THE INTERACTION OF COSOLVENTS, PROTEOLYTIC ENZYMES AND SELECTIVE METAL IONS WITH α-CASEIN - THE STRUCTURE - STABILITY RELATIONSHIP

A THESIS SUBMITTED TO THE UNIVERSITY OF MYSORE, MYSORE, FOR THE DEGREE OF

DOCTOR OF PHILOSOPHY

IN

BIOTECHNOLOGY

By

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September 2006

…… DEDICATED TO

My Beloved Parents

DECLARATION

I hereby declare that this thesis entitled "**THE INTERACTION OF COSOLVENTS, PROTEOLYTIC ENZYMES AND SELECTIVE METAL IONS WITH α-CASEIN - THE STRUCTURE - STABILITY RELATIONSHIP**" which is submitted herewith for the degree of **DOCTOR OF PHILOSOPHY** in **BIOTECHNOLOGY** of the **UNIVERSITY OF MYSORE, MYSORE,** is the result of work done by me in the Department of Protein Chemistry and Technology, Central Food Technological Research Institute, Mysore, India under the guidance and supervision of Dr. V. Prakash during the period 2002 - 2006.

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

Date: September 14, 2006 **Sistla Srinivas**

Place: Mysore, India.

CERTIFICATE

I hereby certify that this Ph.D thesis entitled "**THE INTERACTION OF COSOLVENTS, PROTEOLYTIC ENZYMES AND SELECTIVE METAL IONS WITH α-CASEIN - THE STRUCTURE - STABILITY RELATIONSHIP**" submitted by **Mr. Sistla Srinivas** for the degree of **DOCTOR OF PHILOSOPHY** in **BIOTECHNOLOGY** of the **UNIVERSITY OF MYSORE, MYSORE,** is the result of research work carried out by him in Department of Protein Chemistry and Technology at Central Food Technological Research Institute, Mysore, India under my guidance during the period 2002 - 2006. This work has not been submitted either partially or fully to any other degree or fellowship earlier by Mr. Sistla Srinivas.

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Date: September 14, 2006 **Sistla Srinivas**
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CONTENTS

LIST OF ABBREVIATIONS

%	percent
ε	molar extinction coefficient (M^{-1} cm^{-1})
ΔA	difference in absorbance
$^{\circ}$C	degree centigrade
α-CN	α - casein
ΔG	change in free energy
μg	microgram
ΔH	change in enthalpy
μl	micro liter
μM	micro molar
λ_{max}	lambda maxima
ΔS	change in entropy
A	absorbance
A$^{\circ}$	Angstrom
ACE	angiotensin converting enzyme
ACEI	angiotensin converting enzyme inhibition
AMS	4-acetamido-4'-maleimidyl stillbene-2, 2'-disulphonic acid
ANS	8-anilino-1-naphthalene sulphonic acid
AU	arbitrary units
Cal	calorie
c	concentration of protein

CD	circular dichroism
CHCA	α-cyano-4-hydroxycinnamic acid
CFU	colony forming units
cm	centimeter
CSI	chemical shift index
Cys^{36}	this refers to amino acid cysteine and its position in the sequence as shown in the superscript
DH	degree of hydrolysis
dm	decimeter
dmol	decimole
DMSO	dimethyl sulphoxide
DMSO-d_6	dueteriated dimethyl sulphoxide
DPPH	1, 1-diphenyl-2-picrylhydrazyl
E. coli	*Escherichia coli*
$E^{1\,\%}_{1cm,\,\lambda_{max}}$	absorption coefficient of 1 % solution in 1cm path length
EDTA	ethylene diamine tetra acetic acid, disodium salt
ESI-MS	electro spray ionization – mass spectroscopy
FTIR	Fourier transform infrared
FI	fluorescence intensity
g	gram
gen/h	generations per hour (growth rate)
h	hour(s)

H. pylori	*Helicobacter pylori*
HCl	hydrochloric acid
HEPES	N-2-Hydroxyethylpiperazine-N`-2-ethane sulphonic acid
HPLC	high performance liquid chromatography
IC_{50}	concentration of inhibitor at 50 % inhibition
in silico	using the assistance of software and computer
K	kelvin
k_a	association constant
k_d	dissociation constant
kcal	kilocalorie
kDa	kilo Dalton
kJ	kilo Joule
kv	kilovolts
l	liter
LMCT	ligand to metal charge transfer
m	meter
M	molar concentration
MALDI TOF	matrix assisted laser desorption ionization time of flight
mg	milligram
MIC	minimum inhibitory concentration
min	minutes
mol	mole
MRW	mean residue weight

N	normality
NaCl	sodium chloride
NaOH	sodium hydroxide
ND	not detected
ng	nanogram
nm	nanometer
NMR	nuclear magnetic resonance spectroscopy
NOE	nuclear overhauser effect
NOESY	nuclear overhauser enhancement spectroscopy
OPA	*o*-phthaldehyde
SDS - PAGE	sodium dodecyl sulphate polyacrylamide gel electrophoresis
Pb(II)	lead
PEP	prolyl endo peptidase
PEPI	prolyl endopeptidase inhibitory
Peptide 10	peptide with sequence QKALNEINQF from the α_{S2}- casein f(94-103)
Peptide 14	peptide with sequence TKKTKLTEEEKNRL from α_{S2}- casein f(163-176)
pH	negative logarithm of hydrogen ion concentration
pI	isoelectric point
RP	reverse phase
ROESY	rotating frame overhauser effect spectroscopy
rpm	revolutions per minute

S. aureus	*Staphylococcus aureus*
s	second
SH	thiol
SD	standard deviation
T	absolute temperature
TEA	triethyl amine
TFA	trifluoroacetic acid
T_m	thermal denaturation temperature
TMS	trimethyl silane
TOCSY	total correlated spectroscopy
Tris	tris (hydroxy methyl) amino methane
trp	tryptophan
tyr	tyrosine
UV	ultraviolet
v	volts
W/V	weight /volume
V/V	volume/volume
Zn(II)	zinc

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INTRODUCTION

1. INTRODUCTION

Proteins are complex bio-macromolecules made up of amino acids joined by peptide bonds. Proteins are primary constituents of biological organisms, essential to the structure and function of all living cells. They are important constituents in the structural framework of both plants and animals. Some proteins are enzymes, which catalyze chemical reactions. Functions carried out by proteins include immune response, storage and transport of various ligands. During digestion, proteins are broken down to free amino acids.

1.1. Milk proteins

The role of milk in nature is to nourish and provide immunological protection for the mammalian young. Milk has been a food source for humans since prehistoric times. Milk is a complex food with over thousands of different molecular species (Mulvihill and Fox, 1989). Early studies of milk proteins were directed towards their nutritive and immunological properties. During the last twenty years, there has been a revolutionary change in the understanding of milk protein composition and structure (Holt, 1992; Schmidt, 1982).

Milk is a polyphasic secretion of the mammalian gland containing 3.9% fat, 3.3% protein, 5.0% lactose and 0.7% minerals. The protein fraction of the milk is distributed among caseins (80%) whey proteins (14%) and non-protein nitrogen (6%). Milk proteins

include α_{S1}-casein, α_{S2}-casein, β-casein, κ-casein, β-lactoglobulin, α-lactalbumin, serum albumin, lactotransferrin, immunoglobulins, and β_2-microglobulin (Swaisgood, 1992; Farrell, Jr., 2004). Caseins include α_{S1}-casein, α_{S2}-casein, β-casein and κ-casein. Whey proteins are composed of β-lactoglobulin, α-lactalbumin, bovine serum albumin and immunoglobulins. Whey also contains proteins such as lactoferrin, lactoperoxidase and lysozyme as well as more than 60 different enzymes. In addition, milk contains peptides that exist naturally in the milk or formed from milk proteins, mainly caseins by various chemical or biological treatments (Korhonen and Pihlanto, 2003b).

1.2. Caseins

Casein is the main proteinaceous component of milk, where it accounts for 80% of the total milk protein. Casein in bovine milk exists as large stable micelles (40-300 nm). These “micelles” are 108 kDa. in mass and comprises of four different casein subunits and calcium phosphate (Holt and Horne, 1996). The principal casein fractions are α_{S1}-casein, α_{S2}-casein, β-casein and κ-casein. Casein micelles and submicelles are held together by calcium phosphate - mediated salt bridges (Rollema, 1992). In a casein submicelle, one molecule of κ-casein interacts with four molecules of α_{S1}-casein and one molecule of α_{S1}-casein. The ratios of α_{S1}-casein, α_{S1}-casein, β-casein and κ-casein are 1: 4: 1: 4 (Kumosinski *et al.*, 1987). The relative proportion of these caseins varies among species (Holt, 1992; Holt and Horne,

1996). The distinguishing property of all caseins is their low solubility at pH 4.6. The common feature is that caseins contain phosphorylated serines. These phosphate groups are important to the structure of casein micelle. Binding of calcium by the individual caseins is proportional to the phosphate content (Miller *et al.,* 1990).

The conformation of caseins is like denatured globular proteins. Structurally, caseins are neither globular nor fibrous proteins and do not possess well-defined secondary or tertiary structure. Dunker *et al.,* (2002) has classified caseins as an intrinsically unstructured protein. The high number of proline residues in caseins causes bending of the protein chain and inhibits the formation of ordered secondary structures. Lack of tertiary structure in caseins exposes hydrophobic residues. Caseins have open structure and are hydrated due to a balance between hydrophobic and polar amino acids (Alaimo *et al.,* 1999b). The combination of electrostatic and hydrophobic properties allows the caseins to form large colloidal aggregates (Kumosinski and Farrell Jr., 1994; Alaimo *et al.,* 1999a). In caseins aromatic amino acids and prolines are conserved across species.

Caseins act as molecular chaperones and stabilize proteins (including milk proteins) against aggregation and precipitation under conditions of stress and temperatures (Miller *et al.,* 1990; Bhattacharyya and Das, 1999; Morgan *et al.,* 2005). The food industry

has employed casein and caseinate products for their nutritional and functional value.

1.3. α-Casein

Cow's milk proteins contain about 80% casein. More than 65% of casein is calcium sensitive and called α-casein (Jenness, 1970). The rest is calcium insensitive and is mainly κ-casein. α-Casein is having a molecular weight of 27,300 Da. The physico - chemical properties of α-casein are tabulated (table 1). It is present in all mammals as a random coil protein and is major protein constituent of casein micelle (Swaisgood, 1993). α-Casein is involved in transport of calcium and other metal ions to neonates. The α-casein found to be protective against heat coagulation (Matsudomi *et al.,* 2003), having chaperone like activity, (Nam and Walsh, 2003) binds to membrane receptors and acts as a signal transducer, (Hira *et al.,* 2003) rheomorphic (plasticity), (Syme *et al.,* 2002) having poly (L-proline) II (PPII) helical conformation, (Smyth *et al.,* 2001) transporter of other milk proteins to secretary organelles in the mammalian epithelial cells (Chanat *et al.,* 1999).

The α-casein consists of two proteins α_{S1}-casein and α_{S2}-casein. These two fractions are in the ratio of 4: 1. The α_{S1}-casein (B variant) is a single-chain polypeptide of known sequence with 214 amino acid residues and a molecular mass of 24,529 Da. The amino acid sequence

of α_{S1}-casein is shown in figure 1. The amino acid sequence of α_{S1}-casein (B variant) consists of a major calcium binding peptide (residues 43 – 84) located on the N-terminal half of the molecule. The C-terminal half of the molecule (residues 100 - 199) contains high number of hydrophobic amino acids.

The acidic segment of α_{S1}-casein (residues 43 – 84) contains 7 phosphoserines, responsible for lack of association at low ionic strength through charge repulsions (table 2). The α_{S1}-casein contains 18 proline residues. These proline residues are the pivotal points for two stranded anti- parallel sheet structures, referred to as hydrophobic arms. These hydrophobic portions of the α_{S1}-casein were predicted to be involved in interaction with other proteins within the interior of the micelles (Kumosinski *et al.,* 1991). The chains of native caseins are more extended and flexible which likely prevent strong packing of aromatic clusters in caseins. Aromatic side chain interactions help to stabilize certain secondary structure through intra strand (beta hair pin turns) and inter-strand (helices and beta sheets) contacts (Alaimo *et al.,* 1999a).

α_{S2}-Casein constitutes about 10% of bovine casein fraction. α_{S2}-Casein A is widespread in *Bos taurus* breeds. It has been less studied milk protein because of its weak solubility. The weak solubility of bovine α_{S2}-casein is due to its least hydrophobicity (Chaplin, 1986).

α_{S2}-casein (variant A) consists of 222 amino acid residues with molecular weight of 26,019 Da (Hoagland *et al.,* 2001). The amino acid sequence of α_{S2}-casein is shown in figure 2. It contains 10 proline residues, the negative charges concentrated near N-terminus and positive charges towards C-terminus. This is the most calcium sensitive fraction of bovine milk. For stability in the presence of low calcium concentration, requires an equimolar amount of κ-casein (Snoeren *et al.,* 1980; Haga *et al.,* 1983). Two cysteine residues, cys^{36} and cys^{40} forms an intrachain bridge or dimeric forms with two interchain bridges at higher temperatures (Rasmussen *et al.,* 1994). Purified caseins associate through a large variety of interactions: ionic, hydrophobic interactions, hydrogen, disulphide and calcium bonding (Schmidt, 1982).

1.4. Interaction of proteolytic enzymes with α-casein and release of bioactive peptides

Proteolytic enzymes are complex proteins involved in hydrolysis of proteins. They work optimally at specific temperature and pH. Proteolytic enzymes include pancreatic enzymes, plant-derived enzymes, and fungal-derived enzymes.

Proteases occur naturally in all organisms and constitute 1-5% of the gene content. These enzymes are involved in a multitude of physiological reactions from simple digestion of food proteins to highly

regulated cascades (e.g. the blood clotting cascade, the complement system, apoptosis pathways). Peptidases can hydrolyze either specific peptide bonds (*limited proteolysis*), or break down complete peptide to free amino acids (*complete proteolysis*). These proteases during hydrolysis liberate bioactive peptides from various substrates.

