration.

The effect of various concentrations of GuHCl on the activity of ficin is shown in Fig 42. Ficin looses its half of the activity at around 1 M GuHCl, and the complete inactivation occurs at 3 M concentration. These results indicate that inactivation of ficin occurred very rapidly before major conformational changes occurs in the enzyme molecule. It has been proposed that denaturation of number of enzymes by GuHCl, inactivation occurs before noticeable conformational changes of the enzyme molecule as a whole can be detected (Ma and Tsou, 1991; Tsou, 1993; Zhou *et al*., 1993). It is therefore suggested that the active site of the enzyme is situated in limited and relatively fragile regions whose conformational integrity is more sensitive to denaturation than the molecule as a whole. A slight perturbation to geometry and dynamics of the active site responsible for catalysis destroys enzyme activity before the molecule as a whole (Tsou, 1993; Zhou *et al*., 1993).

GuHCl-induced equilibrium unfolding of ficin was followed by changes in the fluorescence emission maximum and ellipticity values at 222 nm as probes. The data obtained were normalized and analyzed according to the standard equation (Pace, and Scholtz, 1997). The plots of GuHCl concentration versus fraction unfolded are shown in Fig 43. As seen from

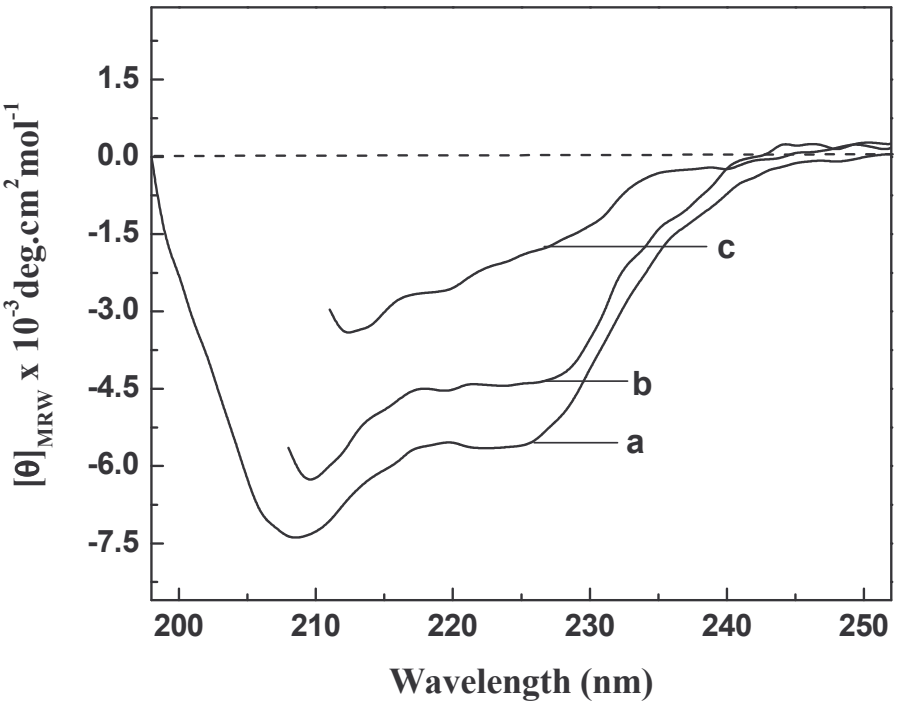

Fig 41: Effect of different concentrations of GuHCl on far-UV CD spectra (representative) of ficin at pH 7.0. a) in absence of urea (only in buffer) and in presence of b) 2 M and c) 3 M GuHCl (Representative spectra are only shown for clarity).

Table 12: Changes in the ellipticity values at 222 nm as a function of GuHCl concentration at pH 7.0

Concentration of GuHCl [M]	MRE values at 222 nm ($deg.cm^2dmol^{-1}$)
Control*	-5623 ± 55
1.0	-5564 ± 45
2.0	-4410 ± 35
2.5	-3135 ± 25
3.0	-2147 ± 20
4.0	-1137 ± 15

** The enzyme is in sodium phosphate buffer, pH 7.0 as control*

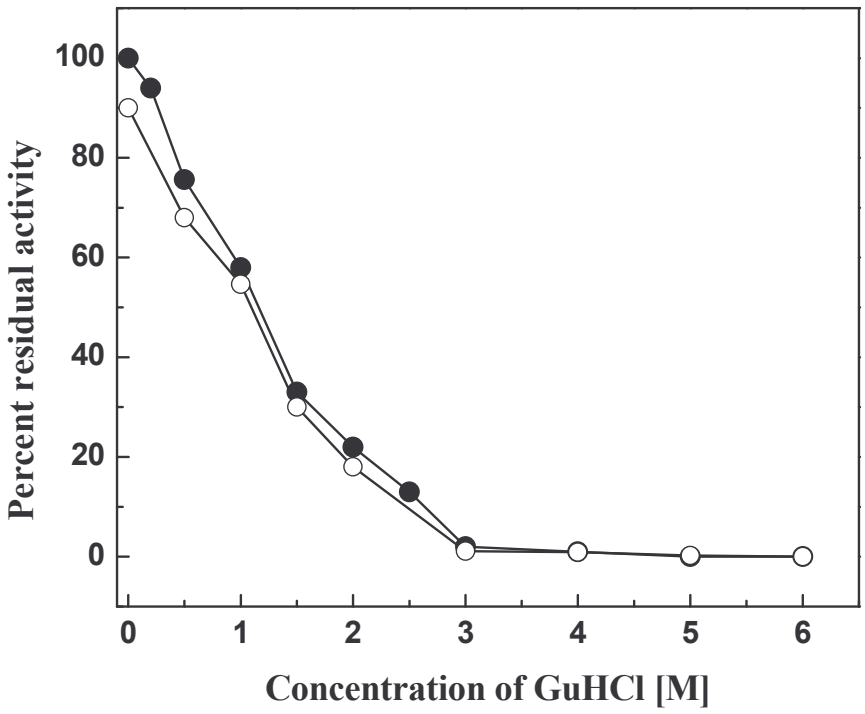

Fig 42: Activity profile of ficin as a function of GuHCl at pH 7.0. Activity of ficin was carried out in presence of various concentrations GuHCl using BAPNA as substrate: unfolding (●) and refolding (○).

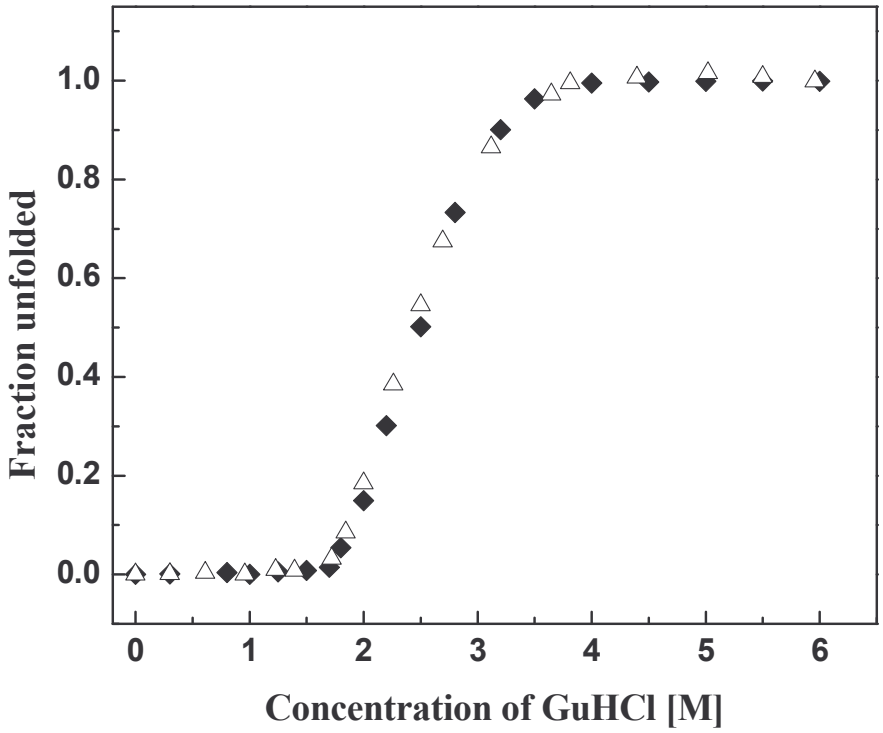

Fig 43: The GuHCl-induced equilibrium unfolding transition curves of ficin at pH 7.0 measured by changes in the CD ellipticities at 222 nm (◆) and fluorescence emission maximum (△).

the figure, the GuHCl unfolding transitions of ficin monitored by both methods are cooperative, coincidental and sigmoidal in nature. The maximum changes occur between 2 and 3.5 M GuHCl with a transition mid point of 2.4 ± 0.1 M as obtained by CD and fluorescence measurements. The refolding was studied by diluting of the protein sample which had been from equilibrium unfolded in 6 M GuHCl, to the desired concentration. The transition curves for the process of refolding was close to that of unfolding curves. Thus, GuHCl-induced unfolding of ficin can be described by two-state model as evident from the coincidental sigmoidal transition curves.

In the present study, the structure-function relationship and folding behaviour of ficin was studied using urea and GuHCl. Several properties which reflect different aspects of the structural integrity of ficin were monitored using different spectroscopic parameters through the course of equilibration denaturation. The results obtained in this study show that ficin, under neutral conditions, as studied by fluorescence and CD spectroscopic measurements, indicated a high rigid nature of the protein molecule towards the unfolding by urea, even at 8 M concentration. However, ficin molecule was more susceptible to unfolding by urea under acidic condition and by GuHCl at neutral condition. In other words, higher extent of denaturation is achieved in acidic pH as shown in some of the earlier studies (Prakash and Nandi, 1977; Dubey and Jagannadham, 2003; Kumar *et al.,* 2009). The equilibrium transition curves as determined by various methods, for both urea (at pH 3.0) and GuHCl (at pH 7.0) induced denaturation were coincidental, suggesting the unfolding of the protein is a simple two-state transition (native ↔ unfolded). At lower pH, an increased level of protonation of acidic groups occurs which disrupts the salt bridges. Hence, the results of present study suggest that the salt bridges may be important in determining the stability of this protein molecule.

Understanding the conformational changes in a protein molecule in various conditions would provide an insight to understand 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 ficin has been carried out. The pH induced denaturation process of ficin is reversible in the pH range 2.4 to 7.0 and irreversible inactivation of the enzyme occurs when the pH decreased further below 2.4. The structural perturbations at extreme pH conditions occur mainly due to disruption of electrostatic interactions, which play a significant role in ficin stability. Maximum stability for structure as well as activity of ficin has been observed at pH 7.0. The results demonstrated that the pH induced denaturation of ficin leads to the formation of partially folded conformation at low pH. At pH 1.4 ficin exists as partially folded conformation with characteristic features of molten globule like intermediate state.

The results from folding and unfolding studies show a differential structural stability of ficin towards denaturation by urea and GuHCl. Ficin molecule is resistant to unfolding by urea under neutral condition. However ficin is susceptible to unfolding at lower pH and the process is cooperative and reversible. These results indicate the significant role of electrostatic interaction in determining the stability of ficin. GuHCl-induced unfolding of ficin at neutral pH is cooperative and follows a simple two-state model with on intermediates. The refolding process is reversible with complete recovery of activity. The results obtained in this study help to gain insight into structure- function relationship of the enzyme in view of its stability towards the denaturation by pH, urea and GuHCl. In the subsequent chapter ficin molecule is understood from the point of thermal inactivation phenomenon and its stabilization using different structure stabilizing cosolvents. These studies help to understand the stability of ficin molecule and mechanism of thermal denaturation.

3. Effect of cosolvents on structure and stability of ficin from *Ficus carica*

Stability of proteins is one of the most important limiting factors hampering the development of potentially large number of bioprocesses based on enzymes. Proteins are only marginally stable due to the delicate balance of stabilizing and destabilizing interactions (Jaenicke, 1991). The regular native conformation of protein molecule is stable at physiological conditions and may easily be destroyed by slight changes in pH, temperature, pressure and ionic strength (Mozhaev and Martinek, 1982). The resistance of the catalytically active protein structure towards these adverse factors is one of the most important criteria for commercial utilization of the enzymes. The analysis of enzyme inactivation and the possibility of enhancing thermal stability are the important considerations in the development of enzyme based applications.

Upon extraction of proteins from their biological sources, they are subjected to different environments, which may bring the conformational changes directly related to protein stability. The introduction of cosolvents such as sugars and polyhydric alcohols into the solvent medium has been found to stabilize biological macromolecules in solutions (Timasheff *et al*., 1976; Arakawa and Timasheff, 1982a; Sathish *et al*., 2007). The cosolvents are known to prevent the loss of enzymatic activities, inhibit irreversible aggregation and increase the thermal transition temperature of macromolecules (Timasheff *et al*., 1976; Gekko and Timasheff, 1981; Rajendran *et al*., 1995; Timasheff and Arakawa, 1997; Sathish *et al*., 2007). Among several ways of protein stabilization, the use of specific cosolvents and additives to stabilize the proteins and enzymes represents the simplest and most practical approach to minimizing protein inactivation.

In order to establish a more generalized mechanism of enzyme stabilization, it is necessary to study the behaviour of various types of proteins and enzymes in presence of variety of additives and cosolvents. The objective of this study is to understand the stability of ficin from the mechanism point of view and to improve the stability of the enzyme from a practical point of view. The cosolvents interact with protein in a diverse way, depending on the physico-chemical properties of the proteins. Hence the selection of the appropriate cosolvents depends on the nature of the enzyme. A variety of additives were tested for their stabilizing influence on ficin. The different additives and their concentrations in this study were chosen based on the knowledge of their stabilizing effect on protein and after the preliminary screenings.

The effect of different cosolvents such as sorbitol, trehalose, sucrose and xylitol on the thermal inactivation of ficin was examined by enzyme activity measurements. Ficin was heated in presence of different concentrations of cosolvents at 70° C for 10 min and the remaining activity was measured. The Figs 44 and 45 show the percent residual activity of ficin in presence of different concentrations of cosolvents. Ficin looses 37% of its activity at 70° C after 10 min incubation and in presence of cosolvents the activity was protected upto different extent. The protection of enzyme activity by cosolvents increased with increasing concentration of each cosolvent. Enhanced thermal stability of enzymes in presence of cosolvents in a concentration dependent manner has been observed in several studies (Rajeshwara and Prakash, 1994; Rajendran *et al*., 1995; Timasheff and Arakawa, 1997; Sathish *et al*., 2007).

In presence of 5% sorbitol the enzyme retained about 69% of its original activity and it increases gradually as the concentration of sorbitol increases (Fig 44A). The enzyme retained about 74%, 87% and 92% of its activity in presence of 10%, 30% and 40% sorbitol, respectively. Similarly, in

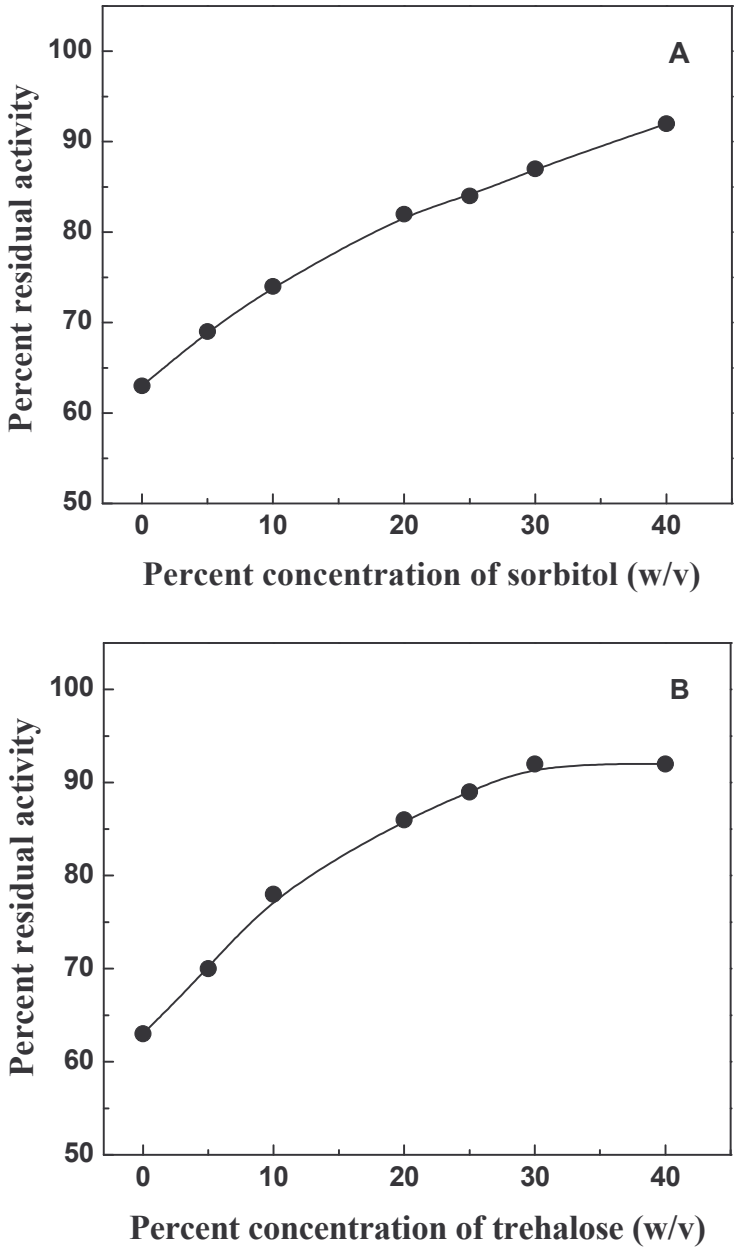

Fig 44: Effect of (A) sorbitol and (B) trehalose on thermal inactivation of ficin. The enzyme was heated at 70° C for 10 min in presence of different concentrations of cosolvents at pH 7.0 and the residual activity was measured using BAPNA as substrate.

