STRUCTURE FUNCTION AND STABILITY OF LOW MOLECULAR WEIGHT PROTEINS FROM SELECTED SEEDS AND DAIRY WHEY

A THESIS SUBMITTED TO THE UNIVERSITY OF MYSORE,

MYSORE, FOR THE DEGREE OF

DOCTOR OF PHILOSOPHY

IN

BIOTECHNOLOGY

BY

P. M. GUNA SEKHAR

DEPARTMENT OF PROTEIN CHEMISTRY AND TECHNOLOGY

CENTRAL FOOD TECHNOLOGICAL RESEARCH INSTITUTE

MYSORE - 570020, INDIA

DECEMBER, 2008

Dedicated to my beloved parents and family

DECLARATION

I hereby declare that the thesis entitled **"Structure function and stability of low molecular weight proteins from selected seeds and dairy whey"** which is submitted herewith for the degree of **Doctor of Philosophy** in **Biotechnology** of the **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 May 2002 - 2007.

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

Date: 29/12/2008 **P. M. Guna Sekhar**

Place: Mysore

CERTIFICATE

I hereby certify that the thesis entitled **"Structure function and stability of low molecular weight proteins from selected seeds and dairy whey"** submitted by **Mr. P. M. Guna Sekhar** for the degree of **Doctor of Philosophy** in **Biotechnology** to the 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 May 2002 - 2007.

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

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

Date: 29/12/2008

Place: Mysore

ACKNOWLEDGEMENTS

With deep sense of gratitude, I owe my sincere respects and heartful thanks to my guide and mentor Padma Shri Dr. V. Prakash, Director, CFTRI, Mysore, India for suggesting the research problem for Ph.D. degree, to make me learn proper planning and execution of experiments and presentation of scientific data, for his support extended to me in my personal and professional level during my research work. I felt it as a great opportunity to work under his able guidance.

My sincere thanks also to him as a Director of the institute for providing the necessary facilities to carry out this investigation and permitting me to submit my Ph.D. work in the form of a thesis.

I extend my sincere thanks to head, staff members and fellow colleagues in the Department of Protein Chemistry and Technology, CFTRI, Mysore both present and past for their encouragement. I also thank all the staff members of central instrumentation facility, pilot plant, solvent shed and administration for their help in the course of my work.

My sincere thanks to Dr. (Mrs.) Purnima Kaul,Dr.(Mrs.)Radha, Dr.P.Ramesh Kumar, Sijo Mathew, Jaykant Yadav, Devaraj, Gangadhara, Gajendra, Anuradha, Srinivas Sistla and all my friends for their co-operation and support during the work.

To build a career in science it is imperative to have assurance and sacrifice from the family. I am greatly indebted to my parents, sisters and brother for their love and encouragement in this endeavor.

My special thanks to the Council of Scientific and Industrial Research, New Delhi, India for providing the financial assistance in the form of Junior and Senior Research Fellowship during the period of this investigation.

Date: 29/12/2008 **P. M. Guna Sekhar**

Place: Mysore

SYNOPSIS

OF

The Ph.D. Thesis Entitled

STRUCTURE FUNCTION AND STABILITY OF LOW MOLECULAR WEIGHT PROTEINS FROM SELECTED SEEDS AND DAIRY WHEY

Submitted to the University of Mysore, Mysore

for the degree

Of

Doctor of Philosophy

In

BIOTECHNOLOGY

By

P. M. GUNA SEKHAR

Department of Protein Chemistry and Technology,

Central Food Technological Research Institute

Mysore - 570020, India

December, 2008

Title of Ph.D. Thesis:

Structure Function and Stability of Low Molecular Weight Proteins from Selected Seeds and Dairy Whey

The knowledge of protein structure, function and stability of the proteins is essential for formulation of various food products. There are different sources of proteins and based on their physicochemical parameters they can be utilized in biological system for different purposes. The bovine milk and plant seeds are the major sources of proteins. Various food formulations and processing involve the undesirable biochemical changes in the proteins. Therefore, it is imperative to study the structure-function relationship of protein and make it more stable under various conditions. In addition, to provide the basic nutrition, many proteins are known to have bioactive peptide sequences, which are released by the digestion process *in vivo* or *in vitro*. Bovine milk and seed proteins are known to be the good sources of bioactive peptides.

Bovine milk contains about 3.5% protein, of which caseins constitutes nearly 80% and remaining 20% is whey. Whey proteins include β-lactoglobulin (β-Lg), α-lactalbumin (α-La), serum albumin, lactotransferrin, immunoglobulin and β_2-micro globulin. The α-La constitutes 4% of the whey proteins. It is also a component of the lactose synthase complex.

Sesame (*Sesamum indicum L.*) seeds contain nearly 25% protein and the defatted flour contains 50% protein. Nearly 25% of total protein is constituted by β-globulin, the second major sesame protein. This low molecular weight protein is rich in α–helical structure and has considerable amount of proteolytic activity with unusually high contents of sulfur containing and acidic amino acids.

The structural stability of native protein is known to affect strongly by a variety of substances used at different concentrations. Cosolvents play an important role in the structural stability of proteins. The mode of stability by these cosolvents is very specific and depends mainly on the nature of cosolvent, structural characteristics of the protein and also the environment in which it is used. Effect of cosolvents on the structure, function and stability of the above two low molecular weight proteins one from bovine milk i.e. α-La and the other from sesame seed i.e. β-globulin were carried out. The mechanism involved in the stability of these two low molecular weight proteins was studied by measuring the partial specific volume and preferential interaction parameters.

Different biologically active peptides, which are encrypted within the sequence of the parent protein, can be released by enzymatic proteolysis. Attempts were made to hydrolyze whey protein concentrate (WPC), α-La from dairy and β-globulin from sesame seed with different enzymes to obtain hydrolysates with potential peptides with various bioactivities. These can be used in lucrative health food market as nutraceuticals, pharma foods or designer foods.

With these objectives the present investigation is undertaken and the results and conclusions are brought out in the form of a thesis entitled "**Structure function and stability of low molecular weight proteins from selected seeds and dairy whey**".

The investigation is divided into the following chapters:

(i) Isolation and characterization of α-lactalbumin from bovine milk and interaction studies with cosolvents.

(ii) Isolation and characterization of β-globulin form sesame seeds and interaction studies with cosolvents.

(iii) Hydrolysis of whey protein concentrate, α-lactalbumin from dairy and low molecular weight protein fraction from sesame seeds.

(iv) Preferential interaction studies of cosolvents with α-lactalbumin and β-globulin.

The results and analysis of the above investigation are presented in the form of a thesis. Brief outlines of the important results obtained in the present investigation are summarized below. The thesis has been divided into introduction, scope & objectives, materials and methods, summary and conclusion and references along with tables and figures.

In **Chapter I,** the isolation, purification and characterization of α-la from bovine milk and also the effect of cosolvents namely sorbitol, glycerol and sucrose on the structural stability of the protein is described. α-Lactalbumin was purified to homogeneity and the purity was evaluated by the SDS-PAGE and RP-HPLC. The amino acid composition of the isolated α-La had a correlation coefficient of 95% with the literature values. The effect of different concentrations of sorbitol, glycerol and sucrose ranging from 10-30% on the protein was investigated. The fluorescence emission spectra of α-La showed an increase in the fluorescence emission intensity with increasing concentrations of sorbitol and glycerol but in presence of sucrose, quenching in the fluorescence emission intensity was observed. There was 2 nm blue shift in the emission maxima in presence of higher concentrations of glycerol and sorbitol. Preferential hydration and conformational change might alter the microenvironment of the aromatic chromophores in presence of cosolvents. These results indicate that there is subtle conformational change of the protein molecule in these cosolvents.

The effect of sorbitol, glycerol and sucrose on the tertiary structural stability of the α-La was examined by near–UV CD measurements. There was marginal change in the tertiary structure of the protein in the presence of different concentrations of cosolvents in the concentration range of 10-30%. The effect of sorbitol, glycerol and sucrose on the secondary structural stability of the protein was examined by far-UV CD measurements. In presence of these cosolvents there was a significant increase in α-helical content but in presence of 30% glycerol there was decrease in the β-structure of the protein by about 12%.

Thermal denaturation of α-La was carried out in presence of cosolvents to determine the stabilization effect. The apparent T_m value of the native protein was found to be 65°C. With increasing concentrations of each cosolvents namely sorbitol, glycerol and sucrose there was an increase in the apparent T_m values for the protein. The efficiency of thermal stabilizing capacity of cosolvents was found to be in the order of sorbitol > sucrose> glycerol. At 40% concentration of sorbitol, sucrose and glycerol the apparent T_m was found to be 71, 71 and 68°C respectively.

In **Chapter II,** the isolation, purification and structural characterization of β-globulin from sesame seeds are described. The protein was purified to homogeneity using salt precipitation method and the homogeneity was evaluated by electrophoretic and gel filtration chromatographic methods. The amino acid composition of the isolated β-globulin was determined and it was well correlated with the literature value with a correlation coefficient value of 97%. The effect of cosolvents on the structure function and stability of β-globulin was studied by CD spectroscopy, fluorescence spectroscopy and differential absorption spectroscopy. The fluorescence emission maximum of β-globulin was found to be 336 nm in absence of cosolvents. The presence of cosolvents namely sorbitol, glycerol and sucrose did not show any changes in the emission maxima

of β-globulin. The results of fluorescence spectra indicate that the presence of cosolvents did not bring any gross conformational change in the protein molecule. The far–UV CD spectra of the protein in absence of cosolvents showed 26 ± 1% α–helix, 38 ± 1%, β-structure and 36 ± 1% aperiodic structures. In presence of different cosolvents namely sorbitol, glycerol and sucrose slight changes in the secondary structural parameters of the protein molecule was observed. There was an increase in the α–helical content of the protein with increasing concentrations of cosolvents. There was a 9% increase in the β-structure in the presence of sorbitol and sucrose at 30% concentration and decrease of 10% in the aperiodic structure with all the cosolvents used.

The effect of cosolvents on thermal stability of β-globulin was determined by measuring thermal denaturation temperature. The apparent transition temperature for β-globulin protein was found to be 78 ± 1°C. The presence of all the cosolvents such as sorbitol, sucrose and glycerol increased the thermal denaturation temperature of β-globulin in a concentration dependent manner. At 10% (w/v) concentration the apparent transition temperature (apparent T_m) increased to 79 ± 1, 80 ± 1 and 82 ± 1°C respectively.

In **Chapter III,** the enzymatic hydrolysis of whey protein concentrate (WPC), α-La from dairy whey and β-globulin from sesame seed are described. The hydrolysates obtained from respective proteins enriched in bioactive peptides with various bioactivities. WPC hydrolysate (38 ± 3%) gave a maximum degree of hydrolysis compared with that of the β-globulin hydrolysate (9 ± 2%). The better degree of hydrolysis of the WPC hydrolysate was due to the maximum susceptibility of the protein for the enzymes used and the broad specificity of the enzymes (fungal protease and pancreatin) used for its

hydrolysis. The least DH% obtained for β-globulin hydrolysate was due to the strong disulfide present in the protein.

The hydrolysates were analysed for their antioxidant, ACE inhibitory and the antimicrobial potential. The WPC hydrolysate was having maximum total antioxidant potential and proton radical scavenging potential compared with the β-globulin hydrolysate. The total antioxidant potential for the whey protein hydrolysate was 5900 ± 210 μmol/g of ascorbic acid equivalents. The IC_{50} values for the WPC hydrolysate for the DPPH radical scavenging action was 0.110 ± 0.01 mg/ml when compared with the standard antioxidant ascorbic acid, which had an IC_{50} value of 0.191 ± 0.01 mg/ml. Thus the obtained hydrolysates can be utilized in *in vitro* models. The total antioxidant and the proton radical scavenging potential are evaluated for WPC hydrolysate, α-La hydrolysate and β-globulin hydrolysate.

The antimicrobial activity of the hydrolysates were checked against microbes namely *Escherichia coli, Bacillus cereus,* and *Listeria monocytogenes.* The inhibitory action of the WPC hydrolysate and the β-globulin hydrolysate was found to be insignificant. But the α-La hydrolysate was effective against *Escherichia coli* and *Bacillus cereus* compared with the *Listeria monocytogenes microbes.* The obtained hydrolysates were found to inhibit angiotensin-converting enzyme. The β-globulin hydrolysate was having maximum ACE inhibitory potential with an IC_{50} value of 380 ± 25 μg/ml. although the hydrolysates were having slightly less ACE inhibitory potential compared with the ramipril (IC_{50} = 215 ± 15 μg/ml) for long term use food sources are advocated for medical purposes rather than chemical source. Thus the above hydrolysates were having antioxidant, antimicrobial and ACE inhibitory potential.

In **Chapter IV** is described the mechanism of interaction of cosolvent-induced stabilization of two low molecular weight proteins namely α-La and β-globulin by measurement of partial specific volume. The cosolvents used in the study are sorbitol, glycerol and sucrose. The preferential interaction parameters calculated from partial specific volume was found to be negative for α-la in the presence of all the cosolvents. These results clearly indicate the phenomenon of preferential hydration or exclusion of the cosolvents from the domain of the protein molecule. The preferential interaction parameter was found to be maximum with -0.237 ± 0.025 g/g at 40% sorbitol and minimum of -0.058 ± 0.015 g/g at 5% sorbitol. The preferential exclusion increased with increasing glycerol concentration, the highest value of -0.299 ± 0.030 g/g was obtained at 40% concentration. The preferential hydration reached the highest value of -0.171 ± 0.016 (g/g) in presence of sucrose at 45% concentration (w/v).

With β-globulin in the cosolvents namely sorbitol, glycerol and sucrose at all concentrations the preferential interaction parameter (ξ_3) was found to be negative. In presence of sorbitol the maximum ξ_3 value of -0.137 ± 0.018 (g/g) was observed at 40% (w/v) concentration and the minimum value of -0.049 ±0.024 (g/g) at 10% (w/v) concentration. The maximum preferential exclusion was obtained at 40 % (w/v) concentration of glycerol with the value of -0.174 ± 0.021 (g/g). The preferential hydration parameter was obviously positive in all the concentrations of glycerol. The preferential interaction parameter reaches a maximum value of -0.110 ± 0.011 (g/g) in presence of sucrose at 40% (w/v) concentration. Thus these values clearly indicate that proteins used in the study are stabilized by preferential hydration of the protein and mutual exclusion of the cosolvent from the domain of the protein, which results in added hydration.

Summary and conclusion gives the salient features of the present investigation in brief. The literature cited in the complete text is arranged in alphabetical order under references, which gives all the relevant details including the title of the paper and pagination.

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

P. M. Guna Sekhar
Candidate

Dr. V. Prakash
Guide and Supervisor
&
DIRECTOR,
Central Food Technological Research Institute,
Mysore - 570020

Place: Mysore
Date: 29/12/2008

CONTENTS

LIST OF ABBREVIATIONS

A	absorbance
A°	angstrom
ANS	8-anilino-naphthalene sulfonic acid
ACE	angiotensin-1-converting enzyme
Asp	aspartic acid
cal	calorie
[C]	concentrations of protein (mg/ml)
cm	centimeter
$CaCl_2$	calcium chloride
°C	degree Celsius
CD	circular dichroism
DPPH	α, α-diphenyl-β- picrylhydrazyl
EDTA	ethylene diamine tetra acetic acid
Fig	figure
FDA	food and drug administration
FPLC	fast protein liquid chromatography
F_u	fraction unfolded
g	gram
GuHCl	guanidine hydrochloride
h	hour
HCl	hydrochloric acid
HPLC	high performance liquid chromatography

kCal	kilocalories
°K	degree Kelvin
kDa	kilo Dalton
KCl	potassium chloride
Kg	kilogram
l	litre
Li_2SO_4	lithium sulfate
M	molar concentration
mA	milli ampere
mg	milligram
min	minute
ml	milliliter
mm	millimeter
mmol	millimoles
mol	moles
MRW	mean residue weight
MW	molecular weight
N	normality
NaCl	sodium chloride
NaOH	sodium hydroxide
ng	nanogram
nm	nanometer
NBS	N- bromosuccinimide

NAL	N-acetyl lactosamine
NAG	N-acetyl glucosamine
PAGE	polyacrylamide gel electrophoresis
P	pressure
rpm	revolutions per minute
RP	reverse phase
pI	isoelectric point
R	universal gas constant
SDS	sodium dodecyl sulfate
SH	thiol
SHMP	sodium hexa metaphosphate
sec	second
SS	disulfide
T	absolute temperature
TCA	trichloro acetic acid
TEA	triethanolamine
TEMED	N, N, N', N'- tetra methyl ethylene diamine
TFA	trifluoro acetic acid
T_m	thermal denaturation temperature of protein
TNBS	2, 4, 6-trinitrobenzene sulfonate
Tris	tris (hydroxymethyl) amino methane
Trp	tryptophan
UV	ultraviolet

v/v	volume by volume
$\bar{v}$	partial specific volume of protein (ml/g) extrapolated to zero protein concentration
$\bar{v}_{app}$	apparent partial specific volume (ml/g) at a single protein concentration
V_e	elution volume
V_{max}	maximal velocity
V_t	total volume
V_0	void volume
w/v	weight/volume
ΔA	difference in absorbance
μ	chemical potential
μg	microgram
μl	microlitre
μmol	micromole
%	percentage
α	alpha
β	beta
γ	gamma
δ	delta
κ	kappa
α-La	α-lactalbumin
β-Lg	β-lactoglobulin
λ_{max}	wavelength of maximum emission

$E^{1\%}_{1cm,\lambda max}$	absorption coefficient of a 1% solution in 1cm path length cell at its absorption maximum
ε	molar extinction coefficient (M^{-1} cm^{-1})
ρ_0	density of solvent buffer
ρ_P	density of protein solution
ξ_3	preferential interaction parameter of protein
ϕ_2^o	isomolal partial specific volume extrapolated to zero protein concentration
$\phi_2'^o$	isopotential partial specific volume extrapolated to zero protein concentration
xg	times acceleration due to gravity
θ_{MRW}	mean residue molar ellipticity
$(\delta g_3/\delta g_2)$	preferential interaction parameter on g/g basis
$(\delta m_3/\delta m_2)$	preferential interaction parameter on mol/mol basis

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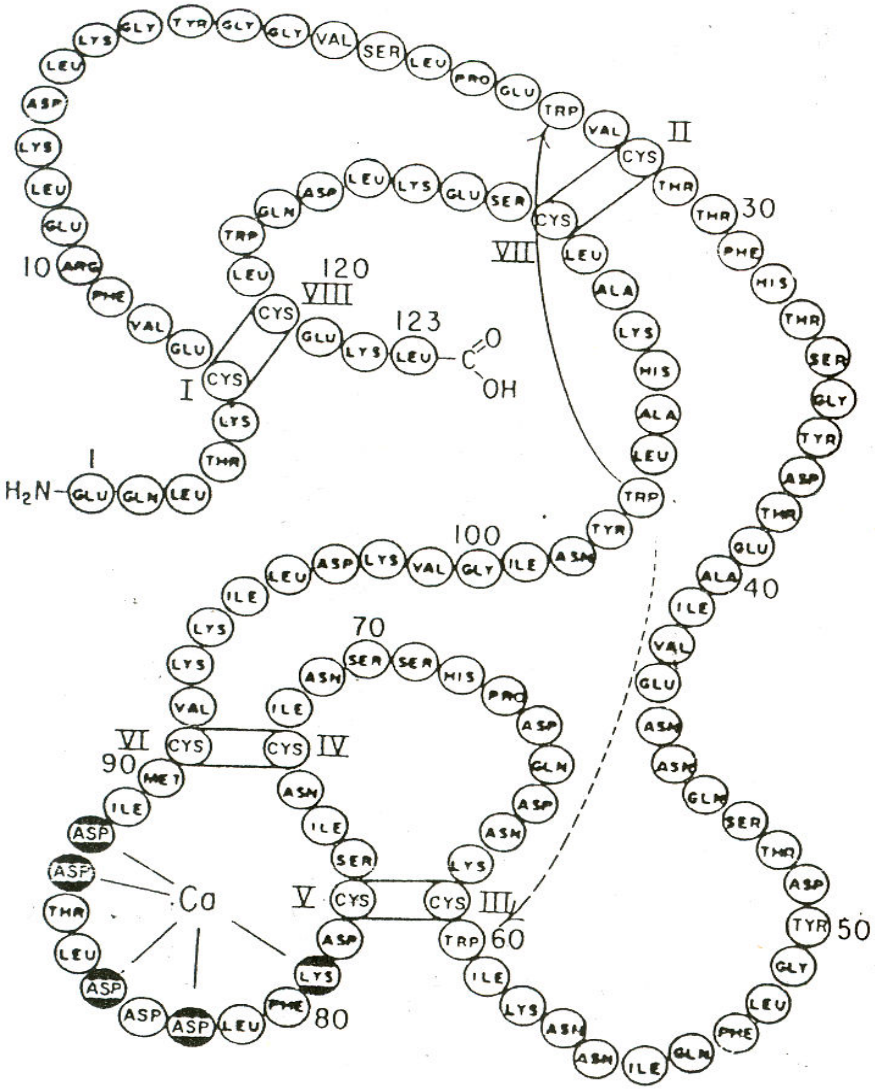

Fig. 1: Linear representation of the amino acid sequence of bovine α-lactalbumin. The shaded residues indicate calcium ion-binding site. [Courtesy: Reproduced from Farell *et al.*, 2004]

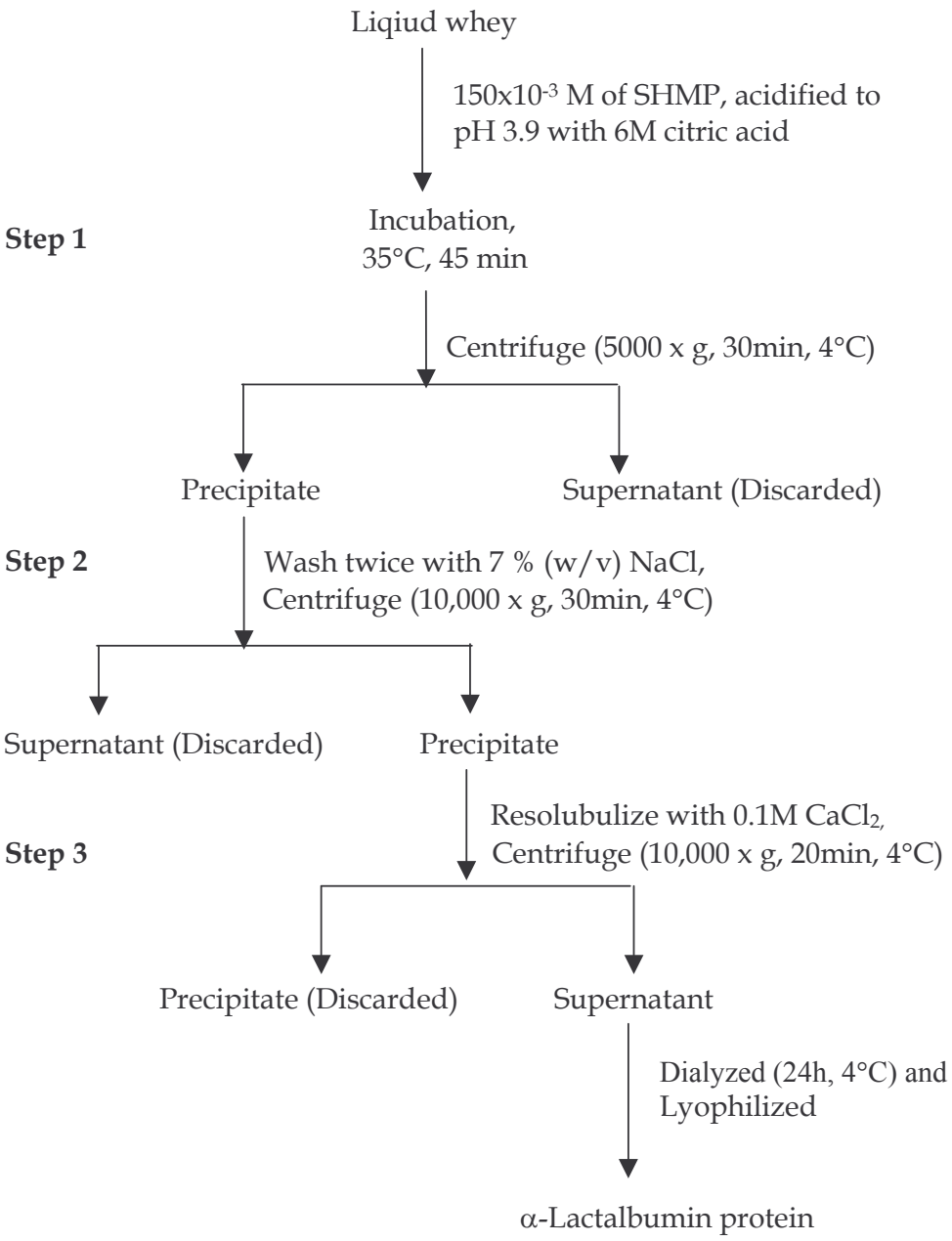

Fig. 2: Flow diagram showing isolation procedure of α-lactalbumin from dairy whey.

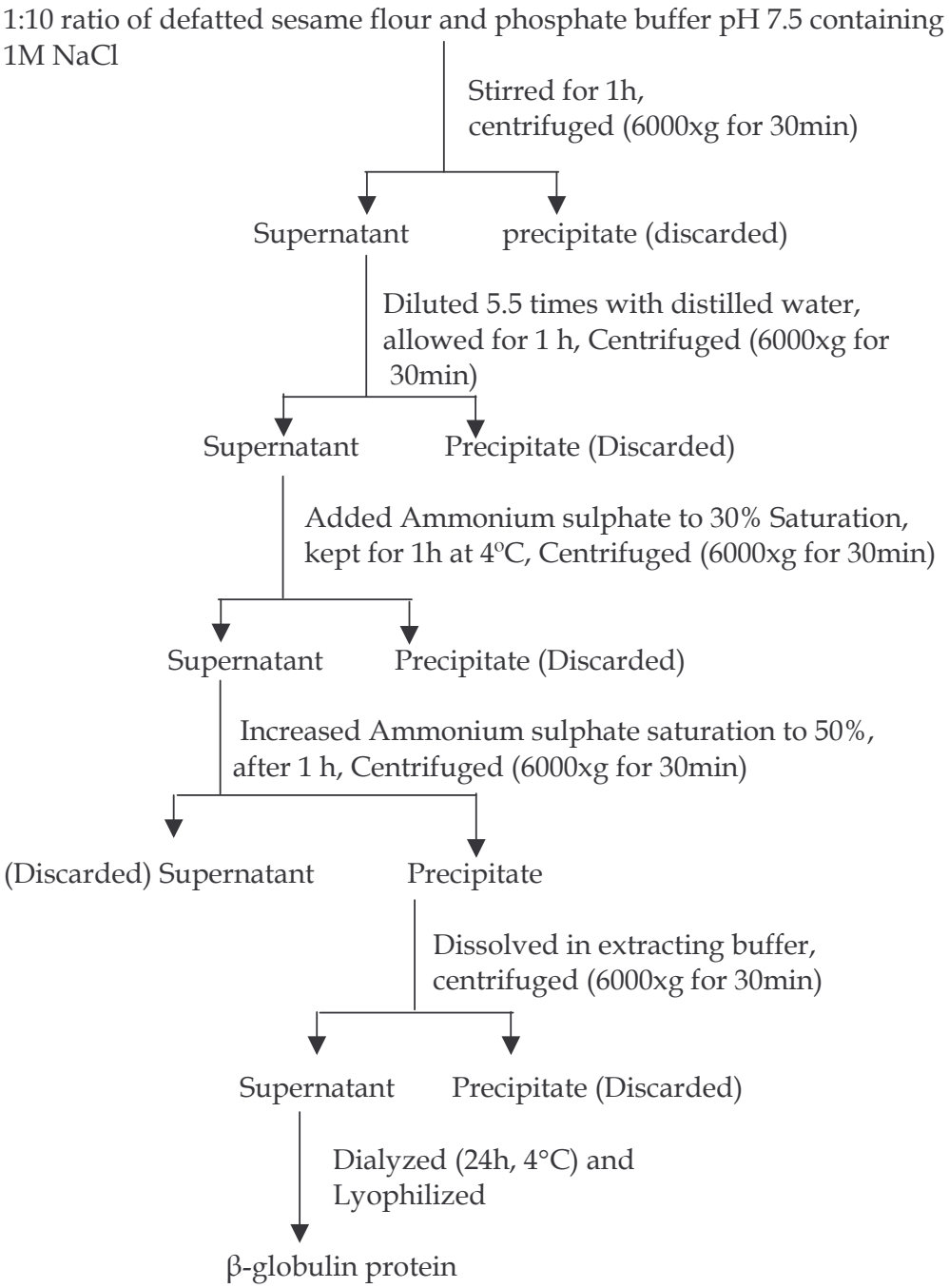

Fig. 3: Flow diagram showing isolation procedure of β-globulin from *Sesamum indicum* (L) seeds.

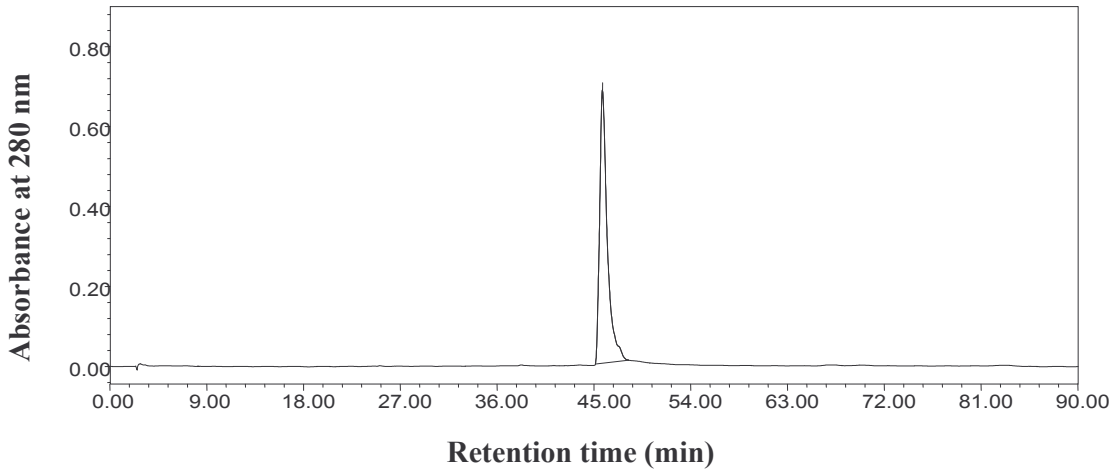

Fig. 4: RP-HPLC profile of isolated α-lactalbumin in C18 reversed phase column (4.6x150mm) using *W*aters HPLC system equipped with diode array, UV-Visible detector. The following two buffer gradient system was used to elute the samples. Buffer A, 0.1% TFA in water and buffer B, 0.05% TFA in 70% acetonitrile.

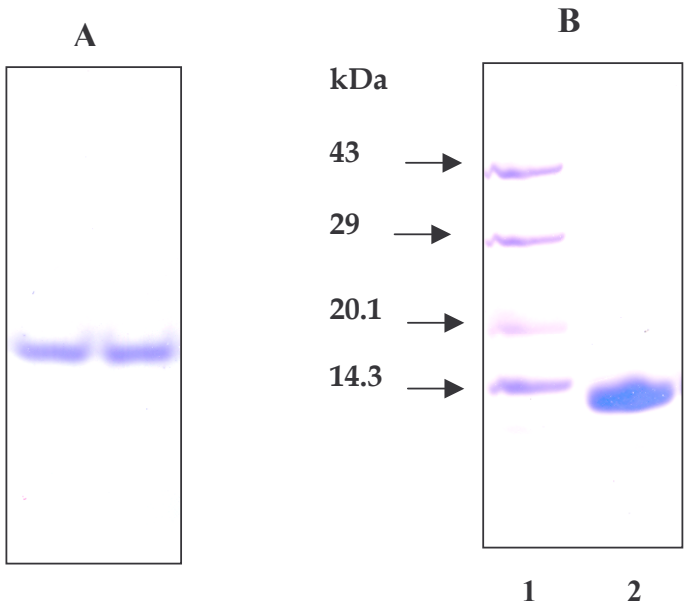

Fig. 5: (A) Native-PAGE pattern of α-lactalbumin isolated from bovine milk. (B) SDS-PAGE of α-lactalbumin isolated from bovine milk. (Lane 1) Standard proteins: ovalbumin (43 kDa), carbonic anhydrase (29 kDa), soybean trypsin inhibitor (20.1 kDa), lysozyme (14.3 kDa). (Lane 2) Isolated α-lactalbumin from bovine milk.