1.4.1. Bioactive peptides

Peptides influence a number of very important physiological and biochemical functions of life. Peptides functions are well known in the central nervous systems, immunological processes, cardio-vascular system and in the intestine. Peptides influence cell-to-cell communication, and are involved in a number of biochemical processes, like metabolism, pain response, reproduction and immune response (Wieland, 1995). Discovery of new peptides is driven by technological improvements; in particular by mass spectrometric methods and bioinorganic tools that detect peptide in picomolar range (Adermann *et al.,* 2004; Miranda *et al.,* 2004). The increasing knowledge in modes of action of bioactive peptides led to increased interest in pharmacology and medical sciences in this class of compounds.

Food-derived bioactive peptides represent a source of health enhancing components that may be incorporated into functional foods and in nutraceutical (Meisel and Fitzgerald, 2003). Although several

animal and plant proteins contain potential bioactive peptide sequences, milk proteins are currently the main source of biologically active peptides (Dziuba *et al.,* 1999). Peptides with different activities like ability to enhance calcium absorption (Reynolds, 1992) opioid (Chiba and Yoshikawa, 1989; Clare and Swaisgood, 2000), hypertensive/ACE inhibitory (Ariyoshi, 1993), immuno-modulating and anti-microbial activities have been isolated from milk proteins (Korhonen and Pihlanto, 2001; Korhonen and Pihlanto, 2003a; Jost, 1993). Intrinsic bioactivities of the peptides encrypted in major milk proteins are latent until they are released and activated by enzymatic hydrolysis or during gastrointestinal digestion. Activated peptides are potential modulators of various regulatory processes in the living system. Enzyme mediated proteolysis is found to be the best source for the release of natural biological peptides (Meisel, 2004).

Three strategies have been used in the identification and characterization of biologically active peptides. (a) Isolation of peptides from *in vitro* enzymatic digests (b) Isolation of peptides from *in vivo* gastrointestinal digests (c) Chemical synthesis of peptides having identical or similar structures to those known to be bioactive (Gobbetti *et al.,* 2002).

The proteins and peptides have biological activities, e.g. ion carriers (caseins, α-lactalbumin), retinal and fatty acid carriers (β-

lactoglobulin and BSA), immunomodulators (α-lactalbumin and lactoferrin) and immune protection (Igs). Some peptides influence gastrointestinal function, while others can stimulate the immune system or effect blood coagulation or blood pressure (Korhonen and Pihlanto, 2003b; Vegarud *et al.,* 2000).

While no specific physiological property has been proposed for the whole casein system, various peptides hidden (or inactive) in the amino acid sequence have been the subject of increasingly intense studies. Due to the lack of secondary and tertiary structures caseins are susceptible to proteolysis and can generate peptides with various biological activities. Some of the peptides are released from intact milk casein during *in vivo* digestion by gastric and pancreatic enzymes while others result from the action of enzymes during food processing (Ariyoshi, 1993; Dziuba *et al.,* 1999; Takano, 1998).

1.4.2. Multifunctional peptides

The multifunctionality of various milk - derived peptides relates to the presence of various bioactivities i.e., specific peptide sequences having two or more biological activities have been reported (Meisel, 2004). Some regions in the primary structure of caseins contain overlapping peptides sequences that exert different biological effects. These regions have been considered as “strategic zones” which are

partially protected from proteolytic breakdown. (Meisel and Bockelmann, 1999).

Bioactive peptides usually contain 3 - 20 amino acid residues per molecule (Korhonen and Pihlanto, 2003a). These peptides express either one of the various biological activities or two or more activities simultaneously. The simultaneous expression of two or more activities by various peptides is called multifunctional peptides (table 3). The relationship between various activities is due to the overlapping sequences present in caseins (Migliore - Samour and Jolles, 1988; Hata *et al.,* 1998; Meisel, 2004; Hou *et al.,* 2003). Some of the properties multifunctional peptides exhibit are as follows:

1.4.2.1. Angiotensin converting enzyme inhibitory peptides

The angiotensin-converting enzyme (EC 3.4.15.1) has a key role in blood pressure regulation. It principally catalyses the conversion of angiotensin-I into angiotensin-II, a powerful endogenous vasoconstrictor; ACE also degrades the vasodilator bradykinin (Tauzin *et al.,* 2002). ACE is a Zn^{2+} metallopeptidase also called peptidyl dipeptidase. By inhibiting ACE, the concentration of the vasoconstrictor angiotensin-II decreases, while that of the vasodilator bradykinin increases, which results in an antihypertensive effect (Messerli, 1999).

Foods containing ACE inhibitory peptides are known to be effective in both the prevention and treatment of hypertension. Many ACE inhibitors have been identified in the enzymatic digests of food proteins, and especially from milk proteins (Meisel, 1993).

Synthetic ACE inhibitors are one group of drugs in the treatment of hypertension. However, these synthetic drugs can have significant side effects due to their high activity and specificity. Peptides with ACE inhibitory activity have already been isolated from different food proteins. These peptides could represent a healthier and natural alternative for the synthetic ACE inhibitory drugs (Pihlanto-Leppala *et al.,* 1998; Yamamoto, 1997).

1.4.2.2. Antioxidant peptides

Antioxidant is an organic molecule that can counteract the damaging effects of oxygen in tissues. The term applies to molecules reacting with oxygen and also with molecules that protect from free radicals (molecule with unpaired electron) (Liu *et al.,* 2004).

Lipidic oxidation is a crucial process in food decay. Food derived peptides protect the food material by preventing the free radical action by scavenging them. The free radicals have a drastic effect on the cells; they bring in oxidation and cell death. Scavenging the free radicals will help to prevent the cell damage. Many food-based

peptides were shown to possess the free radical scavenging ability *in vitro* (Hou *et al.,* 2003; Suetsuna *et al.,* 2000).

Enhancement of the body's antioxidant defenses through dietary supplementation reduces the oxidative stress. This hypothesis has lead to incorporation of various antioxidants like Vitamins E, C, carotenoids and polyphenols. Research into new food ingredients with antioxidant potential is underway. Several studies have described the antioxidant potential of various plant and animal proteins and peptides therein (Cervato *et al.,* 1999). Some of them are milk proteins, maize, zein, wheat gluten egg-white albumin, soy protein and peptide hormones (Tong *et al.,* 2000; Suetsuna *et al.,* 2000).

1.4.2.3. Mineral binding peptides

Minerals and trace elements in cow milk occur as inorganic ions and salts or form complexes with proteins and peptides. The phosphoserine containing peptides from α_{S1}-casein, α_{S2}-casein and κ-casein are of special interest as they are having high amount of negatively charged amino acids and helps in binding of various metals. They can form soluble complexes with divalent cations and form complexes (Vegarud *et al.,* 2000).

All the twenty essential minerals are present in cow's milk compared to human milk (Flynn, 1992). Isolation of mineral binding

peptides includes selective solubilization and precipitation methods by the use of different solvents, chelating complexes, pH and ionic strength. Some of the minerals binding peptides were reviewed by Vegarud *et al.,* (2000). These peptides from α-casein, β-casein and κ-casein can bind to different metal ions like Mg^{2+}, Ca^{2+}, Zn^{2+}, Fe^{3+}, Mn^{2+}, Cu^{2+}, etc. (Schlimmel and Meisel, 1995; Swaisgood, 1992).

1.4.2.4. Prolyl Endopeptidase inhibitory peptides

Prolyl Endopeptidase, (EC 3.4.21.26) is a cytosolic endopeptidase. PEP cleaves peptide bonds on the carboxyl side of proline residues in low molecular weight proteins containing the recognition sequence X-Pro-Y, where X is a peptide and Y is an alcohol. PEP can hydrolyze only small peptides and is thought to be involved in the metabolism of hormones and neuropeptides. However, PEP also degrades many active hormones and neuropeptides, e.g. oxytocin, arginine and vasopressin. Degradation of these proteins affects the social behavior, emotions, stress level and responsivity behavior. Altered PEP activity has been observed in psychiatric disorders such as depression, mania, Alzheimer's disease and schizophrenia (Maes *et al.,* 1995). Increased knowledge of the relationship between peptides derived from food proteins and their PEP inhibition may be important in the development of functional foods as a supplement to pharmaceutical agents in preventing the psychiatric disorders.

1.4.2.5. Antimicrobial peptides

Antimicrobial peptides are the ancient molecules involved in host defense. The antimicrobial action of the peptides is defined as the inhibitory action of peptides on the microbial growth (Pag *et al.,* 2003). The widespread resistance of bacterial pathogens to conventional antibiotics has prompted renewed interest in the use of alternative natural microbial inhibitors such as antimicrobial peptides. Some of these antimicrobial peptides are liberated by the action of proteases on certain proteins. These peptides are potent, broad-spectrum antibiotics, which are potential therapeutic agents. Antimicrobial peptides have been demonstrated to kill Gram-negative and Gram-positive bacteria (including strains that are resistant to conventional antibiotics), mycobacteria, enveloped viruses, fungi and even transformed or cancerous cells. Unlike, conventional antibiotics, antimicrobial peptides have the ability to enhance immunity by functioning as immunomodulators (Yeaman and Nannette, 2003). The modes of action of antimicrobial peptides include: a) inhibition of cell wall formation, b) formation of pores in the cell membrane resulting in the disruption of membrane potential with eventual lysis of the cell c) inhibition of nuclease activity (RNase and/or DNase activity) and d) binding to DNA or RNA (Azorin, 1985).

Antimicrobial peptides are short proteins, generally between 12 and 50 amino acids long. These peptides include two or more positively

charged residues provided by arginine, lysine, histidine, and a large proportion of hydrophobic residues (Papagianni, 2003; Sitaram and Nagaraj, 2002). Many of these peptides are unstructured in solution, and fold into their final configuration upon partitioning into biological membranes. The ability to disrupt cellular membranes is an important feature of antimicrobial peptides. The peptides have a variety of antimicrobial activities ranging from membrane permeabilization to action on cytoplasmic targets (Hancock and Rozek, 2002).

Helicobacter pylori is a Gram -negative, pathogenic bacterium, which specifically colonizes the human gastric mucosa. The bacterium induces chronic atrophic gastritis, gastric diseases including peptic ulcer, gastric adenocarcinoma and mucosa-associated lymphoid tissue lymphoma. *H. pylori* evade host immunological defense mechanisms and persists in untreated patients (Akedo *et al.,* 2004). Among the various antimicrobial peptides available in the literature there is very less information is available on the peptides that prevent the growth of ulcer forming bacteria *H. pylori* (Stromqvist *et al.,* 1995).

1.4.2.6. Anticancer peptides

Members of the Aurora-kinase family have recently emerged as key mitotic regulators required for genome stability. Furthermore, these serine/ threonine kinases are frequently over expressed in human tumours. Aurora-A is frequently amplified in tumours, indicating

that it is important for tumour formation or progression. Auroras are involved in regulating multiple steps of mitosis, including centrosome duplication, formation of a bipolar mitotic spindle, chromosome alignment on the mitotic spindle.

Several reports have been published describing the first generation of small-molecule inhibitors of Aurora kinase activity. Importantly, one of these has shown antitumour activity in *in vivo* models. A great deal has recently been learned about the basic biology of this family of kinases, in the context of the initiation of cancer and in its therapy (Nicholas and Taylor, 2004).

Over expression of Aurora kinase A, B and C were studied in various cancer and tumour cell lines. The aurora kinase inhibitors have been shown recently to be anticancer agents *in vitro* and *in vivo*. Rapid progress made in understanding the fundamental biology of these proteins, and in generating selective inhibitors of their activity. Aurora-kinase inhibitors could soon be promising leads in treating cancer (Nick *et al.*, 2006).

1.4.2.7. Structural studies of peptides

The studies regarding the structure of peptides in solution will help to understand the relationship between the functions of the peptides. The biological activity of the peptides can be related to their

conformation, hydrophobicity, propensity to form different structures in different environments, charge, amphipathicity etc., (Yeaman and Nannette, 2003). The necessary structural insights can be made available through the use of circular dichroism, X-ray crystallography, IR spectroscopy, Raman spectroscopy and NMR. The molecular modeling techniques, aligning softwares, algorithms to calculate the different structural parameters theoretically, helps us to get insight in to the structure function relationships of these bioactive peptides (Hsu *et al.,* 2005).

1.5. Structural and functional stability of proteins and peptides in cosolvents

A variety of attempts have been made to understand the nature of stabilization of proteins. Structural factors such as salt bridges, hydrogen bonding, binding of ligands, hydrophobic interactions, disulfide bonds and amino acid composition also play a major role in the stabilization of proteins/enzymes (Kristjansson and Kinsella, 1991; Mozhaev and Mertinek, 1984).

There are several methods such as immobilization, chemical modification, addition of additives, etc., which can be employed to make proteins/enzymes/peptides thermostable. Based on physicochemical properties, these methods leads to proteins/enzymes/peptides stabilization (Klibanov, 1983).

The problem of stability is of great concern where in proteins/enzymes are used in the biotechnological processes such as therapeutics, diagnostics, bioreactors, biosensors and fine chemicals. There are several reports available in the literature on stabilization of proteins/enzymes/peptides (Schimdt, 1979; Janecek, 1993).

The stability of enzymes and proteins *in vitro* remains a critical issue in biotechnology. Both storage and operational stabilities affect the efficacy of enzyme – based products. Storage stability or shelf life refers to an enzyme maintaining its catalytic abilities in the period between manufacture and eventual use. Operational stability describes the persistence of enzyme activity under conditions of use (Fagain, 2003).

It has been a general practice to stabilize the native conformation of proteins by addition of cosolvents such as sugars and polyhydric alcohols. The cosolvent protein interaction is surface phenomena where more water is structured around the protein molecule, a phenomenon known as preferential hydration, leading to the stabilization of the protein. Here the cosolvent molecule acts as thermodynamic boosters (Timasheff and Xie, 2003; Doi *et al.,* 2003).

The thermodynamic boosters are known to increase the free energy of stabilization of native structure of protein and stabilize the

protein by different mechanisms (Timasheff *et al.,* 1976). Free amino acid stabilizes the protein at low concentrations by increasing the hydrogen bonding (Arakawa and Timasheff, 1983). Salts at low concentration induce stability by preferential binding to hydrophobic sites (Arakawa and Timasheff, 1985). Sugars such as sucrose and trehalose act by increasing the surface tension of the solvent (Lee and Timasheff, 1981). Glycerol increases protein stability by solvophobicity of non-polar residues on protein surface (Gekko and Timasheff, 1981). There is negative binding of cosolvents to the protein and the protein is said to be preferentially hydrated. Using the principles of preferential interaction, many industrial enzymes such as alpha-amylase, alpha-chymotrypsin and carbonic anhydrase are stabilized against thermal inactivation (Rajendran *et al.,* 1995; Gekko and Morikawa, 1981; Timasheff, 2002).