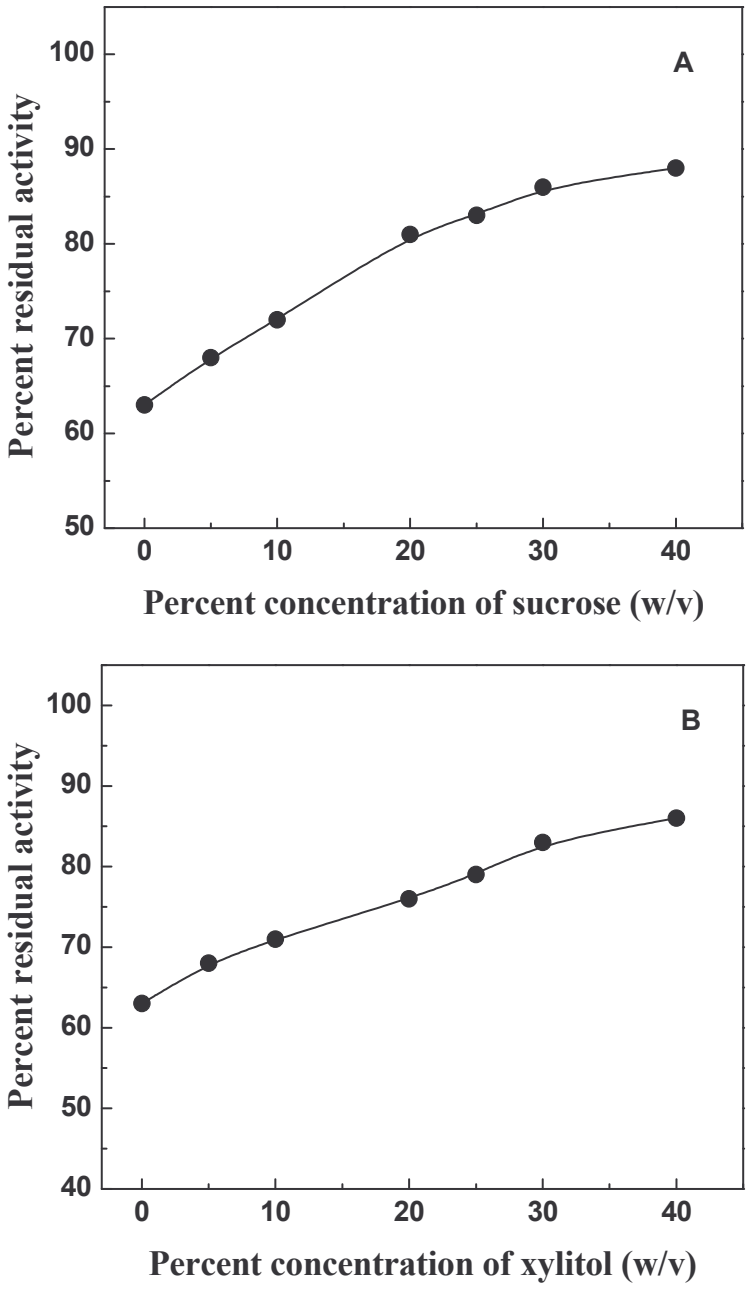

Fig 45: Effect of (A) sucrose and (B) xylitol on thermal inactivation of ficin. The enzyme was heated at 70° C for 10 min in presence of different concentrations of cosolvents at pH 7.0 and the residual activity was measured using BAPNA as substrate.

presence of trehalose, a gradual increase in the activity was observed up to 30% concentration (Fig 44B) and above which it reached a plateau retaining a maximum activity of 92% in presence of 30% and 40% trehalose. In case of sucrose (Fig 45A), the maximum activity of 88% was retained at 40% concentration and minimum of 68% activity was retained in presence of 5% sucrose. In Fig 45B, the effect of xylitol on the thermal stability of ficin is shown. The enzyme retained the activity in a concentration dependent manner. The maximum retaining activity of 86% was observed at 40% concentration. These results indicate that the maximum protection was exhibited by trehalose, followed by sorbitol, sucrose and xylitol.

Ficin was incubated at 70° C at different time intervals in absence and presence of cosolvents (at 30% concentration) to determine the stabilizing capacities of each cosolvents. Ficin looses nearly 50% of its activity after 20 min of incubation at 70° C. In Fig 46 is shown the increased thermal stability of ficin in presence of cosolvents. In presence of trehalose the enzyme retained 85% of its activity after 20 min and 58% activity even after 60 min of incubation. In case of sorbitol, sucrose and xylitol, the enzyme retained the activity of 82, 80 and 78%, respectively after 20 min incubation. The results presented here are most likely that of the cosolvents which stabilize the various proteins and enzymes (Gekko and Timasheff, 1981; Rajendran *et al*., 1995; Timasheff and Arakawa, 1997; Sathish *et al*., 2007).

The enzyme was further evaluated for the reversibility of the activity by exposing the ficin at 70° C for 10 min in presence of cosolvents and after cooling, the cosolvent was removed by gelfiltration chromatography on Shephadex G-25 column. The enzyme fraction eluting in the void volume was used for the reversibility studies. The results of the experiments are summarized and listed in Table 13. From the table, it is clear that trehalose and sorbitol at 40% concentration has the maximum protection against thermal inactivation as evident by the maximum recovery of about 78% and

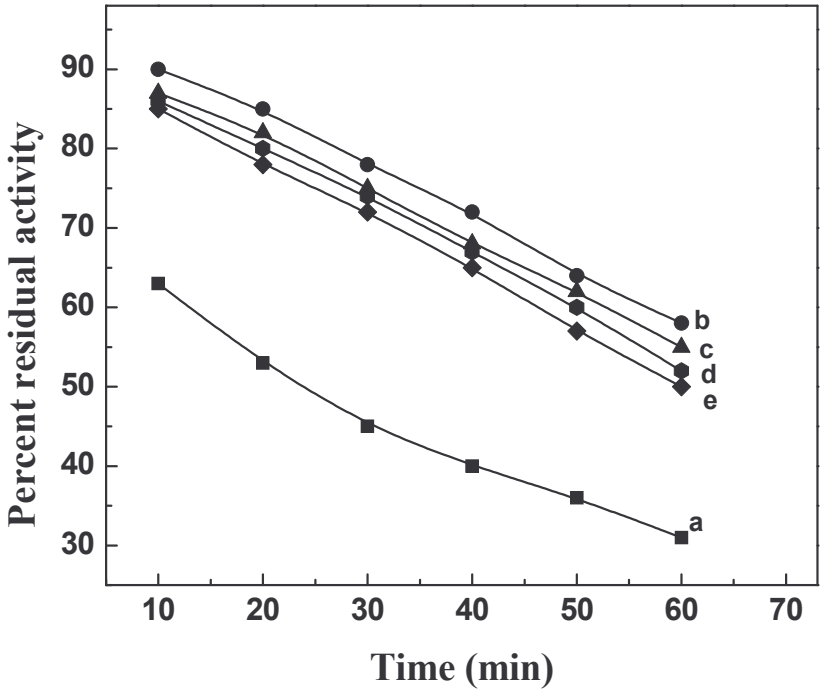

Fig 46: Time course thermal inactivation of ficin at 70° C in presence of cosolvents (at 30% (w/v) concentration): (a) in absence and presence of (b) trehalose, (c) sorbitol, (d) sucrose and (e) xylitol. The enzyme solution was heated at 70° C for different time intervals and the remaining activity was determined.

Table 13: Reversibility of the activity of ficin as a function of cosolvent concentration after exposed to 70° C for 10 min in the presence and after removal of cosolvents by gelfiltration procedure

Cosolvent	Concentration (%) (w/v)	Residual specific activity (%)*	
		In presence of cosolvent	After removal of cosolvent by gel filtration
Sorbitol	10	74 ± 2	68 ± 1
	20	82 ± 2	70 ± 1
	30	87 ± 2	73 ± 2
	40	92 ± 3	75 ± 2
Trehalose	10	78 ± 1	69 ± 2
	20	86 ± 2	72 ± 2
	30	92 ± 2	75 ± 2
	40	92 ± 2	78 ± 2
Sucrose	10	72 ± 2	66 ± 1
	20	81 ± 2	68 ± 2
	30	86 ± 3	70 ± 3
	40	88 ± 3	73 ± 3
Xylitol	10	71 ± 2	65 ± 2
	20	76 ± 2	66 ± 2
	30	83 ± 2	68 ± 2
	40	86 ± 3	70 ± 2

**Residual specific activity of ficin in 0.05 M sodium phosphate buffer, pH 7.0 is 63 ± 2%. The cosolvent concentration shown is in the same buffer as above.*

75% activity, respectively. Xylitol is least effective of all the cosolvents against the thermal inactivation of ficin. Thus from the activity measurement studies, it clearly indicates that ficin is stabilized against thermal inactivation in presence of cosolvents. The presence of cosolvents have been shown to prevent the thermal inactivation of the many enzymes (Rajeshwara and Prakash, 1994; Rajendran *et al*., 1995). The studies of Sathish *et al*. (2007) have shown the stabilizing effect of cosolvents on papain, which is also a cysteine protease. The presence of cosolvents protected the activity of papain at higher temperature and these results are very similar to our results in stabilizing papain against thermal inactivation using different cosolvents.

Effect of temperature on the conformation of ficin in presence and absence of cosolvents was studied by fluorescence measurements. Fluorescence emission signals are very sensitive to the structural changes of a macromolecule. Ficin was exposed at 70° C for different time intervals in presence and absence of cosolvents and their fluorescence emission spectra were recorded. Fig 47 shows a significant reduction in the intensity of intrinsic fluorescence at 347 nm in case of control. The presence of all cosolvents protected this intensity reduction at different time intervals. The maximum protection was shown by trehalose. These results further confirm the best stabilization effect of trehalose on structure-function of ficin. Several studies have showed that the trehalose is a better stabilizer in providing protection to biological macromolecules (Kaushik and Bhat, 2003; Park *et al*., 2005; Zancan and Sola-Penna, 2005).

The effect of cosolvents on the structural integrity of ficin was examined by fluorescence and circular dichroic spectral measurements. Fig 48A and 48B show the fluorescence emission spectra of ficin in presence of different concentrations of sorbitol and trehalose, respectively. As evident

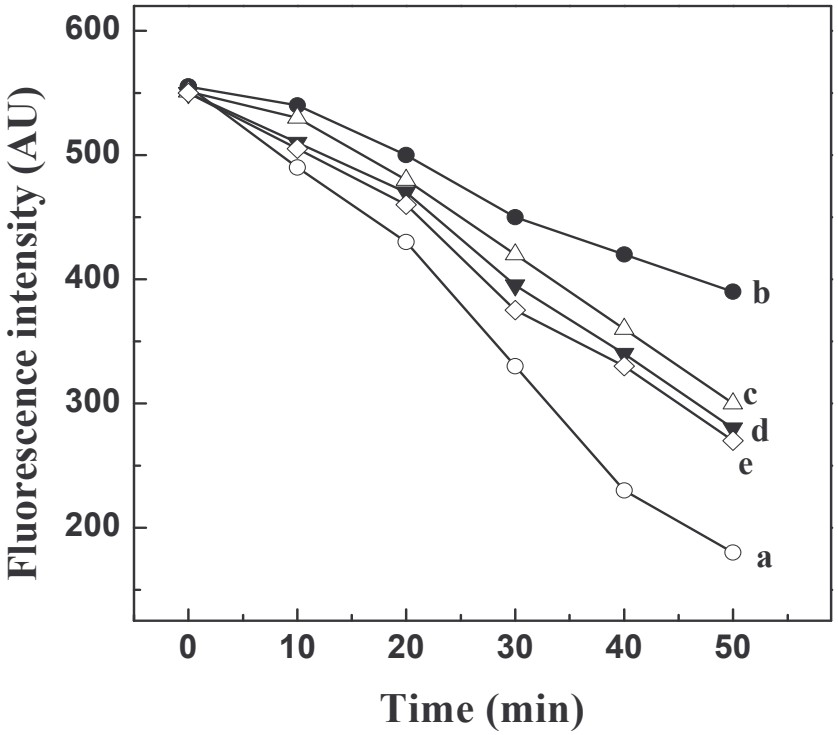

Fig 47: Time course of thermal denaturation of ficin at 70° C in presence of cosolvents (at 30% concentration). The fluorescence intensity at 347 nm was monitored after incubation of ficin for different time intervals, (a) in absence and presence of (b) trehalose, (c) sorbitol, (d) sucrose and (e) xylitol.

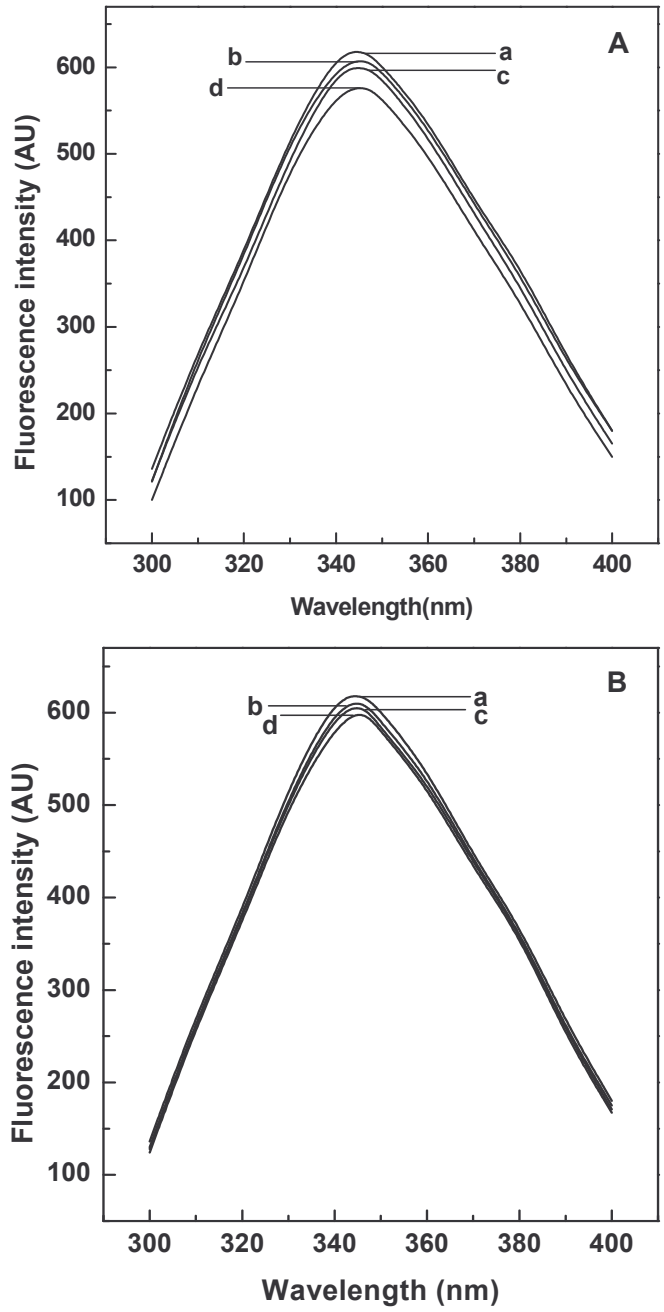

Fig 48: Intrinsic fluorescence emission spectra of ficin in presence of different concentrations of (A) sorbitol and (B) trehalose. (a) Control in pH 7.0 phosphate buffer only, (b) in 10%, (c) in 20% and (d) in 40% cosolvents.

from the spectra, the presence of these cosolvents did not bring any shift in the emission maximum except slight decrease in fluorescence emission intensity. In case of sucrose and xylitol, the spectra experienced a slight increase in fluorescence intensity without affecting the emission maxima. These results suggest that no gross conformational changes of the protein occur in presence of cosolvents. These results are in agreement with the other reports that the cosolvents stabilize proteins without bringing major conformational changes in the protein structure (Lee and Timasheff, 1981; Rajeswara and Prakash, 1995; Gunasekhar and Prakash, 2008).