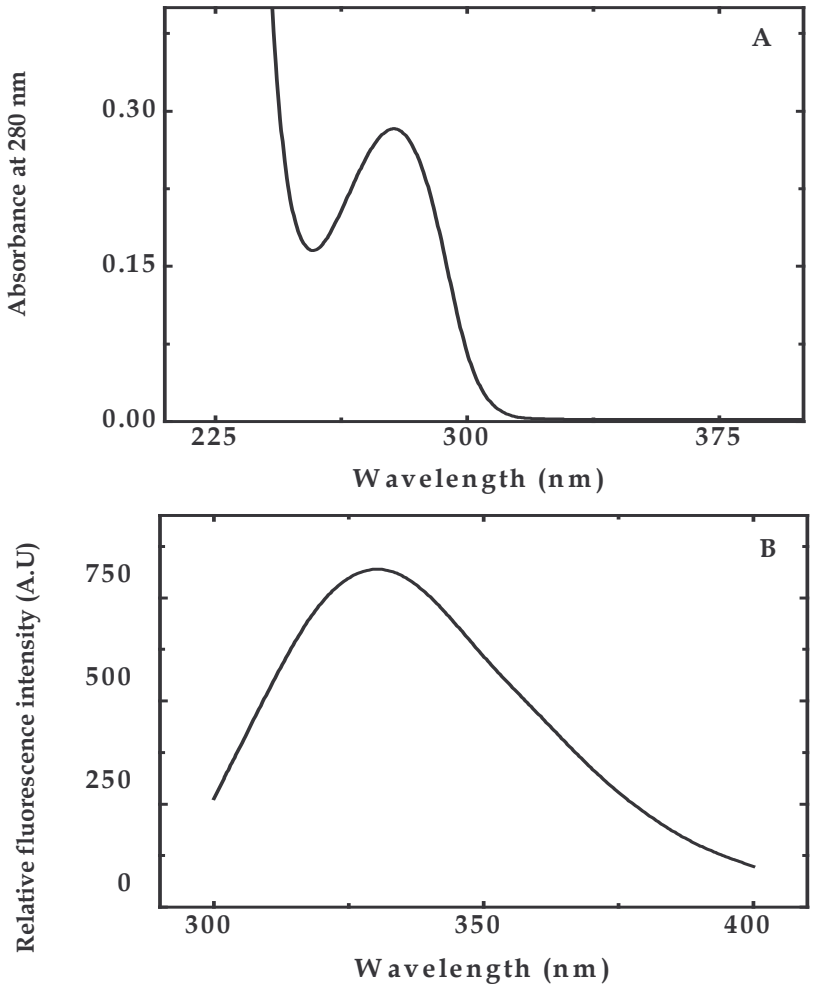

Fig. 6: (A) UV-absorption spectra and (B) fluorescence emission spectra of isolated α-lactalbumin in 0.01 M Tris-HCl buffer at pH 7.5.

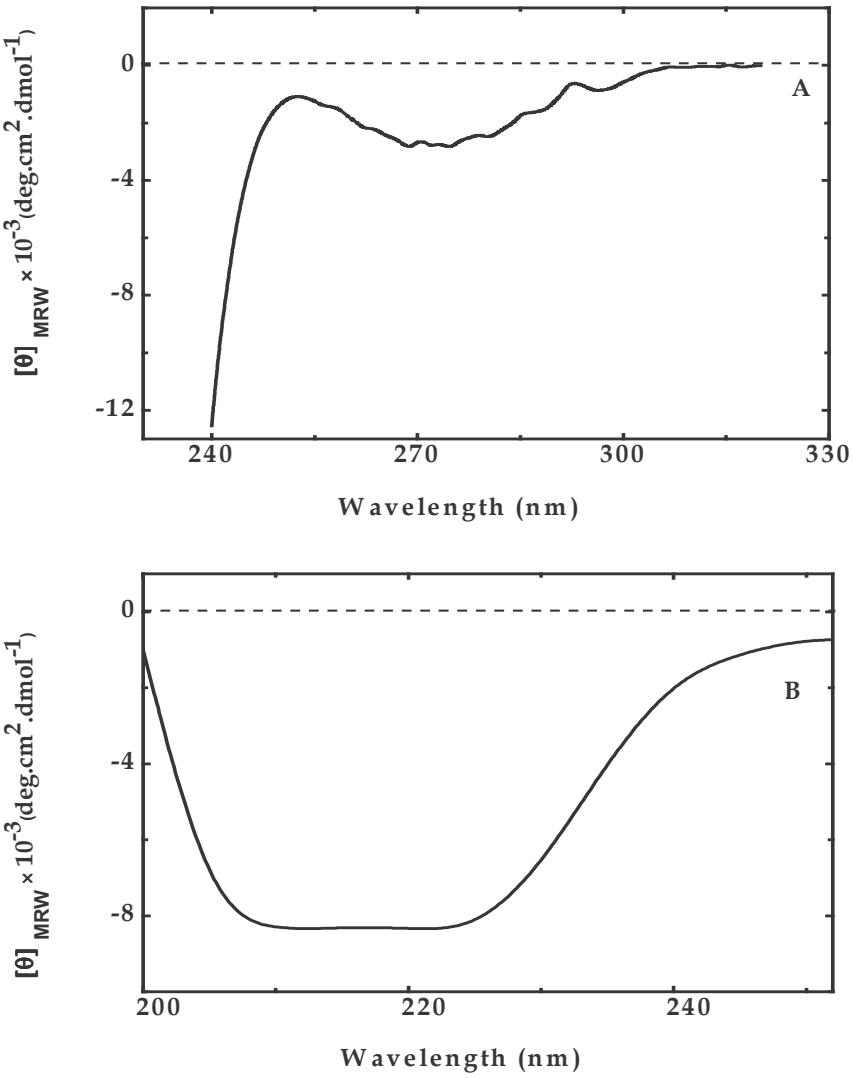

Fig. 7: (A) Near-UV CD and (B) Far-UV CD spectra of isolated α-lactalbumin from bovine milk in 0.01 M Tris-HCl buffer of pH 7.5 at 25°C.

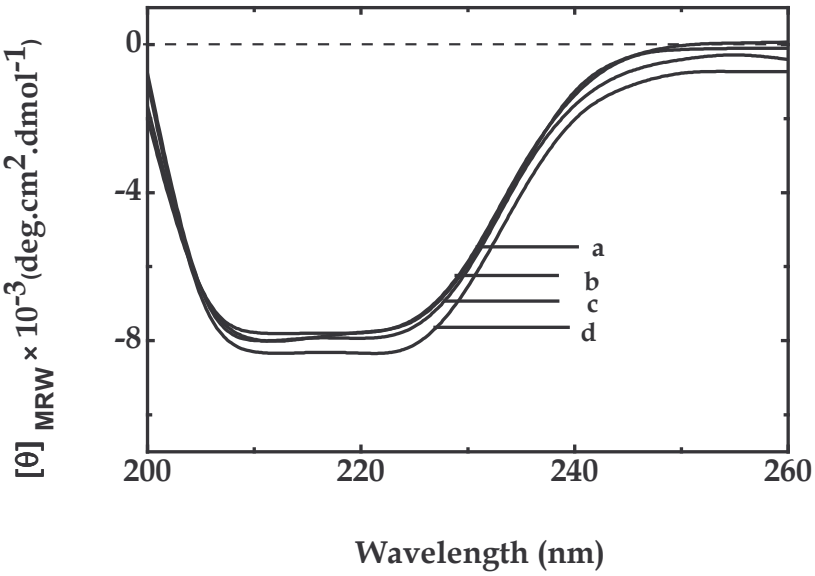

Fig. 8: Far-UV CD spectra of α-lactalbumin in the presence different concentrations of sorbitol in 0.01 M Tris-HCl buffer at pH 7.5. (a) In buffer, (b) in 10%, (c) in 20% and (d) in 30% sorbitol.

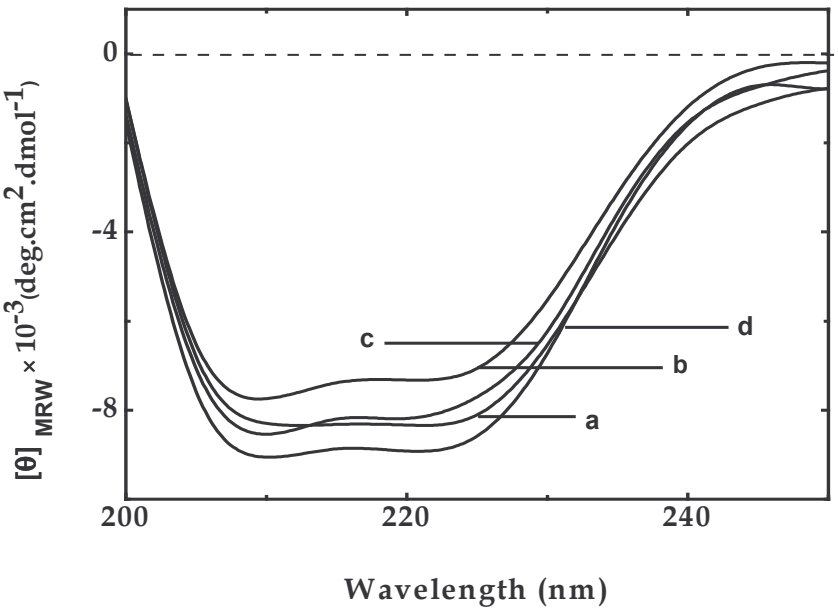

Fig. 9: Far-UV CD spectra of α-lactalbumin in the presence of different concentrations of glycerol in 0.01 M Tris-HCl buffer at pH 7.5. (a) In buffer, (b) in 10%, (c) in 20% and (d) in 30% glycerol.

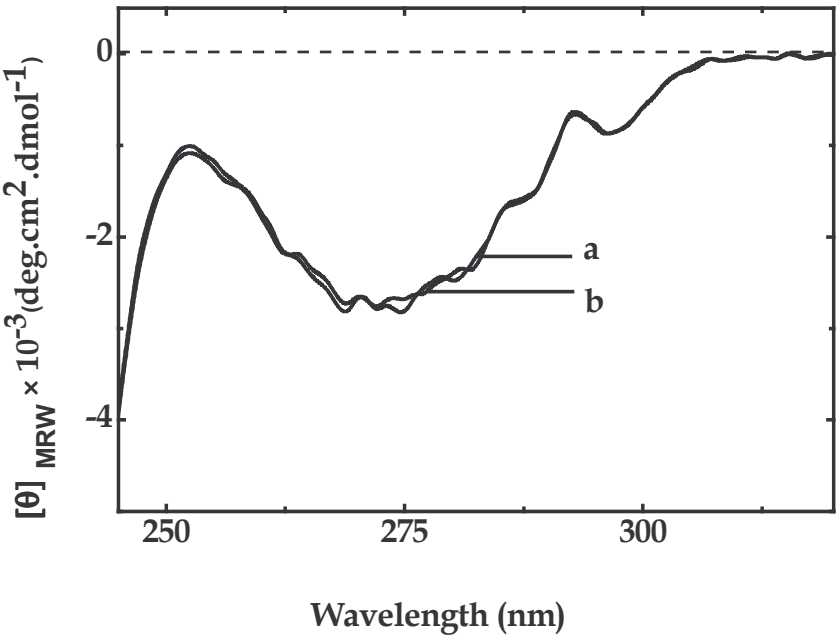

Fig. 10: Near-UV CD spectra of α-lactalbumin in 0.01 M Tris-HCl buffer at pH 7.5. (a) In buffer and (b) in 30% sorbitol.

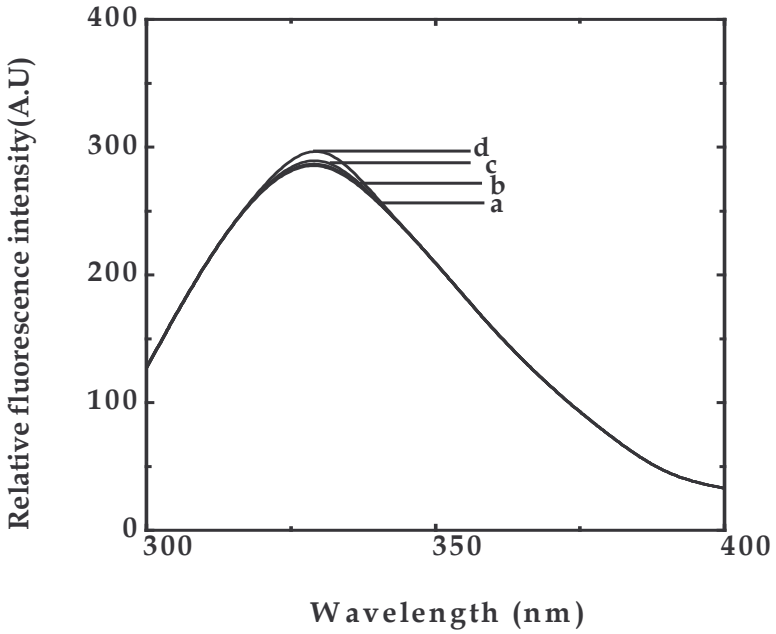

Fig. 11: Fluorescence emission spectra of α-lactalbumin in the presence of different concentrations of sorbitol in 0.01M Tris-HCl buffer at pH 7.5. The protein was excited at 280 nm and emission spectra were recorded in the range of 300-400 nm. (a) In buffer, (b) in 10%, (c) in 20%, and (d) in 30% sorbitol.

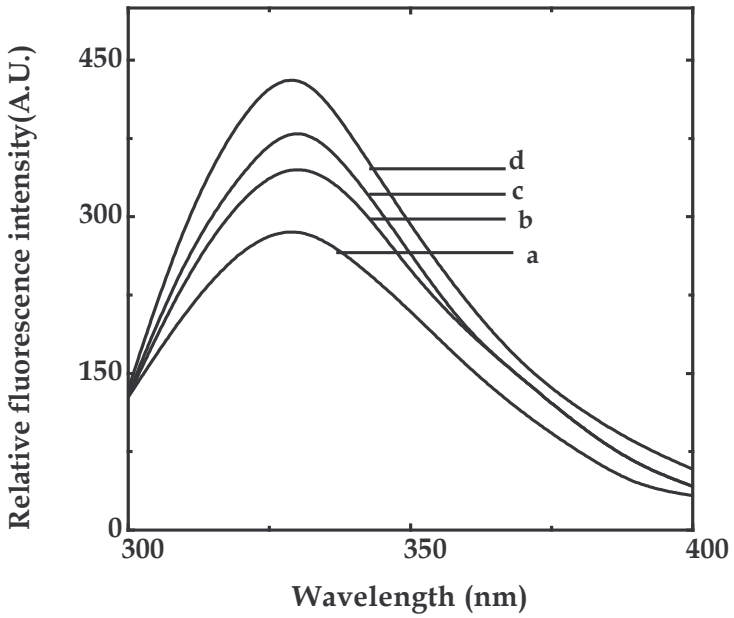

Fig. 12: Fluorescence emission spectra of α-lactalbumin in the presence of different concentrations of glycerol in 0.01M Tris-HCl buffer at pH 7.5. The protein was excited at 280 nm and emission spectra were recorded in the range of 300-400 nm. (a) In buffer, (b) in 10%, (c) in 20% and (d) in 30% glycerol.

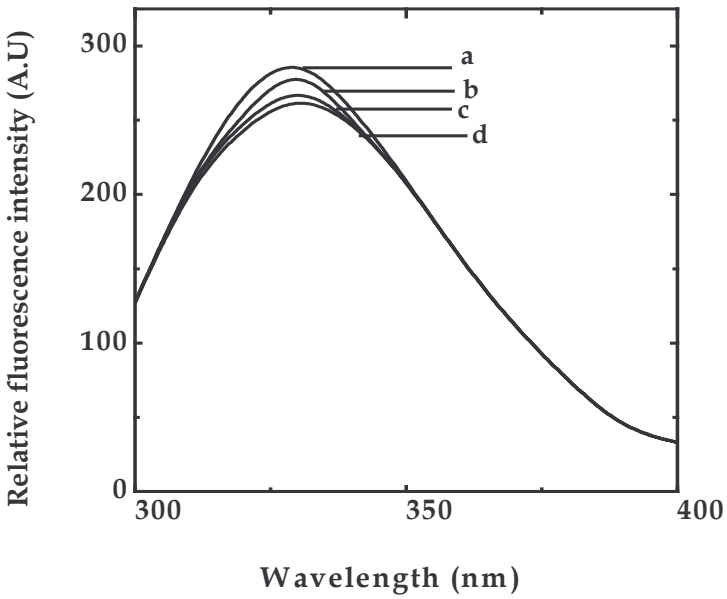

Fig. 13: Fluorescence emission spectra of α-lactalbumin in the presence of different concentrations of sucrose in 0.01M Tris-HCl buffer at pH 7.5. The protein was excited at 280 nm and emission spectra were recorded in the range of 300-400 nm. (a) In buffer, (b) in 10%, (c) in 20% and (d) in 30% sucrose.

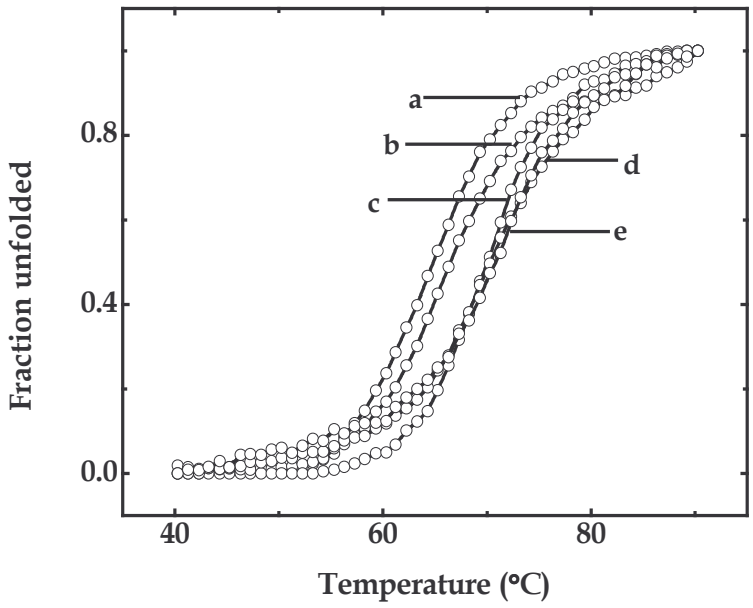

Fig. 14: Apparent thermal denaturation curves of α-lactalbumin in the presence of different concentrations of sorbitol in 0.01M Tris-HCl buffer at pH 7.5. (a) In buffer, (b) in 10%, (c) in 20%, (d) in 30% and (e) in 40% sorbitol. The absorption spectra were recorded as a function of temperature at 287nm.

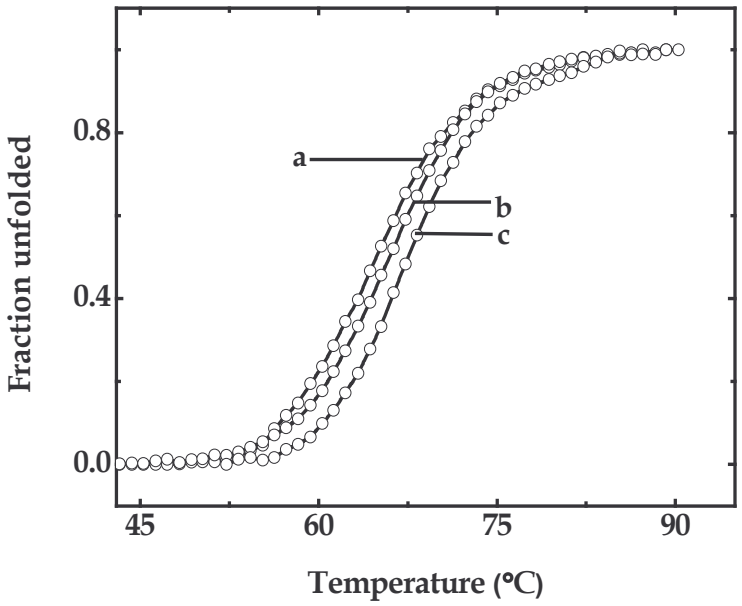

Fig. 15: Apparent thermal denaturation curves of α-lactalbumin in the presence of different concentrations of glycerol in 0.01M Tris-HCl buffer at pH 7.5. (a) In buffer, (b) in 20% and (c) in 40% glycerol. The absorption spectra were recorded as a function of temperature at 287nm.

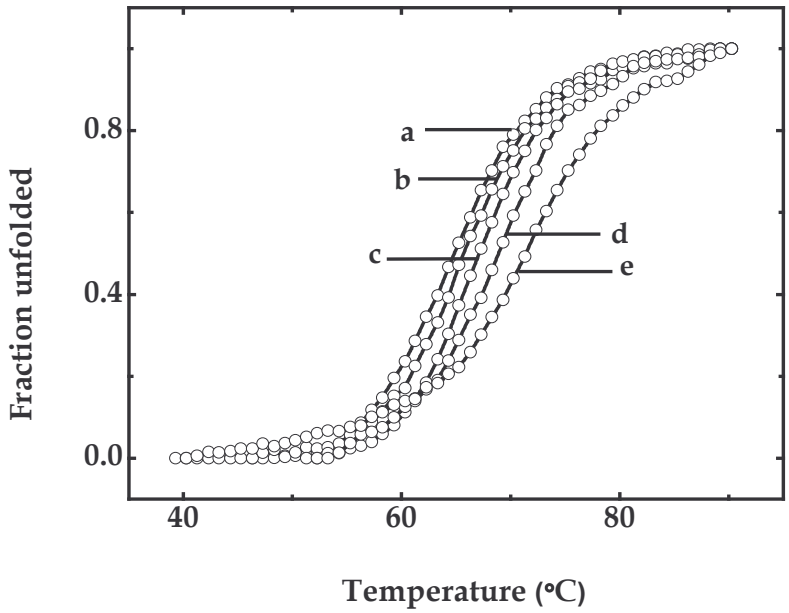

Fig. 16: Apparent thermal denaturation curves of α-lactalbumin in the presence of different concentrations of sucrose in 0.01M Tris-HCl buffer at pH 7.5. (a) In buffer, (b) in 10%, (c) in 20%, (d) in 30% and (e) in 40% sucrose. The absorption spectra were recorded as a function of temperature at 287nm.

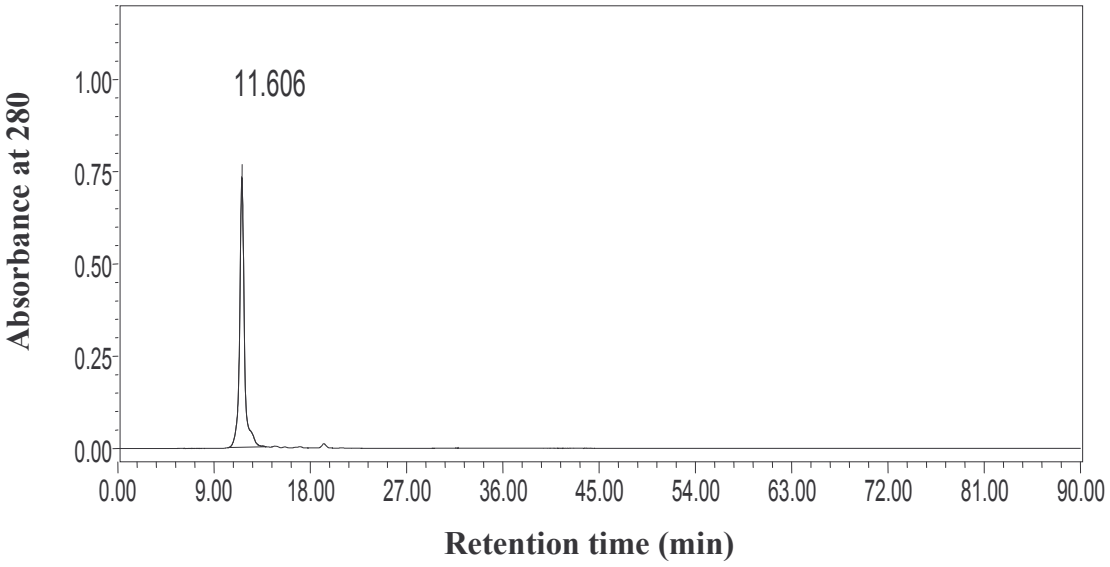

Fig. 17: Gel filtration elution profile of isolated protein β-globulin was performed on Shodex-100 column using a waters HPLC system, equipped with diode array, UV-visible detector. The column was equilibrated with 0.06M phosphate buffer pH 7.5 containing 1M NaCl.

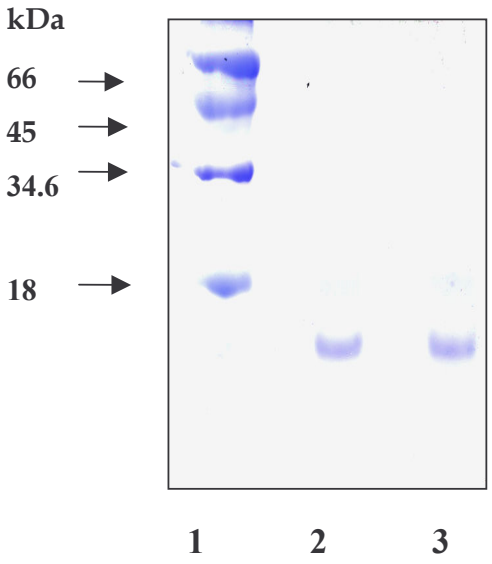

Fig. 18: SDS-PAGE of isolated β-globulin from sesame total protein. (Lane 1) Molecular markers -bovine serum albumin (66 kDa), ovalbumin (45 kDa), carboxypeptidase (34.6 kDa), β-lactoglobulin (18 kDa); (Lane 2) Isolated β-globulin and (Lane 3) Standard protein - α-lactalbumin (14.2 kDa).

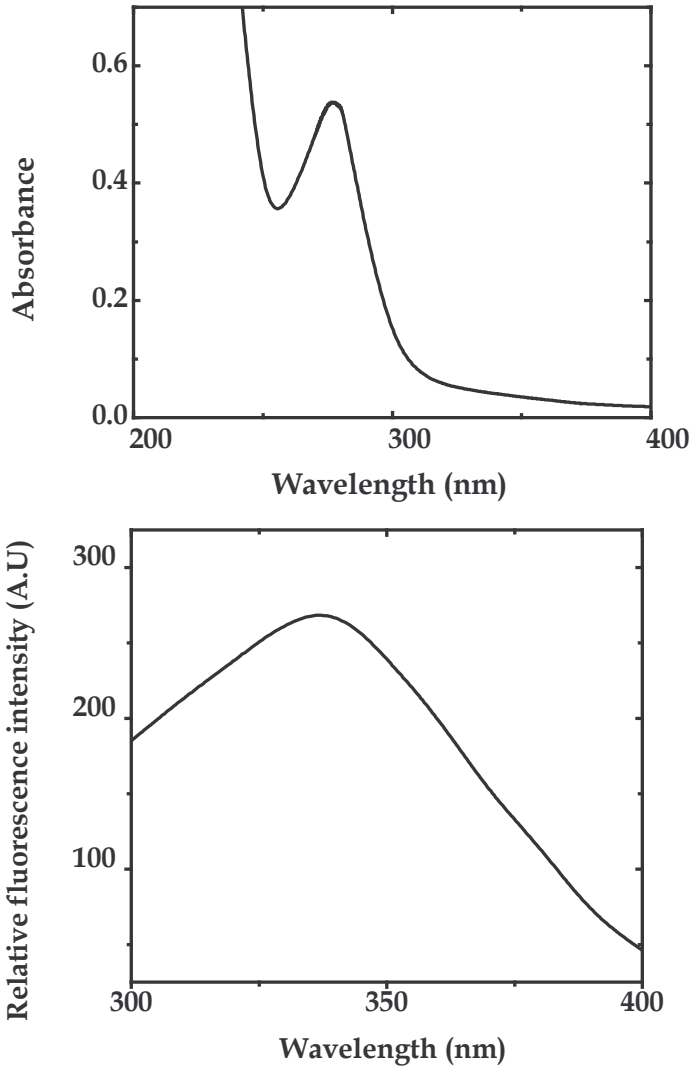

Fig. 19: (A) UV-absorption spectra and (B) fluorescence emission spectra of β - globulin isolated from sesame total proteins in 0.06M phosphate buffer, pH 7.5 containing 1M NaCl.

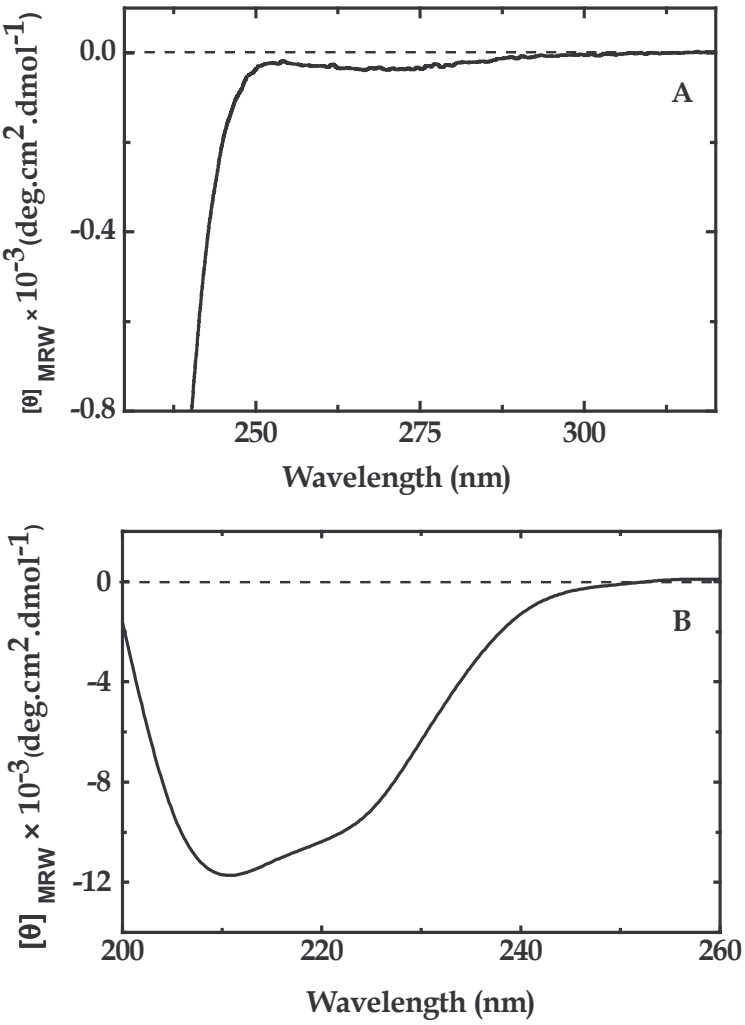

Fig. 20: CD spectra of native isolated β-globulin from sesame total protein in 0.06 M phosphate buffer containing 1M NaCl at pH 7.5. (A) Near-UV CD spectrum and (B) Far- UV CD spectrum.

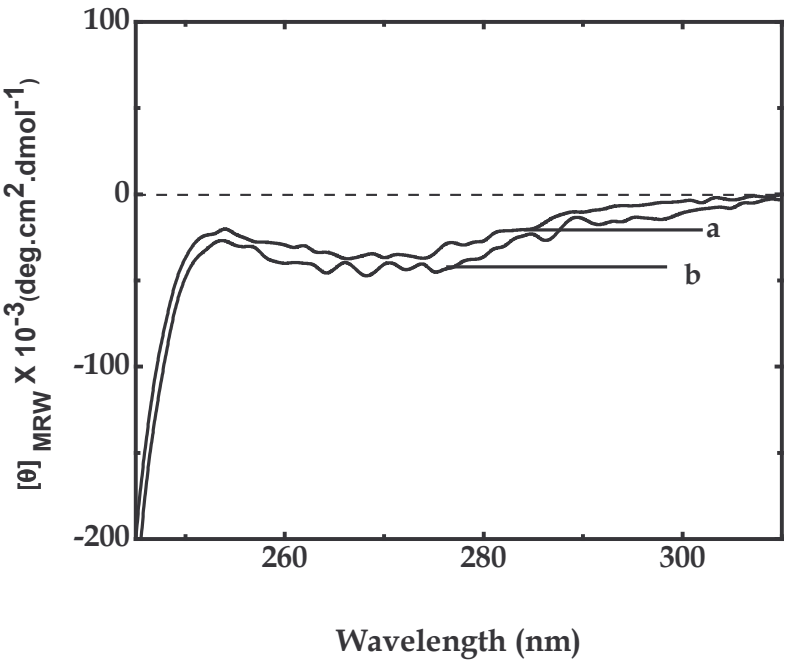

Fig. 21: Near-UV CD spectra of β-globulin in 0.06M phosphate 1M NaCl buffer at pH 7.5. (a) In buffer and (b) in 30% sorbitol.