The successful development of peptide and protein pharmaceuticals requires a comprehensive characterization of the drugs stability in various formulations. Virtually all peptide and protein formulations consist of active ingredients as well as excipients (e.g., cosolvent, surfactant) and buffer components. The physical and chemical stability of a protein drug depends not only on the properties of the protein itself but also on the formulation system and the components. Excipients such as sugars and polyols are common cosolvents added to stabilize the conformation of a protein. They are

widely used as cryoprotectants and lyoprotectants in protein lyophilization (Shihong *et al.,* 1996).

Deamidation of asparagine residues is probably the most common pathway for chemical inactivation of peptide pharmaceuticals. It is now well established that the conformation of the peptide backbone (i.e., the secondary structure), as well as the side chain dihedral angles can affect the rate of deamidation. Therefore, one approach for slowing deamidation might be to control peptide conformation. One way this control might be accomplished through the use of preferentially excluded solutes. These solutes are additives that are selectively excluded from the hydration sphere of the protein (Stratton *et al.,* 2001; Susanne *et al.,* 2004).

1.6. Interaction of metal ions with α-casein

Metals ions serve a variety of functions in proteins, the most important of which are to enhance the structural stability of the protein in the conformation required for biological function and/or to take part in the catalytic processes of enzymes. Metal ions can activate chemical bonds and make them more amenable to reaction. They can take part in trigger and control mechanisms by specifically altering or stabilizing a macromolecular conformation on binding. (Rasmussen, 1990). The atoms and group of atoms that surround a metal ion and which are

close enough to be chemically bonded are termed as ligands. Ligands donate an electron pair to the bond and are generally negatively charged or neutral. The number of such liganding atoms surrounding a central metal ion is termed the coordination number of that metal ion. Important in study of metal–ligand interactions are the polarizabilities of metal ion and the ligand, the number of the ligands around each metal ion, and the stereochemistry of the resulting arrangement.

The major metal ion binding sites in proteins are carboxyl (aspartic acid and glutamic acid), imidazole (histidine), indole (tryptophan), thiol (cysteine), thioether (methionine), hydroxyl (serine, threonine, and tyrosine), and possible amide groups (asparagines and glutamine, although generally via their side-chain carbonyl, rather than amino groups) (Voet and Voet, 1990). About 65% of the various types of amino acid side chains are potential metal-binding groups. In addition protein main chain carbonyl and amino groups bind metal ions. Binding of metal ion to protein can be considered in terms of Lewis acid- base theory (Brown and Skowron, 1990).

1.6.1. Interaction with zinc

Nutritional significance of zinc is well known. Zinc is an essential micronutrient and has a role in neonatal growth and immunity. The major consequences of zinc deficiency leads to growth retardation, skin lesions, iron deficiency anemia, anorexia, wound healing and

neuromodulatory effect (Singh *et al.,* 1989). Zinc deficiencies are also known to cause diarroheal diseases and malaria. Decrease in zinc levels can reduce memory function in brain. Zinc requirement for cell-mediated immunity is also well established (Pekarek, 1979).

Zinc displays coordination numbers of 4 - 6 in small molecule complexes (Cotton and Wilkinson, 1980). In aqueous solution zinc exists as a hexahydrate. In proteins the coordination number 4 is most common, where the zinc ion is typically coordinated in tetrahedral or distorted tetrahedral fashion (Christianson, 1991). The coordination polyhedron of structural zinc is dominated by cysteine thiolates, and the metal ion is typically sequestered from solvent by its molecular environment. The coordination polyhedron of the catalytic zinc is dominated by histidine ligands, and the metal ion is exposed to the bulk solvent and typically binds a solvents molecule (Valle and Auld, 1990).

Transition metal ion zinc has been used in a wide variety of structural and catalytic roles. The properties of zinc that makes it well suited for these roles are: zinc is a good Lewis acid, has a stable +2 oxidation state, and can form a four-coordinate geometry. Zinc has full 3d electron shell unlike Co (II) and Ni (II), there is no difference in ligand field stabilization energy between octahedral and tetrahedral geometries (Klemba and Regan, 1995). In terms of hard-soft-acid-base

theory, zinc is regarded as a borderline acid. (Pearson, 1969; Valle and Auld, 1990; Watt and Hooper, 2003) Zinc is not redox active and spectroscopically silent.

Among the first-row transition metals, zinc is second only to iron in terms of abundance and importance in biological systems. Zinc is an essential requirement to all forms of life. Zinc is an essential trace element in human nutrition playing a role in structure and function of several enzymes. Zinc is tightly bound to metalloproteins and enzymes. The coordination chemistry of zinc is as versatile as its functions in biology. In proteins zinc binds to negatively charges residues (e.g., carboxylates and thiolates) and /or neutral dipolar residues (e.g., carbonyls and imidazoles). The recognition of a transition metal by a protein - binding site requires the discrimination of ionic size, charge and chemical nature (Pearson, 1969). Among the biologically important divalent metal ions, zinc possesses a distinguishable ionic radius and it is typically found in the tetrahedral or distorted protein sites. Zinc is metal of borderline hardness, and it can easily accommodate nitrogen, oxygen and sulphur atom in its biological coordination spectra. In many proteins the large free energy of zinc binding is exploited to stabilize protein structure.

Much attention has been paid in the last few years to zinc absorption by infants fed on different milks. The average zinc content

of bovine milk is 3.5 mg/L and it is associated with skim milk fraction and over 95% is associated with casein micelles (Pekarek *et al.,* 1979; Flynn and Power, 1985; Fransson and Lonnerdal, 1983). Despite the apparent nutritional significance of the zinc-casein interaction the nature of zinc binding by caseins has received little study. Casein is used in many studies as a model system to understand the molecular state during interactions with metal ions. Initial studies on zinc interaction with caseins was studied by Harzer and Kauer, (1982) wherein, they proposed that the zinc binds to phosphorylated serines in caseins. Studies by Singh *et al.,* (1989) have shown the zinc binding capacity of various proteins fractions in bovine milk. They have isolated various milk proteins and shown the number of binding sites for different milk proteins. Further studies in presence of zinc on the structure and functional change in α-casein was not studied in great detail. An attempt has been made to fill these lacunae with the further studies on zinc binding.

1.6.2. Interaction with lead

Lead is an environmental pollutant with severe toxic effects. It has had important industrial uses in paints and in the construction of storage batteries. Lead is used in gasoline (antiknock) as tetraethyl lead. Tetraethyl lead however causes significant pollution and continues to do so. Treatment involves using various lead chelating

agents, but these are usually nonspecific and relativley toxic (Ratcliffe, 1981).

Lead, with its electronic configuration [Xe] $4f^{14}5d^{10}$-$6s^2$, is one of the post- transition elements that exihibits “inert pair effect”. This term refers to the resistance of the pair of outer electrons in covalent or hydrogen bond formation (Gillespie and Hargittai, 1991).

The interaction of lead with proteins may represent a fundamental mechanism underlying lead toxicity (Lanphear, 1998). Mahaffey (1990) has summarized the influence of total food intake and patterns of food intake on lead toxicity. Lead from water and other beverages is absorbed to a greater degree than lead in food. Lead ingested between meals is greatly absorbed than lead with meals. The composition of the diet, particularly high protein content, was shown in experimental studies to decrease lead intake, but there has been no new information regarding this in recent years. Lead interacts or competes with gastrointestinal absorption, tissue deposition and as a cofactor in metallo-enzymes with essential trace elements, particularly calcium, iron and zinc. It has been recognized for many years that lead metabolism mimics calcium metabolism in many ways (Simons, 1994). It is also clear from the animal studies, that calcium deficiency clearly enhances lead absorption and mobilize lead from internal tissue stores and bones (Barltrop, 1969). During lactation, lead appears along with calcium in mothers milk (Bonithon - Kopp *et al.,* 1986).

Lead competes with zinc as a cofactor with heme enzymes, like aminolevulinic acid hydratase and ferrochelatase. Iron deficiency increases erythrocyte protoporphyrin activity which inturn increases lead levels.

Zinc deficiency does not enhance toxicity of parenteral lead. Lead inhibits the synthesis of haemoglobin, causing anemia (Needleman, 1998). Studies regarding the inhibition of D-Xylose isomerase and ferrochelatase enzymes by lead are reported recently (Carell *et al.,* 1989).

Many Studies have been carried out on different type of metals present in milk in order to assess the different bioavailability of micronutrients (Lonnerdal, 1985). The affinity of lead towards caseins has been partially shown from the balance of lead in milk and by-products (Pertoldi and Gabrielli, 1983). The distribution of lead in caseins was studied by Mannino and Bianco, (1988) by potentiometric stripping analysis.

It appears that much more research is required to understand the complexities of reactions of inorganic toxins with proteins. There exists a wide gap in understanding the biochemistry and toxicology of lead (Goering, 1993).

Based on the above literature an indepth study was undertaken to characterize α-casein from milk. The characterization of α-casein from milk is necessary to understand the composition, release of potent peptides, structure and stability of α-casein and interactions with metal ions. It also throws light on the stability of the protein metal complexes formed, such that one can use the information for applications using biotechnological approaches. α-Casein from milk was isolated and characterised for its strutural, functional, enzymatic and biophysical properties. The effect of various proteases was studied for release of multifunctional biologically active peptides. These peptides were characterised for structure and function. The peptides stability in presence of various cosolvents was also undertaken. The interaction of metal ions with α-casein was also studied in detail for understanding the mechanims of interactions.

1 MKLLILTCLV AVALARPKHP IKHQGLPQEV LNENLLRFFV

2 APFPEVFGKE KVNELSKDIG ESTEDQAMED IKQMEAESIS

3 SSEEIVPNSV EQKHIQKEDV PSERYLGYLQ LLRLKKYKVP

4 QLEIVPNSAE ERLHSMKEGI HAQQKEPMIG VNQELAYFYP

5 ELFRQFYQLD AYPSGAWYYV PLGTQYTDAP SFSDIPNPIG

6 SENSEKTTMP LW

Fig.1. Amino acid sequence of α_{S1}- casein (Mercier *et al.,* 1971)

1 MKFFIFTCLL AVALAKNTME HVSSSEESII SQETYKQEKN

2 MAINPSKENL CSTFCKEVVR NANEEEYSIG SSSEESAEVA

3 TEEVKITVDD KHYQKALNEI NQFYQKFPQY LQYLYQGPIV

4 LNPWDQVKRN AVPITPTLNR EQLSTSEENS KKTVDMESTE

5 VFTKKTKLTE EEKNRLNFLK KISQRYQKFA LPQYLKTVYQ

6 HQKAMKPWIQ PKTKVIPYVR YL

Fig.2. Amino acid sequence of α_{S2}-casein (Grosclaude *et al.,* 1979)

Table 1

Physico-chemical properties of α-casein

Property	Total protein
Absorption maximum[1]	278 – 280 nm
Absorption coefficient[1] ($E^{1\%}_{1cm}$ at 280nm)	10.12
Fluorescence emission maximum	345 nm
Phosphorus content[1]	1%
Carbohydrate content[1]	Nil
Proteolytic activity[1]	Nil
Apparent hydrophobicity[2]	4.87 kJ/residue
Molecular weight[1]	27,300 Da
Composition[1]	α_{S1}-casein and α_{S2}-casein in 4:1 ratio
Stokes radius[2]	30Å at pH 7.4
Apolar residues[2]	29%

([1]Chein and Waugh, 1965; [2]Marchesseau et al., 2002)

Table 2

Charged groups in α-Casein

Group	By sequence	
	α_{S1}-Casein[a]	α_{S2}-Casein[b]
Phosphate	8	11
Imidazole	3	3
ε-amino	15	25
Aromatic	20	23
Guanidyl	6	6
Total anionic	32	28
Total cationic	24	34

(Farrell Jr. et al., 2004)

[a] *Charged groups of α_{S1} –casein variant B is taken from swiss prot id-P02662 (CAS1_BOVIN)*

[b] *Charged groups of α_{S2} –casein is taken from swiss prot id-P02663 (CAS2_BOVIN)*

Table 3

Examples of multifunctional bioactive peptides encrypted in bovine α-casein

Peptide sequence	Fragment	Name	Enzyme	Bioactivity
TTMPLW	α_{S1} - CN (f194-199)	α-immunocasokinin	Trypsin	Immunomodulatory, ACE-inhibitory (hypotensive *in vivo*)
QMEAESISSSEEIVPNS VENK	α_{S1} - CN (f59-79) 5P	Caseinophosphopeptide	Trypsin	Mineral binding Immunomodulatory
KNTMEHVSSSEESIISQ ETYKQEKNMAINPSK	α_{S2} - CN (f1-32) 3P	Caseinophosphopeptide	Trypsin	Mineral binding Immunomodulatory

(Meisel, 2004)

SCOPE AND OBJECTIVES

2. SCOPE AND OBJECTIVES

Bovine milk contains about 80% casein, of which 65% is α-casein. α-Casein is the major casein in bovine milk proteins. α-casein refers to a combination of two closely related proteins, α_{S1}-casein and α_{S2}-casein. These proteins have similar properties in their phosphorous content (1%), and molecular weight of 27,300 Da.

α-Casein is easily degradable protein due to their random coil structure. Though there are ample number of studies on the enzymatic hydrolysis of the α-casein and liberated peptides, the hydrolysis behavior and the release kinetics of bioactive peptides by various proteases is few. Secondly, studies on the multifunctional peptides i.e., peptides having more than one biological acitivity were seldom in the literature.

The scope of the present investigation is to study the hydrolysis of α-casein by different enzymes and to compare the release kinetics bioactive peptides. The screening of the enzymatic hydrolysates for the biologically active peptides will be undertaken. The multifunctional activity of the purified/synthesized peptides will be studied and compared in detail for its main biological properties and structure function relationships.

Proteins can be reliably stabilized by a number of means such as use of solvent additives, chemical modification and immobilization. It has been a general practice to stabilize the proteins by keeping them in cosolvents such as sugars and polyols. Depending upon the solvents, the proteins are stabilized by preferential hydration. Studies on structural stabilization of α-casein in presence of cosolvents in literature are less. There are a hardly any reports on the stabilization of peptides in presence of cosolvents. This study includes effect of cosolvents on the tertiary conformation of α-casein and the relationship between the bulk solvent and the protein especially in sugars and polyols. In the present investigation, efforts are directed towards the stabilization of the bioactive peptides in cosolvents for their biological activity. These cosolvents can act as stabilizers and protect the peptides from the extremes of temperature.