The structural integrity of ficin in presence of cosolvents was further evaluated by CD measurements. The far-UV CD spectral measurements were carried out in presence of different concentrations of cosolvents. The representative spectra of ficin in presence of 40% concentration of each cosolvent is shown in Figs 49 and 50. As seen from the spectra, there is no gross difference in the structure of the protein in presence of cosolvents. In Table 14, the secondary structural features of ficin determined from CD spectra in presence of different concentrations of cosolvents is shown. There is not much changes observed in the secondary structural contents of ficin in presence of cosolvents. Several similar kind of observations have been made with many proteins that cosolvents stabilize the protein molecule without affecting the structural integrity (Lee and Timasheff, 1981; Gunasekhar and Prakash, 2008).

Thermal denaturation profile of ficin was studied in presence of cosolvents by monitoring the absorbance at 287 nm as a function of temperature in the range of 30-90° C. These results further demonstrate the stabilization effect of cosolvents. From the change in the absorbance, the apparent thermal melting temperature (T_m) of ficin was calculated from the plot of fraction unfolded versus temperature. The apparent melting temperature of ficin was found to be 72.0 ± 1.0° C. The apparent thermal

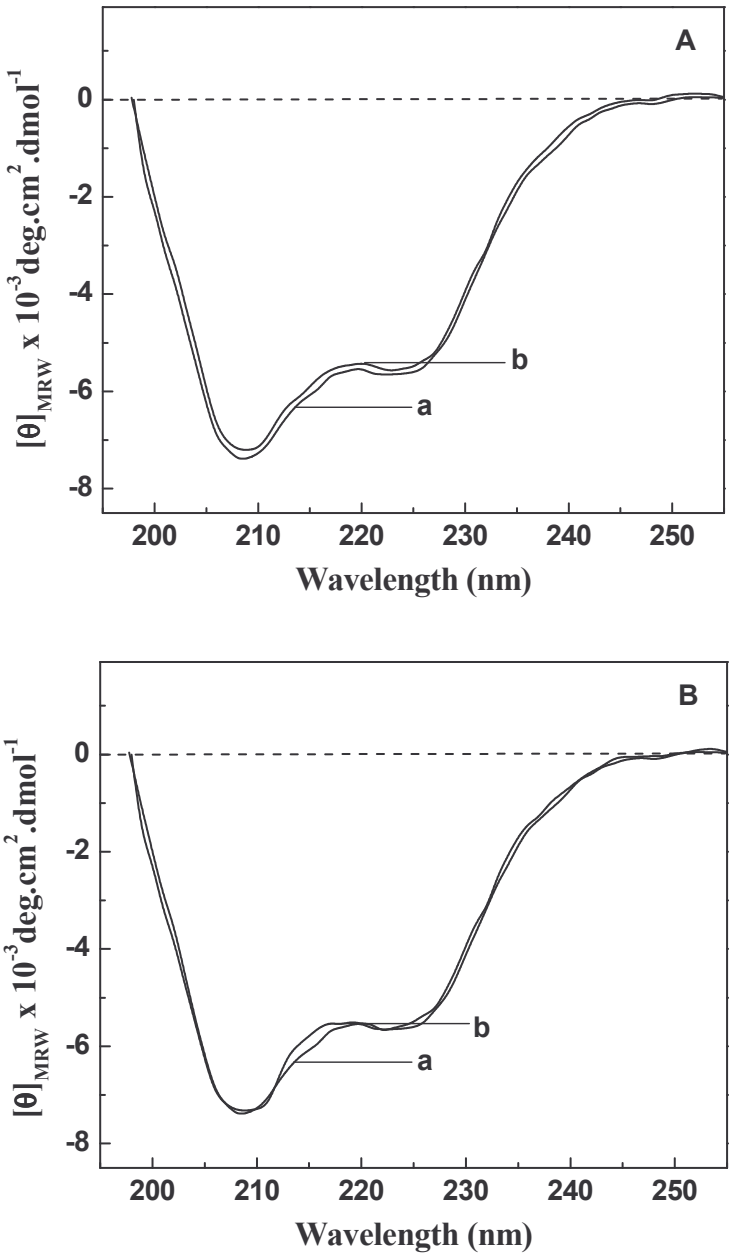

Fig 49: Far-UV CD spectra of ficin in presence of 40% (A) sorbitol and (B) trehalose. (a) The enzyme in absence of cosolvents (only in buffer) and (b) in presence of respective cosolvent in 0.02 M sodium phosphate buffer at pH 7.0 (Representative spectra are only shown for clarity).

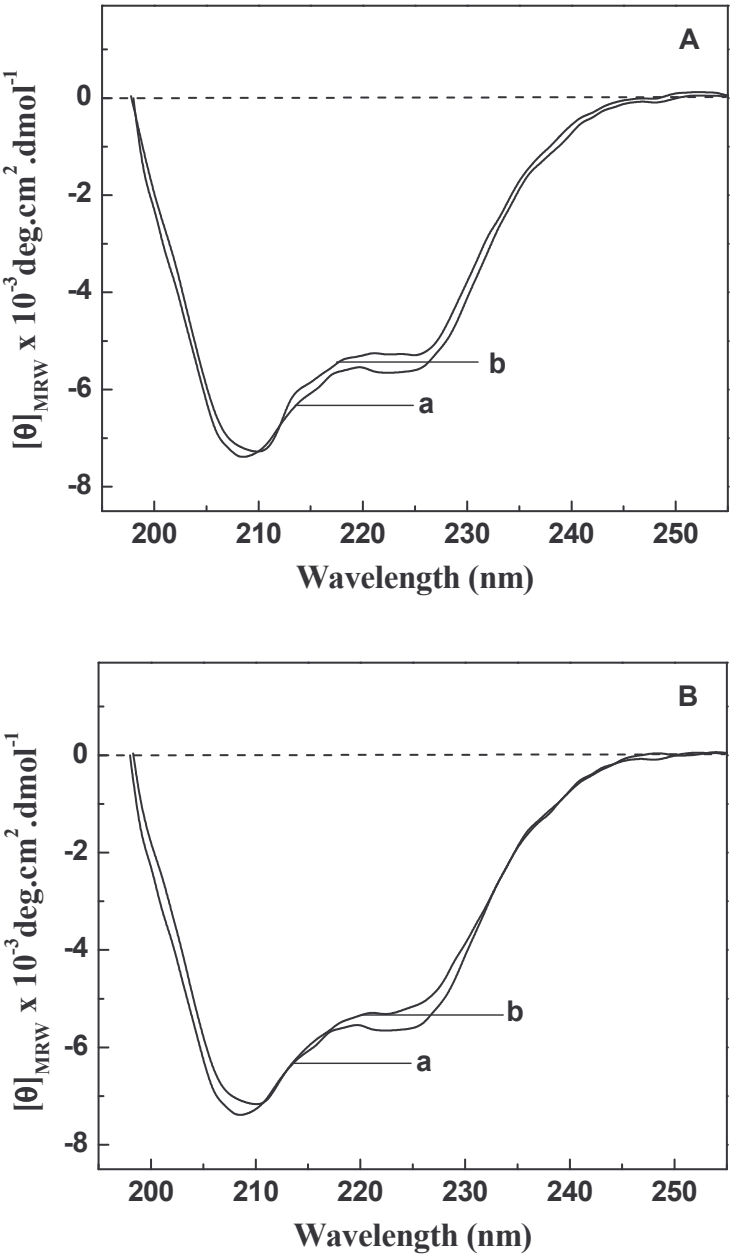

Fig 50: Far-UV CD spectra of ficin in presence of 40% (A) sucrose and (B) xylitol. (a) The enzyme in absence of cosolvents (only in buffer) and (b) in presence of respective cosolvent in 0.02 M sodium phosphate buffer at pH 7.0 (Representative spectra are only shown for clarity).

Table 14: Secondary structural features of ficin in presence of different cosolvents at 40% concentration

Cosolvents	Secondary structures (%)		
	α-Helix	**β-Structures**	**Aperiodic**
Control*	21 ± 1	43 ± 2	36 ± 2
Sorbitol	22 ± 1	44 ± 2	34 ± 2
Trehalose	22 ± 1	45 ± 2	33 ± 2
Sucrose	20 ± 1	44 ± 2	36 ± 2
Xylitol	20 ± 1	43 ± 2	37 ± 2

* *The enzyme is in sodium phosphate buffer, pH 7.0 as control*

melting temperature of the enzyme was increased to different extent in presence of cosolvents and the increase is concentration dependent.

In Fig 51A is shown the thermal denaturation curves of ficin in presence of different concentrations of sorbitol. The analysis of thermal denaturation curves indicates a shift in the apparent thermal melting temperature of ficin as a function of sorbitol. The T_m shifted from the control value of 72° C to 83° C in the presence of 40% sorbitol. The increment in the T_m of ficin in presence of sorbitol is concentration dependent manner. The T_m values of 75, 77 and 81° C were found in presence of 10%, 20% and 30% sorbitol, respectively. In case of trehalose the T_m of ficin was shifted to higher temperature as the concentration increases. The apparent thermal denaturation curves of ficin as a function of trehalose concentration is shown in the Fig 51B. As seen from the figure, the shift in T_m is concentration dependent and the maximum effect was seen in presence of 40% trehalose. The T_m of ficin in the presence of 10%, 20% and 30% trehalose was 75, 78 and 82° C, respectively.

Similarly, the increased thermal stability of ficin was further observed in presence of sucrose and xylitol. In Fig 52A is shown the apparent thermal denaturation curves of ficin as a function of sucrose. The maximum shift in the T_m of ficin was observed in presence of 40% sucrose. The T_m values of 74, 77, 80 and 82° C were determined from the thermal denaturation curves in presence of 10%, 20%, 30% and 40% sucrose, respectively. In case of xylitol, similar kinds of results were obtained and in Fig 52B, the apparent thermal denaturation curves in presence of different concentrations of xylitol are shown. The analysis of curves revealed that the T_m of ficin was increased in presence of different concentrations of xylitol. The T_m shifted to 81° C in presence of 40% xylitol. Even in case of xylitol also the increment in T_m is concentration dependent manner. All the above results indicate that the cosolvents used in these studies were able to increase the apparent T_m of

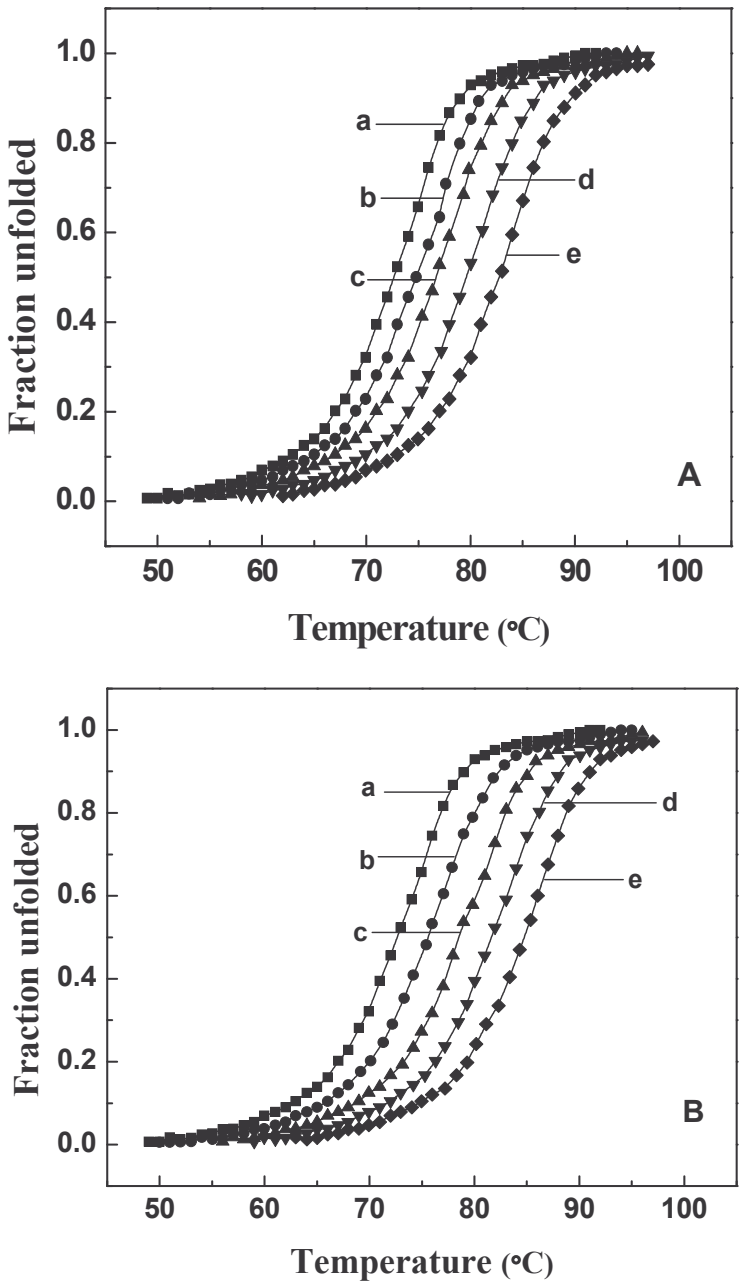

Fig 51: Apparent thermal denaturation curves of ficin in presence of different concentrations of (A) sorbitol and (B) trehalose in 0.02 M sodium phosphate buffer at pH 7.0. The change in the absorbance at 287 nm was monitored as a function of temperature. (a) Control in buffer, (b) in 10%, (c) in 20%, (d) in 30% and (e) in 40% cosolvents of (A) and (B) respectively.