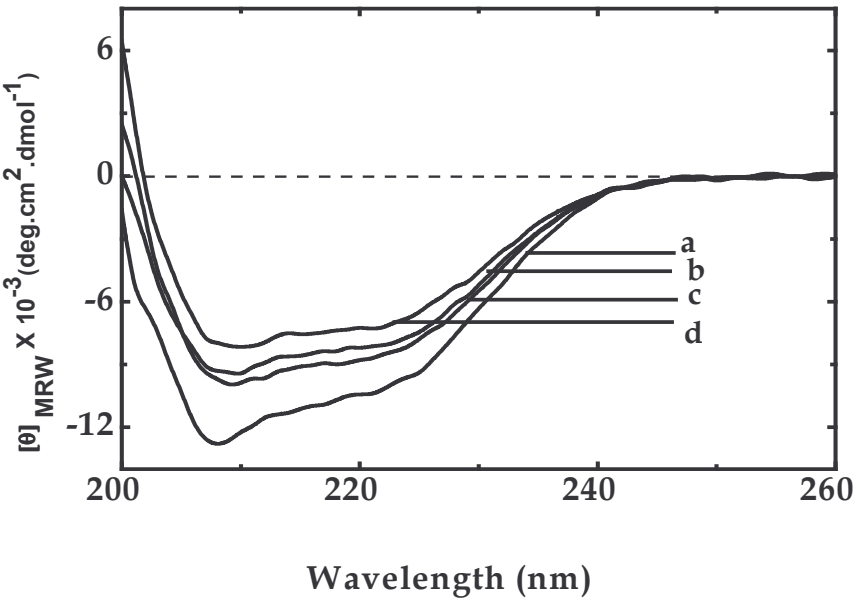

Fig. 22: Far-UV CD spectra of β-globulin in 0.06M phosphate buffer containing 1M NaCl at pH 7.5. (a) In buffer, (b) in 10%, (c) 20% and (d) in 30% sorbitol.

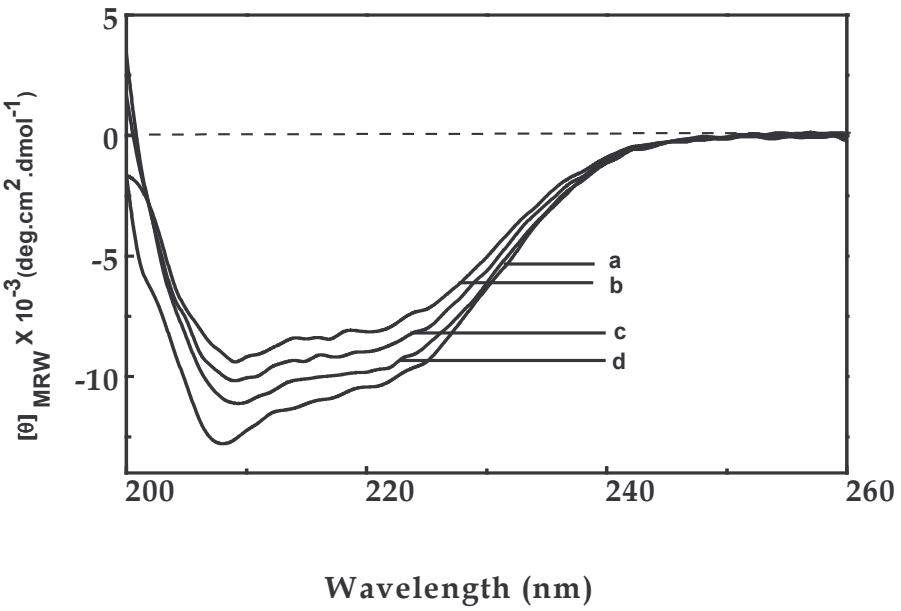

Fig. 23: Far-UV CD spectra of β-globulin in 0.06M phosphate buffer containing 1M NaCl at pH 7.5. (a) In buffer, (b) in 10%, (c) in 20% and (d) in 30% glycerol.

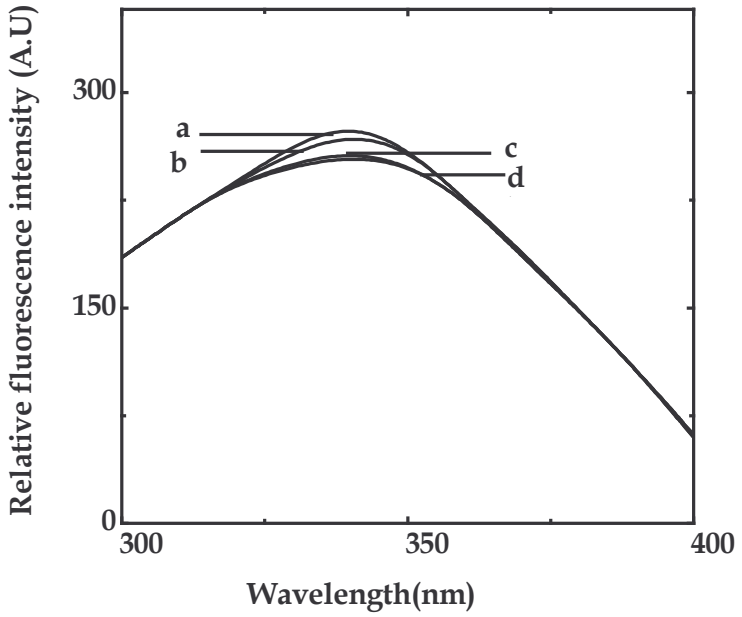

Fig. 24: Fluorescence emission spectra of β-globulin in 0.06M phosphate buffer containing 1M NaCl at pH 7.5. The protein was excited at 280 nm and the emission spectra were recorded in the range of 300-400 nm. (a) In buffer, (b) in 10%, (c) in 20% and (d) in 30% glycerol.

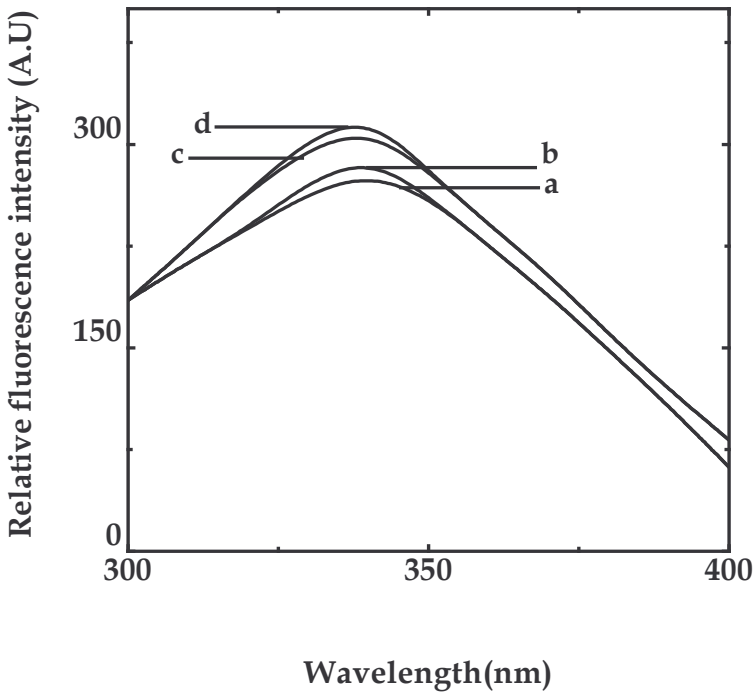

Fig. 25: Fluorescence emission spectra of β-globulin in 0.06M phosphate buffer containing 1M NaCl at pH 7.5. The protein was excited at 280 nm and the emission spectra were recorded in the range of 300-400 nm. (a) In buffer, (b) in 10%, (c) in 20% and (d) in 30% sorbitol.

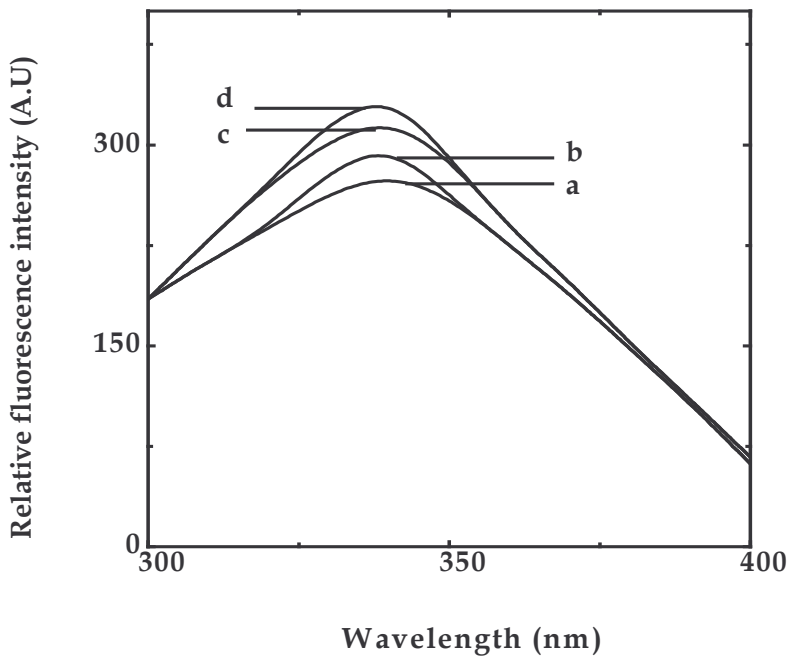

Fig. 26: Fluorescence emission spectra of β-globulin in 0.06M phosphate buffer containing 1M NaCl at pH 7.5. The protein was excited at 280 nm and emission spectra were recorded in the range of 300-400 nm. (a) In buffer, (b) in 10%, (c) in 20% and (d) in 30% sucrose.

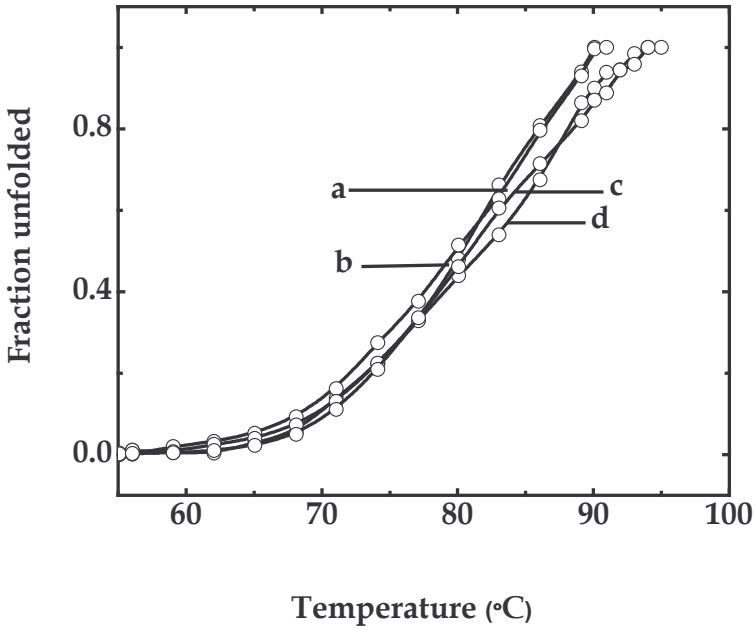

Fig. 27: Apparent thermal denaturation curves of β-globulin in the presence different cosolvents in 0.06 M phosphate buffer containing 1M NaCl at pH 7.5. (a) In buffer, (b) in 10% sorbitol, (c) in 10% glycerol and (d) in 10% sucrose. The absorption spectra were recorded as a function of temperature at 287nm.

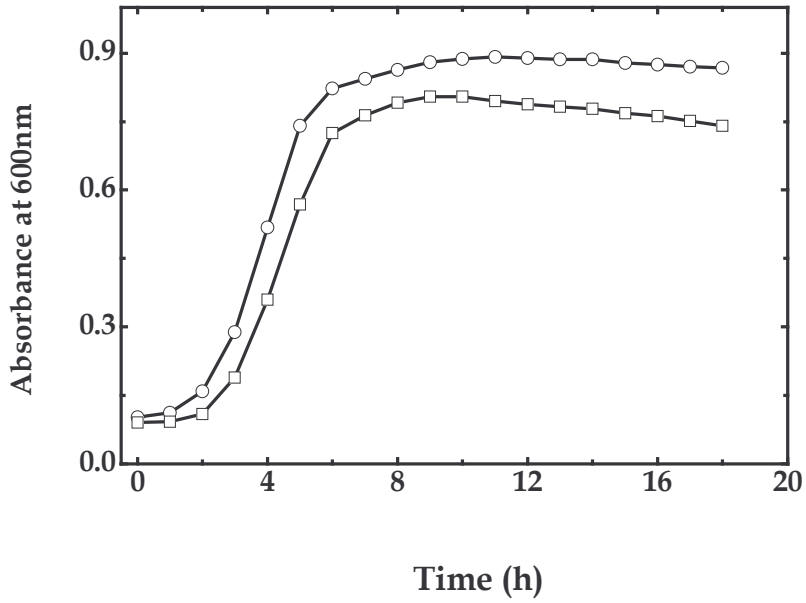

Fig. 28: Antimicrobial activity against *Escherichia coli* of the α-lactalbumin hydrolyzate obtained with double enzyme combination namely fungal protease and pancreatin. (o) Control (□) α-lactalbumin hydrolysate.

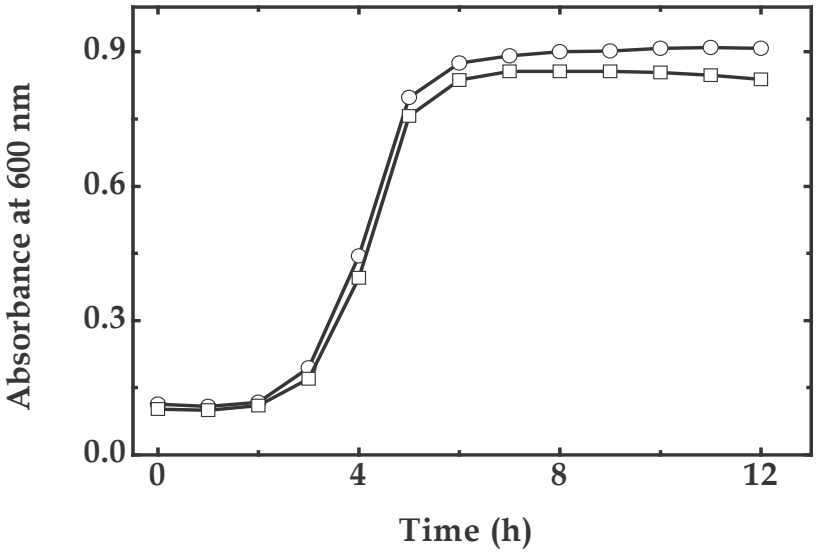

Fig. 29: Antimicrobial activity against *Bacillus cereus* of the α-lactalbumin hydrolyzate obtained with double enzyme combination namely fungal protease and pancreatin. (o) Control and (□) α-lactalbumin hydrolysate.

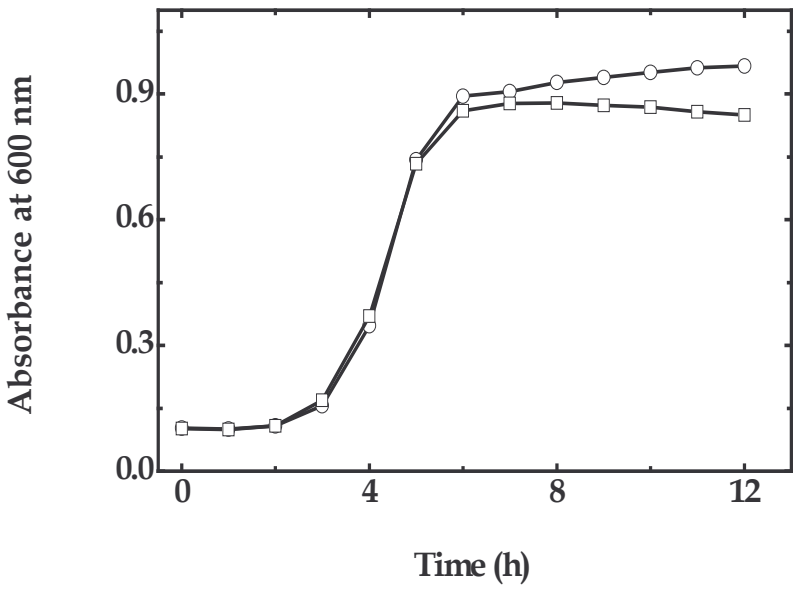

Fig. 30: Antimicrobial activity against *Listeria monocytogenes* of the α-lactalbumin hydrolysate obtained with double enzyme combination namely fungal protease and pancreatin. (○) Control and (□) α-lactalbumin hydrolysate.

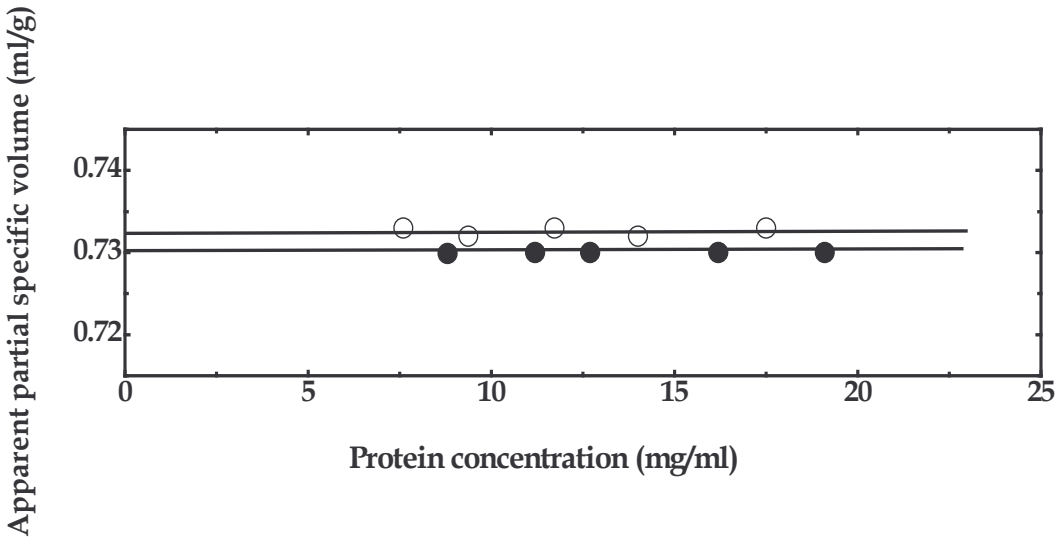

Fig. 31: Apparent partial specific volume of α-lactalbumin in 0.01M Tris-HCl buffer of pH 7.5. (•) In isomolal and (o) in isopotential conditions at 20°C.

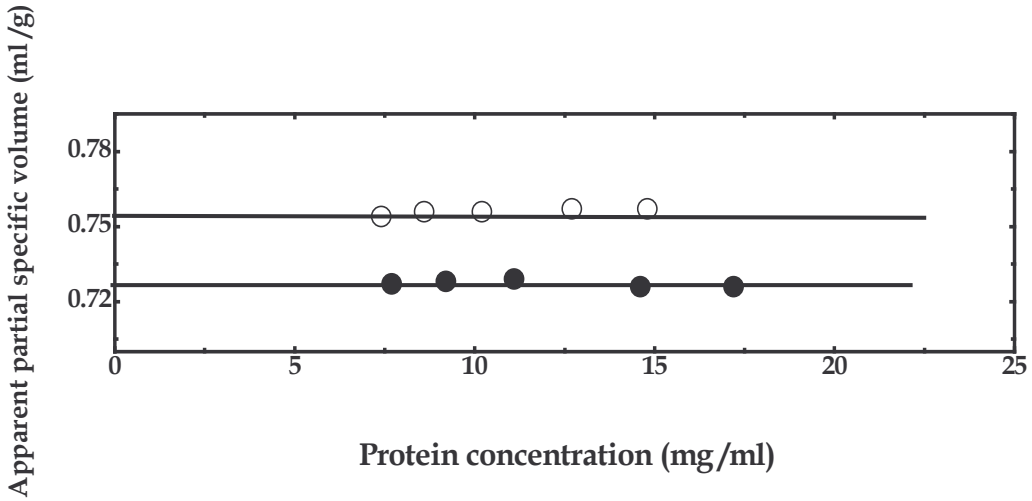

Fig. 32: Apparent partial specific volume of α-lactalbumin in 0.01M Tris–HCl buffer, pH 7.5 in presence of 10%, sorbitol concentrations under (●) isomolal and (o) isopotential conditions at 20°C.

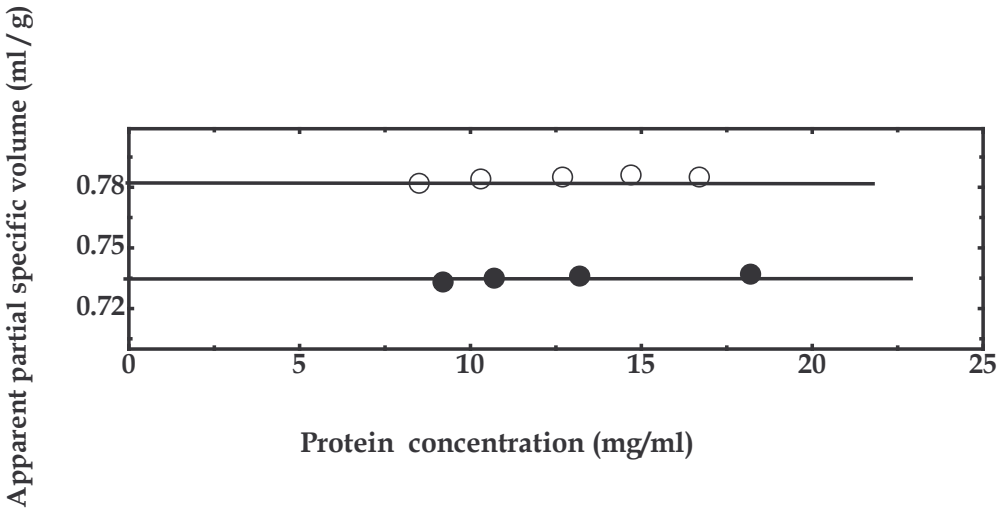

Fig. 33: Apparent partial specific volume of α-lactalbumin in 0.01M Tris–HCl buffer, pH 7.5 in presence of 20% sorbitol concentrations under (•) isomolal and (o) isopotential conditions at 20°C.

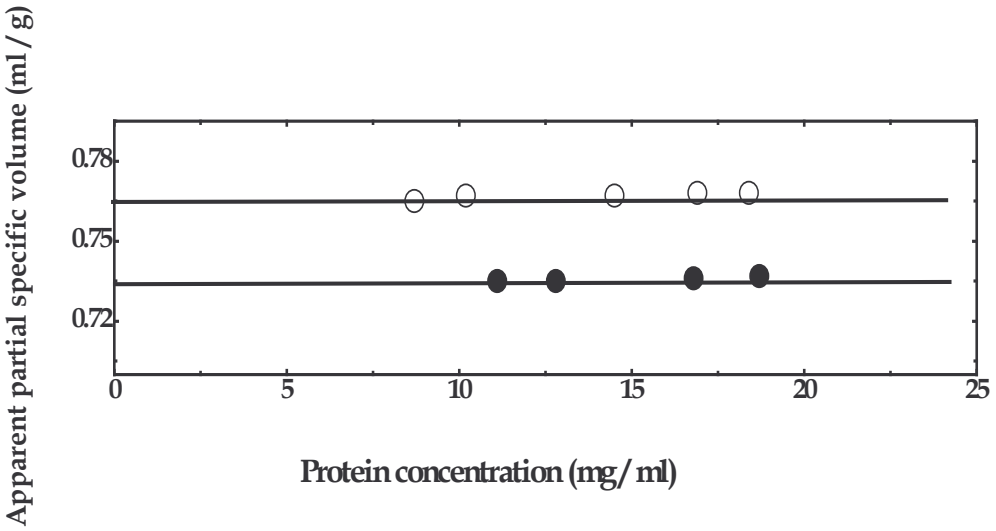

Fig. 34: Apparent partial specific volume of α-lactalbumin in 0.01M Tris–HCl buffer, pH 7.5 in presence of 45% sorbitol concentrations under (•) isomolal and (o) isopotential conditions at 20°C.

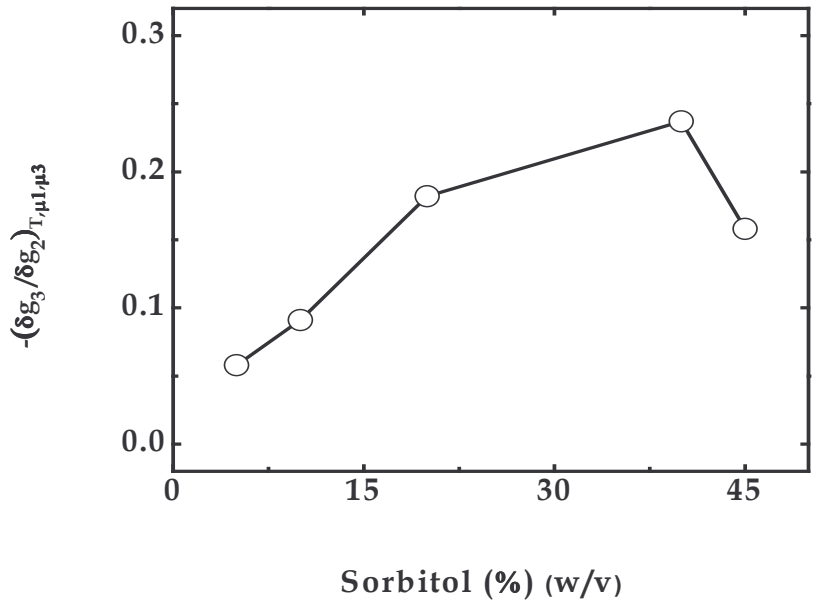

Fig. 35: Effect of sorbitol on the preferential interaction parameter of α-lactalbumin on g / g basis in the concentration range of 0 - 45% (w/v).

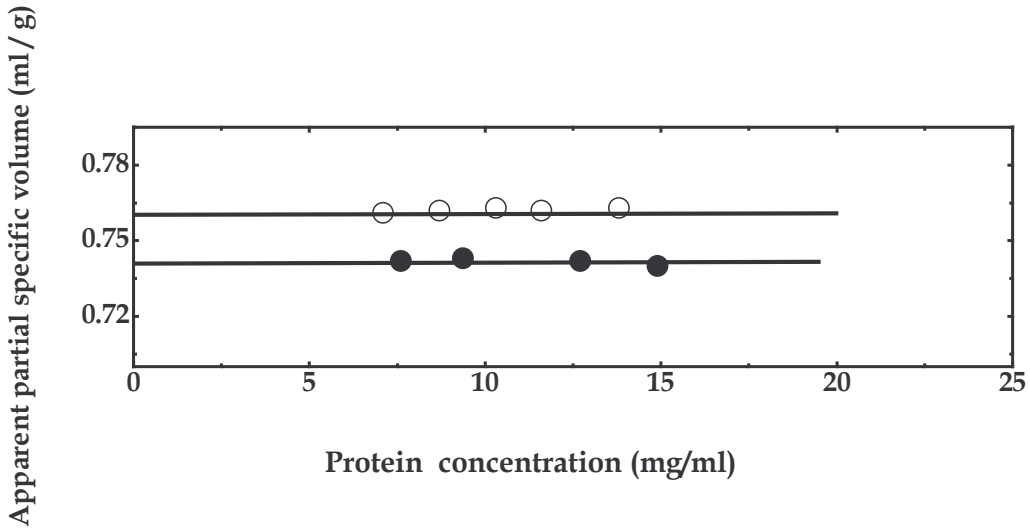

Fig. 36: Apparent partial specific volume of α-lactalbumin in 0.01M Tris–HCl buffer, pH 7.5 in presence of 10% glycerol concentrations under (•) isomolal and (o) isopotential conditions at 20°C.

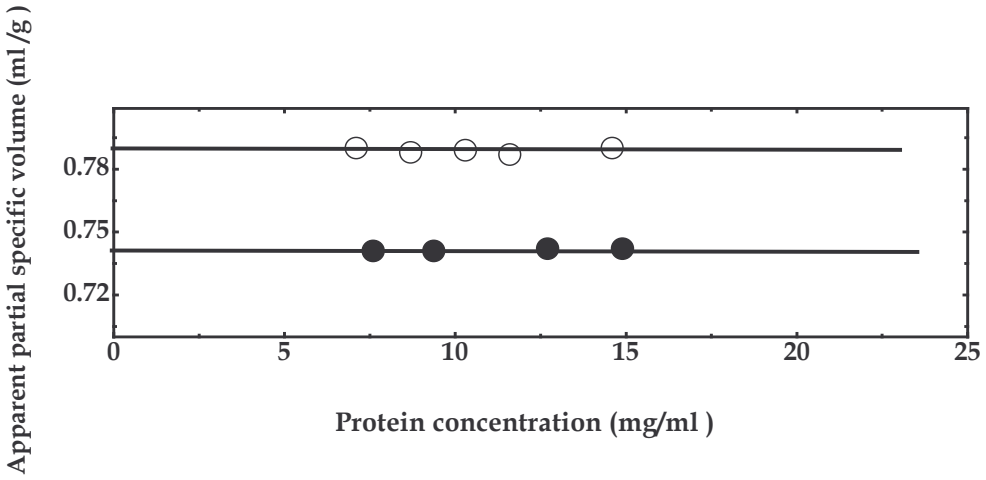

Fig. 37: Apparent partial specific volume of α-lactalbumin in 0.01M Tris–HCl buffer, pH 7.5 in presence of 30% glycerol concentrations under (o) isomolal and (•) isopotential conditions at 20°C.

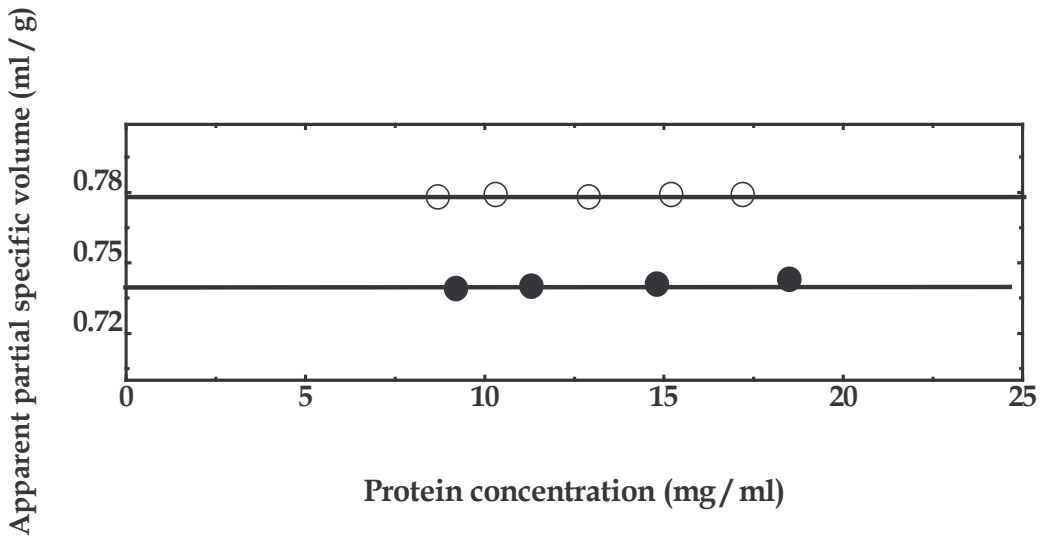

Fig. 38: Apparent partial specific volume of α-lactalbumin in 0.01M Tris–HCl buffer, pH 7.5 in presence of 45% glycerol concentrations under (•) isomolal and (o) isopotential conditions at 20°C.