Metal - protein interactions have emerged as an essential problem of nutrition. Proteins are major metal chelators. They chelate along with essential micronutrients, toxic metals, and makes them bioavailable to humans. However, proper studies were not reported on the binding of heavy metals like zinc and lead with α-casein. Studies on the mechanism of binding with proteins will help us to understand the binding of micronutrients like zinc and toxic metal like lead. This study will be useful in food fortification studies of milk and its byproducts.

Another important strategy would be to prevent the binding of toxic metals like lead with milk proteins.

Based on the above - mentioned information a plan of work has been proposed to study the interactions of α-casein with proteolytic enzymes, cosolvents and metal ions like zinc and lead.

Following are the specific objectives of the present investigation:

Isolation and characterization of biologically active peptides from α-casein. The study includes hydrolysis of α-casein by proteolytic enzymes and isolation, purification and characterization of the biologically active peptides. A study on the screening of peptides is undertaken for multifunctional activity. The peptides structure – function relationships will be identified. The present study involves the use of HPLC, mass spectra, peptide synthesizer, aminoacid sequencer, softwares for identification of peptides and modelling the three dimensional structures of the peptides, bioactivity studies, bacterial membrane polarization studies, peptide – DNA interaction studies, CD, IR sepctroscopy and NMR.

Effect of cosolvents on the stabilization process of α-casein and its biologically active peptides. The stabilization process will be monitored

by fluorescence and UV difference spectroscopy for the tertiary structural changes of α-casein in presence of various cosolvents. The studies on the effect of cosolvents on the biological activity of peptides at different temperatures will be taken up.

Interactions of zinc with α-casein. The study includes identifying the number of binding sites by equilibrium dialysis and fluorescence titration. The secondary structural changes in presence of zinc will be monitored by CD, and IR spectroscopy. The kinetics of formation of the complex is studied by stopped flow spectrophotometer. The tertiary structural changes were studied by ANS surface hydrophobicity measurements. The zinc - α - casein - complex will be studied for its digestibility and reversibility of metal binding.

Interaction of lead with α-casein. This study involves identifying the number of binding sites by spectrophotometric titration and by equilibrium dialysis. Identification of structural perturbations in α-casein will be studied by fluorescence, CD, IR and Raman spectroscopy. The kinetics of the complex formation will be studied by stopped flow. The effect of temperature on the rate kinetics will be studied. Finally chemical modification of the cysteines will assist in understanding the possible sites of interaction of the ligand with protein.

The above studies help us to understand the purification of peptides from the hydrolysates and characterizing them in pure form. With this study, generation of the multifunctional peptides will help us to use them as future nutraceuticals.

The studies on the stability will help us to understand the effect of different stabilizers on the peptides and proteins. This will help us to formulate the required thermodynamic boosters/ stabilizers for effective functioning of the peptides and proteins.

Nature of binding sites on α-casein for zinc and lead will help us in identification of sites and mechanism of interaction. The results from the above studies help to understand the nature of the ligand binding to caseins. Wider understanding of the proteins - ligand interaction in general with an emphasis on developing improved rationale for the removal of undesirable ligands from milk proteins.

MATERIALS AND METHODS

3. MATERIALS AND METHODS

3.1. Sources of chemicals used

The following chemicals Alpha casein (C6780), acrylamide (A3553), bovine serum albumin (A2153), urea (U0631), tryptophan ethyl ester (93690), N, N'- methylene bisacrylamide (294381), sodium dodecyl sulphate (L4390), EDTA (E5134), Zinc Sulphate (ACS reagent 221376), HEPES buffer (H3375), ANS salt (A1028), chymotrypsin (C4129), from bovine pancreas type II, peptidase (P7500) from porcine intestinal mucosa, zinc standard for atomic absorption (207667), lead nitrate (ACS reagent 228621), lead standard for atomic absorption (207233), N,N,N',N'- tetramethylenediamine (T9281), trifluoroacetic acid (T6508), pancreatin (P5575), from Hog pancreas, bacterial protease (P5147), from *Streptomyces griseus,* Type XIV, angiotensin converting enzyme, (A6778) from Rabbit lung, angiotensin converting enzyme inhibitor (A0772) peptide containing p-Glu-Trp-Arg-Pro-Gln-Ile-Prp-Pro-Pro (Mol wt 1101 Da.), Hippuryl-Histidine-Leucine tetrahydrate (H-1635), 2,2-diphenyl-1-picrylhydrazyl (43180), Lactoferrin (L9507) from bovine milk, Kaiser ninhydrin kit (60017), 1,2-ethanedithiol (02390), *O*-phthaldehyde (P0657), L-ascorbic acid (A92902), iodoacetamide (I6125), 2-mercaptoethanol (M6250), α-cyano-4-hydroxy cinnamic acid (C8982), glycerol (G5516), sucrose (S5016), trehalose (T9531), sorbitol (S6021), calf thymus DNA (D1501), d_6 DMSO, D_20, TMS and standard SDS molecular weight marker kit were procured from Sigma Aldrich chemical company, St. Louis, MO, USA.

Dialysis membranes of 23 mm flat width with molecular weight cut off of 6000-8000 and parafilm were obtained from Thomas Scientific, NJ, USA. Fungal protease (*Aspergillus oryzae*) was obtained from Amano Pharmaceuticals, Japan. Carboxypeptidase, from bovine pancreas, was from Millipore Corporation, Freehold, NJ, USA. Coomassie brilliant blue R-250 and coomassie brilliant blue G-250 was obtained from Bio-Rad laboratories, Richmond, USA. Acetonitrile, ethyl acetate and methanol were obtained from E-Merck (India) Limited, Mumbai, India. N-ethyl morpholine and dimethyl sulphoxide were procured from Sisco Research Laboratories, India. Dimethyl formamide, dichloromethane, diethyl ether, isopropanol, piperidine, and N, N-diisopropylethylamine were procured from Spectrochem India Pvt. Limited, Mumbai, India. The Fmoc - aminoacids, Rink amide resin, O-(Benzotriazol-1-yl)- N,N,N,N-tetramethyluronium hexa fluoro phosphate (HBTU), 1-Hydroxybenzotriazole (HOBt) were procured from Chem-impex Limited, Wooddale, IL, USA. Sodium hydroxide, sodium chloride, perchloric acid, hydrochloric acid were procured from S.D Fine Chemicals, Biosar, India. Brain heart infusion broth and nutrient agar, were procured from Hi Media, Labs Pvt. Limited, Mumbai, India. Stillbene AMS, and bis- (1, 3-dibutyl barbituric acid) trimethine oxonol, were procured from molecular probes, USA. All other chemicals were of reagent grade. All glasswares were given nitric acid wash before use. All reagents and buffers were prepared in quartz triple distilled water.

All kinetic, binding and saturation data were analyzed using Origin 7.0 Software (Origin Lab Corporation MA, USA).

Milk was obtained from a Jersey cow and repeatedly procured from the same cow from Mysore, India.

3.2. Protein concentration

Protein concentration of α-casein was determined routinely by taking the ultraviolet absorbance at 278 nm using Shimadzu double beam spectrophotometer (Shimadzu, Japan). The absorbance value was converted into concentration units using the absorption coefficient, $E^{1\%}_{1cm}\, A_{280\ nm}$ value of 10.12. A correction was made for scattering by subtracting 1.7 times the apparent absorbance at 320 nm from the apparent absorbance at 280 nm (Waugh *et al.*, 1971).

3.3. pH measurements

The pH of buffers and protein solutions were routinely measured using a digital Cyber Scan pH meter 510 model (Eutech Instruments Pvt. Ltd., Singapore) with a combined electrode and having automatic temperature compensation facility. pH meter is calibrated weekly with NIST standard buffers (E-Merck India Limited, Mumbai, India). Minor adjustments in pH were done by adding either 0.1 N NaOH or 0.1 N HCl at 25 $\pm$ 2°C. pH of the protein solution was monitored before and after experiments.

3.4. Isolation of α-casein

The procedure for isolation of casein involves the following steps. The fresh milk from cow was taken, defatted and centrifuged at 8,000 rpm for 20 min at 12°C. After centrifugation, the pH of supernatant was adjusted to 4.6 with 1 N HCl by slow stirring. The solution was centrifuged at 8,000 rpm for 30 min and acid casein was separated out as pellet. The acid casein was dissolved in 6.6 M urea. After dissolving, the casein was diluted to 4.63 M, and centrifuged at 3,500 rpm at 25°C for 20 min and the pellet was separated out. The pellet was washed twice with distilled water. This pellet was redissolved in 6.6 M urea containing 0.15M NaCl and diluted to 4.63 M urea. This diluted solution was centrifuged at 3,500 rpm at 25°C for 20 min. The resultant pellet obtained was resuspended again in 4.7 M urea and the residue was separated out by centrifugation, to remove any other casein fraction exists as contaminants. The solution was then dialysed against distilled water to remove excess urea and freeze dried (Hipp *et al.*, 1951).

The isolated α-casein was checked for its purity by SDS-PAGE, gel filtration HPLC and amino acid composition.

3.5. Hydrolysis of α-casein by various enzymes

α-Casein was hydrolyzed by aminopeptidase, carboxypeptidase, bacterial protease, fungal protease and chymotrypsin. For all enzyme

reactions studied, the enzyme to substrate ratio was maintained at 1:150, in 10 mM morpholine-TFA buffer, for 120 min. For aminopeptidase, carboxypeptidase, bacterial protease and α-chymotrypsin the hydrolysis was carried out at 37°C, except for fungal protease the reaction was performed at 45°C. For aminopeptidase and carboxypeptidase the hydrolysis was carried in pH 7.0 and for bacterial protease the reaction was done at pH 7.5. For α-chymotrypsin and fungal protease, the pH was maintained at 8.0. The enzymatic hydrolysis was stopped by keeping at 95°C, for 5 min followed by immediate freezing (Tauzin *et al.*, 2003).

The proteolytic activity of chymotrypsin and fungal protease was measured using denatured hemoglobin as substrate and found to be 5000 tyrosine units/mg protein and 75000 tyrosine units/mg protein respectively.

For peptide digestion studies by pancreatin, the enzyme to substrate ratio was taken as 1:500, at 37°C, for 240 min in 0.01M ammonium bicarbonate buffer, pH 8.15. The pancreatin was estimated for its activity on azocasein at above-mentioned conditions and found to be having 35 AU/mg protein.

3.6. Analytical gel filtration HPLC

The peptides were purified on Shodex Protein KW 803, (8 mm x 300 mm), porous silica gel filtration column with 1,50, 000 exclusion limit, (Showa Denko America, INC, USA) mounted on a Waters HPLC system equipped with Millennium software (Waters, Milford, USA). The 10 μl of hydrolysates was injected and isocratic elution was performed at flow rate of 1.0 ml/min using 10 mM morpholine -TFA buffer, pH 7.8.

3.7. Reverse phase HPLC

The peptides collected from gel filtration were again resolved on reverse phase C_{18} column (4.6 mm x 150 mm column, pore size 5μ Waters Symmetry® column) on Waters HPLC (Waters, Milford MA, USA) system equipped with Millennium version 32 software. The protein was detected at 215, 230 and 280 nm with a flow rate of 0.7 ml/min by waters 2996 Photo diode array detector. A gradient system of A: 0.1% TFA/water and B: 0.01% TFA acetonitrile/water (70/30 V/V) was used for elution. The column was initially washed with a gradient of 0-40% B for 8 min, and further the gradient was expanded from 0-100% in 18 min. Finally the elution was completed with 100% A up to 25 min. The peaks corresponding to peptides were collected and freeze-dried (Tauzin *et al.*, 2003).

3.8. Quantification of peptides

Peptides were quantified by Bradford method. The coomassie G250 dye was prepared as a 0.06% solution in 3% perchloric acid (0.3M) and was filtered through Whatman No.1 filter paper to remove any undissolved material. If necessary, stock solutions were diluted with perchloric acid to give an absorbance of 1.3 - 1.5 at 465 nm. The assay contains adding 0.5 ml of protein or peptide to 0.5 ml of dye solutions. The absorbance of the solutions was measured at 620 nm and was compared with standard curve (Sedmak and Grossberg, 1997).

3.9. Degree of hydrolysis

The degree of hydrolysis was estimated by *o*-phthaldehyde method (Church *et al.,* 1983; Nielsen, *et al*., 2001). The methodology involves preparing the reagent containing 25 ml of 100 mM sodium tetraborate, 2.5 ml of 20% SDS, 40 mg of OPA (dissolved in 1ml methanol) and 100 μl of β-mercaptoethanol. The final volume was made up to 50 ml with distilled water. The reagents were freshly prepared daily. To assay the proteolysis of milk proteins as substrates, a small aliquot containing 10 μl to 50 μl containing 5 to 100 μg protein was added directly to 1 ml of OPA reagent in a spectrophotometer cuvette, solution was mixed briefly by inversion and incubated for 2 min at 25°C and the absorbance was recorded at 340 nm. The values

were then subtracted from the blank and were converted to the percentage degree of hydrolysis by following equation:

$$DH\ (\%) = \frac{\Delta Abs_{340\ nm} \times \text{Mol. Wt (protein)} \times DF}{M_{Leu} \times n \times W\ (g/L)} \qquad (1)$$

where the DF is dilution factor; M_{Leu} is molar extinction coefficient of luecine is taken as 6000 mol^{-1} L^{-1}, n is number of peptide bonds in protein, W initial weight of the protein.

3.10. Mass spectra

The peptide mass fingerprinting was done by MALDI - TOF (Bruker Daltonics, Bruker GMBH, Germany). Peptides were analyzed as a fingerprint and latter the individual peptides were sequenced. The lyophilized samples were dissolved in 10 μl acidified water (0.1% TFA in water). From this 2 μl was taken mixed with 2 μl of the matrix solution. The matrix solution used was 0.05 M α-cyano-4-hydroxy cinnamic acid in acetonitrile /ethanol (1:1). The 2 μl was transferred on to the plate and allowed to dry. The peptides were desorbed and ionized by nitrogen laser. The analysis of the peptides was done on *flex Analysis* software provided with the instrument. The instrument was internally calibrated with the standard peptide mixture (RP pepmix, Bruker Daltonics, Bremen, Germany). The MS/MS ion pattern of the

selected bioactive peptides was generated by fragmentation of the peptide (Tauzin *et al*., 2003; Miranda *et al*., 2004).