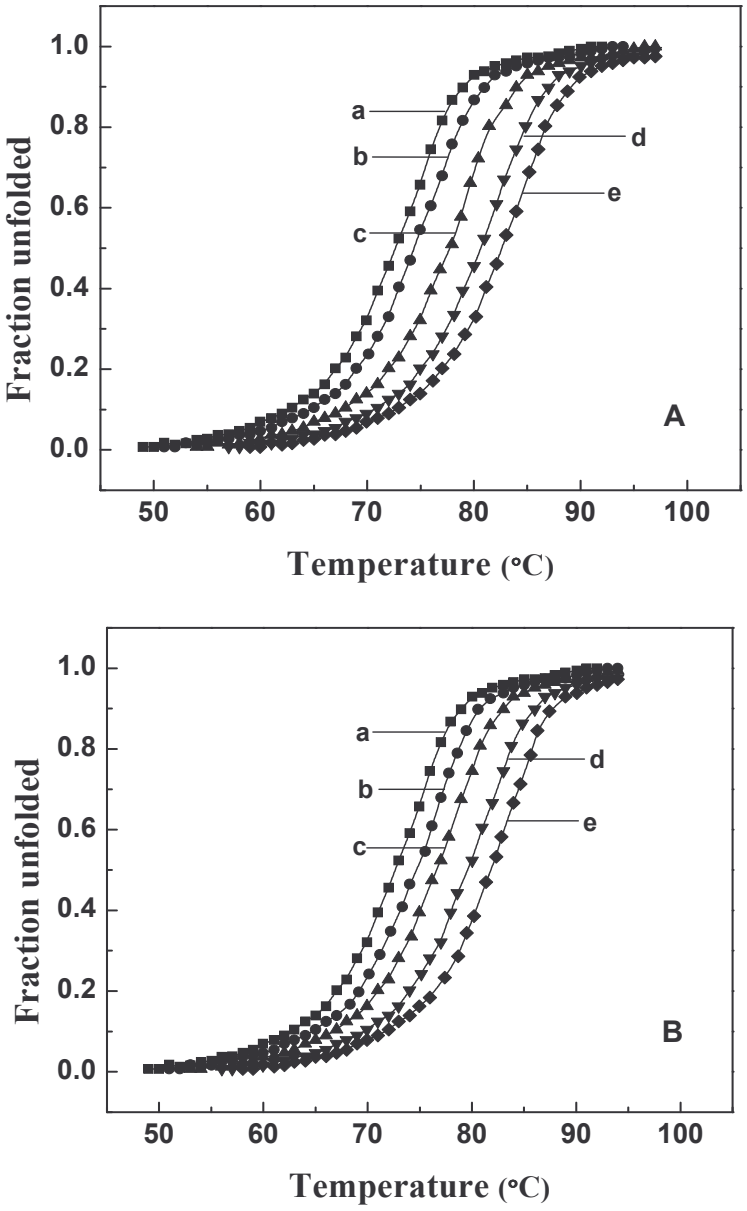

Fig 52: Apparent thermal denaturation curves of ficin in presence of different concentrations of (A) sucrose and (B) xylitol in 0.02 M sodium phosphate buffer at pH 7.0. The change in the absorbance at 287 nm was monitored as a function of temperature. (a) Control in buffer, (b) in 10%, (c) in 20%, (d) in 30% and (e) in 40% cosolvents of (A) and (B) respectively.

ficin and elevated the thermal melting temperature to different extents in a concentration dependent manner (Fig 53). The hierarchy of effectiveness of these cosolvents towards thermal stabilization of ficin is as follows:

Trehalose > Sorbitol > Sucrose > Xylitol.

The apparent T_m of the enzyme in these cosolvents as a function of cosolvent composition is summarized and listed in Table 15. These results clearly indicate that ficin is stabilized by cosolvents against thermal denaturation. The increments in the thermal melting temperature of proteins and enzymes in presence of different cosolvents have been extensively studied (Gekko and Timasheff, 1981; Rajeshwara and Prakash, 1994; Rajendran *et al*., 1995). The increased thermal stability of proteins in cosolvents is interpreted as increase in surface free energy or surface tension of water in presence of cosolvents leading to the preferential hydration of proteins (Lee and Timasheff, 1981; Timasheff and Arakawa, 1997). As surface tension of the solvent increases, the free energy required to form a cavity in the solvent to accommodate the protein molecule increases. For the unfolded state cavity would be larger than for the folded state, hence the folded state is stabilized in presence of cosolvents (Arakawa and Timasheff, 1982b). Among different cosolvents, trehalose has been considered an exceptional stabilizer and it increases thermal melting temperature of proteins to a maximum extent. The higher stabilizing efficiency has been pointed out in various studies with different bio-macromolecules even at very high temperatures (Kaushik and Bhat, 2003; Park *et al*., 2005; Zancan and Sola-Penna, 2005).

All the above results suggest that ficin is stabilized by cosolvents against thermal inactivation and the cosolvents help to retain the structural integrity of protein. The stabilizing effects of these cosolvents depend mainly on the surface properties of the protein as well as the cosolvent molecule itself. To understand the mechanism of cosolvent-mediated thermal stability

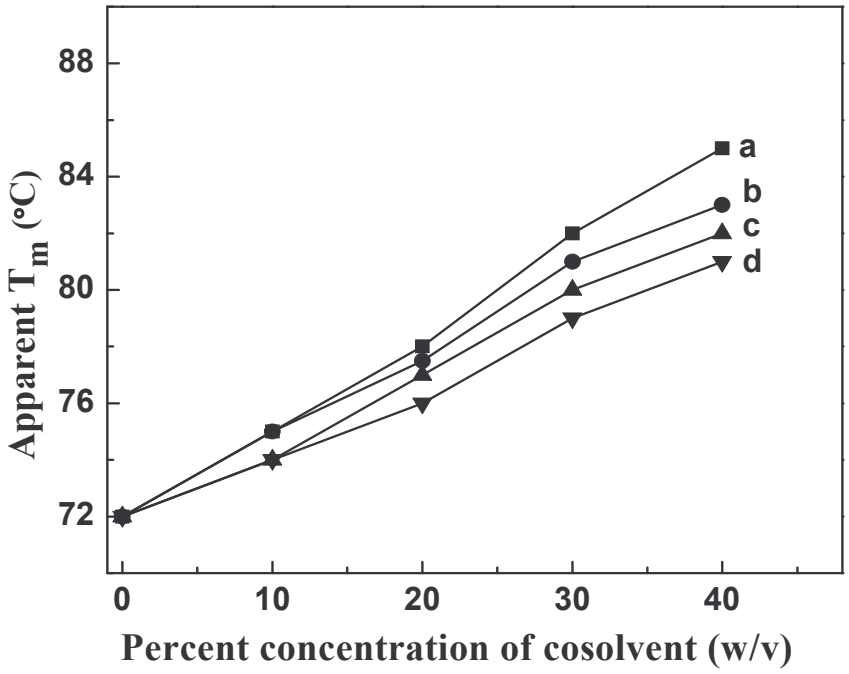

Fig 53: Apparent thermal melting temperature (T_m) of ficin as a function of cosolvents at different concentrations. In presence of (a) trehalose, (b) sorbitol, (c) sucrose and (d) xylitol.

Table 15: Apparent thermal melting temperature (T_m) of ficin in presence of different cosolvents

Cosolvent concentration (%) (w/v)	Apparent T_m (°C)			
	Trehalose	Sorbitol	Sucrose	Xylitol
Control*	72 ± 1	72 ± 1	72 ± 1	72 ± 1
10	75 ± 1	75 ± 1	74 ± 1	74 ± 1
20	78 ± 2	77 ± 1	77 ± 1	76 ± 1
30	82 ± 2	81 ± 2	80 ± 2	79 ± 2
40	85 ± 2	83 ± 2	82 ± 2	81 ± 2

**Ficin in 0.05 M sodium phosphate buffer, pH 7.0 is control.*

of ficin, the interaction of cosolvents with the enzyme was studied by measuring partial specific volume using a precession densitymetry method. The alteration in the partial specific volume is an induction of interaction of cosolvents with protein under given conditions (Mc Clemments, 2002). In a triphasic system water is considered as component one, protein as component two and cosolvent as component three according to the notation of Stockmayer (1950). The presence of third component such as cosolvent can significantly change the solvent structure which in turn affects the preferential interactions of cosolvent and partial specific volume of protein.

The partial specific volume of ficin was measured as a function of protein concentration in 0.02 M sodium phosphate buffer at pH 7.0 under both isomolal and isopotential conditions at 20° C. The isomolal value was measured after 4 hours of solvent addition and the isopotential value was measured after 36 hours of equilibration in the respective solvents. The apparent partial specific volume of 0.726 ± 0.001 mL/g and 0.727 ± 0.001 mL/g were obtained at isomolal and isopotential conditions respectively, in buffer only at pH 7.0. The representative plot of apparent partial specific volume of ficin is shown in Fig 54. These results indicate that there is no significant difference in isomolal and isopotential partial specific volumes.

The preferential interaction of solvent components with ficin as function of cosolvent concentration was determined. Typical plots of apparent partial specific volume against protein concentration in presence of different concentrations of sorbitol are represented in Fig 55. In all the concentrations, there was little or no change in the apparent partial specific volume of ficin under isomolal condition. However under isopotential condition, there was an increase in apparent partial specific volume of ficin as the concentration of sorbitol increases. The apparent partial specific volume values of 0.738, 0.745, 0.751 and 0.754 mL/g were obtained in presence of 10%, 20%, 30% and 40% sorbitol, respectively. The extent of

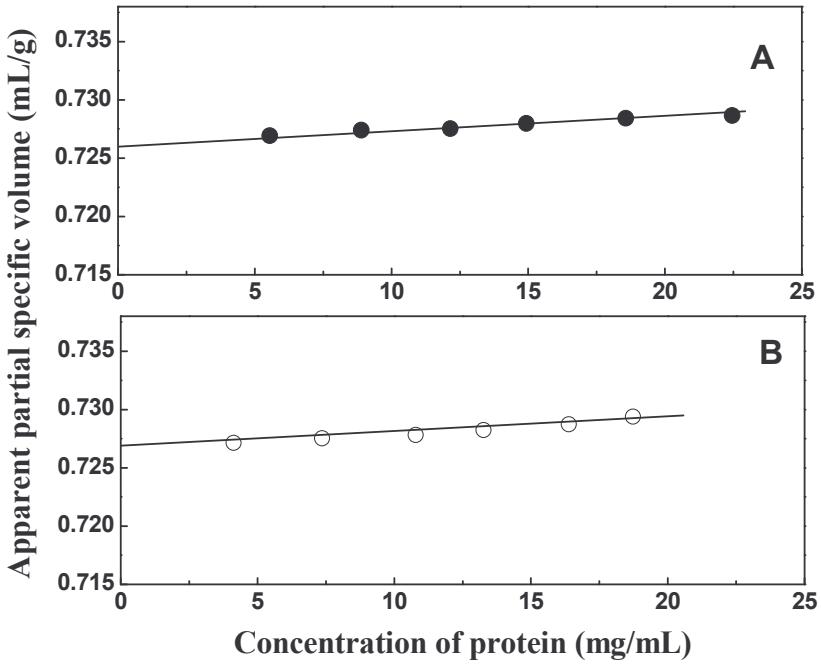

Fig 54: Apparent partial specific volume of ficin in 0.02 M sodium phosphate buffer at pH 7.0 under (A) isomolal and (B) isopotential conditions at 20° C.

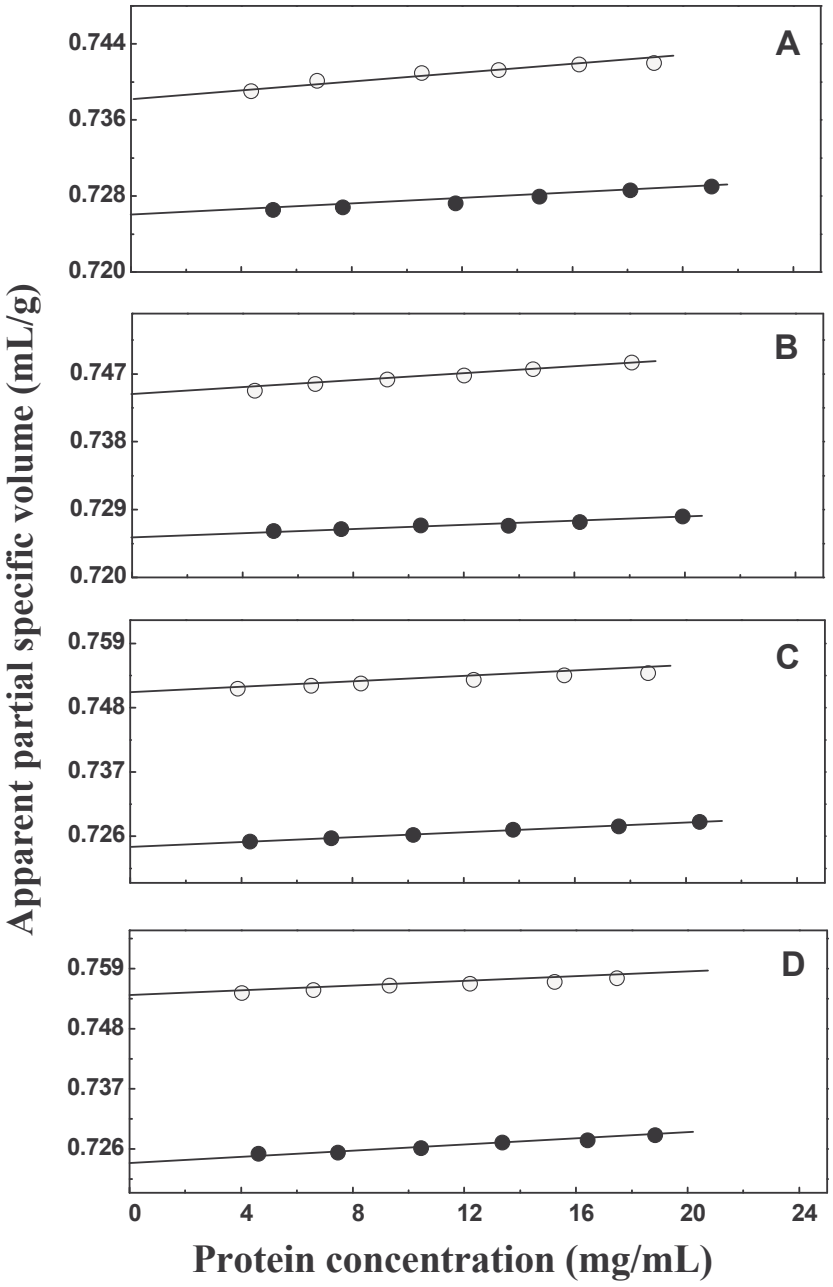

Fig 55: Apparent partial specific volume of ficin in 0.02 M sodium phosphate buffer of pH 7.0 in presence of (A) 10%, (B) 20%, (C) 30% and (D) 40% sorbitol under isomolal (●) and isopotential (○) conditions at 20 °C.

preferential interaction of the solvent components was determined by using partial specific volumes of the protein. The results of partial specific volume measurements and the calculated preferential interaction parameters are shown in Table 16. As seen from the table, the preferential interaction parameter $(\partial g_3/\partial g_2)_{T,\mu_1,\mu_3}$ was found to be negative, indicating the exclusion of sorbitol from the immediate domain of the protein.

In Fig 56, the representative plots of partial specific volume of ficin as function of trehalose concentration is shown. There was no significant change observed in the apparent partial specific volume of ficin under isomolal condition. At isopotential condition there was in increase in the apparent partial specific volume of ficin as a function of trehalose concentration. At 10%, 20%, 30% and 40% concentrations of trehalose the apparent partial specific volumes were determined to be 0.739, 0.743, 0.750 and 0.754 mL/g, respectively. Table 17 summarizes the apparent partial specific volumes and preferential interaction parameters of ficin at different concentrations of trehalose. From the table, it is clear that preferential interaction parameter was found to be negative. The negative interaction indicates the exclusion of trehalose molecules from the protein domain resulting in preferential hydration. An increase in negative interaction from -0.036 to -0.101 on g/g basis was observed as the concentration of trehalose increases from 10% to 40%.

The representative plots of apparent partial specific volume of ficin as function of sucrose concentration are shown in Fig 57. The partial specific volume of ficin in presence of sucrose under isomolal condition was not affected significantly. Under isopotential condition, an increase in the partial specific volume was observed. The apparent partial specific volume values of 0.739, 0.746, 0.753 and 0.758 mL/g were obtained at 10%, 20%, 30% and

Table 16: Apparent partial specific volume and preferential interaction parameters of ficin in presence of sorbitol at 20° C

Parameters	Concentration of Sorbitol (%) (w/v)			
	10	20	30	40
ϕ_2^o (mL/g)	0.726 ± 0.001	0.726 ± 0.002	0.725 ± 0.002	0.725 ± 0.002
$\phi_2^{o'}$ (mL/g)	0.738 ± 0.002	0.745 ± 0.002	0.751 ± 0.002	0.754 ± 0.002
g_3 (g/g)	0.107	0.230	0.374	0.544
m_3 (mol of solvent/ 1000 g H_2O)	0.59	1.26	2.05	2.99
$(\partial g_3/\partial g_2)_{T,\mu_1,\mu_3}$ (g/g)	- 0.037 ± 0.01	- 0.060 ± 0.01	-0.086 ± 0.02	- 0.104 ± 0.02
$(\partial g_1/\partial g_2)_{T,\mu_1,\mu_3}$ (g/g)	0.34 ± 0.01	0.26 ± 0.01	0.23 ± 0.01	0.19 ± 0.01
$(\partial m_3/\partial m_2)_{T,\mu_1,\mu_3}$ (mol/mol)	- 4.63 ± 0.42	- 7.59 ± 0.65	- 10.95 ± 1.01	- 13.16 ± 1.24

The control ∇_{app} values of ficin under isomolal and isopotential conditions are 0.726 ± 0.001 and 0.727 ± 0.001 mL/g, respectively in 0.02 M sodium phosphate buffer, pH 7.0.