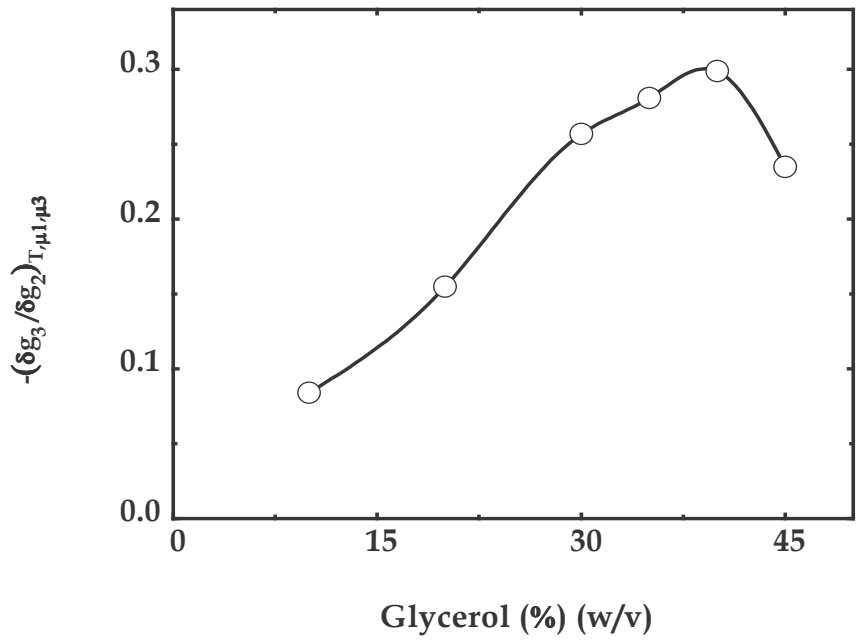

Fig. 39: Effect of glycerol on the preferential interaction parameter of α-lactalbumin on g/g basis in the concentration range of 0-45% (w/v).

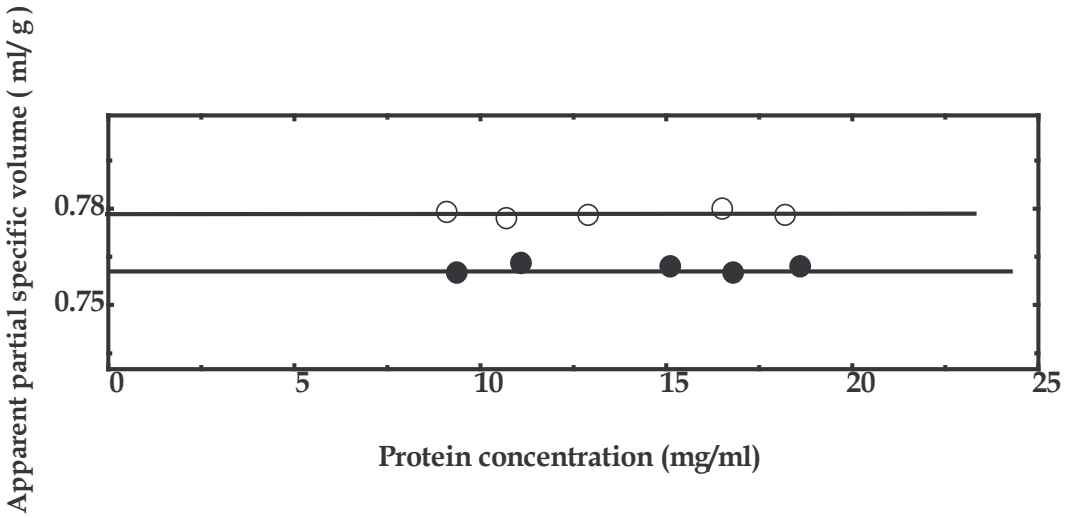

Fig 40: Apparent partial specific volume of α-lactalbumin in 0.01M Tris–HCl buffer, pH 7.5 in presence of 10% sucrose under (•) isomolal and (o) isopotential conditions at 20°C.

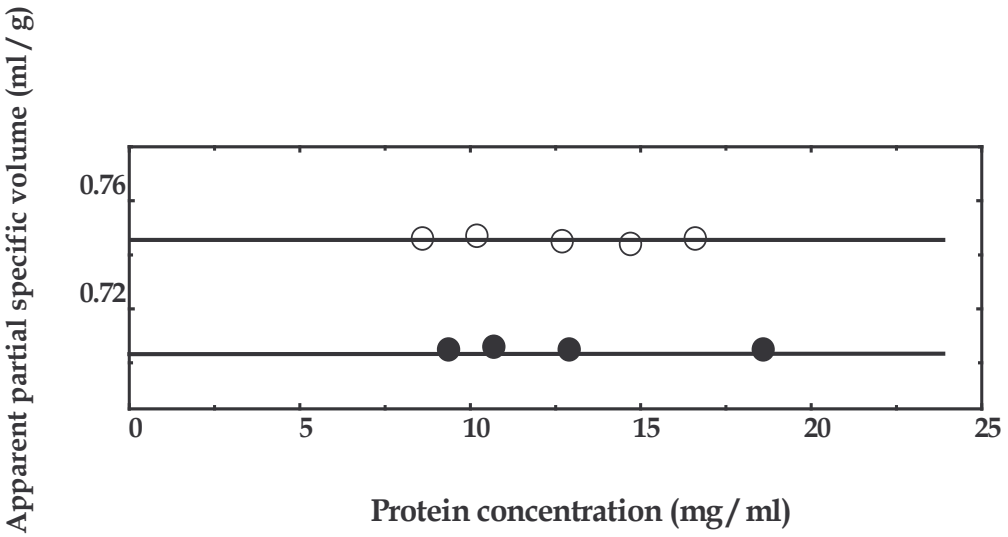

Fig. 41: Apparent partial specific volume of α-lactalbumin in 0.01M Tris–HCl buffer, pH 7.5 in presence of 40% sucrose under (•) isomolal and (o) isopotential conditions at 20°C.

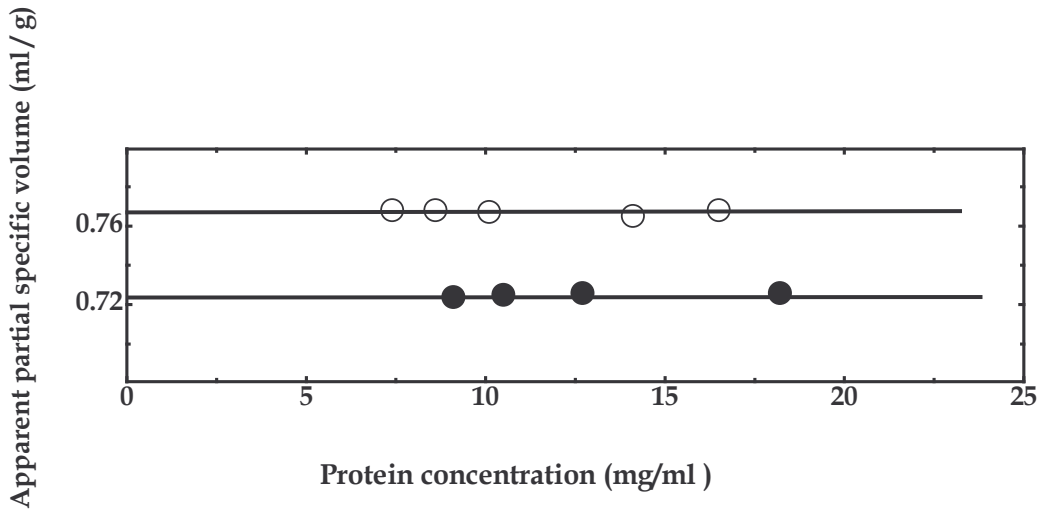

Fig. 42: Apparent partial specific volume of α-lactalbumin in 0.01M Tris–HCl buffer, pH 7.5 in presence of 45% sucrose under (•) isomolal and (o) isopotential conditions at 20°C.

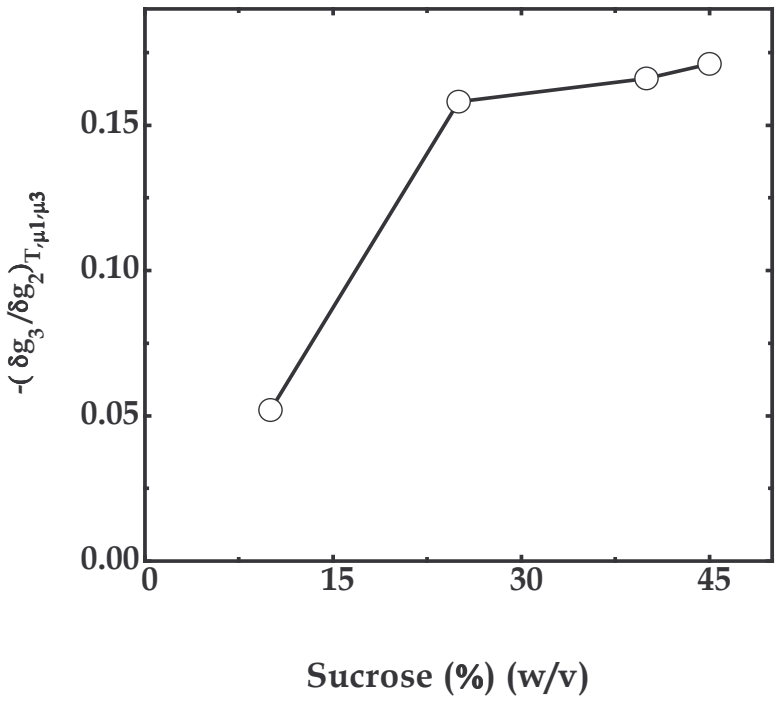

Fig. 43: Effect of sucrose on the preferential interaction parameter of α-lactalbumin on g / g basis in the concentration range of 0 - 45% (w/v).

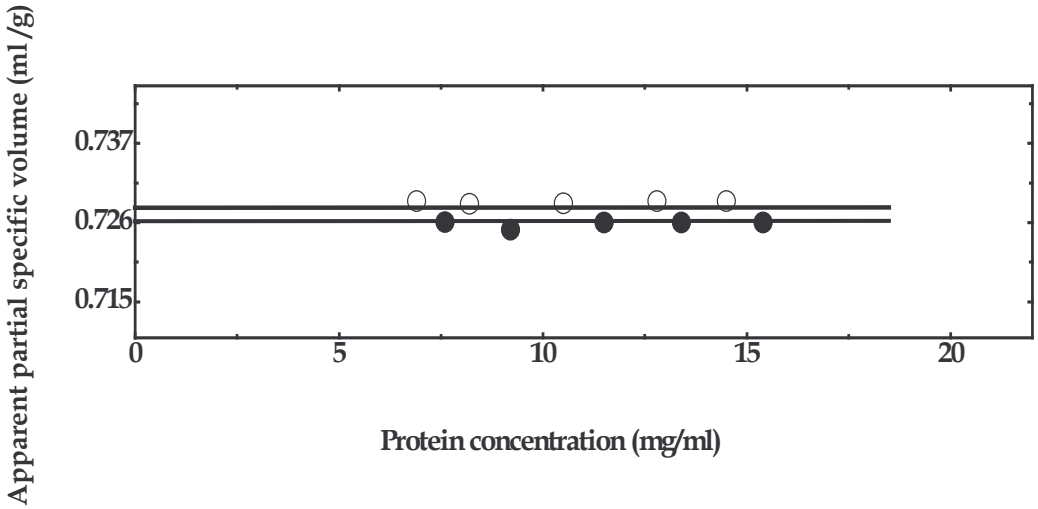

Fig. 44: Apparent partial specific volume of β-globulin in 0.06M phosphate buffer containing 1M NaCl at pH 7.5, under (•) isomolal and (o) isopotential conditions.

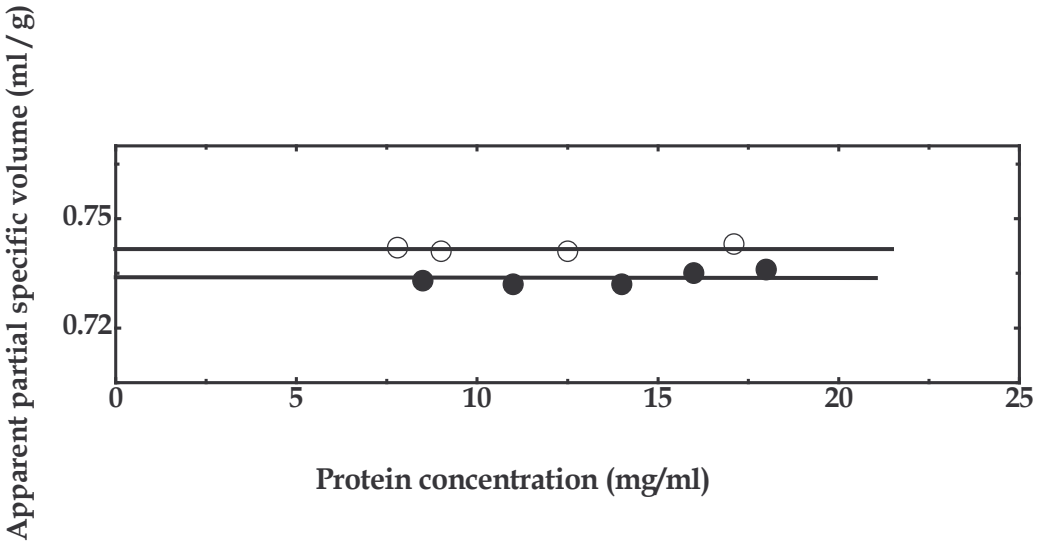

Fig. 45: Apparent partial specific volume of β-globulin in 0.06M phosphate buffer containing 1M NaCl at pH 7.5 in the presence of 10% sorbitol under (•) isomolal and (o) isopotential conditions at 20°C.

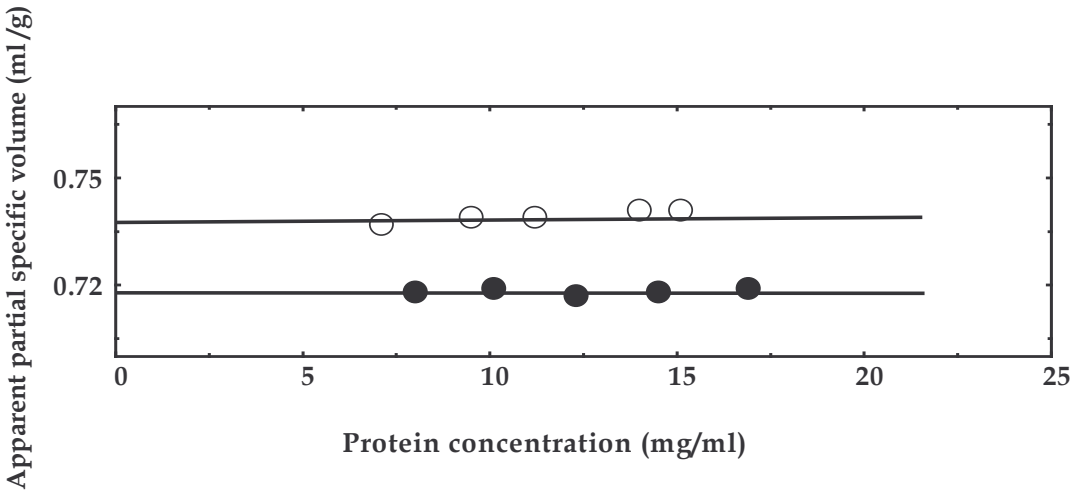

Fig. 46: Apparent partial specific volume of β-globulin in 0.06M phosphate buffer containing 1M NaCl at pH 7.5 in the presence of 20% sorbitol under (●) isomolal and (o) isopotential conditions at 20°C.

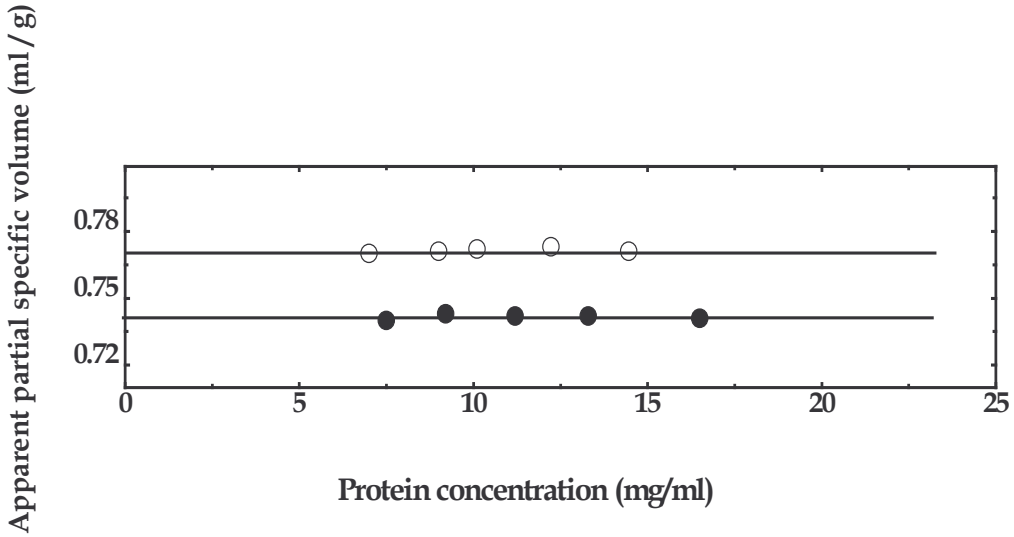

Fig. 47: Apparent partial specific volume of β-globulin in 0.06M phosphate buffer containing 1M NaCl at pH 7.5 in the presence of 40% sorbitol under (•) isomolal and (o) isopotential conditions at 20°C.

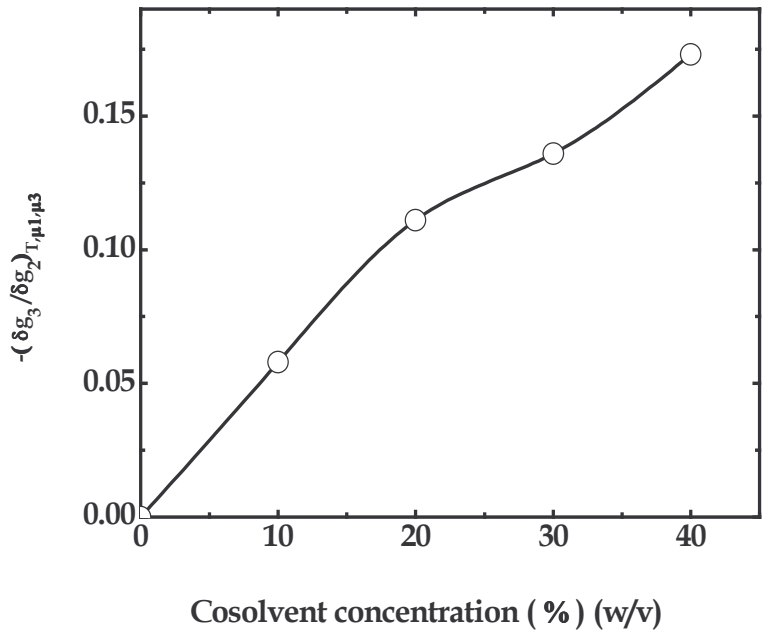

Fig. 48: Preferential interaction parameters of β-globulin in 0.06M phosphate buffer containing 1M NaCl at pH 7.5 in the presence of different concentrations of sorbitol.

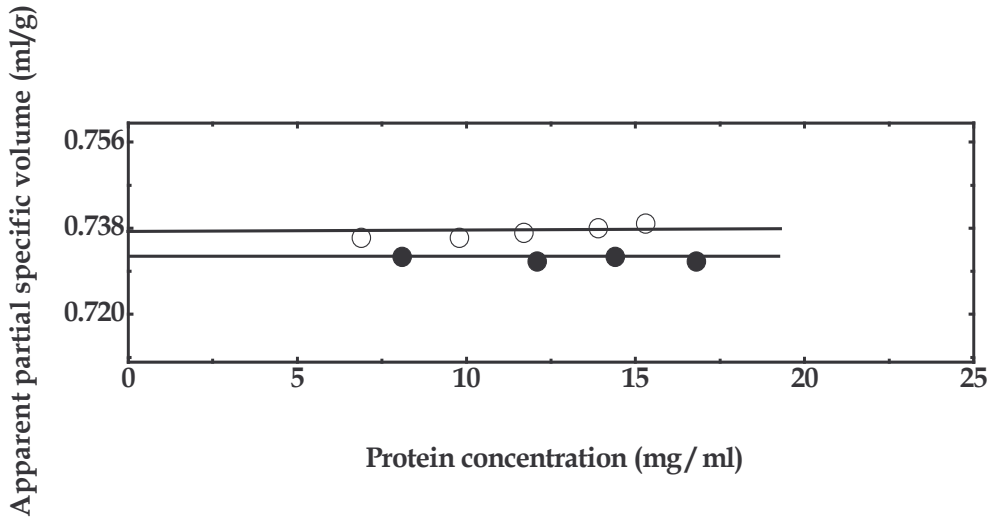

Fig. 49: Apparent partial specific volume of β-globulin in 0.06M phosphate buffer containing 1M NaCl at pH 7.5 in the presence of 10% glycerol under (•) isomolal and (o) isopotential conditions at 20°C.

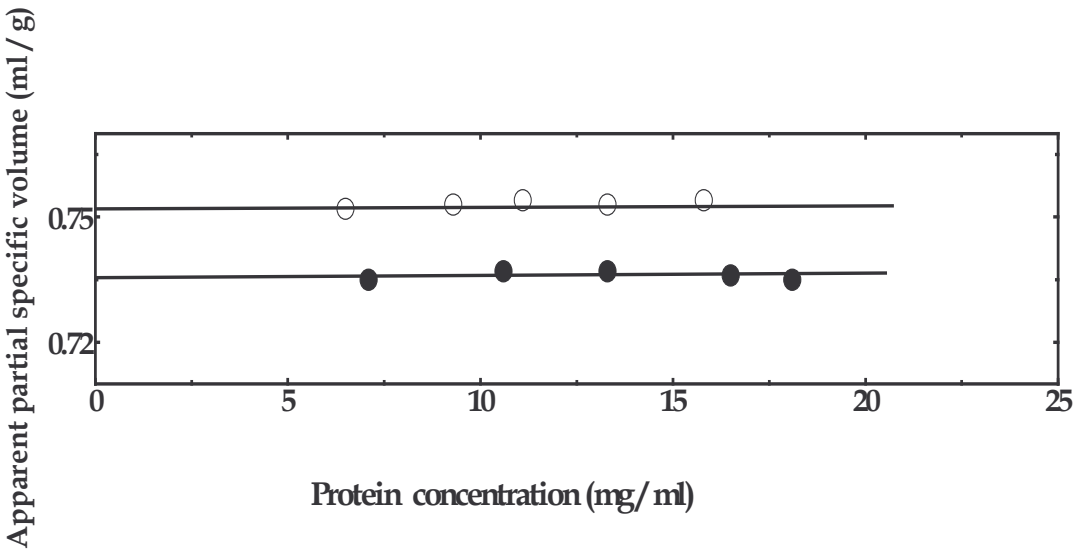

Fig. 50: Apparent partial specific volume of β-globulin in 0.06M phosphate buffer containing 1M NaCl at pH 7.5 in the presence of 20% glycerol under (•) isomolal and (o) isopotential conditions at 20°C.

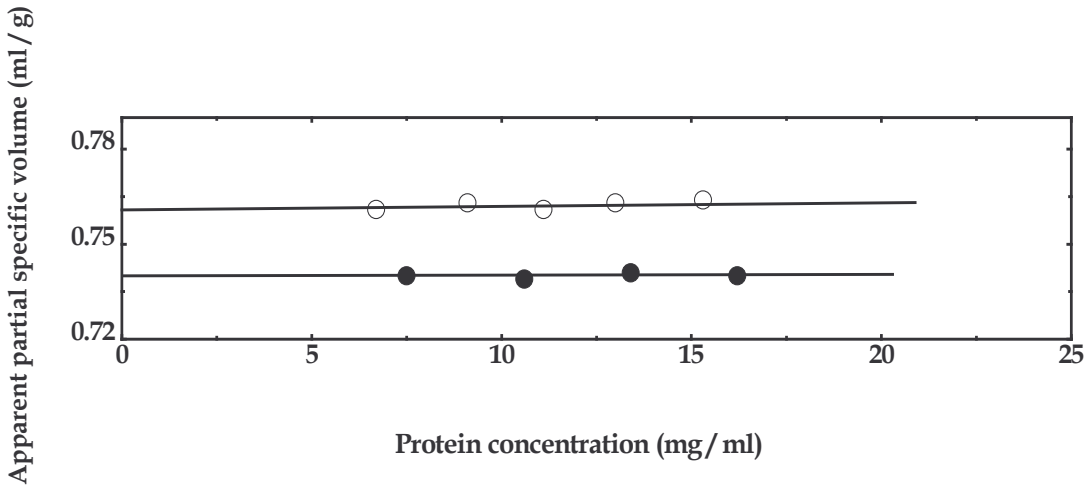

Fig. 51: Apparent partial specific volume of β-globulin in 0.06M phosphate buffer containing 1M NaCl at pH 7.5 in the presence of 30% glycerol under (●) isomolal and (o) isopotential conditions at 20°C.

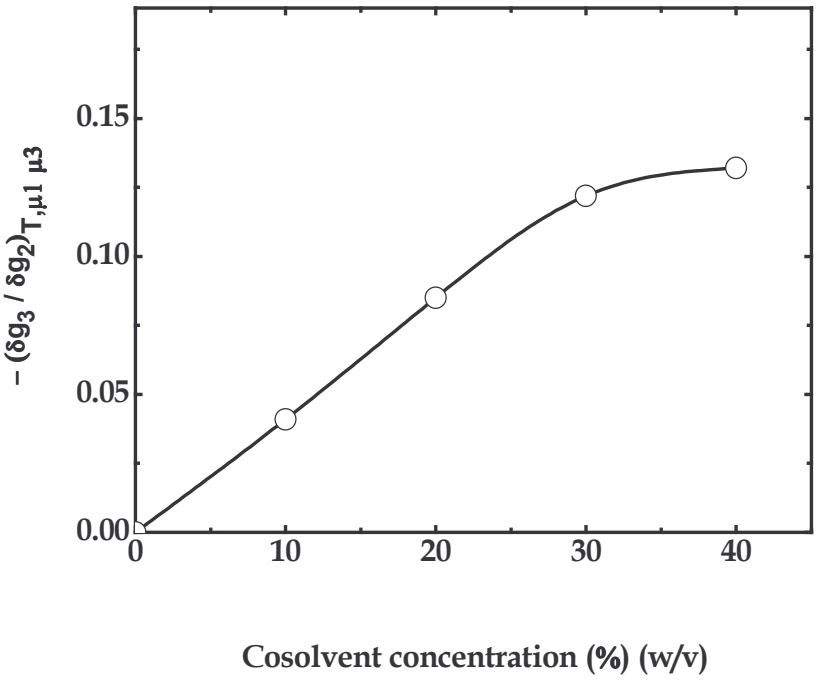

Fig. 52: Preferential interaction parameters of β-globulin in 0.06M phosphate buffer containing 1M NaCl at pH 7.5 in the presence of different concentrations of glycerol.

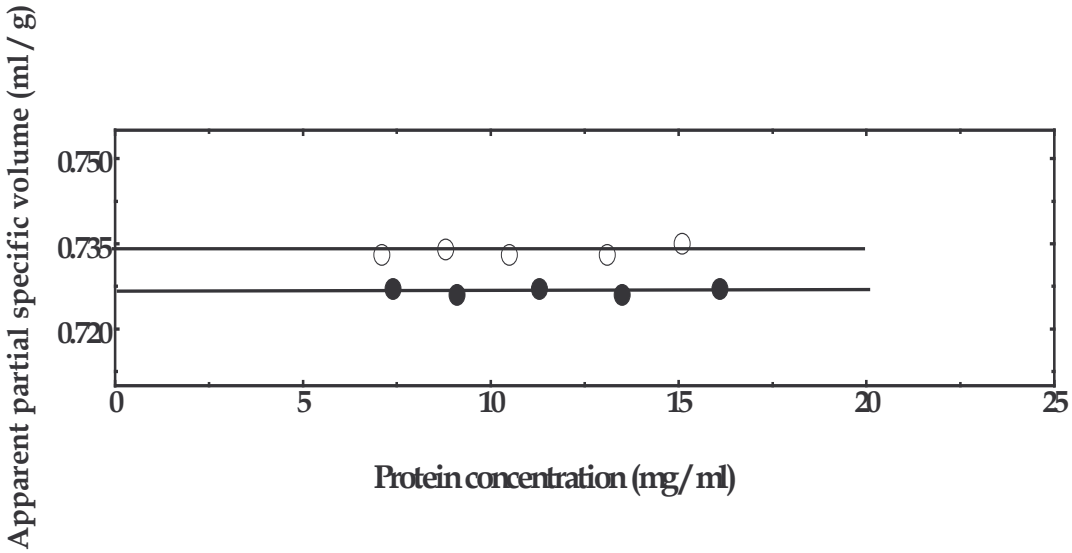

Fig. 53: Apparent partial specific volume of β-globulin in 0.06M phosphate buffer containing 1M NaCl at pH 7.5 in the presence of 10% sucrose under (•) isomolal and (o) isopotential conditions at 20°C.

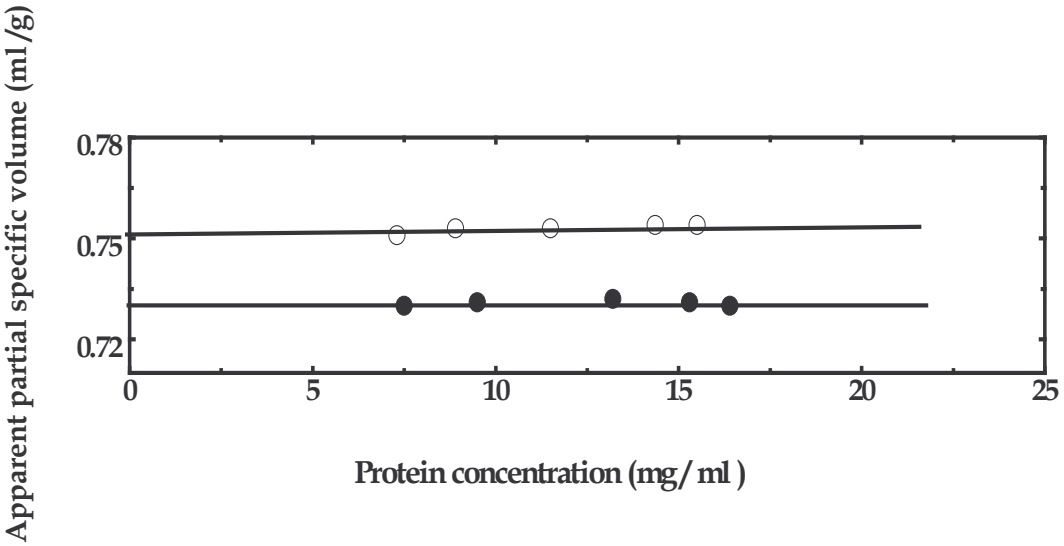

Fig. 54: Apparent partial specific volume of β-globulin in 0.06M phosphate buffer containing 1M NaCl at pH 7.5 in the presence of 20% sucrose under (•) isomolal and (o) isopotential conditions at 20°C.

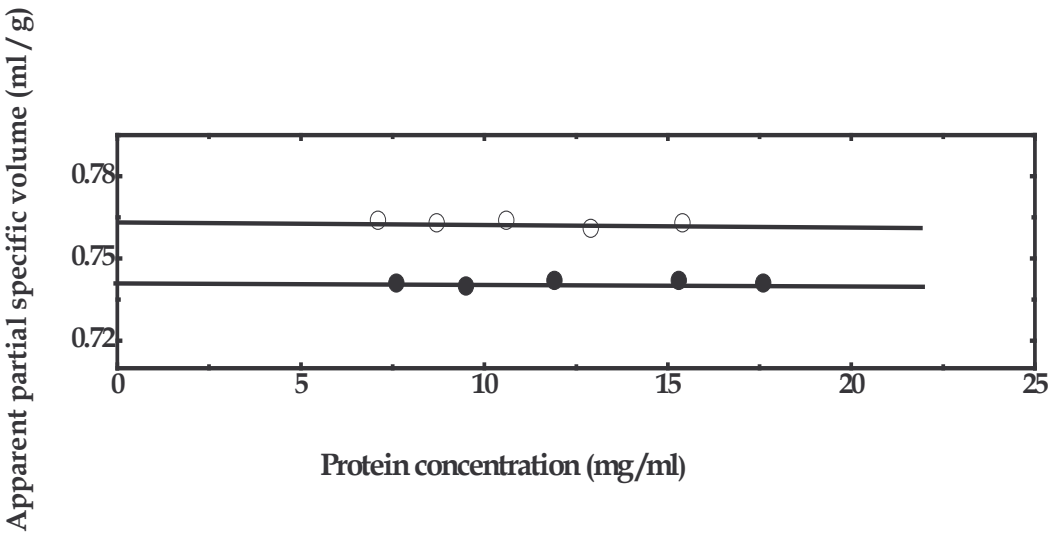

Fig. 55: Apparent partial specific volume of β-globulin in 0.06M phosphate buffer containing 1M NaCl at pH 7.5 in the presence of 30% sucrose under (•) isomolal and (o) isopotential conditions at 20°C.