The synthesized peptides were confirmed for their mass by ESI - MS (Q-TOF Ultima, Micromass, Manchester, UK) and analyzed using *Mass Lynx 3.5* software. The instrument was internally calibrated with the sodium iodide.

3.11. Identification of peptides

The peptide sequence i.e., the monoisotopic masses of the N-terminal *b* -type ions and C-terminal *y* -type ions were generated by using *MS-Seq* program in *protein prospector* software package version 4.0.5 (http://prospector.ucsf.edu, University of California, SF, CA, USA). This software calculates the possible fragment ions and converts them to amino acids resulting from the fragmentation of a peptide in a mass spectrometer. The necessary parameters were given as follows: instrument for analysis– MALDI-TOF-TOF, enzyme– chymotrypsin, missed cleavage– 1, and percentage error in molecular weight– $\pm$ 2.0 Da. The peptide was defined as - hydrogen ending N-terminal and free acid at the C-terminal. The database used was Swiss-Prot database (Clauser *et al*., 1999).

3.12. Synthesis of peptides

The peptides were synthesized on the bench scale continuous CS136 automated solid phase amino acid synthesizer (CS Bio Co., San Carlos, CA, USA). The nitrogen was purged continuously at 10 psi /hour. The Rink amide MBHA (4-methylbenzhydrylamine) resin at substitution of 0.75 mmol was used to synthesize the peptides. The amino acids were F-moc (9- Fluorenyl methoxy carbonyl) protected and secondary side groups were Boc (*tert* – Butyloxy carbonyl) protected. The F-moc protected aminoacids, HBTU, HOBT (where required) were added at 3 times concentration to the substitution capacity of the resin. HBTU, HOBT (where required) were added to the amino acids to prevent the racemization and unwanted oxidation of OH group (containing amino acids) during synthesis. The organic solvents dichloromethane and dimethyl formamide were employed during the synthesis.

The coupling and decoupling was done by using 10% piperidine and 10% N, N-diisopropyl ethylamine. The sequential synthesis of the amino acids was done and the coupling and decoupling steps were monitored using Kaiser ninhydrin reagent. The peptide was washed twice with dimethyl formamide and dichloromethane, weighed and vacuum dried for 24 h. The peptide was dissolved in 5 ml of 98% TFA, 125 μl ethane dithiol and stirred in cold for half an hour. The peptide was then separated and filtered by washing with two volumes of

isopropanol, and two volumes of ice – cold anhydrous distilled diethyl ether. The precipitate was separated and dried under vacuum (Marglin and Merrifield, 1966; Elsayed, *et al.*, 2004).

3.13. Amino acid analysis of α-casein

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

An aliquot of the purified protein (20 μg) was dried under vacuum and 200 μl of constant boiling HCl (5.8 M) containing 1% (v/v) phenol was added. Samples were hydrolyzed in the workstation at 110°C for 24h.

Standard free amino acids, in a mix (Pierce H) containing up to 25 nmol of each amino acid were dried under vacuum after adding 20 μl of ethanol-water-TEA in a ratio of 2:2:1 at 55-60 mtorr vacuum. The derivatization reagent consisted of ethanol-water-TEA-phenylisothiocyanate (PITC) in the ratio of 7:1:1:1. To make 300 μl of derivatization reagent 210 μl of ethanol was mixed thoroughly with 30 μl each of PITC, TEA, and water. Phenylthiocarbamyl amino acids (PTC-amino acids) were formed, by adding 20 μl of derivatization reagent to the dried samples.

The High performance liquid chromatography was monitored at 254 nm to detect the amino acids. The temperature was maintained at 38 ± 1°C with column heater. The sample volume of 5 µl was injected to application specific column (150 mm x 3.9 mm). The solvent system consisted of (1) Solvent A: mixture of an aqueous buffer (0.14 M sodium acetate containing 0.5 ml/L TFA, pH 6.4): acetonitrile (94:6) and (2) Solvent B: 60% acetonitrile in water. The gradient elution with a flow rate of run for the separation at a flow rate 1 ml/min is as follows: 100% A and 0% B (initial), 54% A and 46% B (10 min), 0% A and 100% B (11 min), 0% A and 100% B (13 min), 100% A and 0% B (14 min) and 100% A and 0% B (25 min).

3. 14. Bioactivity studies

The peptides were studied for the following biological activities

3.14.1. Angiotensin converting enzyme inhibition assay

The conditions established in this study to determine ACE activity *in vitro* were as follows: 110 µl of 10 mM Hippuryl Histidine Leucine dissolved in pH 8.3 buffer (0.2 M phosphate buffer; 0.3 M NaCl, 26 mU/ml ACE (dissolved in 50% glycerol) of reaction medium. The total reaction volume was 150 µl. Incubation was done for 80 min at 37°C. The enzyme was inactivated by addition of 110 µl of 1 N HCl. The reaction product was extracted with 1ml ethyl acetate, taking

750 μl of the organic layer, which was dried out. Hippuric acid was redissolved in 1ml water and the absorbance was recorded at 228 nm. The standard peptide was assayed in a similar way for comparison (Cushman and Cheung, 1971; Hernandez – Ledesma, *et al.*, 2003).

3.14.2. Antioxidant activity assay

The antioxidant activity of the peptides was checked by DPPH radical scavenging activity. 0.3 ml of the peptide was taken (6-60 μM) and made up with 0.1 ml of distilled water. To this added 0.6 ml of 100 μM DPPH reagent in methanol. The reaction mixture was incubated for 20 min under dark and the reading was taken at 517 nm. The decrease in absorbance at 517 nm was taken as the antioxidant capacity of the peptide. L - ascorbic acid was taken as standard (Liu *et al.*, 2004).

3.14.3. Prolyl endopeptidase inhibition assay

Prolyl endopeptidase activity from bovine blood serum was measured using *Z*-pro-prolinal as the substrate. Assay samples were prepared by mixing 20 μL of bovine blood serum with 1960 μL of 0–20 μM inhibitor in 20 mM sodium phosphate buffer, 150 mM sodium chloride, pH 7.4. After 30 min at 37°C, the reaction was started by the addition of 20 μL of 5 mM *Z*-pro-prolinal in methanol. The formation of naphtylamine was continuously monitored by fluorescence (4 – 6 min at 37°C; excitation and emission were at 340 and 410 nm, respectively). Enzyme reaction controls were included in the assay.

Three independent experiments were performed, and the average was taken (Sorensen, *et al.*, 2004).

3.14.4. Divalent metal binding assay

Enzymatic hydrolysates and synthetic peptides were incubated with zinc concentrations between 1- 10 mg/ml in 0.01M HEPES buffer, pH 7.3 for 1 h at 37°C with gentle stirring. Samples were determined for bound zinc by atomic absorption spectroscopy (model AA-6701F, Shimadzu) in absorption mode at 213.9 nm, respectively (Dashper *et al.*, 2005).

3.14.5. Antimicrobial studies

3.14.5.1 Microbial growth inhibition

For assay of antibacterial activity, enzymatic hydrolysates were solublized in water and sterile filtered (Millipore, 0.22μ) before use. The assay was carried out in sterile honeycomb micro plates (Honeycomb 2, Thermo labsystems, Oy, FI), and each well contained a total volume of 375 μl. 300 μl of freshly inoculated bacteria was added to each well, together with 75 μl of hydrolysates. The final concentration of the hydrolysates in the wells varied between 0.75-3.0 mg/ml. Growth controls contained 375 μl of sterile growth media. The samples were incubated at 37 °C for 24 h. The absorbance (600 nm) was measured every 15 min by a plate reader (Bioscreen C, Oy growth

curves AB Ltd., FI). The experiments were repeated three times for each bacterial strain, with three parallels of each sample. Data were presented as mean values with calculated standard deviation. Student's t-test (two-sample, assuming unequal variances) was run to compare the different growth curves and the differences were found to be ($p<0.05$) significant.

The growth rate was calculated using the following equation (Deibel and Lindquist, 1981)

$$\text{Growth rate (k)} = \frac{\log_{10} X_t - \log_{10} X_0}{0.301 \times t} \quad \text{.....} \qquad (2)$$

where X_t, is highest value on the logarithmic growth curve, X_0, is lowest value on the logarithmic growth curve and t, is time in hours.

The inhibitory activity of peptides was tested against selected organisms like *E. coli* (MTCC 118), *B. cereus* (F4801), *L. monocytogenes* (Scot A), and *S. aureus* (Fri722). The organisms were initially subcultured by growing them in brain heart infusion broth for 12 – 24 h at 37°C. The cultured broth was centrifuged in clean sterile centrifuge cups at 6,500 rpm for 30 min. The cell pellet was collected and dissolved in sterile 9 ml of phosphate buffered saline, pH 7.0 in a screw cap vial. The serial dilution was done from the stock (2×10^9 CFU /ml) to get the viable cell population of 2×10^4, 2×10^5, 2×10^6 CFU /ml. One ml of viable cultures from the diluted culture test tubes

were taken and added to sterile 5 ml test tubes and 0.1 ml of the peptide at different concentrations from 1.5 - 4.0 mg/ml dissolved in saline and DMSO. The tubes containing cell suspension and the peptides were kept in rotary shaker at 100 rpm for 12 h at 37°C. The growth of the survived organisms was checked by pour plate method. To the sterile petri-plates cell suspension-containing peptides were poured along with nutrient agar (15 ml) and incubated at 37°C for 24 - 72 h. The number of colony forming units survived was counted manually and the survival organisms are expressed population survived in CFU/ml. The appropriate controls along with buffer, saline, DMSO were also checked in the similar way to know their role in inhibition (Jeon *et al.*, 2001; Varadaraj *et al.,* 1993). Student's t-test (two-sample, assuming unequal variances) was run to compare the different growth curves and the differences were found to be ($p<0.05$) significant.

3.14.5.2. Antimicrobial activity against *H.Pylori*

The *H. Pylori* was obtained from the clinical isolate of a patient with peptic ulcer using endoscopy (Karnataka Cardio Diagnostic Center, Mysore) and cultured on Ham's F-12 nutrient agar (Gibco BRL, USA) with 5% fetal bovine serum at 37°C for 24 h (Medium composition: 10 g/L F12 nutrient mixture with 5% fetal bovine serum). *H. pylori* strains were confirmed by Gram staining, colony morphology studies, motility test and urease positivity test. The 100 μl of stock *H. pylori* was added

in to test tubes containing 10 ml broth media and incubated at 37°C for 2-3 days. Sterile filter paper disks containing peptides were applied to the surface of F12 nutrient agar plates (which were seeded with test *H. pylori* from 2-3 days) and incubated at 37°C for 3 days. To each well added 75 μl of peptide and amoxycillin as standard. The assay for each culture was determined in duplicate. After incubation, the plates were observed for the zone of inhibition (diameter of the clear zone around the disk in the bacterial lawn) and were measured and expressed as the antimicrobial activity of the peptides (Stevenson *et al*., 2000).

3.14.5.3. Binding of peptides to DNA

The bacterial genomic DNA of *Bacillus cereus* F4810 strain (Public Health Laboratories, London, UK) was isolated by the method of Schraft and Griffiths, (1995). The studies on binding of peptides with DNA were studied by DNA gel mobility shift assay. The peptides (10 mg/ml) were incubated with *B. cereus* bacterial DNA (100 μg/ml) in 10 mM Tris, 1 mM EDTA buffer, pH 8.0 for 2h and the peptide DNA mixture was loaded on to a 0.8% agarose gel. The electrophoresis was run in TBE buffer at constant voltage of 100V for 45 min. The DNA in the gel was staining with ethidium bromide and visualized by using *Gene snap* software (Hsu *et al*., 2005).

The binding of peptides was studied by time kinetics mode in UV spectrophotometer. The DNA was monitored for 15 min in a time

kinetics mode at 260 nm before and after addition of peptide. The time kinetics was monitored similarly for calf thymus DNA.

3.14.5.4. Membrane polarization studies

Cells of *B. cereus* was grown in half-concentrated nutrient broth to an absorbance (600 nm) of 0.5 and incubated with 1μM of the membrane potential sensitive fluorescent probe bis- (1,3-dibutyl barbituric acid) trimethine oxonol [$DiBAC_4$ (3)], (Molecular probes, USA) for 15 min. The peptide (10 mg/ml) was added to the cell suspension and the change in the relative fluorescence intensity of the oxonol dye was followed at 492 nm and 515 nm excitation and emission wavelengths respectively (Pag *et al*., 2004).

3.15. NMR spectroscopy

For NMR structure elucidation of peptides, 5 mg of peptide 10 was dissolved in 500 μl of the d_6 – DMSO and 8 mg of peptide 14 was dissolved in 500μl of 0.01M sodium phosphate buffer pH 7.95 respectively. The peptide 14 was dissolved as 90% buffer and 10% D_20. The spectra were recorded for the peptide 10 and peptide 14 at 25°C. Trimethyl silane (TMS) was used as an internal standard.

NMR spectra of the peptides were obtained on 500 MHZ Bruker Avance spectrometer equipped with 5 mm TXI probe with a Z gradient coil. Acquisition and analysis of the data was done on *Topspin v1.2*

software available with the instrument. The spectra were recorded in single dimension as ^{1}H NMR spectra. The second dimension was recorded as TOCSY (Donald and Davis, 1985) NOESY (Jeener et al., 1979) and ROESY (Bothner-By et al., 1984) experiments and was used to assign the peptides. Spectra were acquired with a mixing time of 100 ms for TOCSY, and where as NOESY experiments were recorded at 250 ms and 500 ms. The peak assignment was done manually and all the spectras are baseline corrected and zero filled to 1 K in the F1 dimension. The F2 dimension contains the 2K real points. The water signal was suppressed with low power irradiation. A total of 2048 complex data points were taken in the F2 dimension and 1042 in F1 dimension for all the experiments. A sine bell function was applied to data and it was zero filled 2-fold prior to Fourier transformations. Several NOESY spectra were recorded in an attempt to assign ambiguous peaks and clarify structural constraints. The chemical shift assignments were compared to the standard chemical shifts for the structural assignments (Wishart *et al.*, 1991).