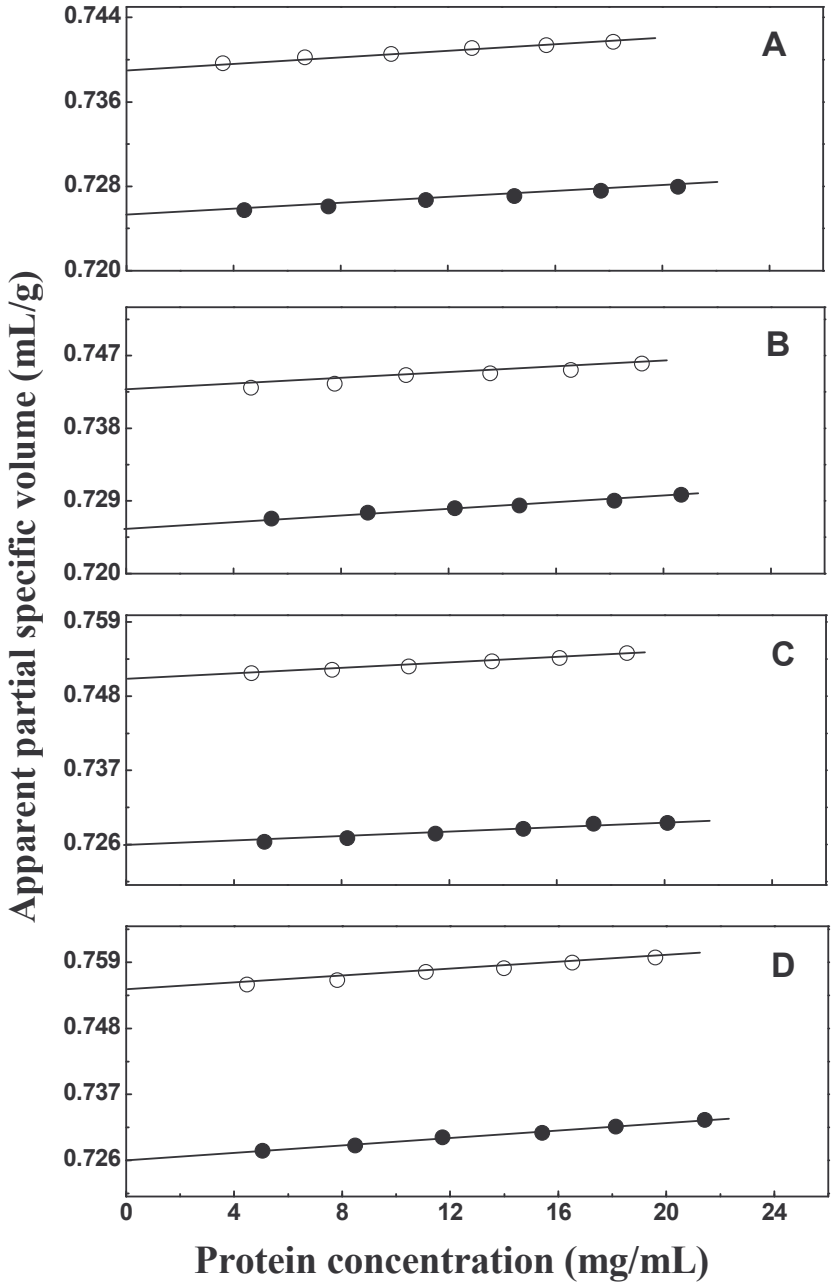

Fig 56: Apparent partial specific volume of ficin in 0.02 M sodium phosphate buffer of pH 7.0 in presence of (A) 10%, (B) 20%, (C) 30% and (D) 40% trehalose under isomolal (●) and isopotential (○) conditions at 20 °C.

Table 17: Apparent partial specific volume and preferential interaction parameters of ficin in presence of trehalose at 20° C

Parameters	Concentration of Trehalose (%) (w/v) 10	20	30	40
ϕ_2^o (mL/g)	0.725 ± 0.001	0.726 ± 0.002	0.726 ± 0.001	0.726 ± 0.002
$\phi_2'^\circ$ (mL/g)	0.739 ± 0.002	0.743 ± 0.002	0.750 ± 0.002	0.754 ± 0.002
g_3 (g/g)	0.107	0.230	0.373	0.542
m_3 (mol of solvent/ 1000 g H_2O)	0.28	0.61	0.99	1.43
$(\partial g_3/\partial g_2)_{T,\mu_1,\mu_3}$ (g/g)	- 0.036 ± 0.01	- 0.057 ± 0.01	-0.081 ± 0.02	- 0.101 ± 0.02
$(\partial g_1/\partial g_2)_{T,\mu_1,\mu_3}$ (g/g)	0.40± 0.01	0.24 ± 0.01	0.21 ± 0.01	0.18 ± 0.01
$(\partial m_3/\partial m_2)_{T,\mu_1,\mu_3}$ (mol/mol)	- 2.62 ± 0.22	- 3.32 ± 0.43	- 4.89 ± 0.86	- 6.00 ± 1.04

The control $\bar{\nabla}_{app}$ values of ficin under isomolal and isopotential conditions are 0.726 ± 0.001 and 0.727 ± 0.001 mL/g, respectively in 0.02 M sodium phosphate buffer, pH 7.0.

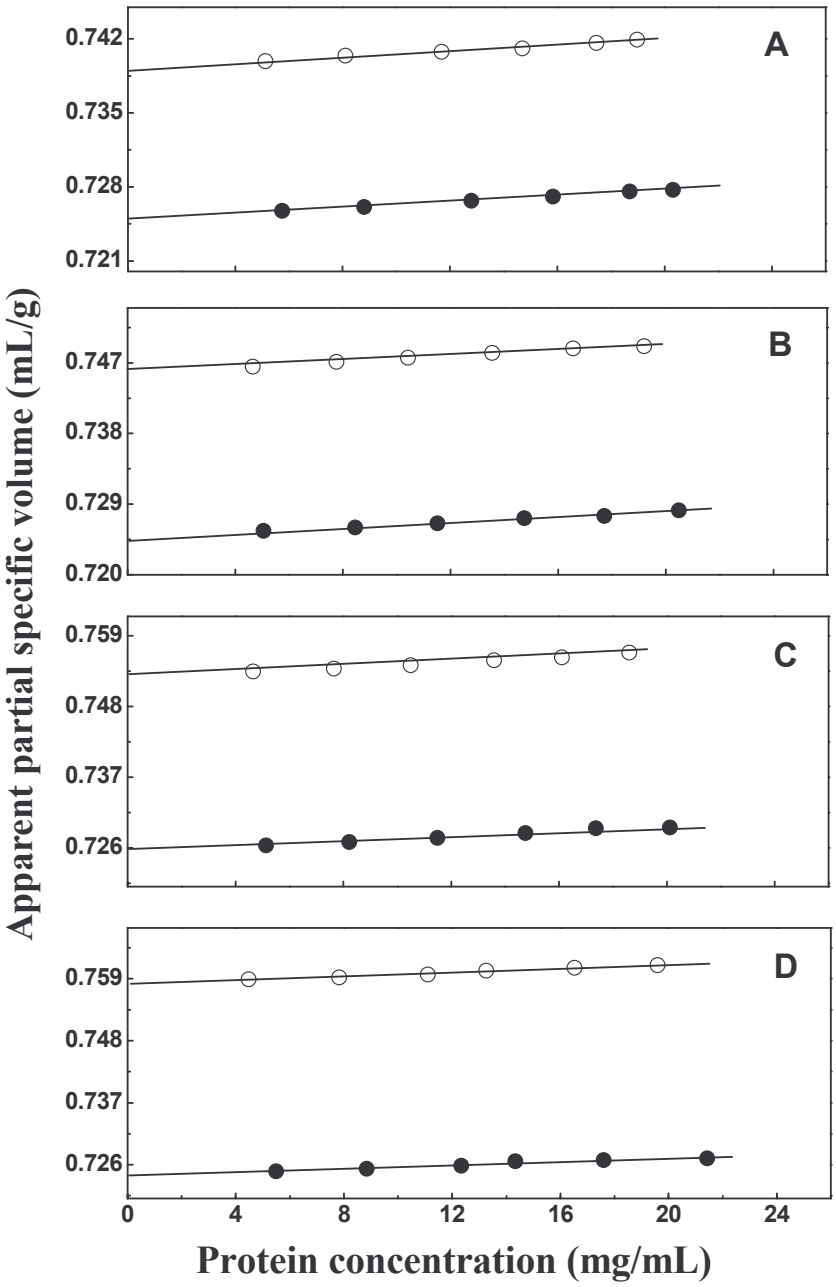

Fig 57: Apparent partial specific volume of ficin in 0.02 M sodium phosphate buffer of pH 7.0 in presence of (A) 10%, (B) 20%, (C) 30% and (D) 40% sucrose under isomolal (●) and isopotential (○) conditions at 20 °C.

40% concentration respectively. The determined preferential interaction parameters are represented in Table 18. The preferential interaction parameter in presence of different concentrations of sucrose was also found to be negative. The negative interaction indicates the exclusion of sucrose molecules from the domain of the protein. A highest value of -0.103 on g/g basis was obtained at 40% concentration.

The partial specific volume measurements of ficin as function of xylitol was further measured and the representative plots are shown in Fig 58. Even in the case of xylitol, the apparent partial specific volume of ficin increases in a concentration dependent manner under isopotential condition. At 10%, 20%, 30% and 40% concentration the determined apparent specific volumes of ficin were 0.736, 0.742, 0.748 and 0.751 mL/g, respectively. The preferential interaction parameters and partial specific volumes are summarized in Table 19. The preferential interaction parameter was found to be negative in all concentrations of xylitol indicating the preferential hydration of the protein molecule. The preferential parameter $(\partial g_3/\partial g_2)_{T,\mu_1,\mu_3}$ on g/g basis was determined to be maximum being -0.102 at 40% concentration.

All the above results of preferential interaction studies suggest that, the cosolvents used in this study are preferentially excluded from the domain of protein molecule. For all the cosolvents the preferential hydration parameter (g_3) was found to be positive and the preferential interaction parameter $(\partial g_3/\partial g_2)_{T,\mu_1,\mu_3}$ was found to be negative indicating the deficiency of cosolvent molecules in the immediate domain of the protein. The extent of this negative interaction increases in monotone fashion with increase of cosolvent concentration (Fig 59). A high value of preferential interaction parameter, being -0.104 g/g in 40% sorbitol and a low value of -0.034 g/g was observed in presence of 10% xylitol.

Table 18: Apparent partial specific volume and preferential interaction parameters of ficin in presence of sucrose at 20° C

Parameters	Concentration of Sucrose (%) (w/v) 10	20	30	40
ϕ_2^o (mL/g)	0.725 ± 0.001	0.725 ± 0.002	0.726 ± 0.002	0.724 ± 0.002
$\phi_2'^o$ (mL/g)	0.739 ± 0.002	0.746 ± 0.002	0.753 ± 0.002	0.758 ± 0.002
g_3 (g/g)	0.106	0.228	0.368	0.531
m_3 (mol of solvent/ 1000 g H_2O)	0.31	0.67	1.07	1.55
$(\partial g_3/\partial g_2)_{T,\mu_1,\mu_3}$ (g/g)	- 0.037 ± 0.01	- 0.058 ± 0.01	-0.078 ± 0.02	- 0.103 ± 0.02
$(\partial g_1/\partial g_2)_{T,\mu_1,\mu_3}$ (g/g)	0.35 ± 0.01	0.25 ± 0.01	0.21 ± 0.01	0.19 ± 0.01
$(\partial m_3/\partial m_2)_{T,\mu_1,\mu_3}$ (mol/mol)	- 2.51 ± 0.22	- 3.92 ± 0.45	- 5.25 ± 0.81	- 6.98 ± 1.04

The control $\bar{\nabla}_{app}$ values of ficin under isomolal and isopotential conditions are 0.726 ±0.001 and 0.727± 0.00 mL/g, respectively in 0.02 M sodium phosphate buffer, pH 7.0.

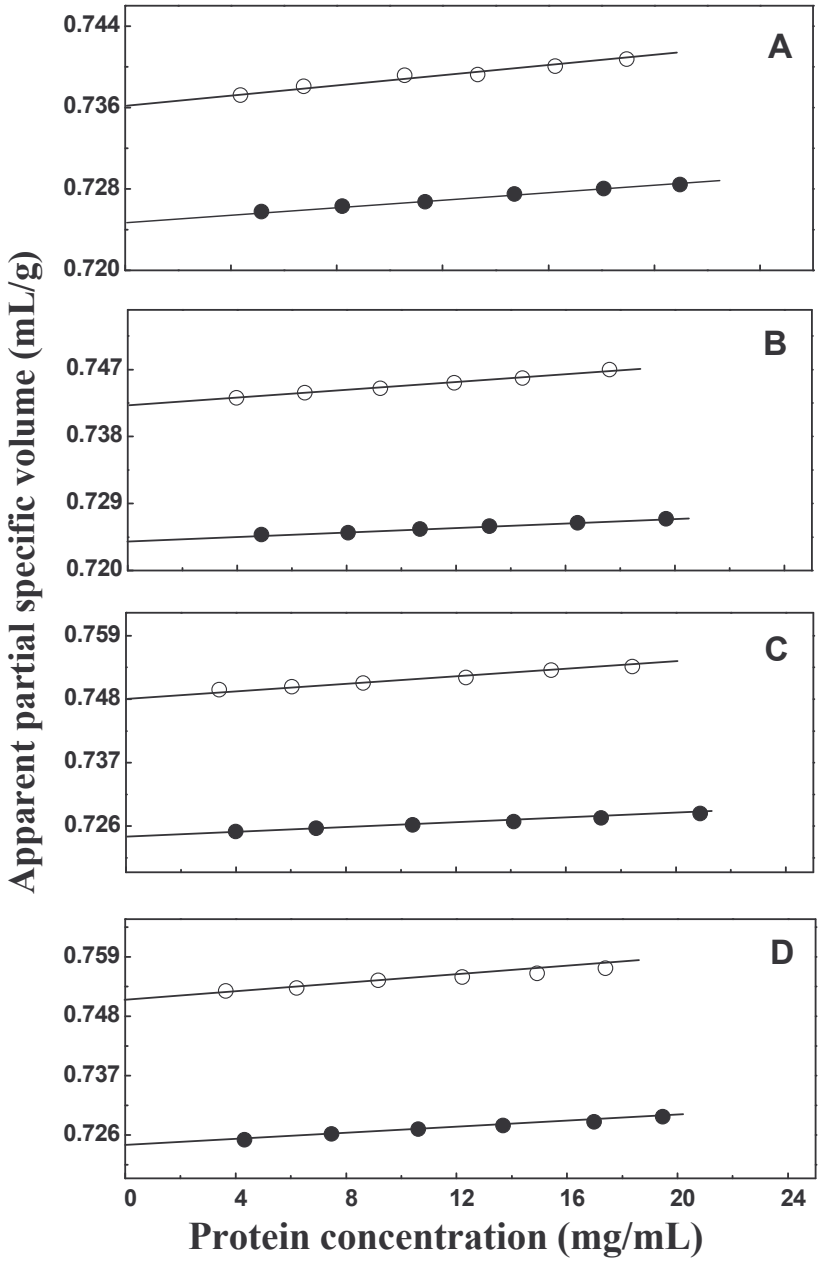

Fig 58: Apparent partial specific volume of ficin in 0.02 M sodium phosphate buffer of pH 7.0 in presence of (A) 10%, (B) 20%, (C) 30% and (D) 40% xylitol under isomolal (●) and isopotential (○) conditions at 20 °C.