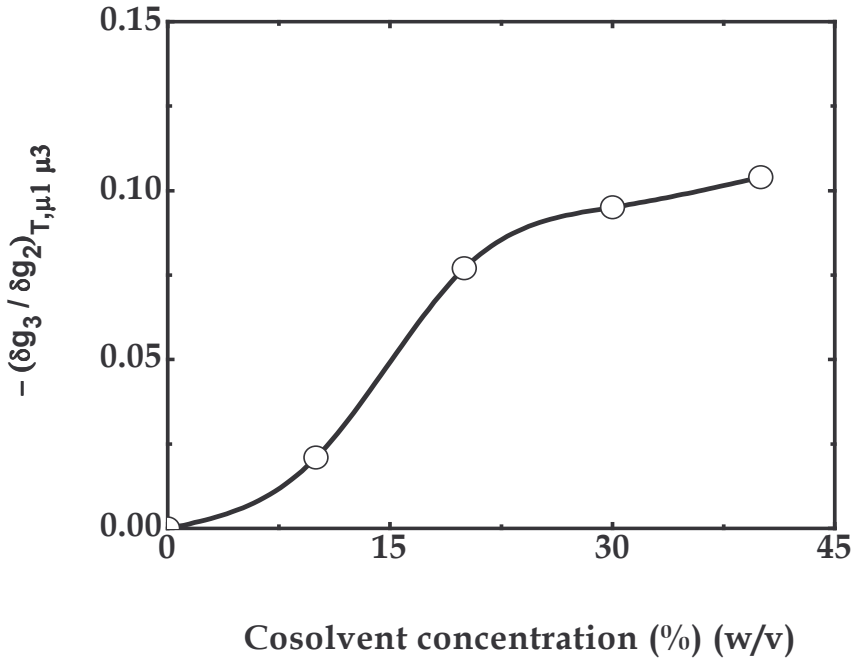

Fig. 56: Preferential interaction parameters of β-globulin in 0.06M phosphate buffer containing 1M NaCl at pH 7.5 in the presence of different concentrations of sucrose.

Table 1: Major categories of bioactivities in whey with their general effects and causative agents.

Bioactivity	Specific effects	Attributed to
Antimicrobial	Inhibition of coliform and pathogen growth	Lactoferrin, lactoperoxidase, lactoferricin
Antiviral	Inhibition of viral infection	Lactoferrin, whey protein concentrate, lactoferricin
Anticarcinogenic	Control the cellular glutathione levels	Lactoferrin, whey protein concentrate, α-Lactalbumin
Antioxidant	Inhibit the formation of free radicals	Lactoferrin, low molecular weight whey protein and peptides
Anti-inflammation	Reduces the release of cytokines by lymphocytes and macrophages	Lactoferrin, Lactoferrin peptides
Immunoregulation	Modulation of lymphocyte differentiation, Enhanced killer cell activity	Lactoferrin, whey protein concentrate, α-Lactalbumin, casein glycomacropeptide
Gastrointestinal functions and digestion	Altered rate of gastric emptying, decreased intestinal motility	Opioid peptides (α-lactorphin), Opioid antagonist peptide (lactoferroxin)

Source: Kinsella and Whitehead (1989); Shah (2000).

Table 2: Physico-chemical characteristics of whey proteins of bovine milk.

Protein component	Molecular mass (Da)	Approximate content in skim milk protein (g/l)
β-Lactoglobulin	18600	7 - 12
α-Lactalbumin	14200	2 - 5
Bovine serum albumin	66000	0.7 - 1.3
Immunoglobulin	15 – 96 x 10^4	1.9 - 3.3
Proteose peptones		
β-CN-5P (1-105)	11500	--
β-CN-5P (1-107)	13000	--
β-CN-4P (1-28)	4100	--
Lysozyme	18000	0.13 - 0.32
Lactoferrin	76500	0.02 - 0.35

Source: Kinsella and Whitehead (1989); Pihlanto and Korhonen (2003).

Table 3: Amino acid composition of isolated α-lactalbumin protein from bovine milk.

Amino acids	Gram (%)
Asp	17.0
Glu	15.7
Ala	1.7
Cys	5.8
Gly	3.3
His	3.2
Ile	7.2
Leu	12.0
Lys	10.4
Met	0.8
Phe	4.3
Pro	2.9
Ser	3.0
Thr	4.3
Tyr	0.8
Val	0.7
Arg	1.2
Trp*	5.3

* Estimated by N-bromosuccinimide method (Spande and Witkop, 1969 a, b)

Table 4: Secondary structural parameters of α–La in presence of different concentrations of cosolvents.

Cosolvents	Concentration (w/v)	Secondary structures (%)		
		α-Helix	β-Structures	Aperiodic
Control*		21±1	46±1	33±1
Sorbitol	10	23±1	43±2	34±1
	20	21±1	49±1	31±1
	30	26±1	38±1	36±2
Glycerol	10	21±2	49±1	30±1
	20	27±1	38±1	34±1
	30	28±2	34±1	37±1
Sucrose	10	22±1	43±2	35±1
	20	20±1	43±1	36±2
	30	26±2	38±1	36±1

*α–Lactalbumin in 0.01M Tris-HCl buffer of pH 7.5. The various concentrations of cosolvents were prepared in the same buffer.

Table 5: Apparent transition temperature (T_m) values of α-lactalbumin in presence of different concentrations of cosolvents recorded at 287nm.

	Apparent T_m (°C)		
Cosolvent (%) (w/v)	**Sorbitol**	**Glycerol**	**Sucrose**
Control*	65±1	65±1	65±1
10%	67 ±1	66±1	66±1
20%	70 ±2	67±1	67±1
30%	71 ±1	67±1	69±1
40%	71 ±1	68±1	71±1

*α–Lactalbumin in 0.01M Tris-HCl buffer of pH 7.5. The various concentrations of cosolvents were prepared in the same buffer.

Table 6: Amino acid composition of β-globulin isolated from sesame seed.

Amino acids	Gram (%)
Asp	3.1
Glu	42.0
Ala	3.7
Cys	3.0
Gly	2.0
His	0.4
Ile	1.3
Leu	6.0
Lys	2.0
Met	1.1
Phe	2.4
Pro	3.2
Ser	3.0
Thr	1.3
Tyr	1.1
Val	2.0
Arg	20.0
Trp*	2.0

* Estimated by N-bromosuccinimide method (Spande and Witkop, 1969 a, b)

Table 7: Secondary structural parameters of β –globulin in presence of different concentrations of cosolvents.

Cosolvents	Concentration (w/v)	Secondary structure (%)		
		α-Helix	β-Structure	Aperiodic
Control*	0	26±1	38±1	36±1
Glycerol	10	25±1	41±1	33±1
	20	28±1	42±1	30±1
	30	27±2	47±1	25±1
Sorbitol	10	24±2	40±1	35±1
	20	27±1	33±1	29±1
	30	29±2	39±1	31±2
Sucrose	10	25±1	41±1	34±1
	20	32±1	43±1	24±2
	30	27±1	39±1	32±1

*β-Globulin in 0.06M Phosphate buffer pH 7.5 containing 1M NaCl. The various concentrations of cosolvents were prepared in the same buffer.

Table 8: Degree of hydrolysis obtained for different protein hydrolysates.

Enzyme used	Substrate	% Degree of hydrolysis for the hydrolysates
Fungal protease and pancreatin	Whey protein concentrate	38 ± 3%
Fungal protease and pancreatin	α-Lactalbumin	26 ± 2%
Subtilisin	β-Globulin	9 ± 2%

Table 9: Bioactivities of the whey protein concentrate hydrolysate and respective IC_{50} values

Assay	IC_{50} values	Standard	IC_{50} values
Antioxidant activity *(Radical scavenging activity using DPPH)*	0.11 ± 0.01 mg/ml	Ascorbic acid	0.191 ± 0.01 mg/ml
Angiotensin converting enzyme	600 ± 35 µg/ml	Ramipril	215 ± 15 µg/ml

Table 10: Bioactivities of the α-lactalbumin hydrolysate and respective IC_{50} values

Assay	IC_{50} values	Standard	IC_{50} values
Antioxidant activity *(Radical scavenging activity using DPPH)*	0.18 ± 0.02 mg/ml	Ascorbic acid	0.191 ± 0.01 mg/ml
Angiotensin converting enzyme	410 ± 30 μg/ml	Ramipril	215 ± 15 μg /ml

Table 11: Bioactivities of the β-globulin hydrolysate and respective IC_{50} values.

Assay	IC_{50} values	Standard	IC_{50} values
Antioxidant activity *(Radical scavenging activity using DPPH)*	0.20 ± 0.02mg/ml	Ascorbic acid	0.191 ± 0.01 mg/ml
Angiotensin converting enzyme	380 ± 25 μg/ml	Ramipril	215 ± 15 μg/ml

Table 12: Apparent partial specific volume of α-lactalbumin in presence of different concentrations of sorbitol at 20°C.

Partial specific volume	Concentration of sorbitol (%) (w/v)				
	5	**10**	**20**	**40**	**45**
ϕ_2^o (ml/g)	0.736 ± 0.001	0.727 ± 0.001	0.733 ± 0.001	0.723 ± 0.001	0.734 ± 0.002
$\phi_2'^o$ (ml/g)	0.755 ± 0.001	0.754 ± 0.001	0.782 ± 0.002	0.774 ± 0.001	0.765 ± 0.001

Table 13: Preferential interaction parameters of α-lactalbumin in presence of different concentrations of sorbitol at 20 °C.

Interaction Parameters	Concentration of sorbitol (%) (w/v)				
	5	**10**	**20**	**40**	**45**
g_3 (g/g)	0.051	0.107	0.233	0.566	0.675
m_3 (mol of solvent per 1000g water)	0.28	0.59	1.28	3.11	3.71
$\delta g_3/\delta g_2$ (g/g)	-0.058 ± 0.015	-0.091 ± 0.021	-0.182 ± 0.030	-0.237 ± 0.025	-0.158 ± 0.015
$\delta m_3/\delta m_2$ (mol/mol)	-4.60 ± 1.2	-7.12 ± 1.6	-14.18 ± 2.31	-18.43 ± 2.0	-12.31 ± 1.8

Table 14: Apparent partial specific volume of α-lactalbumin in presence of different concentrations of glycerol at 20 °C.

Partial specific volume	Concentration of glycerol (%) (w/v)					
	10	**20**	**30**	**35**	**40**	**45**
ϕ_2^o (ml/g)	0.742 ± 0.002	0.733 ± 0.001	0.741 ± 0.001	0.703 ± 0.003	0.718 ± 0.001	0.739 ± 0.002
$\phi_2'^o$ (ml/g)	0.761 ± 0.001	0.765 ± 0.002	0.790 ± 0.001	0.754 ± 0.001	0.770 ± 0.001	0.778 ± 0.001

Table 15: Preferential interaction parameters of α-lactalbumin in presence of different concentrations of glycerol at 20 °C.

Interaction Parameters	Concentration of glycerol (%) (w/v)					
	10	**20**	**30**	**35**	**40**	**45**
g_3 (g/g)	0.108	0.236	0.391	0.480	0.579	0.690
m_3 (mol of solvent per 1000g water)	1.17	2.56	4.25	5.21	6.29	7.49
$\delta g_3/\delta g_2$ (g/g)	-0.084 ± 0.018	-0.155 ± 0.015	-0.257 ± 0.021	-0.281 ± 0.023	-0.299 ± 0.030	-0.235 ± 0.024
$\delta m_3/\delta m_2$ (mol/mol)	-13.01 ± 2.7	-23.90 ± 2.1	-39.57 ± 3.28	-43.36 ± 3.34	-46.15 ± 4.40	-36.29 ± 3.71

Table 16: Apparent partial specific volume of α-lactalbumin in presence of different concentrations of sucrose at 20 °C.

Partial specific volume	Concentration of sucrose (%) (w/v)			
	10	**25**	**40**	**45**
ϕ_2^o (ml/g)	0.760 ± 0.001	0.745 ± 0.001	0.705 ± 0.001	0.724 ± 0.002
$\phi_2'^o$ (ml/g)	0.779 ± 0.001	0.793 ± 0.001	0.746 ± 0.002	0.768 ± 0.001

Table 17: Preferential interaction parameters of α-lactalbumin in presence of different concentrations of sucrose at 20 °C.

Interaction parameters	Concentration of sucrose (%) (w/v)			
	10	**25**	**40**	**45**
g_3 (g/g)	0.107	0.300	0.555	0.658
m_3 (mol of solvent per 1000g water)	0.31	0.87	1.62	1.92
$\delta g_3/\delta g_2$ (g/g)	-0.052 ± 0.012	-0.158 ± 0.021	-0.166 ± 0.024	-0.171 ± 0.016
$\delta m_3/\delta m_2$ (mol/mol)	-2.26 ± 0.50	-6.55 ± 1.1	-6.89 ± 1.4	-7.09 ± 1.8

Table 18: Apparent partial specific volume of β-globulin in presence of different concentrations of sorbitol at 20 °C.

Partial specific volume	Concentration of sorbitol (%) (w/v)			
	10	**20**	**30**	**40**
ϕ_2^o (ml/g)	0.733 ± 0.001	0.718 ± 0.001	0.721 ± 0.001	0.741 ± 0.002
$\phi_2'^o$ (ml/g)	0.742 ± 0.001	0.737 ± 0.002	0.748 ± 0.001	0.770 ± 0.001

Table 19: Preferential interaction parameters of β-globulin presence of different concentrations of sorbitol at 20 °C.

Interaction Parameters	Concentration of sorbitol (%) (w/v)			
	10	**20**	**30**	**40**
g_3 (g/g)	0.103	0.223	0.365	0.533
m_3 (mol of solvent per 1000g water)	0.568	1.22	2.01	2.93
$\delta g_3/\delta g_2$ (g/g)	-0.049 ± 0.024	-0.081 ± 0.013	-0.124 ± 0.021	-0.137 ± 0.018
$\delta m_3/\delta m_2$ (mol/mol)	-3.95 ± 1.32	-6.47 ± 1.24	-9.88 ± 2.0	-10.89 ± 1.12

Table 20: Apparent partial specific volume of β-globulin in presence of different concentrations of glycerol at 20 °C.

Partial specific volume	Concentration of glycerol (%) (w/v)			
	10	**20**	**30**	**40**
ϕ_2^o (ml/g)	0.732 ± 0.001	0.735 ± 0.001	0.740 ± 0.001	0.723 ± 0.001
$\phi_2'^o$ (ml/g)	0.737 ± 0.001	0.752 ± 0.001	0.761 ± 0.001	0.746 ± 0.002

Table 21: Preferential interaction parameters of β-globulin in presence of different concentrations of glycerol at 20 °C.

Interaction Parameters	Concentration of glycerol (%) (w/v)			
	10	**20**	**30**	**40**
g_3 (g/g)	0.104	0.227	0.376	0.558
m_3 (mol of solvent per 1000g water)	1.14	2.50	4.10	6.21
$\delta g_3/\delta g_2$ (g/g)	-0.052 ± 0.010	-0.113 ± 0.017	-0.135 ± 0.014	-0.174 ± 0.021
$\delta m_3/\delta m_2$ (mol/mol)	-8.31 ± 1.15	-17.84 ± 2.01	-21.3 ± 2.8	-27.4 ± 3.1

Table 22: Apparent partial specific volume of β-globulin in presence of different concentrations of sucrose at 20 °C.

Partial specific volume	Concentration of sucrose (%) (w/v)			
	10	**20**	**30**	**40**
ϕ_2^o (ml/g)	0.727 ± 0.001	0.730 ± 0.001	0.741 ± 0.001	0.738 ± 0.001
$\phi_2'^o$ (ml/g)	0.733 ± 0.001	0.751 ± 0.001	0.763 ± 0.001	0.767 ± 0.001

Table 23: Preferential interaction parameters of β-globulin in presence of different concentrations of sucrose at 20 °C.

Interaction parameters	Concentration of sucrose (%) (w/v)			
	10	**20**	**30**	**40**
g_3 (g/g)	0.105	0.220	0.365	0.519
m_3 (mol of solvent per 1000g water)	0.304	0.644	1.07	1.52
$\delta g_3/\delta g_2$ (g/g)	-0.043 ± 0.015	-0.077 ± 0.008	-0.101 ± 0.020	-0.110 ± 0.011
$\delta m_3/\delta m_2$ (mol/mol)	-1.33 ± 0.61	-3.27 ± 0.75	-4.28 ± 1.01	-4.65 ± 1.2

INTRODUCTION

Proteins play a vital role in biological systems. They are present in the living system as enzymes, hormones, immunoglobulins, and carrier molecules for various elements and regulate various metabolic pathways. They form the structural building blocks of the cell, forming the main components of cytoskeleton. Proteins are formed by sequential polymerization of amino acids by the formation of peptide bonds between amino acids. Many are biologically active proteins and generally adopt specific native three-dimensional structures for their specificity.

The structural organization of proteins can be studied at different levels. The primary amino acid sequence of protein mainly determines the final three-dimensional structure of a protein. The secondary structure is formed by formation of local hydrogen bonds. Depending on the orientation of the participating amino acid residues, the secondary structure can be either α-helices or beta-pleated sheets. Tertiary structure is formed by covalent and non–covalent interactions, which brings the distant domains and motifs closer to give the final three-dimensional structures.

The union of several polypeptide chains forms quaternary structure by several types of covalent and non-covalent interactions. The reaction medium of enzyme/protein is very crucial for the determination of their functionality. Based on the solution conditions and presence of various substances in the aqueous medium, protein can acquire numerous conformations. All

biologically active proteins have ordered secondary and tertiary structures. During complete unfolding of protein all the interactions gets weakened and the protein remains as unordered polypeptide chain (Chothia, 1984).

Protein can be hydrolyzed by either chemical hydrolysis or biological hydrolysis using enzymes such as proteases. The complete hydrolysis of protein in living system is essential for their optimum utilization. The protein malnutrition is a serious problem in today's world. For the developed and the developing nations, there is a need for nutritious food containing all the required essential amino acids. The milk proteins are one of the important dietary sources, which have the complete and balanced amount of essential and non-essential amino acids (Aimutis, 2004).

The proteins from oil seeds form a major source of dietary protein, which is the cheaper and easily available across the globe (Khalil *et al.*, 1985). Sesame is one of the major oil seeds, having high concentration of proteins, which are rich in aromatic and sulfur containing amino acids. Many of the seed proteins have a very compact structure and undergo slow hydrolysis (Tasneem and Prakash, 1989). An alternative approach to hydrolyze the protein *in vitro* to obtain the derived peptide(s) in the hydrolysate is very essential. The proper optimization of such processes will have a major impact on the industrial utilization of these proteins in the production of biotechnologically important peptides.

1.1. Proteins from milk

Milk is the first nutrition provided to the mammalian new born. It provides complete nutrition due to its accurate balance of elements that is required for proper nutrition, growth and development (Severin and Wenshui, 2005). Bovine milk is designed for nutrition, growth stimulation and immunological protection of the young calf. Since prehistoric times, human beings have consumed bovine milk either as fluid or as dairy products (Meisel, 2004). Enormous amounts of high quality milk proteins are now available for both functional and nutritional utilization in food and feed products. During the recent decades, phenomenal growth has been observed for the utilization of bovine milk proteins in specialty food products, such as infant formulations, dietetics and other health foods. Bovine milk contains about 3.5% of protein of which casein constitutes about 80% and whey proteins 20%. The concentration changes significantly during lactation, especially during the first few days of postpartum and a significant change occurs in the whey protein fraction.

Casein is the major milk protein, which constitute about 80% of the total milk protein and based on its structure and function it is divided into different fractions such as α-s_1, α-s_2, β, and k-casein. The major function of casein is to provide and transport calcium for the neonatal nutrition. Another less apparent function involves the formation of framework of submiscelles and micelles that are stable during transit and readily degraded during

digestion (Kumosinski *et al.,* 1994; Schmidt, 1979). Raman spectroscopy has demonstrated that caseins have significant amounts of secondary structure with 30% extended beta-sheet conformations and a similar percentage of turns (Byler *et al.,* 1988). Intact casein may mainly modulate B-lymphocyte function. k-Casein, its sub fractions and their hydrolysates have potential modulating affect on T-or B-lymphocytes.

1.2. Whey proteins

Whey proteins constitute 20% of the total milk proteins. Whey can be defined as byproduct of cheese making industries. Traditionally it was not explored due to lack of knowledge about its biological components. In the recent years there is significant accumulation of literature indicating the usefulness of milk whey in health and nutrition. The whey proteins have tremendous potential in the formulation of various functional foods and bioactive compounds (Walzem *et al.,* 2002; Forsum, 1973; Kinsella and Whitehead, 1989).

Nutritional and functional characteristics of whey proteins are related to their structure and biological functions of the proteins. During the recent decades, interest has grown in the nutritional efficiency of whey proteins in infant formula, dietetic and health foods using either native or predigested proteins. These proteins have been implicated in a number of biological effects observed in human and animal studies (Table1).

The suggested physiological role of total whey proteins indicates enormous potential for conventionally processed whey streams to enter new and lucrative health food market as functional food ingredients (also referred to as nutraceuticals, pharma foods or designer foods). These foods offer an identified health benefit when consumed as part of the normal diet, which in addition to nutritional substance confer a specific health advantage. Whey is made up of 5 major fractions and some minor fractions (Table 2) and each of them have a different structural and functional characteristic.

1.2.1. Biological activities of whey proteins

Whey proteins have been used in various processes of food formulations such as emulsification, gelation, water binding, solubulization, and whipping/foaming and viscosity developments. The functionality of the whey proteins is attributed due to its physical and biological characteristics. The above functionalities of the whey proteins are subject to variation depending on the conditions such as temperature, pH, ionic concentration and presence of lipid etc. Whey protein isolate has a great value in several specific applications in food ingredients. The ability of whey protein to bind water and other functional attributes make this protein to find wide applications in the area of functional foods (Shah, 2000). Whey proteins have fairly well balanced amino acid profile as compared to many other proteins. Whey proteins have proportionately more sulfur containing amino acids such as cysteine and methionine.

The high sulfur containing amino acids of whey proteins appear to have the ability to enhance immune function and antioxidant property mediated by sulfur containing tripeptide glutathione. Whey proteins are also rich in branched chain amino acids such as L-isoleucine, L-leucine and L-valine, they are supposed to promote protein biosynthesis in athletes and for individuals seeking optimal lean muscle mass (Yalcin, 2006).

1.2.2. α-Lactalbumin

α-Lactalbumin (α-La) is the second most prevalent whey protein after β-lactoglobulin. It represents 2 - 5% of bovine milk proteins. The gene for α-La is located on chromosome 12. It is calcium–binding metalloprotein having a single polypeptide chain of 123 residues. It plays a major role in the lactose biosynthesis pathway. The isoelectric point of α-La lies between the pH 4.2 and 4.6 based on the solvent conditions. It is highly soluble in water and salt solutions. Recently, it is recognized that α-La is associated with apoptosis of transformed cancer cell lines *in vitro.* Thus this protein is under consideration for the design of new antitumor agents (Hakansson *et al*., 1995). From nutritional point of view α-La appears to be of major importance as it is readily digestible and its amino acid composition meets the essential amino acid requirements of the newborn baby (Lonnerdal and Lien, 2003).

The crystal structures of α-La from various species in different forms and metal complexes are similar to that of the structure of homologous lysozymes. It has a two lobed structure separated by a cleft (Stuart *et al,* 1986;

Acharya *et al.*, 1994; Chandra *et al.*, 1998). The native structure of α-La consists of two domains; a α-helical domain and β-sheet domain linked by calcium-binding loop. It is stabilized by four disulfide bonds (Cys 6-120, Cys 28-111, Cys 61-77, Cys 73-91) (Ewbank and Creighton, 1993). In bovine α-La molecule, Trp 104 is in closer proximity to Trp 26 (Leu in human) than to Trp 60. This alignment places Trp 118 on the surface of bovine α-La and causes Trp 60 to be more exposed as well. The linear structure of bovine α-La is shown in Fig. 1.

Bovine milk has a ratio of whey protein: casein of approximately 20:80, where as it is approximately 60:40 in human milk (Stinnakre *et al.*, 1994). Milk based infant formula is usually made by adding whey protein concentrate to skimmed milk to make the whey protein: casein ratio equivalent to that of human milk. This modification in the ratio results in appropriate amino acid composition, which is suitable for the infants (Lonnerdal and Chen, 1990). α-La is a rich source of essential amino acids such as tryptophan and cysteine but have low levels of arginine.

During the digestion of α-La smaller peptides with several biological activities like angiotensin-1-converting enzyme inhibition (Pihlanto-Leppala *et al.*, 2000), immunomodulation and bactericidal activity (Kee *et al.*, 1998) are formed. Since the amino acid composition of α-La is limited in arginine, it is imperative to introduce the arginine from another source and preferably from seed proteins. Sesame seed is one such very good source of arginine, which

can be used to formulate the nutritional product with an enrichment of the same.

1.2.3. β-Lactoglobulin

β-Lactoglobulin (β-Lg) constitute approximately 50% of the total whey protein in bovine milk. It is capable of binding hydrophobic molecules and may function *in vivo* as a transporter for biologically important molecules such as retinol into small intestine. It also binds fatty acids and may stimulate the activity of pancreatic lipases (Perez *et al.,* 1992). β-Lg has been shown to have potential immunomodulatory properties and has numerous binding sites for minerals, fat-soluble vitamins and lipids. It can be used to incorporate desirable lipophilic compounds such as tocopherol and vitamin A into low fat products (Wong *et al.,* 1998).

In addition to β-Lg and α-La, milk has several biologically important proteins such as; serum albumin, immunoglobulins etc. which are incorporated into milk and are recoverable as minor whey proteins (Table 2). Serum albumin binds fatty acids as well as other small molecules. The immunoglobulins IgG_1, IgG_2, IgA, IgM are major bioactive components in whey. IgG antibodies provide a multitude of functions such as activation of complement mediated bacteriolytic reactions. Another vital function is their ability to augment recognition and phagocytosis of bacterial pathogens by leucocytes (Korhonen, 1998).

1. 3. Sesame proteins

Sesame (*Sesamum indicum L.)* is an important oil seed plant. Sesame seeds contain 25% protein and the defatted meal contains 50% protein. Most of the oil seed proteins and other legume proteins are deficient in methionine but rich in lysine (Khalil *et al*., 1985). Sesame seed protein has been well recognized as a nutritionally important protein source, owing to its richness in sulfur-containing amino acids, particularly methionine (Johnson *et al*., 1979). Sesame seed proteins are divided into four major fraction as; α, β, γ, δ-globulins. α-Globulin is the major protein fraction, which constitutes about 60-70% of the total proteins (Prakash and Nandi, 1978). The β-globulin constitutes 25% and the remaining portion is constituted by γ, δ-globulins of the total proteins. α-Globulin have been isolated, characterized and studied under various solution conditions and its quaternary structure is well understood (Prakash *et al.,* 1980; Prakash and Nandi, 1977a & b). The association dissociation and denaturation phenomenon of α-globulin are studied in detail (Prakash and Narasinga Rao, 1986; Lakshmi *et al.,* 1985).

1.3.1. β-Globulin

β-Globulin is the second major protein fraction constituting 25% of total protein from sesame seed (Rajendran and Prakash, 1988). It is also called as con-sesamin and has a sedimentation coefficient of 2S. It has been isolated and partially characterized. It is a single polypeptide chain of 14000 ± 500 Da. Structural stability in alkaline solution was studied and concluded that the

denaturation of β-globulin is a multi step process. At high pH it attains structurally a stable form (Rajendran and Prakash 1993a). Interaction of myo-inositol hexa phosphate with β-globulin was studied and the binding of the ligand to the protein has been shown to be very specific (Rajendran and Prakash, 1993b). The structural stability of the protein in various cosolvents gives an insight into the mechanism of stabilization of this low molecular weight oil seed protein.

1. 4. Structural stability of proteins

The term stability refers to a protein's resistance to adverse influences such as heat or denaturants, that is to the persistence of its molecular integrity or biological function in the face of high temperatures or other deleterious forces (O'Fagain, 1995). Definition and quantification of the forces contributing to protein folding and stabilization are of major importance. An understanding of the forces that stabilize proteins is essential for understanding and predicting how proteins fold from a random coil into their native conformation.

Protein stability results from the delicate balance between large and opposing entropic and enthalpic effects (Janin, 1984; Kuntz, 1984). Protein unfolding is a highly co-operative process and to an approximation only two states (native and unfolded) are observed for small globular proteins. A delicate balance exists between large stabilizing forces of favorable

intramolecular interactions and destabilization arising from the loss of conformational entropy of the native state (Creighton, 1983; Janin, 1984). A balance between stabilizing and destabilizing forces of the protein in turn determines the final structural stability and integrity of protein (Dill, 1985; Dill *et al*., 1989).

Proteins are only marginally stabilized due to the delicate balance of stabilizing and destabilizing interactions (Janecek, 1993; Burley and Petsko, 1988). A variety of different physicochemical forces play a pivotal role in stabilizing the native structure of a protein. Both the strength and specificity of many of the forces are strongly dependent on the environmental conditions in which the protein is present. The regular native conformation of protein molecule is stable at physiological pH, temperature, salt concentrations and many other conditions and is easily destroyed by slight changes in the environmental conditions such as pH, temperature, pressure and ionic strength. Different non-covalent interactions are associated with protein stability. The important intrinsic factors that play a major role in protein stability are: salt bridges, hydrogen bonding, hydrophobic interactions, disulfide bonds, amino acid interactions and binding of ligands (Tanford, 1961; Creighton, 1983; Baldwin and Eisenberg, 1987; Singleton *et al*., 1977).

The stability of enzymes and proteins *in vitro* remains a critical issue in biotechnology. Elucidation of the mechanisms responsible for stabilization or destabilization of enzymes is of both scientific and commercial importance. There are several ways to achieve stability of protein. For example protein engineering i.e. manipulation of the protein in genetic level, directed evolution, stabilization by polypeptide chain extension, site-specific mutagenesis, chemical modification and use of additives are a few to quote (Feeney, 1987). In many instances the use of cosolvents represents an approach in minimizing protein inactivation due to several bulk solvent conditions.

1. 5. Role of cosolvents in the stabilization of protein structure

There are various methods available today to stabilize the proteins in different conditions of solvents. Cosolvents have been used since long for the stabilization of various protein formulations. Several studies have shown that stability of proteins in aqueous solutions may be increased by addition of sugars and polyols (Lee and Timasheff, 1981; Arakawa and Timasheff, 1982a and b; O'Fagain *et al.,* 1988). Cosolvents do not alter the protein structure covalently but have tremendous effect on the solvent structure in close vicinity of the protein molecules. The stabilizing effect of these additives has been attributed to their effect on water structure, which may indirectly enhance hydrophobic interactions in the proteins. Solubility of N-acetyl ethyl esters of Phe, Tyr and Trp was decreased in solutions of glucose and sucrose.

This decrease in solubility or sugaring-out effect was attributed to increased hydrophobic interactions among amino acids in sugar solutions. Similar effects could explain stabilization of proteins in sugar solutions (Lakshmi and Nandi, 1976).

Stabilizing effects of sugars and polyhydric alcohols on proteins have been extensively studied by different groups (Lee and Timasheff, 1981; Arakawa and Timasheff, 1982a; Gekko and Timasheff, 1981a, b; Arakawa and Timasheff, 1985; Lee *et al.*, 1979). Stabilization of proteins by sugars has implications in sugar-rich food systems as protein transition temperature may be increased considerably. Thus certain additives such as polyhydric alcohols and chaotrophic salts inclusion are an effective way of stabilizing proteins.

In presence of cosolvents, the unfavorable interactions increase free energy of the system, but more so for the unfolded state of the protein because of its larger surface area, resulting in more unfavorable protein-water interactions. Thermodynamically, the native structure of the protein is therefore stabilized, as the folded state is favored energetically in the equilibrium between the folded and unfolded states of the protein (Gekko and Timasheff, 1981a, b). The stabilizing effect of sugars seems to be related to their effect on the surface tension of water. Increase in the surface tension need not be the sole factor responsible for preferential hydration, as glycerol,

which also induces preferential hydration, actually lowers the surface tension of water (Gekko and Timasheff, 1981b; Gekko and Morikawa, 1981a,b).