3.16. Infrared spectroscopy

For infrared (FTIR) measurements α-casein and the α-casein complexes with zinc and lead were taken at 4.2 x 10^{-4} M, in 0.01 M HEPES buffer, pH 6.8. The protein and KBr was mixed and grinded well and the pellets were made. The pellets were kept between two Teflon

spacers and were inserted in to KBr windows and the scans were recorded.

The spectra were obtained on a Nicolet 950 FT- IR spectrometer (Thermo – Nicolet, Madison, WI, USA) operated with Thermo - Nicolet *OMNIC* software (Version 5.2). Resolution was 1 cm^{-1} with 256 double-sided interferograms collected for each spectrum. Two levels of zero filled were employed. The double-sided interferograms were co-added, phase corrected (Mertz), apodized (Happ - Genzel) and fast-Fourier transformed. The spectra were deconvoluted and the nonlinear curve iterative curve fitting procedure was used to fit the Gaussian bands (Kumosinski and Unruh, 1996).

3.17. Raman spectroscopy

For Raman spectroscopy α-casein and α-casein–lead complex were collected at 4.2 x 10^{-4} M concentration. Protein solutions were taken in sealed borosilicate NMR tubes of 1.4 ml capacity and were mounted in sample compartment of the Raman spectrometer. Spectra were recorded on Nicolet FT-Raman 950 spectrometer (Thermo-Nicolet, Madison, WI, USA) excited at 1064 nm laser obtained by NdYAG (Neodydinium Yttrium Argon) laser source and InGaAs (Indium Gallium Arsenide) detector. Laser power at the sample was 1.5 mW. The spectral resolution was recorded at 4 cm^{-1}. The data spacing was at 1.928 cm^{-1}. Spectral interpretations, buffer subtraction and the

difference spectra were done by using Thermo-Nicolet's OMNIC © software available with the instrument (Xing *et al.*, 2003; Krimm and Bandekar, 1986).

The spectra were KBr and baseline corrected. The smoothing was done with Savitsky - Golay 9 point gentle smoothing in order to prevent the peak distortion and peak location. The *OMNIC peak resolve* routine uses Fletcher-Powell-McCormick algorithm. The spectral curve fitting was done as following: the second derivative spectra were generated to identify the peaks. The peaks were fit with Gaussian /Lorenzian 50:50 peak width at half height (PWHH) of 4. The spectral noise was taken in to consideration for whole region. The peaks were fit with the manual fit and then latter iterations were performed to fit the peaks.

3.18. Molecular modelling of peptides

The molecular modelling of the peptides was done using the program *Pepstr:* peptide tertiary structure prediction server available online from the Bioinformatics Center, Institute of Microbial Technology, Chandigarh (Kaur and Raghava, 2003). The *Pepstr* server predicts the tertiary structure of small peptides with sequence length varying between 7 to 25 residues. The method is based on the data set of 77 bioactive peptides with length varying between 9 to 20 residues.

Pepstr is a *de novo* method for prediction of tertiary structure of small peptides. It uses 3 steps: In first step, the regular secondary structure states (helix, β-strand and coil) and β-turn types are predicted using *BetaTurns* software. In second step, initial or starting conformation for a given sequence was generated using the T leap module of *Amber version 6.0* with phi, psi values corresponding to the secondary structure states predicted in step I.

The side chain angles are assigned using standard *Dunbrack backbone dependent rotamer library*. This initial conformation was then subjected to energy minimization and molecular dynamics simulations using *Sander module of Amber*. It consists of few initial cycles of steepest descent minimization followed by dynamics, which was carried out for 25 ps at 300K using 1-femtosecond-time steps. Finally, this was followed by minimization using a combination of steepest descent and conjugate gradient algorithms. The final coordinates are saved in Protein Data Bank format. The peptides were modeled as per the program and were visualized for their 3D structure using molecular graphics software *Pymol* Version 0.99 (DeLano, 2002).

3.19. Spectrophotometric studies

3.19.1. Equilibrium dialysis

The equilibrium dialysis was performed to study the binding of zinc with α-casein in 0.01M HEPES buffer, pH 6.8 containing 0.17 M

NaCl. 5 ml of 1.83 x 10^{-4} M of α-casein was taken in dialysis bags and dialyzed against 10 ml of different concentrations of zinc, ranging from 1x 10^{-6} M to 1 x 10^{-8} M. The necessary blanks were also taken as controls. The solutions were kept at 25 $\pm$ 1°C for 24 h in Innova 4000 incubator shaker (New Brunswick Scientific, NJ, USA) at 75 rpm. After equilibrium, the initial and final solutions in the outer compartment were analyzed for the concentration of zinc by Shimadzu atomic absorption spectrophotometer (AA-6701F) by calibrating it with appropriate standards. Binding parameters were evaluated and analyzed using the following equation (Scatchard, 1949)

$$\nu / [L_{free}] = -k_a \nu + n\, k_a \qquad (3)$$

where ν is the number of moles of ligand bound per mole of α-casein, [L_{Free}] is the molar concentration of unbound or free molar ligand concentration, k_a is the association constant (M^{-1}) and n is the total number of independent binding sites. The thermodynamic parameter, change in the Gibbs free energy ΔG° was calculated from the expression of Tanford (1961).

$$\Delta G^{\circ} = -RT \ln K_a \qquad (4)$$

where R is universal gas constant (8.32 kJ/mol), T is the absolute temperature (in Kelvin) and K_a is the association constant (M^{-1}).

The equilibrium dialysis was performed to study the binding of Pb(II) with α-casein in 0.02 M tris - HCl buffer pH 6.8, containing 0.17 M NaCl. 5 ml of 1.83 x 10^{-4} M of α-casein was taken in dialysis bags and dialyzed against 10 ml of different concentrations of lead, ranging from 1x 10^{-8} M to 1 x 10^{-4} M. The necessary blanks were also taken as controls. The solutions were kept at 25 $\pm$ 1°C for 24 h in Innova 4000 incubator shaker (New Brunswick Scientific, NJ, USA) at 75 rpm. After equilibrium, free concentration of Pb(II) was analyzed by Shimadzu atomic absorption spectrometer (AA-6701F) by calibrating it with appropriate standards. Binding parameters were evaluated and analyzed by the method of Scatchard (1949).

3.19.2. Spectrophotometric titration of lead

The effect of lead on α-casein was studied by spectroscopic titration to find out the various parameters of binding. The lead was prepared in 0.02 M tris - HCl buffer, pH 6.8 at a concentration of 1 x 10^{-4} M and was added in increments of 10 μl to the α-casein (4 x 10^{-5} M) taken in a quartz cuvette of 1 cm path length. The addition was continued up to a total dilution of the protein by 10%. After addition, absorption of the protein was recorded at 277 nm on a double beam Shimadzu spectrophotometer (Shimadzu, Japan). The temperature was kept constant by a circulating water bath connected to the instrument at 25°C. This experiment was done according to the procedure described earlier (Oberfelder and Lee, 1985). Quenching of

absorbance was recorded as a function of lead concentration. The difference in absorbance of protein with and without ligand (ΔA) was plotted as the percentage quench (Q%) versus total lead concentration. Q_{max} was determined by extrapolation of the inverse plot of Q% and the total lead concentration. Using this Q_{max}, bound ligand concentration was calculated. The bound ligand concentration was then converted to moles of ligand bound per mole of protein and analyzed for the association constant of the protein-ligand complex formation by using the following equations of Scatchard (1949).

3.19.3. Ultraviolet difference spectra

UV-difference spectra of α-casein (1.8×10^{-4} M) at different concentrations of Zinc (1×10^{-4} M to 1×10^{-6} M) were recorded against native protein using Shimadzu UV-1604 double beam spectrophotometer at 25°C. The difference spectra were recorded in the range of 230-340 nm using a pair of 1 cm matched quartz cuvettes. The detailed methodology was adopted from Donovan (1973).

UV-difference spectra of α-casein in different concentrations of cosolvents were studied. The absorbance was monitored (200-320 nm) using the protein concentration of 2.2×10^{-4} M.

3.19.4. Circular dichroism spectroscopy

Circular Dichroism measurements were performed from 190-260 nm at 25 ± 1°C using a Jasco J-810 automatic recording circular dichroism spectro-polarimeter fitted with a xenon lamp, calibrated with d -10 - camphor sulphonic acid. The instrument slits are programmed to give 1 nm bandwidth. For CD spectral measurements a protein concentration of 1 x 10^{-5} M, equilibrated with different concentrations of zinc and lead were used. A protein concentration of 3.6 x10^{-5} M was used for near-UV measurements. Peptides were also studied in the range of 0.2-0.3 mg/ml for far-UV CD spectra and 1mg/ml for near UV CD spectra respectively. The mean residue ellipticities were calculated using a mean residue weight of 115 for α-casein and for the peptides based on the amino acid composition (Chein and Waugh, 1965; Waugh *et al.*, 1962).

The far-UV scans were also recorded at 85 °C to analyze the effect of temperature on secondary structure in the presence and absence of zinc. Secondary structure analysis of the data was done according to Yang *et al.*, (1986). The molar ellipticity values [θ] are calculated according to equation:

$$[\theta] = [\theta]_{obs} \times MRW/10 \times d \times c \qquad (5)$$

where $[\theta]_{obs}$ is the observed ellipticity (deg), d is the path length of the cell (cm), c is the protein concentration (g/ml) and MRW is the mean residue weight of the protein.

3.19.5. Fluorescence quenching

Intrinsic fluorescence quenching of α-casein upon zinc binding was followed using a Shimadzu RF-5000 recording spectrofluorimeter having thermo regulatory water bath and magnetic stirrer. α-Casein of 2.2 x 10^{-6} M was titrated in a quartz cuvette of 1 cm path length at 25°C. The 5 x 10^{-7} M zinc concentration of was taken and titrated by adding 10 μl at each fixed interval with 1 min mixing time. The excitation was at 287 nm and emission recorded at 300-400 nm, keeping the excitation and emission slit widths at 5 nm each. Tryptophan ethyl ester was used as internal standard to correct any inner filter effect. The data analysis was done according to Stern and Volmer (1919).

$$F_O/F=1+K_{SV} \quad (6)$$

where K_{SV} is referred to as the Stern–Volmer constant. F_O is the fluorescence quench, in the absence of the quencher Q, while F is the fluorescence quench in the presence of the different concentrations of quencher Q.

3.19.6. ANS induced fluorescence studies

The ANS titration of α-casein was performed on a Shimadzu-RF 5000 recording spectrofluorimeter. α-Casein at a concentration of 2.2 x 10^{-6} M was titrated in a quartz cuvette of 1cm path length at 25°C. The ANS of 1 x 10^{-3} M concentration was taken and titrated by adding 10 μl at each fixed interval (1 min). The fluorescence emission was monitored at 400 – 600 nm by fixing the excitation at 375 nm with an emission and excitation slit width of 5 nm. The corrections due to inner filter effect and the self-emission of ANS were taken care of.

Hydrophobicity measurement of the protein samples using hydrophobic fluorescent probe ANS was done as per the method of Kato and Nakai, (1980). Each protein sample (3 ml) was serially diluted with 0.02 M tris - HCl buffer pH 6.8 to obtain protein concentrations ranging from 0.008-0.03%. Then 10 μl of ANS (10 mM ANS in 0.02M Tris buffer, pH 6.8) solution was added. Fluorescence intensity was measured after 3 h on Shimadzu Spectrofluorimeter RF-5000 at wavelengths excitation at 375 nm and emission at 400 – 600 nm respectively. The slits were maintained at 5 nm each for emission and excitation wavelengths. The net fluorescence intensity at each protein concentration was determined by subtracting fluorescence intensity of each solution without probe from that of probe. The initial slope of the fluorescence intensity versus protein concentration (%) plot, was

calculated by linear regression analysis was used as index of the protein hydrophobicity.

3.19.7. Stopped flow studies

Fast reaction kinetics of interaction of zinc with α-casein was followed at 287 nm using a stopped flow spectrophotometer model SX-18 MV, version 4.4 (Applied Photophysics, Leatherhead, U.K), with a path length of 2 mm and pressure of 125 psi (8 bar), compressed nitrogen at 25 $\pm$ 1°C in 0.01 M HEPES buffer, pH 6.8. For each data set, 1000 data points were collected. All experiments were repeated at least three times for each of the time regimes. The ligand and protein concentration were mixed in such a way to get 1:1 ratio. The data scan was recorded three times on an Acorn Risc computer interfaced with the instrument. The dead time of the instrument is $<$ 1 ms. Data was automatically fitted with the steady state equations/Marquardt Levenberg algorithm available with software (Version 4.36).

Fast reaction kinetics of interaction of lead with α-casein was followed at 250 nm, 277 nm using a stopped flow spectrophotometer. The temperature was controlled by a circulating water bath maintained at 25°C. The ligand and protein in equal volumes were mixed from taking the stock concentrations of 1 x 10^{-4} of lead (in 0.02 M tris - HCl pH 6.8) and 2 x 10^{-5} M of α-casein.

For time resolved spectral studies, rapid kinetic scans were carried out by using the *kinscan* software available with the instrument. These spectral scans were taken from 200- 400 nm on time base of 1s with 10 nm intervals and were spliced to get the time resolved spectra.

The studies on the effect of iodoacetamide on α-casein lead complex were studied by dialyzing the α-casein (2×10^{-5}M) for 12 h against iodoacetamide 0.1 M at pH 6.8, in 0.02 M tris - HCl buffer at 4°C with intermittent changes of the buffer containing idoacetamide (Oinuma *et al*., 2005).

Effect of temperature on the reaction rates of lead with α-casein was studied in the range of temperatures 25 - 40°C. van't Hoff plot was constructed to show the thermodynamic parameters and reaction energetics of binding as studied on stopped flow spectrophotometer.

The slow kinetics of interaction of zinc with α-casein was monitored at 287 nm by using time scan mode in the Shimadzu UV-1601 double beam recording spectrophotometer using 1×10^{-5} M ligand concentration and protein concentration of 2×10^{-5} M were used. The derivative plot analysis was done according to the Gutfreund, (1972).

The slow kinetics of lead with α-casein was monitored at 250 nm by using time scan mode in the Shimadzu UV-1601 double beam recording spectrophotometer using 1 x 10^{-5} M ligand concentration and protein concentration of 1.8 x 10^{-5} M was used.

3.20. Reversibility studies

The reversibility studies were studied by the following method. The 2 x 10^{-5} M solution of α-casein was taken and dialysed against 2 x 10^{-6} M of zinc for 12 h at 4 ± 1°C. After dialysis, the retentate and the dialysate were checked for the zinc present in each fraction. The protein was dialyzed again, against deionized water and subsequently with 10 mM EDTA solution for 12 h at 4°C was rechecked for the zinc present in each fraction (Duranti *et al.*, 2001).