Table 19: Apparent partial specific volume and preferential interaction parameters of ficin in presence of xylitol at 20° C

Parameters	Concentration of Xlitol (%) (w/v)			
	10	20	30	40
ϕ_2^o (mL/g)	0.725 ± 0.001	0.724 ± 0.002	0.724 ± 0.001	0.724 ± 0.002
$\phi_2'^o$ (mL/g)	0.736 ± 0.002	0.742 ± 0.002	0.748 ± 0.002	0.751 ± 0.002
g_3 (g/g)	0.107	0.231	0.375	0.548
m_3 (mol of solvent/ 1000 g H_2O)	0.70	1.52	2.47	3.60
$(\partial g_3/\partial g_2)_{T,\mu_1,\mu_3}$ (g/g)	- 0.034 ± 0.01	- 0.059 ± 0.01	-0.084 ± 0.02	- 0.102 ± 0.02
$(\partial g_1/\partial g_2)_{T,\mu_1,\mu_3}$ (g/g)	0.32 ± 0.01	0.26 ± 0.01	0.22 ± 0.01	0.19 ± 0.01
$(\partial m_3/\partial m_2)_{T,\mu_1,\mu_3}$ (mol/mol)	- 5.18 ± 0.62	- 8.98 ± 1.00	- 12.70 ± 1.68	- 15.42 ± 2.02

The control $\bar{\nabla}_{app}$ values of ficin under isomolal and isopotential conditions are 0.726 ± 0.001 and 0.727± 0.001 mL/g, respectively in 0.02 M sodium phosphate buffer, pH 7.0.

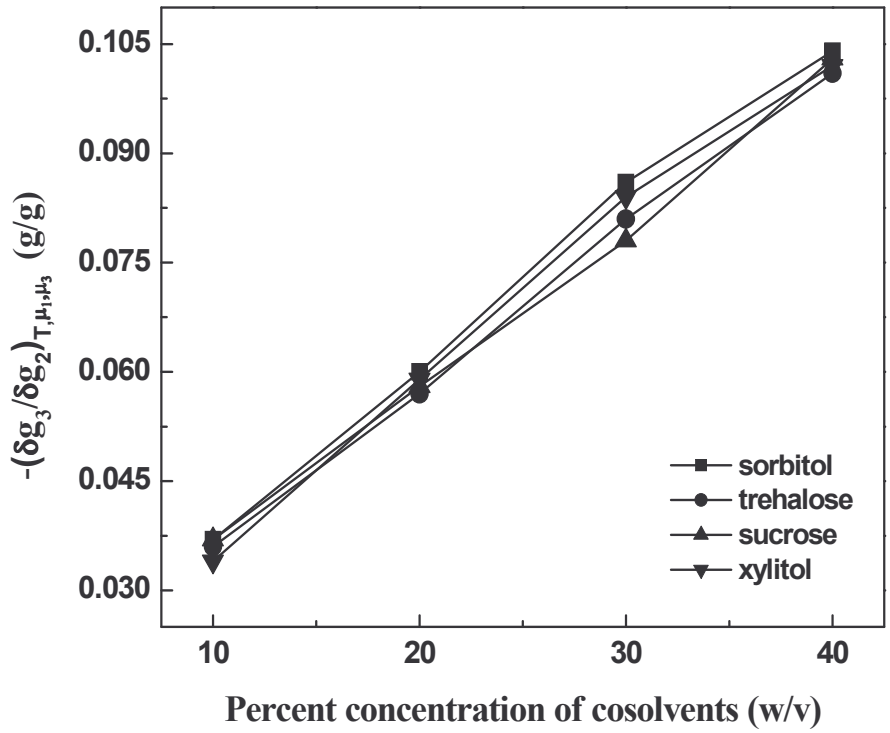

Fig 59: Effect of cosolvents on the preferential interaction parameter of ficin on g/g at different concentrations (0-40% w/v). The values were calculated from the isomolal and isopotential partial specific volume measurements.

A number of studies have showed that variety of proteins have been stabilized in different cosolvents systems (Lee and Timasheff, 1981; Gekko and Timasheff, 1981; Kita *et al*., 1 994). The stabilization effect of these additives has been attributed to their effect on water structure around the domain of protein which in turn enhances the hydrophobic interactions between the non-polar groups of the protein molecule (Back *et al*., 1979). The phenomenon of 'preferential hydration' has been claimed as responsible for a large number of cosolvent-mediated stabilization of proteins. According to this phenomenon cosolvent molecules are preferably excluded from the surface of the protein, which leads to hydration of the protein (Timasheff and Arakawa, 1997). The measurements of the preferential interactions of cosolvents such as sorbitol, trehalose, sucrose and xylitol have clearly indicated that ficin is preferentially hydrated by all cosolvents.

The addition of cosolvents into the protein solution results in a thermodynamically unfavourable condition, and the system becomes destabilized due to either a specific repulsion between the protein and cosolvents or solvent structure effect which excludes the cosolvent from the protein domain (Lee and Timasheff, 1981). This preferential exclusion of cosolvents from the surface of protein is indeed the thermodynamic source in protein stabilization. Addition of cosolvents to protein solution results an increase in the chemical potential of the protein. The degree of exclusion and increase in chemical potential are directly proportional to surface area of the protein (Lee and Timasheff, 1981; Kendrick *et al*., 1997). Since the unfolding of protein is generally accompanied by an increase in surface reactant, the preferential exclusion thermodynamically favors the sate i.e. folded state with the smallest surface area in the equilibrium between folded and unfolded states of protein. The free energy required to unfold the protein in presence of cosolvents also increases due to the increase in the surface of contact between protein and solvent on unfolding.

The stabilization of protein by variety of cosolvents can also be positively correlated to their property of increasing surface tension of water (Lin and Timasheff, 1996). Several studies have demonstrated the positive correlations between positive surface tension and protein stabilization in presence of cosolvents (Arakawa and Timasheff, 1982a, b, 1983 and Kita *et al*., 1994). The stabilizing action of cosolvents is related to its effect on the surface free energy of cavity formation. Since for unfolded protein such cavity would be larger, the free energy would increase (Lee and Timasheff, 1981), hence the folded state of protein is stabilized against unfolding in presence of cosolvents.

The results show that ficin is stabilized by addition of cosolvents such as sorbitol, trehalose, sucrose and xylitol. The activity measurements at higher temperature indicate that the presence of cosolvents prevent loss of enzyme activity against the thermal inactivation of the enzyme. The addition of cosolvents does not induce any significant structural changes in the protein molecule as evidented by structural studies using fluorescence and CD spectroscopic measurements. The thermal denaturation studies indicate that the thermal melting temperature of ficin increases to different extent depending on the nature of the cosolvent. The results obtained from the studies of preferential interaction of cosolvents with ficin show that the cosolvents are preferentially excluded from the domain of protein molecule. The preferential hydration parameter was found to be positive and the preferential interaction parameter is found to be negative in presence of all the cosolvents used. These results indicate that, the principle driving force involved in stabilization of ficin is preferential hydration as indicated by the preferential interaction measurements.

SUMMARY AND CONCLUSIONS

The elucidation of physicochemical characteristics of enzymes helps to understand the structure-function and stability relationship. The name ficin has been used variously to describe the endoproteolytic enzyme activity in tree latex of the genus *Ficus* or the sulfhydryl protease or proteases prepared from the latex of the genus *Ficus*. Hence, the term ficin must be regarded as generic. The most extensively studied ficins are cysteine proteases isolated from the latex of *Ficus glabrata*. However, the proteases (ficins) from other *Ficus* species have not been investigated in detail. In the present study, an attempt has been made to purify and study the ficins from the latex of two different species *Ficus racemosa* and *Ficus carica*.

It is well known that the study of conformational changes induced by various treatments or denaturants would be very useful in understanding the structure-function and stability relationship of proteins and enzymes. The knowledge gained in these studies helps in better understanding the forces that determine the conformation of proteins and to optimize their stabilities. The effect of pH and denaturing agents such as urea and GuHCl on the conformation of ficin has been carried out to understand the structure-stability relationship and denaturation mechanism of ficin.

The problem of stability is of great concern when proteins and enzymes are used for various industrial applications. Hence, the stability with respect to structure and biological activity needs to be considered during production, isolation and purification, storage and ultimate application of the protein product. The introduction of cosolvents such as sugars and polyhydric alcohols into the solvent medium is found to stabilize biological macromolecules in solution. The cosolvents interact with protein in a diverse way, depending on the surface and physicochemical properties of the proteins.

Brief outline of the results obtained in the present investigation are summarized below.

Chapter 1: Isolation, purification and characterization of ficins from the latex of Ficus racemosa and Ficus carica

This chapter principally deals with the characterization of ficins from *Ficus racemosa* and *Ficus carica*. The purified ficin from the latex of *Ficus racemosa* exists as single isoform. The enzyme is a single polypeptide chain protein of molecular mass of 44500 ± 500 Da. The enzyme shows maximum activity at 60 ± 0.5° C and exhibits a broad spectrum of pH optima between pH 4.5–6.5. These results indicate that this protease is distinct from other known ficins with respect to its existence as a single isoform, molecular mass and pH optimum. The enzyme activity is completely inhibited by pepstatin-A indicating that the purified enzyme is an aspartic protease. The cleavage specificity studied indicates that the protease preferably hydrolyzed peptide bonds C-terminal to glutamate, leucine and phenylalanine (at P_1 position). This aspartic protease shares a number of homology with several plant aspartic proteases with respect to pH optimum, molecular mass, inhibition by pepstatin, thermostability and its secondary structure and stability.

The ficin from *Ficus carica* was purified to homogeneity from the commercial crude proteinase mixture preparation. The purified ficin is a single polypeptide chain protein of molecular mass of 23100 ± 300 Da. The enzyme is active in the pH range of 6.5 - 8.5 and shows maximum activity at pH 7.0. The ficin activity is completely inhibited in presence of cysteine group specific inhibitors which clearly confirms the participation of cysteine residue at the active site of the enzyme. The purified ficin contains three disulfide bonds and a single free cysteine residue. The N-terminal sequence has homology with plant proteinases of cysteine proteases family. The determined analytical observations reveal that the enzyme shares several homologies with many other plant cysteine proteases. The enzyme activity of ficin from *Ficus carica* is much higher as compared to the activity of ficin from *Ficus racemosa* and holds greater potential for its study in depth.

Therefore, the ficin isolated from *Ficus carica* 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 structure and stability of ficin from Ficus carica

This chapter describes the effect of pH, urea and GuHCl on the activity, structure and stability of ficin. The pH-induced denaturation of ficin was followed over a range of pH 1.0 to 7.0. The pH-induced transition of ficin is found to follow a simple cooperative two-state transition as studied by far-UV CD measurements at 222 nm. The denaturation process of ficin is reversible in the pH range of 2.4 – 7.0 and irreversible inactivation of the enzyme occurs when the pH is decreased further below 2.4. The maximum stability for structure as well as activity of ficin has been observed at pH 7.0. The results demonstrate that the pH-induced denaturation of ficin leads to the formation of a partially folded conformation at low pH with the characteristic features of a molten globule.

Ficin molecule is resistant to unfolding by urea under neutral conditions as observed by florescence and far-UV CD studies. The enzyme is susceptible to urea unfolding at pH 3.0 and the changes are reversible. The urea unfolding transition of ficin is cooperative and the maximum structural changes occur between 2 to 4 M urea with a transition mid point of 2.5 ± 0.1 M. The results indicate that urea-induced unfolding of ficin at pH 3.0 follows a simple two-state transition. In order to further characterize such an unfolding process, the effect of GuHCl on the structure, function and stability of ficin has been followed under neutral conditions. The GuHCl-induced equilibrium unfolding of ficin indicates that the transition is sigmoidal in nature and follows simple two-state transition without formation of any intermediate state. The results obtained from folding and unfolding studies show a differential structural stability of ficin towards denaturation by urea and GuHCl.

Chapter 3: Effect of cosolvents on structure and stability of ficin from Ficus carica

In this chapter, the effect of cosolvents such as sorbitol, trehalose, sucrose and xylitol on the structure and stability of ficin was studied. Ficin looses 37% of its activity at 70° C after 10 min incubation and in presence of cosolvents the activity is protected up to different extent. The apparent melting temperature (T_m) of ficin is 72.0 ± 1.0° C. All the cosolvents are able to elevate the thermal melting temperature to different extents in a concentration dependent manner. The effect of cosolvents on the structural integrity of ficin was examined by fluorescence and circular dichroic spectral measurements. Fluorescence and far-UV CD spectra of ficin revealed that no gross conformational changes of the protein molecule occur in presence of cosolvents.

In order to understand the mechanism of cosolvent-mediated thermal stabilization of ficin, the partial specific volume and preferential interaction parameters were determined in presence of cosolvents. The apparent partial specific volume of ficin under isomolal and isopotential conditions is 0.726 ± 0.001 mL/g and 0.727 ± 0.001 mL/g, respectively, in buffer only at pH 7.0. The determined preferential interaction parameters showed that, for all the cosolvents the preferential hydration parameter is found to be positive and the preferential interaction parameter $(\partial g_3/\partial g_2)_{T,\mu_1,\mu_3}$ (g/g) is found to be negative indicating the deficiency of cosolvent molecules in the immediate domain of the protein. A high value of preferential interaction parameter, being -0.104 ± 0.2 g/g in 40% sorbitol and a low value of -0.034 ± 0.01 g/g is observed in presence of 10% xylitol. These results show that the principle driving force involved in stabilization of ficin is preferential hydration. Thus, the effect of cosolvent on stability of ficin depends on nature and concentration of cosolvents as well as the solution conditions under which these are added in protection to the enzyme.

REFERENCES

Anfinsen, C.B. (1973) Principles that govern the folding of protein chains. *Science*, **181**, 223-230.

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

Antão, C.M. and Malcata, F.X. (2005) Plant serine proteases: biochemical, physiological and molecular features. *Plant Physiol. Biochem.,* **43**, 637-650.

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

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

Arakawa, T. and Timasheff, S.N. (1982b) Preferential interaction of proteins with salts in concentrated solutions. *Biochemistry*, **21**, 6545-6552.

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

Arakawa, T. and Timasheff, S.N. (1985a) Theory of protein solubility. *Methods Enzymol.*, **114**, 49-77.

Arakawa, T. and Timasheff, S.N. (1985b) Mechanism of poly(ethylene glycol) interaction with proteins. *Biochemistry,* **24**, 6756-6762.

Arima, K., Uchikoba, T., Yonezewa, H., Shimada, M. and Kaneda, M. (2000) Cucumisin-like protease from the latex of *Euphorbia supina*. *Phytochemistry,* **53**, 639-644.

Aune, K.C. and Tanford, C. (1969) Thermodynamics of the denaturation of lysozyme by guanidine hydrochloride. I. Dependence of pH at 25° C. *Biochemistry*, **8**, 4586-4590.

Auton, M. and Bolen, W.D. (2005) Predicting the energetics of osmolyte-induced protein folding/unfolding. *Proc. Natl. Acad. Sci. U.S.A.,* **102** (42), 15065-15068.

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

Barrett, A.J. (1994) Proteolytic enzymes: serine and cysteine peptidases. *Methods Enzymol.*, **244**, 1-15.

Barrett, A.J. (1995) Proteolytic enzymes: aspartic and metallopeptidases. *Methods Enzymol.*, **248**, 183-242.

Barrett, A.J., Rawlings, N.D. and Woessner, J.F. (1998) *Handbook of Proteolytic Enzymes*. Academic Press, San Diego, California.

Belozersky, M.A., Sarbakanova, S.T. and Dunaevsky, Y.E. (1989) Aspartic proteinase from wheat seeds: Isolation, properties and action on gliadin. *Planta,* **177**, 321–326.

Bendicho, S., Marti, G., Hernandez, T. and Martin, O. (2002) Determination of proteolytic activity in different milk systems. *Food Chem.,* **79**, 245-249.

Berger, D. and Altmann, T. (2000) A subtilin like serine protease involved in the regulation of stomatal density and distribution in *Arabidopsis thaliana*. *Gene Dev.,* **14**, 1119-1131.

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.

Blundell, T.L., Cooper, J.B., Sali, A. and Zhu, Z. (1991) Comparisons of the sequences, 3-D structures and mechanisms of pepsin-like and retroviral aspartic proteinases. *Adv. Exp. Med. Boil.*, **306**, 443-453.

Boller, T. Roles of proteolytic enzymes in interaction of plants with other organisms. In: *Plant proteolytic enzymes*; Dalling, M.J., Ed.: Vol.1. CRC Press, Boca Raton, FL, 1986, pp. 67-96.