The thermodynamic parameters for the thermal unfolding were determined for proteins such as lysozyme and chymotrpsinogen (Gekko and Morikawa, 1981a,b). In presence of different polyols the mechanism of stabilization of these proteins appeared to be different.For chymotrypsinogen, free energy gets increased as a result of large decrease in entropy, compensating for a decrease in enthalpy, for lysozyme these parameters increased (Gekko and Morikawa, 1981b). It was found that the driving force in both the cases was a solvent mediated effect or solvent ordering effect. Preferential hydration of proteins also occurs in concentrated solutions of amino acids (Arakawa and Timasheff, 1983).

The most tenable and widely accepted mechanism of protein stabilization in presence of cosolvents in aqueous solution is the preferential interaction of proteins. Preferential interaction means that a protein prefers to interact with either water or an excipient (co-solute/co-solvent). In the presence of a stabilizing excipient, a protein prefers to interact with water (preferential hydration) and the excipient is preferentially excluded from the domain (preferential exclusion). In this case, proportionally more water molecules and fewer excipient molecules are found at the surface of the protein than in the bulk (Arakawa *et al*., 1990a and b; Timasheff, 1998). Preferential interaction of a protein with an excipient has been defined by

preferential hydration $[(\delta g_1/\delta g_2)_{T,\mu 1,\mu 3}]$ or by preferential interactions parameters $[(\delta g_3/\delta g_2)_{T,\mu 1,\mu 3}]$. The numbers 1, 2 and 3 represent the components namely water, protein and the third component like salt, cosolvents etc, respectively and 'μ' is the chemical potential of each of the components (Timasheff, 1993 and 1998). Interaction can be expressed on a gram basis (g) or on a molar basis (m). Preferential interaction of a protein with either water or an excipient is the consequence of a difference in affinity of a protein for water or an excipient. Measurements will be carried out by equilibrium dialysis techniques.

1. 6. Enzymatic hydrolysis of proteins and isolation of bioactive peptides

It is well documented that dietary proteins from both plant and animal origin possess nutritional, functional and biological properties and are often affected by the technological processes used in the food manufacture and processing (Korhonen and Pihlanto, 2003). The role of proteins as physiologically active components in the diet has been increasingly acknowledged in the recent years (Tome and Debabbi, 1998; Walzem *et al.*, 2002; Berrocal *et al.*, 1989). Such proteins and their precursors may occur naturally in raw food materials exerting their physiological action directly or upon enzymatic hydrolysis *in vitro or in vivo*. For example several dietary proteins can act as a source of biologically active peptides (Clare and Swaisgood, 2000). These peptides are inactive within the sequence of the

parent protein and can be released during gastrointestinal digestion or food processing.

At present milk proteins are considered the most important source of bioactive peptides, although other animal and plant proteins are also having potential bioactive sequences. Enzymatic hydrolysis of whole protein molecules has been one of the methods to produce bioactive peptides. Various pancreatic enzymes, preferably trypsin, have been widely used to liberate many known bioactive peptides from milk proteins (Berrocal *et al.*, 1989; Ariyoshi, 1993; Leppala *et al.*, 1998; Meisel, 1997). On the other hand, enzymes and different combinations of enzymes such as proteinases including alcalase, chymotrypsin, pancreatin and pepsin as well as enzymes from bacterial and fungal sources have been applied to produce bioactive peptides from various sources (Yamamoto *et al.,* 1994; Ondetti and Cushman, 1984).

From bovine milk, whey is the by-product of cheese, casein and traditional food manufactures and contains approximately 20% of the valuable milk protein. Whey proteins had been reported to be inhibitors of cancer cell growth (Tsuda *et al.*, 2000; Ushida *et al.*, 1998) and having anti hyper cholestremic effect (Zhang and Beynen, 1993). The growth promoting property of whey protein is well established and it is suggested to be due to enhanced absorption of essential nutrients such as calcium, controlled

delivery of amino acids to particular tissues, direct stimulation of cellular growth and inhibition of inflammatory cytokine.

The interaction between whey components and immune system is one of the most promising nutritional properties of the whey. For ageing adults the decline in immune system poses a serious threat towards various infections and therefore inclusion of whey protein as immune cell maturation supporting factor would be highly beneficial. Whey protein has antibacterial and antifungal properties (Walzem *et al.*, 2002). It has been also used for rapid elimination of toxic substances and microorganisms from the intestinal tract. In addition to these, whey proteins are rich in lysozymes and lactoferrin and their positive interaction as antibacterial make the whey protein essential to the infants (Walzem *et al.*, 2002; Chiba and Hoshikawa, 1989).

Whey components include α-lactalbumin, β-lactoglobulin, bovine serum albumin, immunoglobulins, lactoferrin and lactoperoxidase. Many of these components exhibit biological activity that is valuable in nutraceuticals formulations. Specific digestion products of milk proteins identified as bioactive peptides are shown to have a variety of bioactivities like ACE-inhibitory, antimicrobial, antithrombotic, immunomodulatory, mineral binding, opioid agonist and other activities (Korhonen and Pihlanto, 2003; Meisel, 2004).

The β-globulin from sesame is shown to have considerable amount of proteolytic activity (Rajendran and Prakash, 1988). It may be noted that the major protein fraction, α-globulin, the high molecular weight protein fraction does not show any proteolytic activity (Tasneem and Prakash, 1989). This may have a significant role to play during the germination of seeds. The high molecular weight protein may act as substrate releasing the needed amino acids for the growth of seedlings through specific enzyme action involving the low molecular weight proteins (Prakash and Narasinga Rao, 1986).

Thus the bioactive peptides having different health benefits can be produced by enzymatic hydrolysis. The optimization of enzymatic hydrolysis of various proteins under different condition would be a positive step towards formation of variety of bioactive peptides with specific functions. The stability of these peptides will have a major concern from point of view of their biotechnological application and formulation of food supplements and nutraceuticals.

Based on the above literature and the information available, a detailed investigation was undertaken to study the effects of different cosolvents on the structure and stability of the proteins namely α-lactalbumin from bovine milk and β-globulin from sesame seed protein. The structural stability studies help in understanding the mechanism of interaction of these cosolvents on the proteins, which is of scientific interest and has immense application in various biotechnological areas. The enzymatic hydrolysis of whey protein, isolated α-lactalbumin from bovine

milk and β-globulin from sesame was also carried out to understand the bioactivity of various hydrolysates. This study helps in the utilization of these hydrolysates in various food products, as these are rich in various bioactive peptides. The focus of the study is also to understand the mechanism of preferential interaction of cosolvents with the important protein of whey namely α-lactalbumin and protein of sesame seed, β-globulin. These two proteins are selected as model proteins one from dairy whey another from seed protein family keeping in view of their size, structure and their close molecular weight for comparison and bringing out generalities of structure-stability of proteins in general.

SCOPE AND OBJECTIVES

Dietary proteins from both plant and animal origin possess nutritional, functional and biological properties. Bovine milk contains about 3.5% protein of which 80% is constituted by casein. Whey is the by-product of the dairy industries. It constitutes 20% of the valuable milk protein. Whey proteins have tremendous potential in the formulation of various functional foods and bioactive compounds. α-Lactalbumin is the second major whey protein. It represents 2 - 5% of bovine milk proteins. It is a calcium binding metallo-protein with molecular weight of 14200 ± 200 Da.

Sesame (*Sesamum indicum L.)* seeds contain nearly 25% protein and the defatted flour contains about 50% protein respectively. Essential amino acid composition of sesame seed protein, with the exception of lysine was noted to be comparable to the proteins of beef and casein. α-Globulin is the major protein fraction, which constitutes about 60-70% of total sesame seed proteins. β-Globulin is the second major protein constituting nearly 25% in sesame seed, the remaining portion is occupied by γ, δ-globulins. Based on the deduced amino acid sequence from corresponding gene of β-globulin, the protein is demonstrated to be sulfur rich, which apparently accounts for the high nutritional value of sesame seed. The importance of structure, function and stability of proteins has been a very important subject area of research, both from functional and application point of view. Reports on the structure, function and stability of these low molecular weight proteins namely

α-lactalbumin from bovine milk and β-globulin from sesame seed with cosolvents are limited. The scope of the present investigation would throw more light on the structural stability of these two proteins in presence of selected cosolvents such as sorbitol, glycerol and sucrose. This study would help in understanding the mechanism of structural stability of the above proteins under various conditions, which has immense application in various biotechnological purposes. In this direction investigation was carried out to understand the effect of sorbitol, glycerol and sucrose on the structural stability of these low molecular weight proteins.

The role of proteins as physiologically active components in the diet has been increasingly acknowledged in recent years. Such proteins and their precursors may occur naturally in raw food materials, exerting their physiological action directly or upon enzymatic hydrolysis *in vitro* or *in vivo*. Several dietary proteins are shown to be sources of biologically active peptides. Bovine milk proteins are considered one among them. β-globulin from sesame seed protein, is shown to have proteolytic activity. There are few reports available on the bioactivities of the various hydrolysates obtained from it.

In the present investigation studies on enzymatic hydrolysis of whey protein concentrate, α-lactalbumin and β-globulin will be carried out *in vitro* using different proteolytic enzymes in order to obtain hydrolysates rich in bioactive peptides with different biological activities.

Specific objectives of the present study are:

CHAPTER 1. Isolation and characterization of α-lactalbumin from bovine milk and interaction studies with cosolvents.

The isolation of α-Lactalbumin was carried out using precipitation methods. The biochemical properties of the purified protein were studied by various biophysical and analytical methods. The structure and stability of the purified α-lactalbumin was studied in presence of the selected cosolvents such as sorbitol, glycerol and sucrose by using fluorescence spectroscopy, circular dichroic spectra and thermal denaturation measurements.

CHAPTER 2. Isolation and characterization of β-globulin from sesame seeds and interaction studies with cosolvents.

The isolation of low molecular weight sesame protein, β-globulin was carried out using salt precipitation methods. The biochemical properties of the purified protein were studied by various biophysical and analytical methods. Effect of selected cosolvents such as sorbitol, glycerol and sucrose on the structure and stability of purified β-globulin was evaluated using various spectroscopic and thermal denaturation measurements.

CHAPTER 3. Hydrolysis of whey protein from dairy and low molecular weight protein from sesame seeds.

Enzymatic hydrolysis of whey proteins from dairy and β-globulin from sesame seeds was carried out using different proteolytic enzymes to get hydrolysates, which are rich in various bioactivities. Enzymatic hydrolysates

from these proteins were evaluated for their various bioactivities such as antioxidant, ACE inhibition and antimicrobial activity.

CHAPTER 4. Preferential interaction studies of cosolvents with α-lactalbumin and β-globulin.

The preferential interaction of selected cosolvents with α-lactalbumin from dairy whey and β-globulin from sesame seeds was determined by measuring the partial specific volumes. The preferential interaction parameters thus measured were related to understand the mechanism of their structural stabilization.

The objectives of the present study as indicated above is the focus of the study and the data generated would give more insight in understanding the mechanism of structure-function and structural stability of α-lactalbumin and β-globulin in presence and absence of selected cosolvents. The enzymatic hydrolysis of whey protein concentrate, α-lactalbumin and β-globulin would give more insight into various bioactivities of the hydrolysates obtained from them which has several biotechnological claims and possible applications.

MATERIALS AND METHODS

1. Materials

Sorbitol, glycerol, sucrose, acrylamide, 2-mercaptoethanol, N, N, N′, N′-tetraethylenediamine, tricine, sodium dodecylsulfate, ANS salt, EDTA, TNBS, L-leucine, bovine serum albumin, pancreatin from hog pancreas, angiotensin converting enzyme from rabbit lung, hippuryl-histidine leucine-tetrahydrate, lactoferrin from bovine milk, ascorbic acid, 2-2-diphenyl-1-picrylhydrazyl, ammonium persulphate, ramipril, standard SDS molecular weight marker kit were procured from Sigma-Aldrich chemical company St Louis, MO, USA. Spectrapor molecular porous membrane tubing was from Spectrum Laboratories, Inc, Rancho Dominguez, CA, and USA. Fungal protease (*Aspergillus oryzae*) was obtained from Amino Pharmaceuticals, Japan. Coomassie brilliant blue R-250 was obtained from Biorad Laboratories, Richmond, USA. Acetonitrile, methanol, sodium phosphate, sodium chloride, copper sulfate, ammonium sulphate, sulphuric acid, sodium hydroxide, sodium hydrogen phosphate, sodium dihydrogen phosphate, potassium chloride, bromo-cresol green, methyl red, trichloroacetic acid, selenium dioxide, calcium chloride dihydrate, ferrous sulfate, were procured from E-Merck (India) Ltd., Mumbai, India. Dimethyl sulphoxide was procured from Sisco Research Laboratories, India. Brain heart infusion broth and nutrient agar were procured from Himedia, Labs, Pvt. Limited, Mumbai, India. All the chemicals used in the study were of the highest purity available. Quartz triple distilled water was used throughout all the experiments.

1.1. Sourcing of milk, whey and sesame seeds

Milk was collected repeatedly from a local jersey cow of Mysore, India. The ultra filtered whey protein concentrate (WPC) was obtained from Mahaan proteins Ltd (PROCON 3700, Batch No EREK.N_1) Mathura, India and the WPC was stored in an airtight container under refrigeration. Authentic varieties of sesame seeds (*Sesamum indicum* L.) were obtained from local market. Dry sesame seeds were flaked in a flaking machine and defatted with n-hexane until the fat content was less than 1.0% (six washes). The defatted material was powdered to 60-mesh size and used for routine extraction of protein.

2. Methods

2.1. Isolation of α-lactalbumin and whey from cow milk

The isolation of α-La from cow milk was performed according to the method described by Alomirah and Ali (2004). The milk was defatted by centrifuging at 7500 x g for 45 min at 4°C. Adding 0.1 N HCl with mechanical stirring, adjusted the pH of the obtained skim milk to 4.6. The stirring was continued further for 30 min. The precipitate formed was separated by centrifuging at 6000 x g for 45 min at 30°C. The clear supernatant whey protein fraction was collected separately from precipitated casein protein fraction.

Liquid whey obtained was used as a starting material for the isolation of α-La. Whey was treated with sodium hexa metaphosphate ($150x10^{-3}$ mol/l) and acidified to pH 3.9 using 6M citric acid and the solution was incubated for 45 min at 35°C. The precipitate was removed by centrifugation at 5000 x g for 30 min at 4°C. The precipitate collected was washed, treated with 7% NaCl and centrifuged at 10,000 x g for 20min at 4°C to separate the precipitate. The precipitate was dissolved in 0.1M $CaCl_2$ to obtain the soluble holo-form of α-La. The clear supernatant obtained was dialyzed against water and lyophilized. The flow chart for the various steps in the isolation of α-La from bovine milk is shown in the Fig. 2.

2.2. Isolation of β-globulin from Sesame seeds

From the defatted sesame flour, β-globulin was isolated according to the method of Rajendran and Prakash (1988). Sesame flour was dissolved in phosphate buffer of pH 7.5 containing 1M sodium chloride (extraction buffer) in the ratio of 1:10. The resultant mixture was stirred for 1h. The slurry was centrifuged (6000 x g for 30min) and the supernatant was diluted 5.5 times with distilled water to remove α-globulin (Prakash and Nandi, 1978). The solution was allowed to stand at room temperature for 60 min. The β-globulin precipitate was removed by centrifugation (6000 x g for 30 min). To the clear supernatant, powdered ammonium sulphate was added to 30% saturation with constant stirring. After thorough mixing, the solution was kept at 4°C for 1 h and centrifuged (6000 x g for 30 min, 4°C).

The ammonium sulfate concentration of the supernatant was increased to 50% saturation with constant stirring. After 60 min of standing at 4°C the solution was centrifuged at 6000 x g for 30 min, 4°C. The precipitate obtained was dissolved in extraction buffer, centrifuged and the supernatant was dialyzed against large amounts of distilled water with constant stirring. The dialyzed solution free of salts was lyophilized. The lyophilized material was stored in desiccators at 4°C and was used for all further studies. The steps involved in the isolation of β-globulin are shown in the Fig. 3 as flow chart.

2.3. Preparation of dialysis membrane

The 23mm flat dialysis membrane of 6000-8000 molecular weight cut-off was washed with distilled water thoroughly. The membrane was further treated with 0.2% sodium bicarbonate and 0.2% EDTA and boiled for 30mins.The treated dialysis bags were rinsed thoroughly and boiled thrice in quartz triple distilled water before using in different dialysis experiments.

2.4. Freeze drying

The proteins after the dialysis was freeze dried in virtis freeze dryer. Freeze drying was continued till the complete removal of moisture from the protein samples. Proteins were sealed in airtight containers and stored in desiccators. The freeze-dried protein samples were used for further studies.

2.5. Determination of protein concentration

The protein content were measured using microkjeldhal, spectrophotometric, folin ciocalteu's phenol reagent and Bradford methods as described below.

2.5.1. Total nitrogen estimation

The total nitrogen present in the samples was estimated by the method of micro-kjeldhal (AOAC, 1995). The method consists of 3 steps namely digestion, distillation and titration. During digestion, 0.5 -1 gram of sample was mixed with 100mg of digestion mixture (K_2SO_4: $CuSO_4$: SeO_2: 10:1:0.25). 15 ml of sulphuric acid was added and digested in a Gerhardt digestion system by increasing the temperature gradually up to 400°C. The digestion was continued until the solution in the tubes turns colorless. The digested sample was made up to 50ml in a volumetric flask with triple distilled water.

During distillation of 5 ml sample, 20 ml of 40% NaOH was added and allowed the steam to pass through. The steam distillate liberated was trapped in 10ml of 2% boric acid trapping medium containing 0.01ml of mixed indicator (0.033% (w/v) methyl red and 0.167% (w/v) bromocresol green). After distillation the distillate was titrated with N/70 HCl. Ammonium sulphate was used as standard to determine the acid factor. The total protein in the samples was determined by multiplying the total nitrogen obtained, by a factor of 6.25 (AOAC, 1995).

2.5.2. Protein concentration determination using extinction coefficient

The protein concentration was determined spectrophotometrically in a Shimadzu 1601 UV-Visible double beam spectrophotometer using extinction coefficient ($E^{1\%}_{1cm}$) of 20.1 at 280 nm for α-lactalbumin (Ewbank and Creighton, 1993) and 7.24 at 276 nm for β-globulin (Rajendran and Prakash, 1988).

2.5.3. Protein estimation by Folin and Ciocalteu's phenol reagent method

The protein content was measured according to the procedure of Lowry's (Lowry *et al*., 1951). The reaction mixture consists of the following reagents, Reagent A; 2% Na_2CO_3 in 0.1N NaOH; Reagent B:0. 5% $CuSO_4.5H_20$ in 1% sodium potassium tartarate; Reagent C: Mix 59 ml of reagent A with 1 ml of reagent B. Reagent D: Diluted Folins reagent. To a sample of 5 to 100 μg of protein in 0.2 ml or less in a 3 to 10 ml test tube, 5 ml of reagent C is added. Mix well and allowed to stand for 10 min. 0.5 ml of reagent D was added, mixed rapidly and allowed to stand for 30 min. The absorbance of the solution was recorded at 660 nm and concentration of protein was determined using BSA standard curve (Lowry *et al*., 1951)

2.5.4. Protein estimation by Bradford method

The protein in the solution was estimated by the method of Bradford (1976). A Bradford stock solution of 200 ml 88% phosphoric acid, 350 mg serva blue G was prepared in 95% ethanol and stored for further use. The working solutions consists of 425 ml distilled water, 15 ml 95% ethanol, 30 ml

88% phosphoric acid and 30 ml Bradford stock solution. All the solutions were mixed together and filtered through Whatman No. 1 filter paper and stored in brown glass bottles.1 ml of Bradford working solutions was added to 100 µl of protein solution, mixed well and allowed to stand for 2 min. Then absorbance was recorded at 595 nm. The protein concentration was estimated using a standard curve of BSA (Bradford, 1976).

2.6. Measurement of pH

The pH of the solutions and the buffers were measured using Cyber scan pH meter at 27 °C. The meter was calibrated with standard buffers from E-Merck, India before every measurement.

2.7. Gel-filtration chromatography

The purity of the isolated protein fractions was evaluated using gel-filtration chromatography on HPLC. Gel-filtration was performed in Shodex 100 column using waters HPLC system, equipped with diode array, UV-Visible detector. The column was equilibrated with 0.06M sodium phosphate buffer pH 7.5 containing 1M NaCl at a flow rate of 0.7 ml/min. The isolated protein of known amount was dissolved in the same buffer and loaded directly into the column. Absorbance values at 280 nm obtained was plotted against the elution volume to get the gel filtration profile of the isolated protein.

2.8. Reverse phase-high performance liquid chromatography (RP-HPLC)

The purity of isolated α-La was also evaluated by RP-HPLC using a C18 Symmetry Shield column (4.6 × 150 mm, 5 cm) on Waters HPLC system equipped with a 1525 binary pump and Waters 2996 photodiode array detector. The solvents used were water containing 0.1% TFA (A) and 70% acetonitrile containing 0.05 % TFA (B). A linear gradient traversing from 0 to 70% B in 75 min at a flow rate of 0.7 ml/min was employed. The eluate was monitored at 210 nm.

2.9. Amino acid analysis

Amino acid analysis was performed according to the method of Bidlingmeyer *et al*., (1984) using a Waters Associate Pico-Tag amino acid analysis system. 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.

The protein (≈20 μg) was dried under vacuum and to it 200 μl of constant boiling HCl (6M) containing 1% (v/v) phenol was added. In the workstation the samples were hydrolyzed at 110°C for 24 h. This step is important in getting the pH of the derivatization mixture up to the required level. The redrying solution consists of 2:2:1 mixture (by volume) of ethanol: water: triethylamine. The 20 μl of redried solution was added to the samples and to the standard free amino acids in a mix (pierce H), which contains up to

25 nmoles of each amino acid, dried under vacuum at 55-60 mtorr. The derivatization consists of 7:1:1:1 solution (by volume) of ethanol: water: triethylamine: phenylisothiocyanate (PITC). To prepare 300 µl of derivatization reagent 210 µl of ethanol were mixed with 30 µl of PITC, TEA and water.

To the standards and the samples 20 µl of the above reagent was added, which leads to the formation of phenyl thiocarbamyl amino acids (PTC-amino acids). The samples are ready for RP-HPLC analysis when the vacuum in the workstation reaches 50 - 60 mtorr. The column used was an application specific Pico-tag column (150 x 3.9 mm). The HPLC system (Water Associations) consists of specific solvent delivery system and a fixed wavelength detector (at 254 nm). The system is controlled with an M 660 solvent programmer attached to a column heater to maintain the temperature at 38 ± 1°C fitted with a model U6K injection system. The samples were injected in volumes ranging from 5-50 µl. The solvent system consists of Solvent A: Mixture of an aqueous buffer (0.14 M sodium acetate containing 0.5 ml /l TEA, pH 6.4) and Solvent B: 60% acetonitrile in water The gradient run for the separation at a flow rate of 1 ml/min consists 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% (13 min), 100% A and 0% B (14 min) and 100% A and 0% B (25 min).

2.10. Estimation of tryptophan

N-Bromosuccinimide addition results in the selective oxidation of indole form of tryptophan residues to oxindole form, which is a much weaker chromophore of tryptophan at the 280 nm. The tryptophan content of the protein, β-globulin (3 ml of 0.6 mg/ml protein solution in 0.05 M acetate buffer, pH 4.0) in native condition and α-La (3.0 ml of 0.6 mg/ml protein solution, 0.01 M phosphate buffer pH 7.0 in 8 M Urea) was estimated spectrophotometrically using NBS (Spande and Witkop, 1967 a and b; Bell *et al.*, 1975). To the protein solutions NBS was added in 10 µl aliquots through syringe with constant stirring. NBS addition was carried until no further decrease in the absorbance was observed. The number of tryptophan residues per mole of protein 'N' was calculated by the equation

$$N = (\Delta A \times 1.31 \times MW) / A_i \times a_f \times 5500 \text{ ---------- (1)}$$

where ΔA is the corrected absorbance decrease at 280 nm, A_i is the initial absorbance of the protein used at its wavelength maxima, 'a_f' is the absorptivity factor to convert absorbance of each protein used to mg of protein, MW is the molecular weight of the protein and 5500 is the molar extinction coefficient of tryptophan at 280 nm.

2.11. Electrophoretic methods

2.11.1. Sodium dodecyl sulfate-polyacrylamide gel electrophoresis

The sodium dodecyl sulphate polyacrylamide gel electrophoresis (SDS-PAGE) of protein samples was performed in mini slab gels using discontinuous system (Laemmlli, 1970). The acrylamide concentration of the gel used for the separation was made up of 15% T and 4% C. Samples was dissolved in sample buffer (0.125M Tris-HCl, 4% SDS, 2% 2-mercaptoethanol, 20% Glycerol, 0.02% Bromophenol blue) in the ratio of 1:1 and boiled for 2 min, cooled and centrifuged at 6000 x g for 5 min. The supernatant was loaded in the wells of the stacking gel and the electrophoretic run was carried out at a constant voltage of 100 volts. When the tracking dye reaches the bottom of the gel the run was ended. After the electrophoretic run the gel was stained using coomassie brilliant blue (CBB) R-250 dye. The staining solution consists of 0.1% CBB R-250 (w/v) in 40% methanol (v/v), 10% acetic acid (v/v). After the dye gets dissolved the staining solution was filtered. The gel was stained in excess of staining solution for 1h in an incubator shaker maintained at 25 °C. The destaining solution consists of 40% methanol and 10% acetic acid. Destaining solution was changed until the background has been removed satisfactorily. Molecular weight of sample proteins was determined by measuring the relative mobility of the standard proteins used as markers (Weber and Osborne, 1969) using following equation

$$R_f = (D_P X L_2) / (L_1 X D_d) \text{ ----------(2)}$$

where D_p is distance moved by protein band from top (cm), D_d is distance moved by indicator dye (cm), L_1 is length of gel after distaining, L2 is length of gel before staining and R_f is relative mobility. From the plot of log molecular weight of the standard proteins versus R_f values, the molecular weight of proteins was determined.

2.11.2. Non-denaturing polyacrylamide gel electrophoresis

Non-denaturing page (Native-PAGE) was carried out in discontinuous slab gel system (Laemmlli, 1970). Separating gel of 10% and stacking gel of 4% was prepared from a 30% stock solution of acrylamide–bisacrylamide (29.2:0.8 %). The samples (15-30 µg) were prepared in the sample buffer containing 10% glycerol and 0.02% bromophenol blue. After initial pre run for 30min, the samples were loaded in the wells of the stacking gel and the electrophoretic run was continued at 100volts for 3 hours. The gels were stained in Coomassie brilliant blue R-250 in 50% methanol, 10% acetic acid for 1 hr and destained with 50% methanol in 10% acetic acid.

2.12. Spectroscopic methods

2.12.1. Ultraviolet difference spectroscopic measurements

The Ultraviolet (UV) absorption spectral measurements of the protein solutions were recorded in Shimadzu 1601 UV-Visible double beam spectrophotometer against respective blanks in the region from 200-350 nm.

2.12.2. Measurement of extinction coefficient in different solutions

The extinction coefficient of the proteins varies with the third component. To obtain the $E^{1\%}$ in presence of third component, identical aliquots of native enzyme stock solution were mixed volumetrically to identical extents with native buffer as well as mixed solvents. The ultraviolet difference spectra were recorded in Shimadzu UV 1601 UV-Visible double beam spectrophotometer. From the ratio (Absorbance of native/Absorbance in mixed solvent) with corresponding blanks it is possible to calculate the $E^{1\%}$ of proteins in mixed solvents.

2.12.3. Intrinsic fluorescence measurements

The intrinsic fluorescence measurements were recorded for the proteins α-lactalbumin and β-globulin in Shimadzu RF-5000 spectrofluorometer with different concentrations of cosolvents. The extinction wavelengths were set at 280 nm and emission spectra were recorded from 300-400 nm with extinction and emission slit width of 5nm. Corrections were applied both for inner filter effect for protein concentration and solvent effect.

2.12.4. Circular dichroic measurements

The Circular dichroic measurements for α-lactalbumin was performed in Tris-HCl buffer pH 7.5 (0.01M) and β-globulin in sodium phosphate buffer pH 7.5 (0.06M) containing 1M NaCl at 25°C using an Jasco-J-810 automatic recording spectropolarimeter, fitted with a xenon arc lamp. Near-UV

measurements were made in the wavelength range of 250-300 nm with 5 mm path length and far-UV measurements from 200-250 nm with 1mm path length. The instrument was calibrated with aqueous solution of d-camphor sulphonic acid. The dry nitrogen gas was purged continuously into the instrument before and during the measurements. The protein concentrations were maintained at 0.2 ± 0.05 mg/ml in the far-UV region measurements and 1 ± 0.05 mg/ml in the near-UV region measurements respectively. The rotations were converted to molar ellipticity values by using a mean residue 115 for the proteins. The molar ellipticity values were evaluated at every 1nm interval by using the equation (Alder *et al*., 1973)

$$[\theta]_{MRW} = \frac{[\theta]_{obs} \times MRW}{10 \times d \times c} \qquad \text{----------(3)}$$

where $[\theta]_{obs}$ is the observed ellipticity (deg), d is the path length of the cell used (cm), C is the protein concentration (g/ml) and MRW is the mean residue weight of the protein. The secondary structural analysis was performed according to the built in computer program of the instrument (Yang *et al.*, 1986).

2.12.5. Thermal denaturation measurements

The thermal denaturation studies were performed for α-La and β-globulin in the native state and in the presence of various concentrations of cosolvents namely sorbitol, glycerol and sucrose using Cary 100 Bio

UV-Visible spectrophotometer (Mulgrave Victoria, Australia) using a scan rate of 1°C per min at 287 nm in the temperature range of 25-95°C. Corresponding blanks were used in the reference cell. The apparent thermal denaturation temperature (T_m) was calculated from the absorbance. From the thermal denaturation profile, fraction unfolded was calculated using the standard equation (Pace and Scholtz, 1997)

$$F_u = \frac{Y_F - Y}{Y_F - Y_U} \qquad \text{----------(4)}$$

where Y_F is the absorbance of protein solution in the native state, $Y_{U\ is}$ the absorbance of protein solution in unfolded state, Y is the absorbance of the protein solution at different temperatures and F_u is the fraction unfolded. The apparent thermal denaturation temperature is defined as the temperature at which the value of F_u is 0.5. The results are the average of three independent measurements.

2.13. Enzymatic hydrolysis of protein

2.13.1. Enzymatic hydrolysis of whey proteins

The enzymatic hydrolysis of whey protein concentrate and α-La was performed using a double enzyme combination of fungal protease and pancreatin with enzyme to substrate ratio of 1:50 (w/w) and 1:100 (w/w) respectively for different periods of time (Pintado *et al.,* 1999). Respective proteins were first hydrolyzed with pancreatin followed by treatment with fungal protease maintaining optimum conditions for the respective enzymes.

The samples were withdrawn at regular intervals during hydrolysis and the degree of hydrolysis was measured (Alder-Nissen, 1979). The reaction was stopped by keeping the samples in boiling water bath for 5 min and immediately freezed at -20°C (Pintado *et al.,* 1999).

2.13.2. Enzymatic hydrolysis of β-globulin

The enzymatic hydrolysis of β-globulin with the enzyme subtilisin was performed with an enzyme to substrate ratio of 1:50 (w/w) maintaining optimum conditions for the enzyme (Castro *et al.,* 1996). The samples were withdrawn at regular intervals and the degree of hydrolysis was measured. The reaction was stopped by keeping the samples in boiling water bath for 5 mins and immediately freezed at -20°C (Castro *et al.,* 1996).

2.14. Degree of hydrolysis

Degree of hydrolysis of the samples was determined by Trinitrobenzene sulfonic acid method (Alder-Nissen, 1979). 0.250 ml of sample containing between $0.25x10^{-3}$ and $2.5x10^{-3}$ amino equiv/l was mixed in a test tube with 2 ml of phosphate buffer at pH 8.2. Then 0.2 ml of 0.10% TNBS solution is added and tubes were incubated at 50 ± 1°C for 60 min in a shaking water bath in dark. After incubation, 4 ml of 0.1N HCl is added to terminate the reaction and the test tube is allowed to cool at room temperature for 30 min before taking the absorbance. The absorbance of samples was measured at 340 nm against water. The reactions of the blank

and the standard solutions are carried out by replacing the sample with 1% SDS and 1.5 x 10^{-3} M L-leucine in 1% SDS, respectively.