3.21. Rate of hydrolysis

α-casein with a concentration of 1 x 10^{-5} M was dissolved in 0.01M HEPES buffer and dialyzed against same concentration of zinc. The dialysis was done at 4°C for 12 h. The α-casein was dialyzed against distilled water under identical conditions as control. The α-chymotrypsin at enzyme to substrate ratio of 1:100 was used to hydrolyze α-casein at 37°C for 2 h. The peptides formed were analyzed by *O*-Phthalaldehyde method (Church *et al.*, 1983). The same experimental setup was used with fungal protease at 45°C at pH 8.0.

3.22. Modification of cysteines

The modification of cysteines present in α-casein was done using AMS (4-acetamido-4'- maleimidyl stillbene-2, 2'-disulphonic acid), a reagent specific for free cysteines. The free cysteines bound to AMS and migrate on a gel with a higher molecular mass compared to an unmodified protein. In the reaction 60 μl of 0.025 M AMS, 6.25% SDS in 0.01M MOPS (4-Morpholine propane sulphonic acid) buffer pH 6.8, 0.1 M NaCl were added to 100 μl of 4×10^{-4} M α-casein to a final concentration of 2.5×10^{-4} M α-casein, 1×10^{-2} M AMS, 2% SDS. The reactions were carried out in dark at 25°C with vigorous stirring. After 2 h, the samples were dialyzed in water and analyzed by non-reducing SDS-PAGE on 15% Tris - glycine gel (Abajain *et al.,* 2004).

RESULTS AND DISCUSSION

CHAPTER – I

ISOLATION AND CHARACTERIZATION OF BIOLOGICALLY ACTIVE PEPTIDES FROM α-CASEIN

4.1. ISOLATION AND CHARACTERIZATION OF BIOLOGICALLY ACTIVE PEPTIDES FROM α-CASEIN

Many bioactivities of milk are latent and inactive within the protein sequence, requiring enzymatic proteolysis for release. Bioactive peptides can be produced *in vivo* following intake of milk proteins, or can be produced *in vitro* by the proteolytic enzymes. These peptides are separated by HPLC methods and characterized by mass spectra. Activated peptides are potential modulators of various regulatory processes in the living system like immunomodulatory peptides, stimulate the activities of cells of the immune system; cytomodulatory peptides, inhibit cancer cell growth; antimicrobial peptides, kill sensitive microorganisms; ACE inhibitory peptides, exert an hypotensive effect; opioid peptides, opioid receptor ligands which can modulate absorption processes in the intestinal tract; mineral binding peptides, function as carriers for different minerals. Many milk-derived peptides are multifunctional, i.e., specific peptide sequences having two or more different biological activities. Milk protein-derived bioactive peptides are health-enhancing components that can be used to reduce the risk of disease or to enhance a certain physiological function (Meisel, 2004; Silva and Malcata, 2005).

The aim of this chapter is to obtain novel peptides from α-casein, which shows multifunctional activities. These novel peptides

were characterized based on the structure and the interrelationships between the structure and function of the peptides was studied.

α-Casein was isolated by the method of Hipp *et al.,* (1951). The procedure involves the differential precipitation of casein with urea to separate α-casein. The purity of the total casein and the α-casein was analyzed by SDS-PAGE (figure 3). The total casein shows three prominent bands and was identified asα, β- and κ caseins respectively, where as α-casein shows two bands and can be identified as α_{S1}-casein and α_{S2}-casein. The α-casein was analyzed for its purity on the size exclusion chromatography and found to be having two peaks with retention times at 10.5 min and 8.9 min, which are again due to the α_{S1}-casein and α_{S2}-casein respectively (figure 4). The amino acid composition of α-casein was analyzed and expressed as gram% in table 4. The amino acid composition shows 94% purity. The isolated α-casein was correlated with standard (sequence based) and found to be comparable.

The characterization of the α-casein was done by UV absorption, fluorescence and CD spectroscopy. The protein has the absorption maxima at 278 nm and the $E^{1\%}$ was found to be 10.12. The fluorescence emission maximum was found to be 340 nm when excited at 278 nm. The far UV CD shows 2 ± 0.5 % α - helix, 63 ± 0.5 % β - structures, and 35 ± 2 % aperiodicity. The circular dichroism spectra of

α-casein was compared with the literature and found to be similar (Aoki *et al.,* 2000).

Peptides are liberated by the action of proteolytic enzymes. Different proteolytic enzymes are employed to generate novel peptides. They are aminopeptidase, carboxypeptidase, fungal protease, bacterial protease and chymotrypsin. These broad and narrow specificity enzymes from animal and microbial sources were employed to hydrolyze the α-casein. Enzymatic hydrolysis was carried out at optimum temperature and pH, for a fixed time. Peptides, thus liberated were collected and characterized by DH. Estimation of DH was performed for each enzyme hydrolysates, to analyze the number of peptide bonds hydrolyzed. This will help us to select the best enzyme for the hydrolysis.

The figure 5 compares DH profiles of enzymatic hydrolysates of α-casein. The figure shows, the DH of 0.7% with aminopeptidase, 0.4% with carboxypeptidase, 4.6% with chymotrypsin, 4.2% with bacterial protease and 70% with fungal protease respectively. The DH with aminopeptidase and carboxypeptidase is less as they hydrolyze proteins from the N and C-terminal ends. Due to their narrow specificity, they are less productive in liberating peptides. The DH was more with bacterial and fungal protease due to their broad specificity of action. Fungal protease is having a mixture of endo and

exopeptidase activity, the reason for the increase in DH. Chymotrypsin is selected for its specificity to hydrolyze peptide bonds containing aromatic and hydrophobic amino acids (Adler-Nissen, 1986). The chymotrypsin cleaves C-terminal side of Phe, Tyr, and Trp containing amino acids. Chymotrypsin hydrolysates show 4.6 % DH. Amiot *et al.,* (2004) has shown, hydrolysis of whole milk with chymotrypsin shows DH of 4.8%. The DH in case of chymotrypsin is less and is due to the inhibition of enzyme by peptides or peptide – peptide interactions (Adler-Nissen, 1986; Pouliot *et al.,* 2000). Most bioactive peptides so far identified from milk hydrolysates contain four to ten amino acids (Xu, 1998; Miller *et al.,* 2000). Amiot *et al.,* (2004) has shown that 80% of the peptides released by chymotrypsin appears to be in the range of 300-1200 Da. Chymotrypsin hydrolysates were selected for further study as they can liberate smaller peptides.

Dunn and Lewis, (1921) reported a comparative study on casein with various proteolytic enzymes. They used pepsin, trypsin and erepsin enzymes and compared the hydrolysis pattern of the casein. But characterization of the liberated peptides by these enzymes was not studied.

In order to isolate potential bioactive peptides from the enzymatic hydrolysates, they were initially screened against ACE inhibitory activity. ACE inhibitory activities of different enzymatic

hydrolysates were shown in the figure 6. From the figure, the chymotryptic peptides were having 64% inhibition, whereas the enzymatic hydrolysates from aminopeptidase, carboxypeptidase, bacterial and fungal protease were found to be inhibiting ACE at 0%, 4%, 52% and 61% respectively. The ACE inhibition was found to be maximum for chymotryptic hydrolysates. The chymotrypsin hydrolysates were further employed for isolation and characterization of novel multifunctional peptides.

During the course of enzymatic hydrolysis, the peptide fractions were collected at different time intervals and they were purified by HPLC. The hydrolysates were separated from unhydrolysed protein and the crude peptides were tested for the ACE inhibitory activity. The figure 7 shows the comparison of ACE inhibition and degree of hydrolysis of the liberated peptides, from α-casein at different time intervals. From the figure, it is evident that first 10 min of hydrolysis, the ACE inhibition of the liberated peptides was found to be high. The ACE inhibition is 85% within 10 min of hydrolysis, which reduces, to 30% after 25 min of hydrolysis time. From 25 min to 150 min the ACEI activity of the hydrolysates increases as a function of hydrolysis time from 35% to 65%. From the figure, the fractions at the 105 min were considered for further studies, as their ACE inhibiting activity is high (65%). These fractions were considered for isolation of peptides, as there will not be any further change in the DH%. The peptides

released during the first 10 min of hydrolysis were not considered due to the further hydrolysis into smaller peptides. The study on release kinetics of peptides will help us to understand the optimum time required for hydrolysis to get potential active peptides (Tauzin *et al.,* 2003).

The 105 min fraction was analyzed for the multifunctional biological activities. Activities like PEP inhibitory, antioxidant and antimicrobial activities against pathogenic food borne bacteria like *B. cereus* (gram positive), *E. coli* (gram negative) and *L. acidophilus* (probiotic bacteria). The table 5 shows multifunctional activity of the crude peptides. The crude peptides show more than one activity along with ACE inhibition. The IC_{50} values for ACE inhibition was found to be 0.1 mg/ml as compared to ramipril with IC_{50} values of 0.025 mg/ml. Ramipril is a well known orally administered hypotensive drug that acts on the ACE enzyme. IC_{50} values for PEP inhibition was 1.3 mg/ml as compared with the standard, Z-prolyl prolinal with an IC_{50} of 0.165 mg/ml. The peptides were found to be scavenging the oxygen free radicals with IC_{50} values of 1.25 mg/ml as compared with the standard, L-ascorbic acid of 0.175 mg/ml. These peptides do not show amylase inhibitory activity and cytotoxic activity, as studied by the agglutination of red blood cells. The peptides show zinc binding activity. These peptides have zinc binding capacity of 2.3 mg/g of hydrolysate

compared with the bacitracin standard of 45 mg/g as reported by Ming and Epperson, (2002).

The bioactivities of peptide are compared with the respective standards. The IC_{50} values of peptides are less compared to the standards. This can be due to the presence of different kinds of peptides in crude mixture. The decrease in IC_{50} values compared to standard IC_{50} values could be due to the peptide - peptide interactions. The reasons for the decrease in activity could also be due to the dilution of peptides in the mixtures (Meisel, 1993; Meisel and Bockelmann, 1999). This study is an indication of the presence of multifunctional activities in crude mixtures.

The antimicrobial activity of the hydrolysates was studied using the bacterial growth curve method. Growth of *E. coli, B. cereus, and L. acidophilus* were followed in presence of hydrolysates and were compared with the lactoferrin standard. Lactoferrin is a known antimicrobial protein from bovine milk. The growth rate of bacteria in presence and absence of hydrolysates and lactoferrin were calculated and represented in the table 6.

The growth rate of *B. cereus* shows 2.4 gen/h compared to presence of hydrolysates (0.75 mg/ml) of 1.2 gen/h. In the presence of lactoferrin (0.75 mg/ml) the generation time of *B. cereus* changed

from 2.4 gen/h to 1.4 gen/h. The results showed that the hydrolysates and the lactoferrin have the similar effect on the *B. cereus*. The optimum concentration required is 0.75 mg/ml compared to other concentrations studied. Similarly the growth rate of *E. coli* was 0.96 gen/h and in the presence of hydrolysates (0.75 mg/ml) the growth rate has reduced to 0.45 gen/h. The presence of lactoferrin growth rate of *E. coli* has changed from 1.2 gen/h to 0.72 gen/h. The lag in the growth rate shows hydrolysates are more potent than the lactoferrin. The above study concludes that the hydrolysates are effective on Gram positive and Gram-negative bacteria.

The growth rate for the *L. acidophilus* is 0.04 gen/h and in the presence of hydrolysates the growth rate has increased to 2.7 gen/h, which indicated that the peptide promotes the growth of probiotic *L. acidophilus* while inhibiting the pathogenic bacteria (table 6). The lactoferrin does not have any effect on the growth of probiotic bacteria. Student's t-test (two-sample, assuming unequal variances) was run to compare the different growth curves and the differences were found to be ($p<0.05$) significant. The presence of antibacterial activity in contrast to cytotoxic activity can be reasoned due to the penetration of peptides in to negatively charged bacterial membranes compared to neutral vesicles mimicking eukaryotic membranes (RBC).

In order to identify and characterize peptides, from crude hydrolysates isolation and purification was attempted. The peptides were purified by size exclusion chromatography using analytical HPLC system. The size exclusion chromatography profile of the 105 min fraction is shown in figure 8. Different fractions were collected at different retention time intervals in the order of elution and the fractions were again checked for their ACE inhibitory pattern. The peak collected at the retention time of 21.6 min shows ACEI of 98%. This fraction is collected and resolved on a reverse phase chromatography.

Reverse phase column chromatography profile of the peptides eluted at different time intervals for the 21.6 min peak is shown in figure 9. Fractions were collected at different retention time intervals and checked for their ACE-inhibitory activity. The peak collected at the retention time of 15 min shows ACEI activity of 99.6 %. These 15 min peptide fraction was characterized using mass spectra.

Difficulties in peptide identification limit the knowledge available on the formation of bioactive peptides and their release from the precursor proteins. Several chromatographic steps are often necessary to purify the peptides of interest and finally they are identified by mass spectrometry or by MS/MS. The development of MALDI made it possible to analyze peptides present in complex mixtures without the need to separate into individual components. The routine analysis is

generally done by comparing the fragmentation pattern with parent peptide masses, thereby allowing the unambiguous identification of peptides from the enzyme digests of proteins (Papayannopoulos, 1995).

The mass spectroscopy is used to characterize peptides on the basis of the molecular weight (Carr *et al.,* 1991). The 15 min fractions were subjected to ionizing laser and the masses of the individual peptides were obtained. The mass spectral data of the 15 min fraction was shown in the figure 10. The fingerprinting of the 15 min fraction shows 9 peptides with different molecular masses. Peptides identified from the mass spectra were compared with the theoretical masses of the peptides. Theoretical masses of the peptides were obtained by *in silico* hydrolysis of the α-casein with chymotrypsin using software *MS–Digest* from protein prospector. The peptides that match with the theoretical masses were selected for further the sequencing and characterization (Clauser *et al.,* 1999). The peptides with molecular weights of 1204 Da. and 1718 Da. were selected as they match with the theoretical mass. The comparison with the theoretical masses will help us to selectively pick peptides, which are released from the particular enzyme. The nonspecific and the autocatalytic peptides generated from the enzyme digest can be avoided using this match. The peptide mass fingerprint pattern for particular protein with an enzyme is unique.