Bradbury, S.L. and Jakoby, W.B. (1972) Glycerol as an enzyme stabilizing agent: Effect on alcohol dehydrogenase. *Proc. Natl. Acad. Sci. U.S.A.*, **69**, 2373-2376.

Bradford, M.M. (1976) A rapid and sensitive method for the quantification of microgram quantities of protein utilizing the principle of protein-dye binding. *Anal. Biochem.,* **72**, 248-254.

Brandy, C. J. (1985) Fruit ripening. *Annu. Rev. Plant Physiol.,* **38**, 155-178.

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

Buttle, D.J. Ficain. In: *Handbook of Proteolytic Enzymes,* 2nd edn (Barrett, A.S, Rawlings, N.D. and Woessner, F.J., Eds.; Vol. 2, Elsevier London, 2004, pp. 1141-1142.

Caffini, N.O., Lopez, L.M.I., Natalucci, C.L. and Priolo, N.S. (1988) Proteases of higher plants. General features, physiological roles and applications. *Acta. Farm. Bonaerense,* **7**, 195-313.

Carra, J.H., Elizabeth, A.A. and Privalov, P.L. (1994) Three-state thermodynamic analysis of the denaturation of staphylococcal nuclease mutants. *Biochemistry,* **33**, 10842-10850.

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 multicomponent solutions. *Adv.Protein Chem.*, **19**, 287 - 395.

Chapman, H.A., Riese, J.R. and Shi, G.P. (1997) Emerging roles for cysteine proteases in human biology. *Annu. Rev.Physiol.*, **59**, 63–88.

Collins, K.D. (2004) Ions from the Hofmeister series and osmolytes: Effects on proteins in solution and in the crystallization process. *Methods,* **34**, 300–311.

Colón, W. and Roder, H. (1996) Kinetic intermediates in the formation of the cytochrome c molten globule. *Nat. Stuct. Biol.,* **3**, 1019-1025.

D'Hondt, K., Bosch, D., Van Damme, J., Goethals, M. Vandekerckhove, J. and Krebbers, E. (1993) An aspartic protease present in seeds cleaves Arabidopsis 2S albumin precursor *in vitro*. *J. Biol. Chem.,* **268**, 20884-20891.

Davie, E.W. and Fujikawa, K. (1975) Basic mechanism in blood coagulation. *Annu. Rev. Biochem.,* **44**, 799-829.

Davies, D.R. (1990) The structure and function of the aspartic proteinases. *Annu. Rev. Biophys. Chem.,* **19**, 189-215.

Devaraj, K.B., Gowda, L.R. and Prakash, V. (2008a) An unusual thermostable aspartic protease from the latex of *Ficus racemosa* (L.). *Phytochemistry,* **69**, 647-655.

Devaraj, K.B., Kumar, P.R. and Prakash, V. (2008b) Purification, characterization and solvent-induces thermal stabilization of ficin from *Ficus carica*. *J. Agric. Food Chem.,* **56**, 11417-11423.

Devaraj, K.B., Kumar, P.R. and Prakash, V. (2009) Characterization of acid-induced molten globule like folding intermediate state of ficin. *Int. J. Biol. Macromol.,* **45**, 248-254.

DiPaola, G. and Belleau, B (1978) Polyol–protein interactions. Thermodynamical evidence for a selective solvation of glycerol and hexitols by aqueous β-lactoglobulin. *Can. J. Chem.,* 56, 848–850.

Domingos, A., Cardoso, P.C., Xue, Z., Clemente, A., Brodelius, P.E. and Pais, M.S. (2000) Purification, cloning and autoproteolytic processing of an aspartic proteinase from *Centaur calcitrapa*. *Eur. J Biochem.,* **267**, 6824-6831.

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.

Dunn, B.M. (1992) *Structure and function of the aspartic proteinases.* (Dunn, B.M. Ed.) Plenum Press, New York, U.S.A.

Edwin, F. and Jagannadham, M.V. (1998) Sequential unfolding of papain in molten globule state. *Biochem. Biophys. Res. Commun.*, **252**, 654-660.

Eftink, M.R. and Ghiron, C.A. (1981) Fluorescence quenching studies with proteins. *Anal. Biochem.,* **114**, 199-227.

Eftink, M.R. and Selvidge, L.A. (1982) Fluorescence quenching of liver alcohol dehydrogenase by acrylamide. *Biochemistry,* **21**, 117-125.

El Moussaoui A., Nijs, M., Paul, C., Wintjens, R., Vincentelli, J., Azarkan, M. and Looze, Y. (2001) Revisiting the enzymes stored in the laticifers of *Carica papaya* in the context of their possible participation in the plant defence mechanism. *Cell Mol. Life Sci.,* **58**, 556–570.

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

Elpidina, E.N., Dunaevsky, Y.E. and Belozersky, M.A. (1990) Protein bodies from buckwheat seed cotyledonds: isolation and characterization. *J. Exp. Bot.*, **41**, 969-977.

Englund, P.T., King, T.P., Craig, L.C. and Walti, A. (1968) Studies on ficin I. Its isolation and characterization. *Biochemistry,* **7**, 163-175.

Fastrez, J. and Fersht A.R. (1973) Demonstration of the acyl-enzyme mechanism for the hydrolysis of peptides and anilides by chymotrypsin. *Biochemistry,* **12**, 2025-2034.

Felicioli, R., Garzelli, B., Vaccari, L., Melfi, D. and Balestreri, E. (1997) Activity staining of protein inhibitors of proteases on gelatin-containing polyacrylamide gel electrophoresis. *Anal. Biochem.*, **244**, 176-179.

Fersht, A.R., Matouschek, A. and Serrano, L. (1992) The folding of an enzyme. I. Theory of protein engineering analysis of stability and pathway of protein folding. *J. Mol. Biol.,* **224**, 771-782.

Finer, E.G., Franks, F. and Tait, M.J. (1972) Nuclear magnetic resonance studies of aqueous urea solutions. *J. Am. Chem. Soc.*, **94** (13), 4424-4429.

Fink, A.L. (2005) Natively unfolded proteins. *Curr. Opin. Struct. Biol.*, **15**, 35-41.

Fink, A.L., Calciano, L.J., Goto, Y., Kurotsu, T. and Palleros, D.R. (1994) Classification of acid denaturation of proteins: Intermediates and unfolded states. *Biochemistry*, **33**, 12504-12511.

Frazao, C., Bento, I., Costa, J., Soares, C.M., Verissimo, P., Faro,C., Pires, E., Cooper, J. and Carrondo, M.A. (1999) Crystal structure of cardosin A, a glycosylated and Arg-Gly-Asp-containing aspartic proteinase from the flowers *of Cynara cardunculus* L. *J. Biol. Chem.,* **274**, 27694–27701.

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

Fruton, J.S. (1976) Mechanism of the catalytic action of pepsin and related acidic proteases. *Adv. Enzymol.,* **44**, 1-36.

Gekko, K. and Morikawa, T. (1981a) Preferential hydration of bovine serum albumin in polyhydric alcohol-water mixtures. *J. Biochem.,* **90**, 39-50.

Gekko, K. and Morikawa, T. (1981b) Thermodynamics of polyol-induced thermal stabilization of chymotrypsinogen. *J. Biochem.,* **90**, 51-60.

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

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

Glazer, A.N. and Smith, E.L. (1971) Papain and other plant sulfhydryl proteolytic enzymes. In: *The Enzymes* (Boyer, P.D., Ed.), Academic Press, New York, pp. 501-546.

Goto, Y., Takahashi, N. and Fink, A.L. (1990a) Mechanism of acid-induced folding of proteins. *Biochemistry*, **29**, 3480-3488.

Goto. Y., Calciano, L.J. and Fink, A.L. (1990b) Acid-induced folding of proteins. *Proc. Natl. Acad. Sci. U. S. A.*, **87**, 573-577.

Greene, R.F and Pace C.N. (1974) Urea and guanidine hydrochloride denaturation of ribonuclease, lysozyme, α-chymotrypsin and β-lactoglobulin. *J. Biol. Chem.*, **249**, 5388-5393.

Grzelakowska-Sztaber, B. (1998) Molecular mechanisms of apoptosis induced by activation of membrane receptors from the TNF-R superfamily. *Postepy. Biochem.*, **44**, 8–21.

Gunasekhar, P.M. and Prakash, V. (2008) Interaction of selected cosolvents with bovine α-lactalbumin. *Int. J. Biol. Macro. Molec.*, **42** (4), 348-355.

Habeeb, A.F.S.A. (1972) Reaction of protein sulfhydryl groups with Ellman's reagent. *Methods Enzymol.*, **25**, 457-464.

Halfman, C.J. and Nishida, T. (1971) Nature of the alteration of the fluorescence spectrum of bovine serum albumin produced by the binding of dodecyl sulfate. *Bichem. Biophys. Acta.*, **243** (2), 294-303.

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

Hartley, B.S. (1960) Proteolytic enzymes. *Annu. Rev. Biochem.*, **29**, 45-72.

Hedwing, G.R., Lilley, T.H. and Linsdell, H. (1991) Calorimetric and volumetric studies of the interactions of some amides in water and in 6 mol dm^{-3} aqueous guanidinium chloride. *J. Chem. Soc. Faraday Trans.*, **87**, 2975-2982.

Hibbs, M.S., Hasty, K.A., Seyer, J.M. Kang, A.H. and Mainardi, C.L. (1985) biochemical and immunological characterization of the secreted forms of human neutrophil gelatinase. *J. Biol. Chem.*, **260**, 2493-2500.

Hill, R.L., Schwartz, H.C. and Smith, E.L. (1959) The effect of urea and GuHCl on activity and optical rotation of crystalline papain. *J. Biol. Chem.*, **234**, 572-575.

Hoccart, X. and Turrell, G. (1993) Raman spectroscopic investigation of the dynamics of urea-water complexes. *J. Chem. Phys.*, **99** (11), 8498-8503.

Husain, S.S. and Lowe, G. (1968) Evidence for histidine in the active sites of ficin and stem-bromelain. *Biochem. J.*, **110**, 53-57.

International Union of Biochemistry. (1992) *Enzyme nomenclature*. Academic Press, Inc., Orlando, Fla.

Jaenicke, L. (1974) A rapid micromethod for the determination of nitrogen and phosphate in the biological material. *Anal. Biochem.*, **61**, 623-627.

Jaenicke, R. (1991) Protein stability and molecular adaptation to extreme conditions. *Eur. J. Biochem.*, **202**, 715-728.

Janeček, Š. (1993) Strategies for obtaining stable enzymes. *Process Biochem.*, **28**, 435-445.

Jones, I.K. and Glazer, A.N. (1970) Comparative studies on four sulfhydryl endopeptidases (Ficins) of *Ficus glabrata* latex. *J. Biol. Chem.*, **245**, 2765-2772.

Juneja, J and Udgaonkar, J.B. (2003) NMR studies of protein folding. *Current Sci.*, **84**, 157-172.

Kaneda, M. and Tominaga, N. (1975) Isolation and characterization of a proteasefrom the sarcocarp of melon fruit. *J. Biochem. (Tokyo)*, **78**, 1287–1296.

Kaneda, M., Yonezawa, H. and Uchikoba, T. (1997) Purification and some properties of a protease from the sarcocarp of musk melon fruit. *Biosci. Biotech. Biochem.,* **61**, 2100-2102.

Kaushik, J. K. and Bhat, R. (2003) Why is trehalose an exceptional protein stabilizer? *J. Biol. Chem*., **278**, 26458-26465.

Kay, M.S. and Baldwin, R.L. (1996) Packing interactions in the apomyglobin folding intermediate. *Nat. Struct. Biol.,* **3**, 439-445.

Kembhavi, A.A., Buttle, D.J., Knight, C.G. and Barrett, A.J. (1993) The two cysteine endopeptidases of legume seeds: purification and characterization by use of specific fluorometric assay. *Arch. Biochem. Biophys.,* **303**, 208-213.

Kendrick, B.S., Chang, B.S., Arakawa, T., Peterson, B., Randolph, T.W., Manning, M.C. and Carpenter, J.F. (1997) Preferential exclusion of sucrose from the recombinant interleukin-1 receptor antagonist: Role in restricted conformational mobility and compaction of native state. *Proc. Natl. Acad. Sci. U.S.A.,* **94**, 11917– 11922.

Kervinen, J., Tobin, G.J., Costa, J., Waugh, D.S., Wlodawer, A. and Zdanov, A. (1999) Crystal structure of plant aspartic proteinase prophytepsin: inactivation and vacuolar targeting. *EMBO J.*, **18**, 3947–3955.

Kim, K., Baek, S.H, Hong, Y., Kang, M., Ha, D.B., Goldberg, A.L. and Chung, C.H. (1995) Purification and Characterization of Protease Ci, a Cytoplasmic Metalloendoprotease in *Escherichia coli*. J. Biol. Chem.,* **270**, 29799-29805.

Kim, P.S. and Baldwin, R.L. (1990) Intermediates in the folding reactions of small proteins. *Annu. Rev. Biochem.,* **59**, 631-660.

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

Klimov, D.K., Straub, J.E. and Thirumalai, D. (2004) Aqueous urea solution destabilizes Abeta (16-22) oligomers. *Proc. Natl. Acad. Sci. U.S.A.,* **101** (41), 14760-14765.

Konno, K., Hirayama, C., Nakamura, M., Tateishi, K., Tamura, Y., Hattori, M. And Kohno, K. (2004) Papain protects papaya trees from herbivorous insects: role of cysteine proteases in latex. *Plant J.,* **37**, 370–378.

Kortt, A.A., Hamilton, S., Webb, E.C. and Zerner, B. (1974) Ficins (EC 3.4.22.3). Purification and characterization of the enzymatic components of the latex of *Ficus glabrata*. *Biochemistry*, **13**, 2023-2028.

Kramer, D.E. and Whitaker, J. R. (1964) Ficus enzymes II. Properties of the proteolytic enzymes from the latex of *Ficus carica* variety kodata. *J. Biol. Chem.*, **239**, 2178-2183.

Kristjansson, M.M. and Kinsella, J.E. (1991) Protein and enzyme stability: structural, thermodynamic and experimental aspects. *Adv. Food Nutr. Res.,* **35**, 237-316.

Kumar, S.S.R., Singh, S.A. and Rao, A.G.A (2009) Conformational stability of α-amylase from malted sorghum (*Sorghum bicolor*): Reversible unfolding by denaturants. *Biochimie*, **91,** 548-557.

Kunitz, M. (1947) Crystalline soybean trypsin inhibitor: General properties. *J. Gen. Physiol.*, **30**, 291-310.

Kuwajima, K. and Arai, M. The molten globule state: the physical and biological significance. In: *Mechanism of protein folding*; Pain, R.H., Ed.; Oxford University Press, New York, 2000, pp. 138-174.

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

Lee, J.C. and Lee, L.L.Y. (1981) Preferential solvent interaction between proteins and polyethylene glycols. *J. Biol. Chem.,* **256**, 625-631.

Lee, J.C. and Timasheff, S.N. (1974) The calculation of partial specific volumes of proteins in guanidine hydrochloride. *Arch. Biochem. Biophys.*, **165**, 268-273.

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, K.L., Albee, K.L., Bernasconi, J. and Edmundus, Y. (1997) Complete amino acid sequence of ananain and a comparison with stem bromelain and other plant cysteine proteases. *Biochem. J.*, **337**, 199-202.

Leisola, M., Jokela, J., Pastinen, O., Turunen, O. and Schoemaker, H. (2001) *Industrial use of enzymes.* Eolas Publisher, Oxford.

Leung-Toung, R., Li, W., Tam, T.F. and Karimian, K. (2002) Thioldependent enzymes and their inhibitors: a review. *Curr. Med. Chem.*, **9**, 979-1002.

Liener, I.E. and Friedenson, B. (1970) Ficin. *Methods Enzymol.*, **19**, 261-273.

Liener, I.E. and Friedenson, B. (1972) The active site sequence of multiple forms of ficin. *Arch. Biochem. Biophys.*, **149**, 169-174.

Lin, T.Y. and Timasheff, S.N. (1996) On the role of surface tension in the stabilization of globular proteins. *Protein Sci.*, **5**, 372-381.

Lowe, G. and Williams, A. (1965) Direct evidence for an acylated thiol as an intermediate in papain- and ficin-catalyzed hydrolyses. *Biochem. J.,* **96**, 189-193.

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.