2.15. Bioactivity measurements

2.15.1. Determination of antioxidant activity by phosphomolybdenum method

The total antioxidant activity of the samples was estimated by phosphomolybdenum method (Prieto *et al.*, 1999). In brief 0.1ml of the individual samples were combined with 1.0ml of the reagent solution (0.6M sulphuric acid, 28 mM sodium phosphate and 4 mM ammonium molybdate). The tubes were capped and incubated on a boiling water bath at 95°C for 90 min. The samples were then cooled and the absorbance of the aqueous sample was measured at 695 nm against a blank containing the reagent solution and the respective solvent. For the samples of unknown concentration the antioxidant activity was expressed as equivalents of vitamin E (μmol/g sample).

2.15.2. Radical scavenging activity using DPPH

Radical scavenging activity of the samples was determined using the free radical (Yamaguchi *et al.,* 1998). Briefly 0.1 ml of the sample with different concentrations was made up to 0.4 ml with methanol and made to react with 0.6 ml of 0.1mM methanolic solution of DPPH and incubated at 27°C for 20 min. Control was prepared as above without sample. The absorbance of the samples was measured at 517 nm. L-ascorbic acid was used

as the positive control. Radical scavenging activity (RSA) of the samples was expressed as the inhibition percentage and was calculated using the following formula

$$\% \text{ RSA} = [1 - A_{\text{sample (517nm)}} / A_{\text{control (517nm)}}] \times 100 \text{ ----------(5)}$$

where 'A' represents the absorbance of the sample solution and the control solution respectively.

2.15.3. ACE inhibitory activity

The samples were assessed for ACE inhibitory activity (Cushman and Cheung, 1971). Different concentrations of sample are incubated at 37°C for 80 mins with 110 µl of 20mM (Hip-His-Leu) substrate in 0.1M sodium acetate buffers with 150 mM NaCl (pH 8.3) and with 25 µl of ACE (dissolved in 50% glycerol) of the reaction medium. The reaction was stopped by adding 110 µl of 1N HCl. The hippuric acid formed in the enzymatic process was extracted with ethylacetate. The ethyl acetate was evaporated to dryness on a water bath and residue was determined at 228 nm. The concentration of protein required to inhibit 50% of ACE activity was defined as IC_{50}. The potency of the samples was compared with the commercially available hypotensive peptide ramipril.

2.15.4. Anti-microbial activity

The antimicrobial activity of the samples was performed against *Escherichia coli, Bacillus cereus* and *Listeria monocytogenes* microbes. The

hydrolysates were solubulized in water before use. The assay was carried out in sterile honeycomb micro plates and each well contained a total volume of 250 µl. A 200 µl of freshly inoculated microbes was added to each well; together with 50 µl of the protein solution. The final concentration of the hydrolysates in the wells varied between 0.75 - 3.0 mg/ml. Growth controls contained 250 µl of sterile growth media. The mixtures were incubated at 37°C for 24 h. The absorbance at 600 nm was measured every 15 min by a plate reader (Haug, 2002).

2.16. Partial specific volume measurements

The densities of the solvents and protein solutions were determined using Anton Paar DMA-58 precision densitymeter at 20.00 ± 0.02°C and the apparent partial specific volume, ϕ (ml/g) was calculated according to the standard procedures using the following equation (Casassa and Eisenberg, 1961,1964)

$$\phi = 1/\rho_0 [1- (\rho- \rho_0)/ C] \text{--------} (6)$$

where ρ and ρ_0 are the densities of the protein solution and the solvent respectively and C is the concentration of protein. The partial specific volume of protein 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 in isomolal (ϕ_2^o) and isopotential ($\phi_2'^o$) conditions and the preferential interaction parameters were calculated using

the established procedures (Lee *et al*., 1974; Prakash and Timasheff, 1985). The standard notations here for components 1, 2 and 3 are water, protein and additive respectively (Scachard, 1946; Stockmayer, 1950).

In a typical three-component system the preferential interaction parameters (ξ_3) is calculated using the equation (Casassa and Eisenberg, 1961; 1964)

$$\xi_3 = 1/\ \rho_0\ (\phi_2^{o} - \phi_2'^{o})\ /(1 - \bar{\nu}_3\ \rho_0) \text{----------} (7)$$

where g is the concentration of component 'i' in gram per gram of water, μ is its chemical potential, ρ_0 is the density of the solvent and T is the thermodynamic temperature. The ϕ_2^{o} and $\phi_2'^{o}$ are the partial specific volume of the protein at isomolal and isopotential conditions and $\bar{\nu}_3$ is the partial specific volume of the component 3. The total amount of solvent component (A_3) bound to the protein is related to by the equation (Scachard, 1946; Stockmayer, 1950). The same parameter on mol/mol basis is calculated from the following equation

$$(\delta m_3 / \delta m_2)_{T,\mu 1,\mu 3} = M_2 / M_3 \times (\delta g_3 / \delta g_2)_{T,\mu 1,\mu 3} \text{----------} (8)$$

where m_i is the molal concentration of component 'i', M_2 is the molecular weight of the protein and M_3 is the molecular weight of the co-solvent.

1. Isolation and characterization of α-lactalbumin from bovine milk and interaction studies with cosolvents

α-Lactalbumin is the protein that is found commonly in the milk of all mammals. Bovine α-La constitutes about 20% of the whey proteins. The maintenance of α-La native structure in a thermodynamically stable form is of prime importance in biological processes and in various biotechnological applications. α-La is the second most prevalent whey protein and is a monomer. Its molecular weight is 14200 ± 200 Da with a calcium-binding site. Calcium ion plays a major role in the stability of α-La (Chrysina *et al.*, 2000). α-La is also a component of lactose synthase complex.

Intact α-La as well as various forms of the protein such as multimers and fragments resulting from partial digestion, has various biological activities. α-La has an amino acid composition that contributes substantially to meet the essential amino acid requirements of newborn infants (Heine *et al.*, 1991). Therefore researchers have suggested that development of infant formula having increased levels of α-La with various degrees of purity (having decreased levels of β-Lg) would result in an amino acid composition more similar to that of breast fed infants (Kiesner *et al.*, 2000).

α-La was isolated from bovine milk using salt precipitation method. Bovine milk was subjected for acid treatment to separate out the casein fraction of milk protein. The remaining whey fraction was used for the

isolation of α-La. The isolation of α-La was followed by a three-step procedure of acid treatment followed by salt precipitation. The α-La fraction thus obtained as precipitate was separated by centrifugation and lyophilized. The homogeneity of the isolated α-La was established by using RP-HPLC, native and SDS-PAGE.

The chromatographic elution profile of α-La was carried out with reverse phase high performance liquid chromatography (RP-HPLC) to confirm the purity of the preparation. The RP-HPLC profile of the isolated α-La showed a single peak (Fig. 4) eluting at 45 min, indicating the isolated protein is homogeneous. The homogeneity of the isolated fraction was further confirmed by using native- and SDS-PAGE (Fig. 5). The isolated α-La showed a single band on SDS-PAGE. The molecular weight was determined to be 14200 ± 200 Da using standard molecular weight markers. The native PAGE of isolated α-La also revealed a single band, which shows that α-La is a monomer with no polymerization of α-La in the native condition.

The amino acid composition of the isolated α-La is shown in Table 3. The obtained values are in agreement with the literature values with a correlation coefficient of 95% (Farrell *et al.,* 2004). The protein contains considerably good amount of essential amino acids. α-La contains four tryptophan residues as determined by NBS method and contains eight cysteine residues involved in the formation of four disulphide bonds.

The spectral behavior of isolated α-La was studied using UV–visible absorption spectra, fluorescence and circular dichroic spectra. Fig. 6A shows the UV-absorption spectrum of isolated α-La having absorption maximum at 280 nm. The concentration of the isolated protein α-La was determined by extinction coefficient ($E^{1cm}_{1\%}$, 280 nm) value of 20.1 (Ewbank and Creighton, 1993). The intrinsic fluorescence spectrum of the protein is an indication of overall domination of the positioning of tryptophanyl residues in the protein structure and the solvent accessibility of tryptophan residues. The wavelength of maximum emission at pH 7.5 was found to be 331 nm as shown in Fig. 6B. Lower wavelength of emission maxima is an indication of the tryptophans being present in the non-polar environment of the protein.

Circular dichroic spectra of α-La were recorded in near and far-UV region at pH 7.5. Fig. 7A represents the near-UV spectrum of α-La. The ellipticity bands are negative and the spectra are characterized by a small peak centered near to 296 nm, a trough near 252 nm and a broad spectral envelope extending from about 255 nm and a maximum near 272 nm. The shoulders at 290, 280 and 267 nm respectively indicate the protein is rich in tryptophan and tyrosine (Alder *et al.,* 1973). Fig. 7B represents the far-UV CD spectrum of α-La, which is characterized by a negative peak located near 208 nm and a relatively flat region of negative ellipticity extending from about 212-223 nm. The analysis of the spectrum revealed that α-La contains 21%

α-helix, 46% β-structure and 33% aperiodic structures. Further the study on the effect of cosolvents on the structure of isolated α-La was carried out.

Various additives such as sugars and polyhydric alcohols have been used to stabilize proteins. The extent of interaction of these additives is specific depending on the nature of protein, which result either in stabilizing or destabilizing the native structure, or has no effect at all on the protein structure (Arakawa *et al*., 1990 a and b; Timasheff, 1998). There are very few reports available on the stabilization of α-La in presence of cosolvents. In the present study, the effect of cosolvent on the stabilization of α-La has been discussed. The mechanism of interaction of various additives with α-La will help in the utilization of the protein for various biotechnological applications. The mechanism involved in the interaction of cosolvents is explained in the subsequent chapters.

In order to understand the effect of cosolvents on the conformation of the protein, the structural aspects of α-La with cosolvents was studied by circular dichroic and fluorescence measurements. The effect of cosolvents on the thermal stability of the protein was also studied further. The effect of sorbitol, glycerol and sucrose on the secondary structural content of the protein was examined by far-UV CD measurements. The secondary structural parameter of α-La in presence of cosolvents is shown in Table 4. As seen from the table the presence of these cosolvents increases the α-helical

content of the protein at all the concentrations. The far-UV CD spectrum of α-La in the presence of 10-30% sorbitol is shown in Fig. 8. There was no significant change in the structure of the protein with sorbitol. In the Fig. 9 is shown the far-UV CD spectra of the protein in presence of glycerol. As the concentration of glycerol increases from 10-30% there was little change in the structure of the protein. These results show that there is no major perturbation occurred in the far–UV CD spectra of the protein in the presence of these cosolvents. The effect of sorbitol, glycerol and sucrose on the tertiary structure of α-La was examined by near-UV CD measurements. There was no significant change in the near-UV CD spectra of α-La with different concentrations of glycerol and sucrose. Fig. 10 shows the near–UV CD spectra of α-La in the presence of sorbitol, at 30% concentration. These spectral results indicated that there is no significant change in the tertiary structure of the protein in the presence of all the cosolvents.

The intrinsic fluorescence measurements of α-La in presence of different concentrations of cosolvents such as sorbitol, glycerol and sucrose were done in order to know the extent of change in the microenvironment of tryptophan residues of the protein. The fluorescence emission measurement of α-La with different concentrations of sorbitol is shown in Fig. 11. In presence of 10-30% sorbitol, there is an increase in the fluorescence emission intensity, but the emission maximum of the protein at 331 nm has not changed. There was no shift observed in the emission maxima even at higher

concentrations. These results indicated that the presence of sorbitol did not have any significant effect on the fluorescence spectra of α-La.

Fig. 12 shows the effect of glycerol on the fluorescence emission spectra of α-La. As seen from the figure the presence of glycerol increases the fluorescence intensity with increase in concentration with 2 nm shift in the emission maxima of 331 nm till 30% concentration. Fig. 13 shows the effect of sucrose at different concentrations on the fluorescence emission spectra of α-La. In case of sucrose at concentrations from 10-30%, the emission intensity of α-La decreased without any shift in the emission maxima. These results indicate no gross conformational change of the protein molecule in these cosolvents. Further the effect of these sugars and polyhydric alcohols on thermal stability of α-La was evaluated.

The thermal stability of α-La in the presence and absence of cosolvents was determined by the measurement of apparent thermal denaturation temperature. The difference spectra were recorded at 287 nm over a temperature range of 25-90°C. From the changes observed in the spectra, the apparent thermal denaturation temperature was calculated from the plot of fraction unfolded versus temperature.

The thermal denaturation profile of α-La in the presence of different concentrations of sorbitol is shown in Fig. 14. There is an increase in the

apparent thermal transition temperature of the protein in presence of sorbitol with increasing concentrations. There was an increase in thermal transition temperature to 71 ± 1°C from the control value of 65 ± 1°C at 40% sorbitol concentration. Similarly the increased thermal stability of α-La was observed in the presence of other cosolvents. Fig. 15 shows the thermal denaturation profile of α-La in presence of glycerol. The maximum thermal stability was obtained in presence of 40% glycerol, where the apparent thermal transition temperature was 68 ± 1°C. Fig. 16 shows the effect of sucrose on the thermal stability of α-La. With increasing concentration of sucrose from 10-40% there was an increase in the apparent T_m from 65 ± 1 to 71 ± 1°C. The apparent thermal transition temperature value of α-La with all the cosolvents used is represented in the Table 5. From the table it can be observed that as the cosolvent concentration is increased, the thermal transition temperature of α-La differs to different extents and depends upon the nature of the cosolvent. It is clear from the results that maximum thermal stability for the protein α-La was obtained in the presence of sorbitol and sucrose and least in presence of glycerol.

The increase in the T_m values of α-La with the above cosolvents may be attributed to the preferential hydration of the protein in their presence. Sugars and polyols stabilize proteins against heat denaturation. Work on different proteins namely ovalbumin, conalbumin, chymotrypsinogen and lysozyme with respect to denaturation indicated that sucrose and glycerol

strengthened the pair wise interaction between hydrophobic groups, however they reduced the tendency for complete transfer of hydrophobic groups from an aqueous to a non-polar environment (Back *et al.,* 1979). Due to the different influences of these cosolvents on the structure of water, the extent of stabilization of a particular cosolvent for the protein differs (Timasheff, 1998). There was a linear relationship between the quantity and shift in the apparent T_m, thus the dominant mechanism by which sugars and polyols stabilize proteins against heat denaturation is through their effect on the structure of water, which, in turn determines the strength of hydrophobic interactions (Back *et al*., 1979).

The stabilization of proteins by a variety of cosolvents can be related to their property of increase in the surface tension of water. It is demonstrated that, during the thermal unfolding of proteins, this increase of the surface tension can be overcome by the increase in the temperature of the solution at the midpoint of the transition, T_m and the weak binding of the cosolvent molecules (Lin and Timasheff, 1996). Recently, work done on papain with respect to increase of thermal stability in the presence of cosolvents is attributed to the preferential hydration (Sathish *et al*., 2007). The thermal stability of wheat germ lipase increased in the presence of cosolvents, the reason was due to increase of preferential hydration with cosolvents, which in turn prevent the thermal inactivation (Rajeshwara and Prakash, 1996). There are various other factors which contributes to the thermal stability of

the protein in cosolvents like the hydrogen bonds between sugar and protein molecules followed by additional hydration (Baier and McClements, 2001) solvent viscosity and the hydroxyl groups present in the glucose units (Creighton, 1983)

Thus from spectroscopic studies, sorbitol, glycerol and sucrose at different concentrations do not affect the structure of α-La. There was an increase in the thermal stability of α-La with the above cosolvents, indicating the unfolding of α-La is greatly disfavored in presence of sorbitol, glycerol and sucrose used in the medium. The mechanism of thermal stabilization of α-La is analysed by using partial specific volume measurements and determination of preferential interaction parameters of cosolvents with the protein as explained in subsequent chapters.

In summarizing the results of this chapter, α-lactalbumin isolated from bovine milk has been shown to be pure and homogeneous. The effect of cosolvents such as sorbitol, glycerol and sucrose on the structure and stability was evaluated. The results demonstrated that in presence of these cosolvents there was no change in the structure of the protein at different concentrations. There was an increase in the thermal stability of the protein to various extents in different cosolvents. This attribute of increased thermal stability of α-lactalbumin with the above additives can be used in different biotechnological applications.

2. Isolation and characterization of β-globulin form sesame seeds and studies in the presence of cosolvents

β-Globulin is one of the major protein fractions of sesame seed, which constitutes nearly 25% of the total protein. The behavior and stability of the protein in acidic and alkaline conditions are very well understood. In alkaline conditions from pH 7-13, β-globulin undergoes denaturation and it is a multistep process. During denaturation it affects the hydrodynamic properties of the protein that is decrease in sedimentation coefficient, partial specific volume and increase of reduced viscosity (Rajendran and Prakash, 1993a). In acidic conditions, by reducing the pH values from 7.0 to 2.0, β-globulin undergoes several transitions, undergoing an expansion due to hydration up to pH 5.0 and a further increase in hydrogen ion concentration results in folding of the protein (Rajendran and Prakash, 1992).

During the isolation of β-globulin from sesame seed the total proteins were subjected to stepwise precipitation with ammonium sulphate. The final supernatant thus obtained was centrifuged, dialyzed and lyophilized to get the pure protein, β-globulin. The homogeneity of the protein was evaluated by gel filtration chromatography and SDS - PAGE. The elution of the purified protein was carried out on HPLC using column eluted with phosphate buffer of pH 7.5. The flow rate was maintained at 0.5 ml/min. The gel filtration profile of the protein is shown in Fig. 17. As seen from the figure the protein got resolved into a single symmetrical peak at a retention time of 11.6 min.

The homogeneity of the isolated fraction was further confirmed by SDS - PAGE. Disulphide bonds play a major role in determining the structure of β-globulin. Fig. 18 shows the SDS - PAGE pattern of the purified protein in the presence of reducing agent. Single band appeared in the SDS - PAGE indicates that β-globulin is a monomer made up of single polypeptide chain having no subunits in it. The molecular weight of the monomer was determined to be 14000 ± 500 Da and was found to be in agreement with earlier reported values (Rajendran and Prakash, 1988).

The amino acid composition of the isolated β-globulin was determined and represented in Table 6. These determined values are in well agreement with the reported values having a correlation coefficient value of 97%. As seen from the composition, β-globulin is rich in arginine residues and hydrophobic amino acids.

The spectral properties of isolated β-globulin were determined using UV-absorption spectra, fluorescence and circular dichroic spectra. Fig. 19A shows the UV-absorption spectrum of the isolated protein. The UV-absorbance maximum of the isolated protein was determined to be 276 nm. In the absorption spectra of protein solutions the UV absorption maxima at 250 nm indicate the presence of disulphide bonds and the strong absorbance below 230 nm is due to peptide bonds. The absorbance maxima in the range

of 275-280 nm indicate the presence of aromatic amino acid residues (Plummer, 1971; Schmidt, 1979).

The fluorescence emission spectra of β-globulin from sesame proteins showed typical tryptophan fluorescence spectra with an emission wavelength maximum of 336 nm at pH 7.5, when excited at 280 nm (Fig. 19B). It has been reported that phenylalanine, tyrosine and tryptophan residues of proteins exhibit fluorescence emission in the range of 320-330 nm, where the dominance of fluorescence by tryptophan is present (Cantor and Schimmel, 1980). It is well known that proteins show fluorescence in the 300-400 nm ranges after excitation in the range of 280-290 nm. If there is any structural change, there will be changes in the fluorescence spectra. Change in the microenvironment of tryptophan residues or a change in the exposure of these residues lead to a drastic change in the fluorescence profile of the protein.

The circular dichroic spectra of the isolated β-globulin both in far- and near-UV region were studied. Fig. 20A show the near-UV CD spectrum, where the minimum was at 268 nm, indicating the specific orientation of the aromatic chromophores in an unusual hydrophobic interior. Studies on the far-UV CD spectrum of the protein, with various additives give a clear indication of the change in the secondary structure and also the structural stability of the protein. Far-UV CD spectrum of isolated β-globulin at pH 7.5

recorded in the region of 200-250 nm is shown in Fig. 20B. A major negative band around 211 nm and a well-defined shoulder at 222 nm characterizes the spectrum. This is indicative of a typical α-helix rich protein CD spectrum (Greenfield and Fasman, 1969). The analysis of data revealed that protein is having 26% α-helix, 38% β-structures and 36% aperiodic structures. The α-helical and β-structure contents of β-globulin, reported here are very similar to the values reported for the low molecular weight proteins from other oil seeds like mustard, sunflower and safflower (Prakash and Narasinga Rao, 1986). The purified protein was further utilized for the structural stabilization studies with selected cosolvents.

The protein native conformation is controlled by intramolecular interactions between protein functional groups and intermolecular interactions between protein and solvent molecules. There are several factors, which affect the protein native conformation, which in turn affects the protein stability, such as temperature, pressure, changes in pH or the addition of other compounds. Addition of cosolvents such as polyhydric alcohols and sugars to the solvent medium has been found to stabilize biological macromolecules in solution, which has huge biotechnological applications. Solvent additives can affect the macromolecular structure by direct interaction with the macromolecules, by indirect action by affecting the on the structure and properties of the solvent or by a combination of both these mechanisms (Creighton, 1993).

The information regarding the structural stability of β-globulin in a three-component system of protein, buffer and cosolvents is lacking. In the present study, the interaction between cosolvents and protein, will give a better understanding on the structural stability of the protein in cosolvents. The mechanism involved in the interaction of these cosolvents is discussed in later chapters.

In order to see the effect of different concentrations of cosolvents on the tertiary structure of β-globulin, the near-UV CD spectra in presence of cosolvents was monitored. In Fig. 21 is shown the effect of 30% sorbitol on near-UV CD spectra of β-globulin. As seen from the figure there was no significant change in the spectra of the protein. The results indicate that these cosolvents were not able to bring any change in the tertiary structure of the protein.

The effect of sorbitol, sucrose and glycerol at concentrations from 10-30% on the secondary structure of β-globulin has been studied using far-UV CD spectra. The far-UV CD spectra of β-globulin in the presence of 10-30% sorbitol concentration are shown in Fig. 22. The native β-globulin has 26% α-helix, 38% β-structure and 36% aperiodic structures. The presence of sorbitol resulted in increase in the α-helical content in a concentration dependent manner. The presence of glycerol on the secondary structure of β-globulin is shown in Fig. 23. There was a marginal change in the secondary structural elements in presence of different concentrations of glycerol and

sucrose as shown in the Table 7. There was 9% increase in the β-structure in the presence of 30% sorbitol, sucrose and there was 10% decrease in the aperiodic structure with all the cosolvents used. At a maximum concentration of 30% concentration of each of the cosolvent there was no major changes in the secondary structural content of the protein. These results indicate that the cosolvents do not bring significant difference in the tertiary and secondary structure of the protein.

The effect of cosolvents on the extent of change in the microenvironment of tryptophan residues was monitored by intrinsic fluorescence measurements. It is evident from the fluorescence spectra that the tryptophan groups of β-globulin are experiencing either a more polar or nonpolar environment in the presence of cosolvents such as glycerol, sorbitol and sucrose. The fluorescence emission spectra of β-globulin, in the presence of glycerol were measured in order to obtain information on the cumulative effect of glycerol at various concentrations on the tryptophan environment. In Fig. 24 it is shown the fluorescence emission spectra of β-globulin in presence of glycerol. The protein was excited at 280 nm and emission spectra were recorded in the range of 300-400 nm. The emission maximum of protein was found to be 336 nm and there was no apparent shift in it in presence of different concentrations of glycerol. It also indicates that the tryptophan residues of β-globulin did not experience any perturbation with the addition of this cosolvent.

The fluorescence emission spectra of β-globulin in the presence of different concentrations of sorbitol are shown in Fig. 25. There is a continuous increase in the emission intensity with increasing concentrations of sorbitol. In presence of 20 and 30% sorbitol there was a blue shift in the emission maximum by 2 nm. This indicated a modification of the environment of the tryptophan residues in the presence of sorbitol. Fig. 26, shows the emission spectra of β-globulin, as a function of sucrose concentration. The fluorescence intensity was found to increase with a blue shift of 3 nm in presence of 30% sucrose.

The exposure of hydrophobic residues might have been hindered by interaction of OH groups of the cosolvent molecules. To lower the surface free energy, the hydrophobic residues orient more readily than less hydrophobic ones at the surface with their polar groups directed towards the aqueous phase (Kato and Nakai, 1980). In protein dynamics solvent viscosity plays a major role and it is highly influenced by presence of different additives. In case of myoglobin the rate of oxygen and carbon monoxide interaction with protein depends on solvent viscosity and are more sensitive to the solvent at lower viscosity. The dominant factor, which markedly reduces the rate of conformational change in myoglobin at low temperatures, is the high viscosity of glycerol-water mixtures (Ansari *et al.*, 1992). Thus viscosity of glycerol would have played an important role in stabilization of structure of β-globulin.

Similar results were also obtained in case of sorbitol with β-globulin. There was an increase in emission intensity with a shift in the emission maxima of the protein. It indicates that the tryptophan residues are in a more hydrophobic environment. The preferential hydration and the conformational change that has taken place in the β-globulin molecule must be in the region where aromatic chromphores are absent. These results indicate gross conformational change in the protein conformation.

Sucrose stabilizes the protein by shifting the conformational equilibria perhaps towards the more compact state. Thus the results clearly indicate that there is no change in the structural features of β-globulin, in the presence of sucrose. From fluorescence studies of the protein one can get information about the microenvironment of the protein. The fluorescence caused by tyrosine and tryptophan residues is sensitive to their electronic environment. The cosolvent can change the dielectric constant around the microenvironment of the tryptophan residues in order to bring either stabilization/destabilization and hence fluorescence spectra cannot be correlated. In a defined system one can use fluorescence, especially in the presence of cosolvents only to get an indication of the change in tryptophan microenvironment and must be interpreted with great caution.

The fluorescence emission λ_{max} of a native protein is related to the polarity of the environment of the tryptophan residue and can change with

the residues in apolar microenvironments exhibit a blue emission. This is fair enough to conclude that these cosolvents did not significantly alter the structure of β-globulin as judged by fluorescence spectra. This is a fully established and sensitive probe used in protein chemistry for understanding the structural stability of tryptophanyl peptides and proteins. There are different reports to explain the effect of cosolvents on the structural stabilization of proteins. Sugars (such as sucrose and glucose) and polyols (e.g. glycerol) generally stabilize the proteins primarily through preferential interaction and steric exclusion mechanism (Timasheff, 1993, 1998). A number of studies on the effect of different cosolvents on the structure of proteins indicate the phenomenon of preferential hydration. The stabilizing effect of these cosolvents is due to preferential hydration of the protein in the presence of these additives (Arakawa and Timasheff, 1982a and b; Rajeshwara and Prakash, 1996).

The thermal stability of β-globulin was studied by differential temperature scanning UV-visible spectra in the temperature range of 25-95°C. The apparent thermal denaturation temperature was obtained after normalization of data and fraction unfolded was plotted using the equation in the materials and methods section. The presence of cosolvents has significant effect on the thermal stability of β-globulin. In Fig. 27 is shown the fraction unfolded versus temperature of β-globulin protein. The apparent transition temperature obtained was 78 ± 1°C. At 10% (w/v) concentration of

different cosolvents namely sorbitol, sucrose and glycerol the apparent transition temperature (T_m) increased from 78 ± 1°C to 79 ± 1°C, 80 ± 1°C and 82 ± 1°C respectively. Thus from the above results it can be concluded that the cosolvents at low concentrations caused the increase in the apparent T_m of the protein molecule.

Varieties of cosolvents have been shown to successfully increase the thermal transition temperature of proteins (Mac Donald *et al*., 1996 a and b; Carvajal *et al*., 1999; Gerlsma and Stuur, 1974). The physicochemical basis in the action of these cosolvents is believed to be similar in many cases, that the cosolvents favor the folded state over the unfolded state, but it is highly dependent on the temperature and pressure (Carpenter and Crowe, 1988; Mac Donald and Lanier, 1994; Dondero *et al*., 1996). The variation in these physical parameters may change the transfer free energy of proteins in some protein-cosolvent systems (Mc Clements, 2002).

The stabilization of protein by a variety of cosolvents can be related to their property of increasing the surface tension of water also. During thermal unfolding of proteins this increase of the surface tension can be overcome by the increase in the temperature of the solution at the midpoint of the transition temperature (T_m) and the weak binding of cosolvent molecules (Lin and Timasheff, 1996).

The increase in the protein surface on denaturation is actually generated by the exposure of nonpolar residues buried in the interior of globular protein and by breaking peptide-peptide hydrogen bonds of the protein. The resultant increase in the nonpolar residues and hydration on the protein surface would cause enhanced preferential hydration of denatured protein in polyhydric aqueous solutions, compared with the native protein (Gekko and Morikawa, 1981a and b).

Studies with various proteins on the effect of cosolvents on the structure of them indicated that there is a hydration layer around the protein, which cannot be penetrated by cosolvent molecules because of the strong force of attraction between water and the protein surface and is explained by preferential hydration (Timasheff, 1998). This is also supported by the fact that the cosolvents molecules are bulkier than the water molecules and hence cannot approach protein as closely as water molecules (Timasheff, 1993). The possibility of ordering of a water-cosolvent mixture around the protein would have a strengthening effect on the hydrophobic interaction. Also the micro phase separation could be due to an increase in the water surface free energy or surface tension, resulting in depletion of co solvent molecules at the interface, leading to additional preferential hydration.

In summarizing the results of this chapter, β-Globulin from sesame total protein isolated was found to be homogeneous as evidenced from the chromatographic, electrophoretic and other techniques. The effect of cosolvents namely sorbitol, glycerol and sucrose on the structure and stability of β-globulin were determined. The results indicate that there was no change in the secondary and tertiary structure of β-globulin in presence of cosolvents at various concentrations used. The thermal stability of β-globulin increased in presence of cosolvents as indicated by increase in the thermal melting temperature. Therefore such approaches of addition of cosolvents at various concentrations to β-globulin and the results obtained can be utilized for different biotechnological approaches in stabilizing macromolecules.

3. Hydrolysis of whey protein from dairy and low molecular weight protein from sesame seeds

In the recent years, dietary proteins are known to have a wide range of nutritional, functional and biological properties. Many of these properties are attributed to physiologically active peptides encrypted in protein molecules. Some of the proteins from both animals and plants are important sources of bioactive peptides (Miyoshi *et al.,* 1995; Yano *et al*., 1996; Gobbetti *et al.,* 1997). Growing interest has been shown in recent years for bioactive peptides derived from dietary proteins. Such peptides have been found to exert various bioactivities both *in vitro* and *in vivo*. They may have protective functions, regulation of digestion, nutrient uptake and metabolic or physiological role in the biological system. Milk proteins are one of the important sources from animals containing potential bioactive peptides (Smacchi and Gobbetti, 2000). Whey proteins from milk traditionally was defined as a byproduct of dairy industries with little or no commercial value. This view has changed radically with increasing number of technical and nutritional evaluation of the whey (Clare and Swaisgood, 2000; FitzGerald and Meisel, 2000; Korhonen, 2002; Walzem *et al*., 2002).

Bioactive peptides are inactive when they are present within the sequence of the parent protein and can be released by enzymatic hydrolysis with digestive enzymes or by fermentation. In case of milk proteins, bioactive peptides can be released by the action of proteolytic enzymes obtained from

various microorganisms (Foegeding *et al.,* 2002; Silvestre, 1997). The enzymatic hydrolysis has been one of the most common ways to produce bioactive peptides. Hydrolysate peptide composition and consequently their properties are dependent on protein and enzyme used, as well as, on the conditions of hydrolysis (such as temperature, pH, enzyme to substrate ratio and reaction time etc). There are limited reports on the whey protein hydrolysates having different biological activities. Many casein-derived ACE inhibitory peptides have also been reported to have the bioactive peptides (Karaki *et al*., 1990; Sekiya *et al*., 1992; Yamamoto *et al*., 1994; Nakamura *et al*., 1995; Maeno *et al*., 1996; Chiba and Yoshikawa, 1991).

Some of the proteins from plant sources are also rich source of bioactive peptides (Miyoshi *et al*, 1995; Yano *et al*, 1996). From plant source, oil seeds mainly the sesame proteins are rich in sulphur containing amino acids and lysine. There are reports of proteolytic activity in the sesame protein mainly β-globulin (Tasneem and Prakash, 1989; Rajendran and Prakash, 1988). Compared to α-globulin, the protein β-globulin has better degree of proteolytic hydrolysis (Tasneem and Prakash, 1992). Therefore, β-globulin was selected for hydrolysis to produce the hydrolysates with different bioactivities. In the present chapter hydrolysis of whey protein concentrate and α-lactalbumin from bovine milk and β-globulin from sesame seed was carried out to obtain hydrolysates rich in peptides.

The enzymatic hydrolysis of whey protein concentrate, α-lactalbumin from bovine milk and β-globulin were carried out with enzymes of narrow and broad specifities in single and double enzyme combinations. The enzymes fungal protease, pancreatin and subtilisin were used for the study. The enzymatic hydrolysis of whey protein concentrate and α-lactalbumin was carried out with fungal protease and pancreatin enzymes and the hydrolysis of β-globulin was done with subtilisin enzymes. The degree of hydrolysis thus obtained for the various hydrolysates is given in Table 8. From the table it is clear that the whey protein hydrolysate is having a better degree of hydrolysis compared with the other hydrolysates. The β-globulin hydrolysate was having least degree of hydrolysis (9 ± 2%). This is due to the presence of strong disulfide bonds in the protein molecule (Tai *et al.,* 1999). The better degree of hydrolysis obtained for the whey protein concentrate is due to the broad specificity of enzymes used for the hydrolysis.

The hydrolysates thus obtained were assessed for various biological activities. The antioxidant potential of the WPC hydrolysates was checked by DPPH radical scavenging activity using ascorbic acid as standard antioxidant. The antioxidant activity of the hydrolysate was obtained by determining the IC_{50} values. The IC_{50} is the concentration of hydrolysate at which the 50% radical scavenging activity is achieved. A high radical scavenging activity was observed in the whey hydrolysates in a concentration dependent manner, the IC_{50} values were found to be 0.110 ± 0.01 mg/ml and

for ascorbic acid it was found to be 0.191 ± 0.01 mg/ml as mentioned in Table 9. The WPC hydrolysate was also checked for their total antioxidant potential and it was found to be 5900 ± 210 μmol/g of ascorbic acid equivalents. Whey hydrolysates obtained above was checked for angiotensin converting enzyme inhibitory activity also. The IC_{50} value of the whey hydrolysates against ACE was 600 ± 35 μg/ml versus 215 ± 15 μg/ml for ramipril, which is a known standard ACE inhibitor. The antibacterial activity of the whey hydrolysate was estimated against the microbes namely *Escherichia coli, Listeria monocytogenes* and *Bacillus cereus* and the inhibitory activity of the hydrolysate were found to be insignificant.

α-Lactalbumin is the second most prevalent whey protein. Enzymatic hydrolysis of α-lactalbumin with double enzyme combination namely fungal protease and pancreatin was carried out. The α-lactalbumin hydrolysate thus obtained was checked for antioxidant activity by DPPH radical scavenging action using ascorbic acid as standard. A high radical scavenging activity was observed for the α-lactalbumin hydrolysate in a concentration dependent manner and the IC_{50} values was found to be 0.18 ± 0.02 mg/ml and for ascorbic acid it was found to be 0.191 ± 0.01 mg/ml (Table 10). The total antioxidant activity was found to be 3100 ± 120 μmol/g ascorbic acid equivalents. The α-lactalbumin hydrolysate obtained in the above experiments was also checked for angiotensin converting enzyme inhibitory activity. The IC_{50} value of the α-lactalbumin hydrolysate against ACE was

found to be 410 ± 30 μg/ml compared to 215 ± 15 μg/ml for ramipril. α-Lactalbumin hydrolysate was checked for the inhibitory activity against *Escherichia coli, Bacillus cereus,* and *Listeria monocytogenes.* The pattern of inhibition by the α-lactalbumin hydrolysate against the different microbes namely *Escherichia coli, Bacillus cereus,* and *Listeria monocytogenes* is shown in Fig. 28-30 respectively. From the figures it can be seen that the hydrolysate thus obtained was having better anti-microbial activity against the above microbes.

The whey protein concentrate and α-lactalbumin protein hydrolysates were shown to have lower ACE inhibitory effect compared to the synthetic antihypertensive ramipril (IC_{50} = 215 ± 15 μg/ml). This does not negate against the application of the above protein-derived hydrolysates in the treatment/prevention of hypertension. It is to be expected that milk protein-derived ACE inhibitory peptides obtained from food source, unlike ramipril, would have no undesirable side effects. As a consequence, whey protein derived ACE inhibitory peptides/hydrolysates may find application as a nutraceutical in various physiologically functional foods. The antioxidant activity shown by the whey protein derived hydrolysates is much better compared to the ascorbic acid. Therefore, these hydrolysates can play a role as a potential and natural (food derived) antioxidants. These hydrolysates can be used in various foodstuffs and can be further utilized to prevent and cure various physiological diseases.

There are reports of sesame protein having proteolytic activity (Rajendran and Prakash, 1988). Studies on the hydrolysates obtained from this protein are limited. The protein was hydrolyzed by using protease namely subtilisin from *Bacillus subtilis*. Enzymatic preparation obtained from *Bacillus subtilis* contains two kinds of proteases (Millet, 1970), which have different catalytic mechanisms and therefore could be of interest in this process. Moreover, this is an adequate enzymatic preparation for food technology purposes, as well as being simpler and less expensive since two enzymes are contained in the same extract. The enzyme to substrate ratio was maintained at 1:20 (w/w) ratio. The details are explained under materials and methods section and the degree of hydrolysis was found to be 9 ± 2%. The hydrolysates thus obtained were checked for various bioactivities *in vitro*.

Antioxidant potential of the β-globulin hydrolysate was investigated by DPPH radical scavenging activity using ascorbic acid as standard. A high radical scavenging activity was observed in the β-globulin hydrolysate in a concentration dependent manner, the IC_{50} values were found to be 0.20 ± 0.02 mg/ml (Table 11). The total antioxidant activity was found to be 1600 ± 80 μmol/g tocopherol equivalents. The β-globulin hydrolysates obtained above were also checked for angiotensin converting enzyme inhibitory activity. The IC_{50} value of the β-globulin hydrolysate against ACE was 380 ± 25 μg/ml compared to 215 ± 15 μg/ml for ramipril. The antibacterial activity of the

β-globulin hydrolysate against the microbes namely *Escherichia coli*, *Listeria monocytogenes* and *Bacillus cereus* were found to have very marginal effect.

In summarizing the results of this chapter, the proteins WPC, α-La, β-globulin hydrolysates had lower ACE inhibitory potencies than the synthetic antihypertensive Ramipril (IC_{50} = 215 ± 15 μg/ml) and does not negate against the application of the above protein-derived hydrolysates in the treatment/prevention of hypertension. It is to be expected that the protein-derived ACE inhibitory peptide rich hydrolysates obtained from food source, unlike Ramipril, would have no undesirable side effects. The protein derived ACE inhibitory peptides/hydrolysates may find application as a nutraceutical in various 'physiologically functional foods'. The antioxidant activity shown by these protein-derived hydrolysates is much better compared to the standard antioxidant. So the obtained hydrolysates can play a role as potential food derived antioxidant. These hydrolysates can be used in various foodstuffs or can be further purified to know the peptide in the fraction, which is showing the bioactivity and can be used for the development of peptides in pharma and other applications.

4. Preferential interaction studies of cosolvents with α-lactalbumin and β-globulin

The term stability refers to a protein's resistance to adverse influences such as extreme conditions of acidic, alkaline pH, denaturants and high temperatures. The persistence of its molecular integrity or biological function at high temperatures or other deleterious influences determines the over all stability of the proteins. The maintenance of protein/enzyme structure in a thermodynamically stable form in its native state is essential for their application in the formulation of therapeutics and diagnostics. Yet the structural differences among different proteins are so significant that generalization of universal stabilization strategies has not been successful. Very often, proteins have to be evaluated individually and stabilized on trial and error basis.

Since long it is a general practice to use sugars and polyhydric alcohols in solvent medium in order to stabilize biological macromolecules in solution (Arakawa and Timasheff, 1982 a and b; Gekko and Morikawa, 1981a and b). There are various enzymes of clinical and industrial importance and for some of the standard proteins stabilization studies have been carried out (Gekko and Timasheff, 1981a and b). Under different conditions these sugars and polyols such as sucrose, sorbitol and glycerol are used to achieve thermal stability and to retain structural integrity of proteins. The stabilizing efficiency of these additives depends mainly on the nature of the protein,

hydrophobicity/hydrophilicity and surface charge density properties, degree of interaction of the additive, the microenvironment and the nature of the cosolvent itself (Arakawa *et al.,* 1990a and b; Shulgin and Ruckkenstein, 2005).

A protein normally has both hydrophilic and hydrophobic surface depending upon its amino acid sequence, composition and structure. The solvent component in bulk interacts with the surface of the protein. In three-component aqueous system water, protein and cosolvent are considered as component 1, 2 and 3 respectively according the notion of Stockmayer (1950). When the third component such as a cosolvent is added which may in turn alter the bulk solvent structure, the surface tension or the interaction with the hydrophilic and hydrophobic surfaces, two things can happen preferential exclusion or binding. Cosolvents generally affect the behavior of proteins by interacting with the bulk solvent, which subsequently affect the protein stability. The interaction of cosolvents with protein is mainly dependent on the nature of cosolvents and proteins.

The mechanism of structural stabilization of α-lactalbumin in the presence of sorbitol, sucrose and glycerol has high significance in its application. These sugars and polyhydric alcohols either by interaction with protein or by indirect action through effects on the structure and properties of the solvent or by combination of both these mechanisms can effect the macromolecular structure of the protein. The cosolvent may be preferentially

interacted or excluded from protein surface depending on the nature of interaction. The favorable interaction of cosolvents has tremendous effect on the structure and stability of proteins. The main aim of this chapter is to understand the mechanism of cosolvent-induced stabilization of α-lactalbumin isolated from bovine milk and β-globulin from sesame seeds.

Interaction of cosolvents with α-lactalbumin

The interaction of cosolvents with isolated α-lactalbumin was studied by measuring the partial specific volumes under isomolal and isopotential conditions. The alteration in the partial specific volume is an indication of interaction of cosolvents with protein under given conditions (Mc Clemments, 2002). The presence of third component such as cosolvents significantly changes the solvent structure, which in turn affects the preferential interaction of cosolvents and partial specific volume of protein.

The partial specific volume of the α-lactalbumin was measured as a function of protein concentrations in 0.01 M Tris-HCl buffer at pH 7.5 and is shown in Fig. 31. Under both isomolal and isopotential conditions the partial specific volume was determined to be 0.730 and 0.732 ml/g respectively. Apparently no significant difference was observed between the isomolal and isopotential partial specific volumes of α-lactalbumin, which indicates that protein, can be easily equilibrated in the aqueous medium.

Addition of cosolvents such as sorbitol, glycerol and sucrose has significant effect on the partial specific volume of the protein under isomolal and isopotential conditions. The apparent partial specific volumes in presence of sorbitol are shown in Fig. 32 - 34 respectively. In isomolal conditions the partial specific volume values were 0.736, 0.727, 0.733, 0.723 and 0.734 ml/g in presence of 5, 10, 20, 40 and 45% of sorbitol respectively. Where as in isopotential conditions the partial specific volume values were 0.755, 0.754, 0.782, 0.774 and 0.765 ml/g respectively. Table 12 summarizes the apparent partial specific volume at all concentrations of sorbitol used. There is an increase in partial specific volume in presence of all concentrations of sorbitol, indicating hydration of the protein. Table 13 summarizes the preferential interaction parameters of α-La in presence of sorbitol. The preferential interaction parameters with different concentrations of sorbitol are represented in Fig. 35.

Thus, the preferential interaction parameter was found to be negative at all the concentration of sorbitol used with α-La. In presence of sorbitol the maximum preferential hydration of –0.237 ± 0.025 g/g was obtained at 40% concentration and minimum of –0.058 ± 0.015 g/g at 5% concentration. In all the concentrations, the preferential hydration parameter was found to be positive indicating preferential hydration and mutual exclusion of the cosolvent from the protein domain. The interaction parameter on a mol/mol

basis calculated showed a highest value of –18.4 ± 2.0 mol/mol at 40% concentration and minimum value of -4.6 ± 1.2 mol/mol at 5% sorbitol.

The apparent partial specific volumes in presence of glycerol are shown in Fig. 36 - 38 respectively. In isomolal conditions the partial specific volume values were 0.742, 0.733, 0.741, 0.703, 0.718 and 0.739 ml/g in presence of 10, 20, 30, 35, 40 and 45% of glycerol respectively. Where as in isopotential conditions the partial specific volume values were 0.761, 0.765, 0.790, 0.754, 0.770 and 0.778 ml/g respectively. Table 14 summarizes the apparent partial specific volume at all the concentrations of glycerol used with the protein. There is an increase in partial specific volumes in presence of all concentrations of glycerol indicating the hydration of protein. Table 15 shows the preferential interaction parameters of α-La in presence of glycerol.

The preferential interaction parameters in different concentrations of glycerol are shown in Fig. 39. The preferential interaction parameters on g/g basis were found to be negative, indicating the depleting of glycerol in the immediate domain of the protein, resulting in preferential hydration. The preferential exclusion increased with increasing glycerol concentration, the highest value of -0.299 ± 0.030 g/g was obtained at 40% and in 45% concentration of the cosolvent preferential hydration value was found to decrease.

The apparent partial specific volumes in presence of sucrose are shown in Fig. 40 - 42 respectively. In isomolal conditions the partial specific volume values were 0.760, 0.745, 0.705 and 0.724 ml/g in presence of 10, 25, 40, 45% sucrose respectively. Where as in isopotential conditions the partial specific volume values were 0.779, 0.793, 0.746 and 0.768 ml/g respectively. Table 16 summarizes the apparent partial specific volume at all the concentrations of sucrose used with the protein. There is an increase in partial specific volumes in presence of all concentrations of sucrose indicating the hydration of protein. Table 17 represents the preferential interaction parameters of α-La in presence of sucrose. The preferential interaction parameters with different concentrations of sucrose are represented in Fig. 43. The preferential exclusion reaches the highest value of -0.171 ± 0.016 g/g at 45% concentration. This indicates that sucrose is excluded from the domain of the protein and the solvent within the protein domain is richer in water than the bulk solvent. The above studies on the interaction of bovine α-La with cosolvents namely sorbitol, glycerol and sucrose by partial specific volume measurements suggest that the cosolvents at various concentrations bring stability to the protein by preferential hydration.

Interaction of cosolvents with β-globulin

The isolation, characterization and the effect of cosolvents on the structure of β-globulin were explained in previous chapter. There was no change in the aromatic chromophores, secondary and tertiary structure of the

protein, β-globulin as revealed from fluorescence and circular dichroic spectral measurements in presence of cosolvents. The interaction of cosolvent is protein specific. There is no data available on the interaction of cosolvent with β-globulin, with respect to quantization and mechanism of interaction.

In order to understand the mechanism of thermal stabilization of β-globulin in cosolvents, the preferential interaction parameters were determined. The partial specific volume measurement of proteins in any particular solvent is dependent on the nature of the protein, type of cosolvent, temperature of measurements and also concentration of the protein. Water plays an important role in this three-component system so interpretation of results is cautiously attempted. Thus, in the present chapter partial specific volume measurements of the protein with various cosolvents namely sorbitol, glycerol and sucrose in isomolal and isopotential conditions were carried out in 0.06 M phosphate buffer at pH 7.5 using precision densimetry. These measurements help in understanding the extent of preferential interaction or preferential hydration as compared to its native condition. The partial specific volume of β-globulin in isomolal and isopotential conditions in pH 7.5 phosphate buffer 0.06 M, are 0.726 and 0.728 ml/g respectively (Fig. 44).

The apparent partial specific volumes in presence of sorbitol are shown in Fig. 45 - 47 respectively. In isomolal conditions the partial specific volume values were 0.733, 0.718, 0.721, 0.741 ml/g in presence of 10, 20, 30

and 40% sorbitol respectively. Where as in isopotential conditions the partial specific volume values were 0.742, 0.737, 0.748 and 0.770 ml/g respectively. Table 18 summarizes the apparent partial specific volumes at all the concentrations of sorbitol used with the protein. There is an increase in partial specific volumes in presence of all concentration of sorbitol indicating the hydration of protein. Table 19 shows the preferential interaction parameters of β-globulin in presence of sorbitol. The maximum value of preferential exclusion, -0.137 ± 0.018 (g/g) was obtained in presence of 40% (w/v) and the minimum value of -0.049 ± 0.024 (g/g) was obtained for 10% (w/v) sorbitol (Fig. 48).

The apparent partial specific volumes in presence of glycerol are shown in Fig. 49-51 respectively. In isomolal conditions the partial specific volume values were 0.732, 0.735, 0.740 and 0.723 ml/g in presence of 10, 20, 30 and 40% glycerol respectively. Where as in isopotential conditions the partial specific volume values were 0.737, 0.752, 0.761 and 0.746 ml/g respectively. Table 20 summarizes the apparent partial specific volume at all the concentrations of glycerol used with the protein. From the table it is clear that isomolal and the isopotential values has changed from the values of protein in native condition and also, compared to isomolal value, the isopotential value increased with corresponding increase in cosolvent concentrations. From the partial specific volume data of the protein at isomolal and isopotential conditions the preferential interaction parameters were calculated at all concentrations and the values are given in Table 21.

Thus the preferential interaction parameters are negative at all concentrations. With increasing concentrations of glycerol the ξ_3 values increased in a sigmoid pattern. The maximum preferential interaction was obtained at 40% (w/v) concentration with the value of -0.174 ± 0.021 (g/g) and a minimum value was obtained at 10% glycerol concentration that is -0.052 ± 0.010 (g/g) (Fig. 52). The preferential hydration parameter was positive in all the concentrations of glycerol. The preferential interaction parameters on mole basis are also correspondingly maximum (-27.4 ± 3.1 mol/mol) for 40% glycerol concentration. Thus indicating preferential hydration and mutual exclusion of glycerol from the domain of the protein.

The preferential interaction parameter (ξ_3) was obtained from partial specific volumes of the protein in isomolal and isopotential conditions with various concentrations of sucrose used. The apparent partial specific volumes in presence of sucrose are shown in Fig. 53 - 55 respectively. In isomolal conditions the partial specific volume values were 0.727, 0.730, 0.741 and 0.738 ml/g in presence of 10, 20, 30 and 40% sucrose respectively. Where as in isopotential conditions the partial specific volume values were 0.733, 0.751, 0.763 and 0.767 ml/g respectively. The values are given in Table 22. Corresponding preferential interaction parameters were calculated and represented in Table 23. Negative value of ξ_3 was obtained at all concentrations indicating that sucrose is preferentially excluded from the domain of the protein.

In explaining the results, perhaps the excess water around the protein, which in turn is surrounded and protected by cosolvent is indirectly responsible for either the preferential interaction or exclusion. The value of preferential interaction parameter reaches a maximum value of -0.110 ± 0.011 (g/g) at 40% (w/v) concentration of sucrose (Fig. 56). In all the concentrations of sucrose used from 10-40% (w/v) the preferential hydration was found to be positive, indicating that sucrose is excluded from the domain of the protein molecule and the cosolvent within the protein domain is richer in water than the bulk solvent. With glycerol and sucrose also there was an increase in the preferential hydration with increasing cosolvent concentrations.

The above studies on the interaction of bovine α-La and β-globulin with cosolvents namely sorbitol, glycerol and sucrose by partial specific volume measurements suggest that the cosolvents at various concentrations bring stability to these proteins. The preferential hydration parameter was found to be positive with all the concentrations in presence of different cosolvents. This indicates that there is a strong force of attraction between the water and protein surface, where the water forms a layer around the protein, which cannot be penetrated by the cosolvent molecules. In a three-component system, preferential hydration parameter is a good indicator to monitor the cosolvent-induced stability of the macromolecule.

The steric exclusion contribution to the transfer of protein molecules from pure solvent to a cosolvent solution is thermodynamically unfavorable, This may be because of the free energy required to maintain the concentration gradient between the local domain and the bulk solution (Timasheff, 1993). Simple sugars (such as sucrose and glucose) and polyols (e.g. glycerol) use to stabilize proteins primarily through preferential hydration and steric exclusion mechanism (Timasheff, 1993, 1998; Ebnel *et al.*, 2000). An alternative mechanism may stem from the increase in the free energy of cavity formation induced by the introduction of sugars, i.e., the surface tension effect. The cohesive force of sugars responsible for the increase in the surface tension of water is a very important factor governing the preferential interaction of proteins with solvent components in aqueous sugar systems and hence the stabilization of proteins. Protein structure stabilizing action of sorbitol is driven by preferential exclusion from the unfolded protein than from the native structure (Xie and Timasheff, 1997a and b).

However, the surface tension effect of cosolvent is not the sole factor involved in the protein-solvent interactions. Increasing the surface tension of water by an excipient does not necessarily mean automatic protein stabilization. On the other hand, cosolvents, which decrease the surface tension of water, may still stabilize a protein. Glycerol is known to decrease the surface energy of water but induce preferential hydration of lysozyme and Bovine serum albumin (Gekko and Timasheff, 1981a and b). Reports on

the stabilization of proteins namely chymotrypsinogen, α-chymotrypsin and ribonuclease A with glycerol (Gekko and Timasheff, 1981a and b; Priev *et al.*, 1996) suggests that glycerol (essentially hydrophilic) interact strongly with water and slightly decrease the surface tension of water. There is a fine balance between attraction from polar groups and repulsion from the non-polar regions of the protein surface. Addition of glycerol increases the chemical potential of the protein. The stabilization of the protein by glycerol may be due to the enhancement of the structural network of the medium or of the solvation layer of the protein molecule.

During stabilization process water structure plays an important role. Water molecules are smaller than the cosolvent molecules and therefore comparatively strong force of attraction is experienced between protein and water molecules than cosolvent molecules. As a result of this a layer of hydration is formed around the protein molecules, which cannot be penetrated by the cosolvent molecules. Thus, the cosolvent molecules are excluded from the domain of the protein and thereby bring stability to the native structure of bovine α-La and β-globulin. Thus the cosolvents namely sorbitol, sucrose and glycerol stabilizes the proteins α-La and β-globulin primarily by preferential hydration.

In summarizing the results of this chapter, it is clear that the cosolvents used at various concentrations in the study enhance the stability of α-lactalbumin and β-globulin. These cosolvents selected namely sorbitol, glycerol and sucrose structurally stabilize the proteins to various extents depending on the nature and type of the cosolvent used. There was an increase in the thermal stability of the proteins to different extent in the presence of these cosolvents depending on the nature of cosolvents. The net preferential hydration of the proteins in presence of cosolvents is predominantly due to the excess of the cosolvent from the protein domain. However the cosolvent do not have any significant effect on the structure of the protein in the detectable level. The role of cosolvent in the stability of protein structure is predominantly due to the strengthening of the nonpolar-nonpolar interactions, which is perhaps the driving force in the process of stabilization of these proteins in presence of cosolvents.

SUMMARY AND CONCLUSIONS

The knowledge of protein structure, function and stability of the proteins is essential for formulation of various food products. There are different sources of proteins and based on their physicochemical parameters they can be utilized in biological system for different purposes. The bovine milk and plant seeds are the major sources of proteins. Various food formulations and processing involve the undesirable biochemical changes in the proteins. Therefore, it is imperative to study the structure-function relationship of protein and make it more stable under various conditions. In addition, to provide the basic nutrition, many proteins are known to have bioactive peptide sequences, which are released by the digestion process *in vivo* or *in vitro*. Bovine milk and seed proteins are known to be the good sources of bioactive peptides.

Bovine milk contains about 3.5% protein, of which caseins constitutes nearly 80% and remaining 20% is whey. Whey proteins include β-lactoglobulin (β-Lg), α-lactalbumin (α-La), serum albumin, lactotransferrin, immunoglobulin and β_2-micro globulin. The α-La constitutes 4% of the whey proteins. It is also a component of the lactose synthase complex.

Sesame (*Sesamum indicum L.*) seeds contain nearly 25% protein and the defatted flour contains 50% protein. Nearly 25% of total protein is constituted by β-globulin, the second major sesame protein. This low molecular weight

protein is rich in α–helical structure and has considerable amount of proteolytic activity with unusually high contents of sulfur containing and acidic amino acids.

The structural stability of native protein is known to affect strongly by a variety of substances used at different concentrations. Cosolvents play an important role in the structural stability of proteins. The mode of stability by these cosolvents is very specific and depends mainly on the nature of cosolvent, structural characteristics of the protein and also the environment in which it is used. Effect of cosolvents on the structure, function and stability of the above two low molecular weight proteins one from bovine milk i.e. α-La and the other from sesame seed i.e. β-globulin were carried out. The mechanism involved in the stability of these two low molecular weight proteins was studied by measuring the partial specific volume and preferential interaction parameters.

Different biologically active peptides, which are encrypted within the sequence of the parent protein, can be released by enzymatic proteolysis. Attempts were made to hydrolyze whey protein concentrate (WPC), α-La from dairy and β-globulin from sesame seed with different enzymes to obtain hydrolysates with potential peptides with various bioactivities. These can be used in lucrative health food market as nutraceuticals, pharma foods or designer foods.

With these objectives the present investigation is undertaken and the results and conclusions are brought out in the form of a thesis entitled "**Structure function and stability of low molecular weight proteins from selected seeds and dairy whey**".

The investigation is divided into the following chapters:

(i) Isolation and characterization of α-lactalbumin from bovine milk and interaction studies with cosolvents.

(ii) Isolation and characterization of β-globulin form sesame seeds and interaction studies with cosolvents.

(iii) Hydrolysis of whey protein concentrate, α-lactalbumin from dairy and low molecular weight protein fraction from sesame seeds.

(iv) Preferential interaction studies of cosolvents with α-lactalbumin and β-globulin.

In **Chapter I,** the isolation, purification and characterization of α-la from bovine milk and also the effect of cosolvents namely sorbitol, glycerol and sucrose on the structural stability of the protein is described. α-Lactalbumin was purified to homogeneity and the purity was evaluated by the SDS-PAGE and RP-HPLC. The amino acid composition of the isolated α-La had a correlation coefficient of 95% with the literature values. The effect of different concentrations of sorbitol, glycerol and sucrose ranging from 10-30% on the protein was investigated. The fluorescence emission spectra of α-La

showed an increase in the fluorescence emission intensity with increasing concentrations of sorbitol and glycerol but in presence of sucrose, quenching in the fluorescence emission intensity was observed. There was 2 nm blue shift in the emission maxima in presence of higher concentrations of glycerol and sorbitol. Preferential hydration and conformational change might alter the microenvironment of the aromatic chromophores in presence of cosolvents. These results indicate that there is subtle conformational change of the protein molecule in these cosolvents.

The effect of sorbitol, glycerol and sucrose on the tertiary structural stability of the α-La was examined by near–UV CD measurements. There was marginal change in the tertiary structure of the protein in the presence of different concentrations of cosolvents in the concentration range of 10-30%. The effect of sorbitol, glycerol and sucrose on the secondary structural stability of the protein was examined by far-UV CD measurements. In presence of these cosolvents there was a significant increase in α-helical content but in presence of 30% glycerol there was decrease in the β-structure of the protein by about 12%.

Thermal denaturation of α-La was carried out in presence of cosolvents to determine the stabilization effect. The apparent T_m value of the native protein was found to be 65°C. With increasing concentrations of each cosolvents namely sorbitol, glycerol and sucrose there was an increase in the

apparent T_m values for the protein. The efficiency of thermal stabilizing capacity of cosolvents was found to be in the order of Sorbitol > Sucrose> Glycerol. At 40% concentration of sorbitol, sucrose and glycerol the apparent T_m was found to be 71, 71 and 68°C respectively.

In **Chapter II,** the isolation, purification and structural characterization of β-globulin from sesame seeds are described. The protein was purified to homogeneity using salt precipitation method and the homogeneity was evaluated by electrophoretic and gel filtration chromatographic methods. The amino acid composition of the isolated β-globulin was determined and it was well correlated with the literature value with a correlation coefficient value of 97%. The effect of cosolvents on the structure function and stability of β-globulin was studied by CD spectroscopy, fluorescence spectroscopy and differential absorption spectroscopy. The fluorescence emission maximum of β-globulin was found to be 336 nm in absence of cosolvents. The presence of cosolvents namely sorbitol, glycerol and sucrose did not show any changes in the emission maxima of β-globulin. The results of fluorescence spectra indicate that the presence of cosolvents did not bring any gross conformational change in the protein molecule. The far-UV CD spectra of the protein in absence of cosolvents showed 26 ± 1% α–helix, 38 ± 1%, β-structure and 36 ± 1% aperiodic structures. In presence of different cosolvents namely sorbitol, glycerol and sucrose slight changes in the secondary structural parameters of the protein molecule was observed. There was an increase in

the α–helical content of the protein with increasing concentrations of cosolvents. There was a 9% increase in the β-structure in the presence of sorbitol and sucrose at 30% concentration and decrease of 10% in the aperiodic structure with all the cosolvents used.

The effect of cosolvents on thermal stability of β-globulin was determined by measuring thermal denaturation temperature. The apparent transition temperature for β-globulin protein was found to be 78 ± 1°C. The presence of all the cosolvents such as sorbitol, sucrose and glycerol increased the thermal denaturation temperature of β-globulin in a concentration dependent manner. At 10% (w/v) concentration the apparent transition temperature (apparent T_m) increased to 79±1, 80±1 and 82±1°C respectively.

In **Chapter III,** the enzymatic hydrolysis of whey protein concentrate (WPC), α-La from dairy whey and β-globulin from sesame seed are described. The hydrolysates obtained from respective proteins enriched in bioactive peptides with various bioactivities. WPC hydrolysate (38 ± 3%) gave a maximum degree of hydrolysis compared with that of the β-globulin hydrolysate (9 ± 2%). The better degree of hydrolysis of the WPC hydrolysate was due to the maximum susceptibility of the protein for the enzymes used and the broad specificity of the enzymes (fungal protease and pancreatin) used for its hydrolysis. The least DH% obtained for β-globulin hydrolysate was due to the strong disulfide present in the protein.

The hydrolysates were analysed for their antioxidant, ACE inhibitory and the antimicrobial potential. The WPC hydrolysate was having maximum total antioxidant potential and proton radical scavenging potential compared with the β-globulin hydrolysate. The total antioxidant potential for the whey protein hydrolysate was 5900 ± 210 μmol/g of ascorbic acid equivalents. The IC_{50} values for the WPC hydrolysate for the DPPH radical scavenging action was 0.110 ± 0.01 mg/ml when compared with the standard antioxidant ascorbic acid, which had a IC_{50} value of 0.191 ± 0.01 mg/ml. Thus the obtained hydrolysates can be utilized in *in vitro* models. The total antioxidant and the proton radical scavenging potential are evaluated for WPC hydrolysate, α-La hydrolysate and β-globulin hydrolysate.

The antimicrobial activity of the hydrolysates were checked against microbes namely *Escherichia coli, Bacillus cereus,* and *Listeria monocytogenes.* The inhibitory action of the WPC hydrolysate and the β-globulin hydrolysate was found to be insignificant. But the α-La hydrolysate was effective against *Escherichia coli* and *Bacillus cereus* compared with the *Listeria monocytogenes microbes.* The obtained hydrolysates were found to inhibit angiotensin-converting enzyme. The β-globulin hydrolysate was having maximum ACE inhibitory potential with an IC_{50} value of 380 ± 25 μg/ml. although the hydrolysates were having slightly less ACE inhibitory potential compared with the ramipril (IC_{50} = 215 ± 15 μg/ml) for long term use food sources are advocated for medical purposes rather than chemical source. Thus the above

hydrolysates were having antioxidant, antimicrobial and ACE inhibitory potential.

In **Chapter IV** is described the mechanism of interaction of cosolvent-induced stabilization of two low molecular weight proteins namely α-La and β- globulin by measurement of partial specific volume. The cosolvents used in the study are sorbitol, glycerol and sucrose. The preferential interaction parameters calculated from partial specific volume was found to be negative for α-la in the presence of all the cosolvents. These results clearly indicate the phenomenon of preferential hydration or exclusion of the cosolvents from the domain of the protein molecule. The preferential interaction parameter was found to be maximum with -0.237 ± 0.025 g/g at 40% sorbitol and minimum of -0.058 ± 0.015 g/g at 5% sorbitol. The preferential exclusion increased with increasing glycerol concentration, the highest value of -0.299 ± 0.030 g/g was obtained at 40% concentration. The preferential hydration reached the highest value of -0.171 ± 0.016 (g/g) in presence of sucrose at 45% concentration (w/v).

With β-globulin in the cosolvents namely sorbitol, glycerol and sucrose at all concentrations the preferential interaction parameter (ξ_3) was found to be negative. In presence of sorbitol the maximum ξ_3 value of -0.137 ± 0.018 (g/g) was observed at 40% (w/v) concentration and the minimum value of -0.049 ± 0.024 (g/g) at 10% (w/v) concentration. The maximum preferential

exclusion was obtained at 40 % (w/v) concentration of glycerol with the value of -0.174 ± 0.021 (g/g). The preferential hydration parameter was obviously positive in all the concentrations of glycerol. The preferential interaction parameter reaches a maximum value of -0.110 ± 0.011 (g/g) in presence of sucrose at 40% (w/v) concentration. Thus these values clearly indicate that proteins used in the study are stabilized by preferential hydration of the protein and mutual exclusion of the cosolvent from the domain of the protein, which results in added hydration.

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