Lynn, K.R. (1979) Purification and some properties of two proteases from papaya latex. *Biochem. Biophys. Acta.,* **569**, 193-201.

Lynn, K.R. and Clevette-Radford, N.A. (1986) Ficin-E, a serine centered protease from *Ficus elastica*. *Phytochemistry,* **25**, 1559-1561.

Ma, Y.Z. and Tsou, C.L. (1991) Comparison of the activity and conformational changes of lactate dehydrogenase H_4 during denaturation by guanidinium chloride. *Bichem. J.,* **277**, 207-211.

Marini-Bettolo, G.M., Angeletti, P.U., Salvi, M.L., Tentori, L. and Vivaldi, G. (1963) Ficin I. *Gazz. Chim. Ital.,* **93**, 1239-1251.

Matlu, A. and Gal, S. (1999) Plant aspartic proteinases: enzymes on the way to a function. *Physiol. Plantarum.*, **105**, 569-576.

Matsudaira, P. (1987) Sequence from picomole quantities of proteins electroblotted onto polyvinylidene difluoride membranes, *J. Biol. Chem.,* **262**, 10035-10038.

Matthews, C.R. (1993) Pathways of protein folding. *Annu. Rev. Biochem.,* **62**, 653-683.

Mc Clemments, D.J. (2002). Modulation of globular protein functionality by weakly interacting cosolvents. *CRC Crit. Rev. Food Sci. Nutr.,* **42**, 417–471.

McGrath, M.E. (1999) The lysosomal cysteine proteases. *Annu. Rev. Biophys. Biomol. Struct*., **28**, 181–204.

Metrione, R.M., Johnson, R.B. and Seng, R. (1967) Purification, partial characterization, and sequence around a reactive sulfhydryl of ficin. *Arch. Biochem. Biophys.,* **122**, 137-143.

Meyers, J.S. and Jakoby, W.B. (1975) Glycerol as an agent eliciting small conformational changes in alcohol dehydrogenase *J. Biol. Chem.,* **250**, 3785-3789.

Mok, K.H., Nagashima, T., Day, I.J., Hore, P.J. and Dobson, C.M. (2005) Multiple subsets of side chain packing in partially folded states of α-lactalbumins. *Proc. Natl. Acad. Sci. U. S. A.,* **102**, 8899-8904.

Monteiro, P.V. and Prakash, V. (1994) Effect of proteases on arachin, conarachin I and conarachin II from the peanut (*Arachis hypogaea* L). *J. Agric. Food Chem.,* **42**, 268-273.

Moqlich, A., Krieger, F. and Kiefhaber, T. (2005) Molecular basis for the effect of urea and guanidinium chloride on the dynamics of unfolded polypeptide chains. *J. Mol. Biol.,* **345** (1), 153-162.

Mountain R.D. and Thirumalai, D. (2003) Molecular dynamics simulations of end-to-end contact formation in hydrocarbon chains in water and aqueous urea solution. *J. Am. Chem. Soc.,* **125** (7), 1950-1957.

Mozhaev, V.V. and Martinek, K. (1982) Inactivation and reactivation of proteins (enzymes). *Enzyme Microb. Technol.*, **4**, 299-309.

Nakamura, K and Soejima, M. (1970) The effect of urea and guanidine hydrochloride on activity of papain. *Agric. Biol. Chem.,* **34**, 489-479.

Naseem, F., Ahmad, B., Ashraf, M.T. and Khan, R.H. (2004) Characterization of molten globule state of fetuin at low pH. *Biochim. Biophys. Acta.,* **1699**, 191-199.

Neurath, H. (1984) Evolution of proteolytic enzymes. *Science,* **224**, 350-357.

O'Brien, E.P., Dima, R.I., Brooks, B. and Thirumalai, D. (2007) Interactions between hydrophobic and ionic solutes in aqueous guanidinium chloride and urea solutions: lessons for protein denaturation mechanism. *J. Am. Chem. Soc.*, **129** (23), 7346-7353.

Ohgushi, M. and Wada, A. (1983) 'Molten-globule state': a compact form of globular proteins with mobile side-chains. *FEBS Lett.,* **164**., 21-24.

Okada, Y., Nagase, H. and Harris, E.D. (1986) A metalloproteinase from human rheumatoid synovial fibroblast that digests connective tissue matrix components. Purification and characterization. *J. Biol. Chem.,* **261**, 14245-14255.

Otto, H.H. and Schirmeister, T. (1997) Cysteine proteases and their inhibitors. *Chemical Rev.*, **97**, 133–71.

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

Pace, C.N. (1990) Conformational stability of globular proteins. *Trends in Biochem. Sci.*, **15**, 14-17.

Pace, C.N. and Scholtz, J.M. Measuring the conformational stability of a protein. In *Protein Structure: A Practical Approach*, 2nd ed.; Creighton, T. E., Ed.; IRL Press: Oxford, U. K., 1997, pp 299-321.

Pal, G. and Sinha, N.K. (1980) Isolation, crystallization and properties of Calotropis D I and D II from *Calotropis gigentea*. *Arch. Biochem. Biophys.*, **202**, 321-329.

Park, H. Kidman, G. and Northrop, D.B. (2005) Effect of trehalose on pressure-induced inactivation of yeast alcohol dehydrogenase. *Protein Pept. Lett.*, **12**, 597-599.

Pearl, L.H. (1987) The catalytic mechanisms of aspartic proteinases. *FEBS Lett.*, **214**, 8-12.

Phadatare, S.U., Rao, M. and Deshpande, V.V. (1997) A serine alkaline proteases from the fungus *Conidiobolus coronatus* with a distinctly different structure than the serine protease subtilisin Carlsberg. *Arch. Microbiol.*, **166**, 414-417.

Polaina, J. and MacCabe, A.P. (2007) *Industrial enzymes- structure, function and applications.* Springer Publications, The Netherlands.

Prakash, V. and Nandi, P.K. (1977) Association-dissociation and denaturation behaviour of an oligomeric seed protein alpha-globulin of *Sesamum indicum* L. in acid and alkaline solutions. *Int. J. Pept. Protein Res.*, **9**, 319-328.

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

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

Prakash, V. and Timasheff, S.N. (1981) The calculation of partial specific volumes of proteins in 8M urea solution. *Anal. Biochem.*, **117**, 330-335.

Prakash, V., Loucheux, C., Sheufele, 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. (1996) Intermediate state in protein folding. *J. Mol. Biol.,* **258**, 707-725.

Privalov, P.L. (1979) Stability of proteins. *Adv. Protein Chem.,* **33**, 167-241.

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

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

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

Rao, M.B., Tanksale, A.M., Ghatge, M.G. and Deshpande, V.V. (1998) Molecular and biotechnological aspects of microbial proteases. *Micro. Mol. Biol. Rev.*, **62**, 597-635.

Rawlings, N.D. and Barrett, A.J. (1995) Families of aspartic peptidases, and those unknown catalytic mechanism. *Methods Enzymol.,* **248**, 105-120.

Redfield, C., Smith, R.A.G. and Dobson, C.M. (1994) Structural characterization of a highly-ordered 'molten globule' at low pH. *Nature Struct. Biol.*, **1**, 23-29.

Richter, G., Schwarz, H.P., Dorner, F., Turecek, P.L. (2002) Activation and inactivation of human factor X by proteases derived from *Ficus carica*. *British J. Heamatol*., **119**, 1042-1051.

Robbins, B.H. (1930) A proteolytic enzyme in ficin, the antihelminthic principle of leche de higaeron. *J. Biol. Chem.,* **87**, 251-257.

Robinson, D.R. and Jenks, W.P. (1965) The effect of compounds of the urea–guanidinium class on the activity coefficient of acetyltetraglycine ethyl ester and related compounds. *J. Am. Chem. Soc.,* **87**, 2462-2470.

Rodrigo, I., Vera, P. and Van Loon, L.C. (1991) Degredation of tobacco pathogenesis-related proteins. *Plant Physiol.,* **95**, 616-622.

Runeberg-Roos, P. and Saarma, M. (1998) Phytepsin, a barley vacuolar aspartic proteinase, is highly expressed during autolysis of developing tracheary elements and sieve cells. *Plant J.,* **15**, 139–145.

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

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

Schechter, I. and Berger, A. (1967) On the size of the active site in proteases. I. Papain. *Biochem. Biophy. Res. Comm*., **22**, 157-162.

Schellman, J.A. (1987) The Thermodynamic Stability of Proteins. *Annu. Rev. Biophys. Chem*., **16**, 115-137.

Schellman, J.A. (2002) Fifty years of solvent denaturation. *Biophys. Chem.,* **96**, 91–101.

Schmitz, K.S. and Schurr, J.M. (1972) The Role of orientation constraints and rotational diffusion in bimolecular solution kinetics. *J. Phys. Chem.,* **76**, 534-545.

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

Sengupta, A., Bhattacharya, D., Pal, D. and Sinha, N.K. (1984) Comparative studies on Calotropis D I and D II from latex of *Calotropis gigentea*. *Arch. Biochem. Biophys.,* **232**, 17-25.

Sgarbieri, V.C., Gupte, S.M., Kramer, D.E. and Whitaker, J.R. (1964) Ficus enzymes I. Separation of the proteolytic enzymes of *Ficus carica* and *Ficus glabrata* lattices. *J. Biol. Chem*., **239**, 2170-2177.

Shannon, J.D., Baramova, E.N. Bjarnason, J.B. and Fox, J.W. (1989) Amino acid sequence of a *Crotalus atrox* venom metalloprotease which cleaves type IV collagen and gelatin. *J. Biol. Chem.,* **264**, 11575-11583.

Shortle, D. and Ackerman, M.S. (2001) Persistence of native-like topology in a denatured protein in 8 M urea. *Science,* **293**, 487-489.

Simpson, R.B. and Kauzmann, W. (1953) The kinetics of protein denaturation. I. The behavior of the optical rotation of ovalbumin in urea solutions. *J. Am. Chem. Soc.,* **75**, 5139-5152.

Singer, P.T., Smalås, A., Carty, R.P., Mangel, W.F. and Sweet, R.M. (1993) The hydrolytic water molecule in trypsin, revealed by time-resolved Laue crystallography. *Science,* **259**, 669-673.

Sommers, P.B. and Kronman, M.J. (1980) Comparative fluorescence properties of bovine, goat, human and guinea pig α-lactalbumin: characterization of the environments of individual tryptophan residues in partially folded conformers. *Biophys. Chem.,* **11**, 217-232.

Song, J., Bai, P., Luo, L. and Peng, Z. (1998) Contribution of individual residues to formation of the native-like tertiary topology in the α-lactalbumin molten globule. *J. Mol. Biol.,* **280**, 167-174.

Sousa, M.J., Ardo, Y. and McSweeney, P.L.H. (2001) Advances in the study of proteolysis during cheese ripening. *Int. Dairy J.,* **11**, 327-345.

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

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

Storer, A.C. and Menard, R. (1994) Catalytic mechanism in papain family of cysteine peptidases. *Methods Enzymol.,* **244**, 486-500

Stryer, L. (1965) The interaction of naphthalene dye with apomyoglobin and apohemoglobin: A fluorescence probe on non-polar binding sites. *J. Mol. Biol.,* **13**, 482-540.

Stryer, L. (1968) Fluorescence spectroscopy of proteins. *Science,* **162**, 526-540.

Tanford, C (1970) Protein denaturation. Part C: Theoretical models for the mechanism of denaturation. *Adv. Protein Chem.*, **24**, 1-95.

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

Tanh, V.H. and Shibasaki, K. (1976) Major proteins of soybean seeds. A straight forward fractionation and their characterization. *J. Agric. Food Chem.*, **24**, 1117-1121.

Terp, N., Thomsen, K.K., Svendsen, I., Davy, A. and Simpson, D.J. (2000) Purification and characterization of hordolisin, a subtilisin-like serine endopeptidase from barley. *J. Plant Physiol.,* **156,** 468-476.

Tiao-Yin, L. and Timasheff, S.N. (1996) On the role of surface tension in the stabilization of globular proteins. *Protein Sci.,* **5**, 372-381.

Timasheff, S. N. and Arakawa, T. (1988). Mechanism of protein precipitation and stabilization by co-solvents. *J.Crystal Growth,* **90**, 39-46.

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

Timasheff, S.N. and Arakawa, T. Stabilization of protein structure by solvents. In *Protein Structure: A Practical Approach*, 2nd ed.; Creighton, T.E., Ed.; IRL Press: Oxford, U. K., 1997**,** pp 349-364.

Timasheff, S.N. and Xie, G. (2003) Preferential interactions of urea with lysozyme and their linkage to protein denaturation. *Biophys. Chem.,* **105**, 421-448.

Tirado-Rives, J., Orozco, M. and Jorgensen, W. L. (1997) Molecular dynamics simulations of the unfolding of barnase in water and 8 M aqueous urea. *Biochemistry,* **36**(24), 7313-7329.

Towbin, H., Staehelin, T. and Gordon, J. (1979) Electrophoretic transfer of proteins from polyacrylamide gels to nitrocellulose sheets: Procedure and some applications, *Proc. Natl. Acad. Sci. U.S.A.,* **76**, 4350–4354.

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

Turk, B., Turk, V. and Turk, D. (1997) Structural and functional aspects of papain like cysteine proteases and their protein inhibitors. *Biol. Chem*., **378**, 141-150.

Uhlig, H. Description of enzymes: Proteases. In *Industrial Enzymes and Their Applications*; Linsmaier-Bednar, E.M. Ed.; John Wiley & Sons, Inc: New York, 1998, pp 146-179.

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. (2002) What does it mean to be natively unfolded? *Eur. J. Biochem.,* **269**, 2-12.

Uversky, V.N., Karnoup, A.S., Segal, D.J., Sheshadri, S., Doniach, S. and Fink, A.L. (1998) Anion-induced folding of *Staphylococcal* nuclease: characterization of multiple equilibrium partially folded intermediates. *J. Mol. Biol.*, **278**, 879-894.

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.

Vanzi, F., Madan, B. and Sharp, K. (1998) Effect of the protein denaturants urea and guanidinium on water structure: A structural and thermodynamic study. *J. Am. Chem. Soc.*, **120**, 10748-10753.

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

Verissimo, P., Faro, C., Moir, A. J., Lin, Y., Tang, J. and Pires, E. (1996) Purification, characterization and partial amino acid sequence of two new aspartic proteases from fresh flowers *Cynara cardunculus* L. *Eur. J. Biochem.*, **235**, 762-768.

Walti, A. (1938) Crystalline ficin. *J. Am. Chem. Soc.*, **60**, 493-494.

Weaver, L.H., Kester, W.R. and Matthews, B.W. (1977) A crystallographic study of the complex of phosphoramidon with thermolysin. A model for the presumed catalytic transition state and for the binding of structures. *J. Mol. Biol.*, **114**, 119-132.

Williams, D.C. and Whitaker, J.R. (1969) Multiple molecular forms of *Ficus glabrata* ficin: Their separation and relative physical, chemical, and enzymatic properties. *Plant Physiol.*, **44**, 1574-1583.

Williams, D.C., Sgarbieri, V.C. and Whitaker, J.R. (1968) Proteolytic activity in the genus *Ficus*. *Plant Physiol.*, **43**, 1083-1088.

Wong, K.P. and Tanford, C. (1973) Denaturation of bovine carbonic anhydrase B by guanidine hydrochloride. *J. Biol. Chem.*, **248**, 8518-8523.

Yadav, S. C., Pandey, M.P. and Jagannadham, M.V. (2006) Highly stable glycosylated serine protease from the medical plant *Euphorbia* milii. *Phytochemistry,* **67**, 1414-1426.

Yamagata, H., Masuzawa, T., Nagaoka, Y., Ohnishi, T. and Iwasaki, T (1994) Cucumisin, a serine protease from melon fruit, shares structural homology with subtilisin and is generated from a large precursor. *J. Biol. Chem.,* **269**, 32725-32731.

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

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.

Zhou, H.M., Zhang, X.H., Yin, Y. and Tsou, C.L. (1993) Conformational changes at the active site of creatine kinase at low concentrations of guanidinium chloride. *Bichem. J.,* **291**, 103-107.

Zou, Q., Habermann-Rottinghaus, S.M. and Murphy, K.P. (1998) Urea effects on protein stability: Hydrogen bonding and the hydrophobic effect. *Protein: Struct. Funct. Genet.,* **31**, 107-115.

****