The peptides with molecular masses of 1204, and 1718 Da. were used for the MS/MS analysis as shown in the figure 11 and figure 12. The mass spectra contain the parent ion with various daughter ions. These daughter ions are nothing but the unassigned amino acids. The mass spectrum is assigned for the amino acids and the sequence in which the amino acids are assigned gives us peptide sequences. The individual masses of the various ions are now converted into their respective amino acids by using the *MS-Seq* from *protein prospector* (Clauser *et al.,* 1999).

The parent and the daughter ion masses were converted in to their sequence by employing *MS-Seq* program (Clauser *et al.,* 1999). The program requires the various ions generated by mass spectrum as input, which in turn converts them in to amino acid sequence by comparing them to the user provided milk protein sequences. The mass and the product ions generated upon collision induced dissociation of the parent ions, agreed well with the N-terminal *b*-type ions and C-terminal *y*-type ions expected from the theoretical MS/MS fragmentation pattern of the peptides and they corresponded to the sequences QKALNEINQF, TKKTKLTEEEKNRL from α_{S2}-casein f(94-103) and α_{S2}-casein f(163-176) respectively. The parent α_{S2}-casein and the peptide sequences are shown in the figure 13.

The above-mentioned novel peptide, i.e., the peptides with 10 and 14 amino acids containing sequences QKALNEINQF and TKKTKLTEEEKNRL from α_{S2}-casein was synthesized. The peptide was synthesized on Fluorenyl methyloxycarbonyl (F moc) group chemistry using automated solid phase peptide synthesizer. The peptides were synthesized on the Rink amide resin. The synthesized peptides were having the amide adduct on the C-terminal end as $–CONH_3$. After synthesis, the sequencing of the synthesized peptides was done and amino acids were found to be correctly incorporated and were 98% pure. The peptides were checked for their purity on reverse phase HPLC column. The profiles show that peptide 10 and peptide 14 elute at retention times of 3.8 min and 5.3 mins respectively. There was no other compound coeluting with the peptides.

The peptides were analyzed for their purity by amino acid composition. The amino acid composition shows that the peptides are 98% pure. These peptides were checked for the purity by ESI-MS spectra. The spectra of the two synthesized peptides are present in the figure 14. The ESI-MS spectra of the peptide shows that the mass of the peptide 10 is 1198 Da. and expected is 1204 Da. Similarly the peptide 14 shows the mass of 1827 Da. and expected is 1718 Da. The increase in the mass of peptide 14 could be due to the presence of TFA bound to the peptide. The trifluoroacetic acid is having the molecular

weight of 114 Da. The presence of TFA in the peptide is due to the use of TFA as a deprotector in the peptide synthesis.

The synthesized peptides were analyzed for multifunctional biological activity. The synthesized peptides and their activities are summarized in the table 7. ACEI activity of the peptide 10 is having an IC_{50} value of 20 nM compared to ramipril with IC_{50} values of 60 nM. The peptide is three times more potent compared to the standard ramipril. The antioxidant activity of peptide 10 is 50 nM compared to ascorbic acid with 150 nM. The peptide 10 is 3 times more potent compared to ascorbic acid, a well-known antioxidant.

Similar experiments with peptide 14 show IC_{50} values for ACE inhibition and antioxidant activity as 37 nM and 6 nM respectively. The IC_{50} values are 60 nM and 150 nM respectively for ramipril and ascorbic acid. The peptide is 1.6 times and 25 times more potent compared to standards.

The peptides were studied for the inhibition of PEP activity. The IC_{50} values of peptides were depicted in table 7. The peptides 10 and 14 shows inhibition with an IC_{50} values of 6 nM and 58 nM respectively. The standard, Z-prolyl-prolinal shows 500 nM. The peptides are efficient compared to standards in inhibiting PEP enzyme. The peptides are 10 times and 13 times more effective compared to standard. These

studies on the PEP inhibition will help us to identify novel peptides to cure psychological disorders (Yanai *et al.,* 2003).

The peptide 10 was checked for the aurora kinase enzyme inhibition. The peptide 10 is having an IC_{50} value of 0.01 nM compared to the inhibitor hesperidin with an IC_{50} value of 250 nM. The peptide 10 is thousand times more active against hesperidin. These peptides can be promising as future anticancer drugs (Nicholas and Taylor, 2004).

The above study shows that the peptides are having simultaneously multiple inhibitory activities (Fiat *et al.,* 1993). The peptide shows simultaneously antioxidant and the ACE inhibitory activities. The hydrophobicity is also one of the reasons for the activity of the peptides as reported by Schlimmel and Meisel (1995). Peptide shows potential ACE inhibition, which can be reasoned due to the presence of aromatic amino acids at the C-terminal end of the peptides (Cheung *et al.,* 1980; Eriksson *et al.,* 2002; Sipola, *et al.,* 2002). The presence of phenylalanine at the C-terminal end is responsible for the potential ACE inhibitory activity as in case of peptide 10.

The peptides were analyzed for the growth inhibiting potential of the microorganisms. The peptide was studied for antimicrobial activity against *B. cereus, E. coli, S. aureus, H. pylori* and *L.*

monocytogenes. The peptides were incubated with the bacterial culture for 12 h and the effect of peptide on the bacterial growth was checked after incubation. The suspensions were poured on to plates and were incubated for 24 - 72 h and the number of colonies grown are counted and expressed as population survived after incubation. The table 8 summarizes the growth inhibitory effect of peptide 10 on various microorganisms. The peptide 10 is taken in different concentrations i.e., 1.5 mg/ml to 4 mg/ml and the population survived after incubation is shown. The table 8 shows peptide 10 is having the bactericidal effect on *B. cereus* at 2 x 10^4 and 2 x 10^5 CFU/ml. The population survived after incubation was less than 0.5 % at peptide concentration of 1.5 mg/ml and indicates efficient bactericidal effect. At higher cell numbers of 2 x 10^6 CFU/ml the peptide is still effective in preventing the growth of bacteria.

The MIC of the peptide is calculated based on the lowest concentration required to efficiently inhibit the growth of the bacteria and shown in the table 11. The MIC value of the peptide 10 is 0.87 μM. The peptide 10 is not effective on *E. coli*. The population survived after incubation are 100%, which indicates all the bacteria, survived after incubating the peptide with the bacteria. The effect of peptide 10 on the *S. aureus* shows 1.0 % survival after treatment. The peptide has no effect on the *Listeria* as seen from the table with 100 % survival of the bacteria after treatment with the peptide. The peptide is active on

B. cereus and *S.aureas,* but not on *Listeria* and *E. coli*. The MIC of the peptides is shown in the table 11.

Similar studies by peptide 14 on the different bacteria was studied and shown in the table 9. The peptide 14 shows inhibition on *B. cereus*, and *S.aureas* respectively. There is comparatively more inhibition in case of *L. monocytogenes* compared to peptide 10. There is no effect of peptide 14 on *E. coli*. The peptide MIC values were shown in the table 11.

These studies were supported by standard bacitracin, where in the effect of bacitracin was also studied simultaneously and compared with peptides. The table 10 shows the population survived after incubation of the bacteria in presence of bacitracin. Bacitracin, (is a peptide antibiotic with 14 amino acids residues and is commercially available) was selected from large group of antimicrobial peptides as standard (table 10). The bacitracin is effective on all the bacteria studied. The MIC values for the bacitracin were calculated from the population survived after incubation and shown in the table 11. Microbial activities associated with milk proteins are known from the literature (Keith *et al.,* 1997). The multiple antimicrobial activities of the milk casein peptides were reported earlier (McCann *et al.,* 2005 and McCann *et al.,* 2006; Coventry, 2004). The mode of action gives us

the insights into the antimicrobial nature of peptides on various bacteria (Epand and Vogel, 1999).

The table 11 shows the antimicrobial activity of peptides as studied on *H. pylori*. The table shows that the peptide 10 is showing the *H.pylori* inhibition with an IC_{50} activity of 0.083 - 0.25 μM. The peptide 14 has no effect on the bacteria. The standard amoxycillin shows an IC_{50} value 0.13 μM. Peptide 10 shows anti ulcer activity whereas peptide 14 is devoid, which could be due to the presence of hydrophobic residues in peptide 10. Synthesized peptides do not show cytotoxic activity, as studied by the agglutination of red blood cells.

The peptides were also studied for their antifungal activity. The peptides were incubated with different food borne pathogenic fungi like *Aspergillus*, *Fusarium*, and *Pencillium* and the activity of the peptides were monitored on the growth of fungi. The peptides were not having any antifungal activity as shown in the table 11. The peptides were not shown any antifungal activity could be due to thick chitinaceous cell wall. These peptides don't have any affect on fungi in turn contribution to the growth of fungi. The peptides were showing very less growth inhibition as monitored for *Fusarium*. The peptides were also studied for their inhibiting against liberating the *Aspergillus* aflatoxins (mycotoxins). There is no significant change in the pattern of release

of toxins in the presence of peptide by *Aspergillus*. Further studied were discontinued due to these results.

The antimicrobial peptides were studied for their mode of action. The peptides were studied for their bacterial cell penetrating behavior in presence of fluorescence membrane potential sensing probe bis- (1,3-dibutyl-barbituric acid) trimethine oxonol [$DIBAC_4(3)$] to monitor the effect of peptides on the bacterial membranes (Epps, *et al.,* 1994). The dye enters depolarized bacterial cells and binds to intracellular proteins or membranes and exhibit enhanced fluorescence. Increased depolarization results in more influx of the anionic dye and thus an increase in fluorescence. Conversely, hyperpolarization is indicated by a decrease in fluorescence. The significance of this dye is that it senses the changes in the membrane potential, which is an indication of the movement of the peptide along the cell membrane in to cytoplasm. The cells were incubated in presence of peptide and the changes in the membrane polarization were studied as function of time. The effects of peptides on *B.cereus* cytoplasmic membranes were studied using fluorescent probe.

The figure 15 shows changes in the membrane potential of *Bacillus* in presence of peptides. During the study, the fluorescence of the probe increases as a function of time in presence of peptide 10. The fluorescence intensity increased from 162 AU to 190 AU in 15 min

time. The change in the fluorescence is 20%, which is indication of the changes in the location of the dye and peptide. Peptide 14 is not showing any change in fluorescence. The increase in the fluorescence is due to penetration of probe in to membrane due to changes in the electrostatic potential across membrane. The figure shows peptide 10 is bringing changes in the membrane potential i.e., depolarization as seen in the increase in fluorescence. This shows that the peptide 10 is damaging the membrane (depolarized) of the cells under study (Pag *et al.,* 2004). This indicates that the peptide 10 moves across the cell membrane. The cell penetrating behavior of the peptide could be due its amphipathic nature.

In order to study the effect of peptide 14 on DNA, bacterial genomic DNA, was isolated and incubated with the peptide 14 for 2h. The peptides after incubation were run on an agarose gel electrophoresis and the shift in the mobility of the DNA is observed. The gel shift mobility assay shows changes in the mobility of the DNA and DNA peptide complex. The gel is shown as inset in figure 16. The gel shows that the control DNA shows no change in the mobility, where as the DNA incubated with peptide 14 is retained in the well. The retardation of DNA by the peptide 14 shows that peptide binds to DNA, which is an indication of intrinsic DNA binding ability of peptide (Hsu *et al.,* 2005).

The DNA binding activity of the peptide was further studied by the time kinetics mode on UV spectrophotometer. The figure 16 shows the binding of peptide 14 to *B. cereus* gDNA and calf thymus DNA as studied by time kinetics mode on spectrophotometer. The kinetics is monitored at 260 nm for the changes in the DNA up on binding. The study was done till 15 min and the change in the absorbance is observed over time. There is a decrease in the absorbance of DNA of 0.22 and 0.24 units respectively. The absorbance was fit with first order equation and the resultant rates for *B. cereus* gDNA and calf thymus DNA were identified as 7 s^{-1} and 1.5 s^{-1} respectively. The binding to calf thymus DNA by peptide 14 is much faster compared to binding to microbial DNA. The decrease in the absorbance of the DNA can be due to the binding of peptide 14 with DNA. This can be due its cationic nature (Komiyama *et al.,* 1983). The presence of lysines and arginine in the peptide 14 can leads to binding of peptide 14 to DNA as observed by the studies using the gel shift mobility and the time kinetics studies. The change in the rate upon binding to two different DNA could be due to the differences in the GC content of bacterial and calf thymus DNA (33% and 42% GC content respectively) or could be due to the complexity in the coiling of the mammalian and bacterial DNA (Zhang, 1996; Azorín, 1985).

Multifunctional peptides are the peptides having more than one biological activity and this is due to the overlapping sequences present

in the parent protein. The present peptides QKALNEINQF, TKKTKLTEEEKNRL are also a peptide having more than one biological activity. Multifunctional peptides were also reported in the literature (Miesel, 2004; Meisel and Bockelmann, 1999). The possible explanation for such behavior is due to the overlapping peptide sequences that exert different biological activities. The regions are called as 'strategic zones', which are partially protected from proteolytic breakdown (Fiat and Jolles, 1989; Meisel 1998).

The peptides should be stable once given orally as a nutraceutical or incorporated in to functional foods. In order to understand the ability of peptides to withstand the hydrolytic enzymes in the gastrointestinal tract (Ohtani *et.al.*, 2003), the hydrolysis of the peptides were studied by pancreatin. The peptides were hydrolyzed by pancreatin enzyme and their degree of hydrolysis was followed as a function of time. From the study it can be concluded that the peptide is stable in presence of proteolytic enzymes. The peptide 10 was found to be having 1 % DH after incubating with pancreatin for 240 min. This is equivalent to 0.1 % peptide bonds hydrolyzed, is very insignificant and found to be stable. Similarly the peptide 14 was found to be having 0.332% DH% which is equivalent to 0.05% peptide bonds hydrolyzed after 240 min, which is very insignificant and found to be stable. The evasion of hydrolysis of the 1204 and 1718 Da. peptide could be due to the amidylated C-terminal. The other reason could be that the small

peptides itself may be inhibiting the enzyme. Lien and Lowman, (2003) has shown that the peptides as smaller as 10 –12 amino acids are structured and rigid and due to their rigidity in structure they are resistant to hydrolysis by peptidases.

The structural features of the peptides were studied. The peptides were characterized by far UV-circular dichroism spectroscopy. The figure 17 shows the CD spectra of the peptides. The CD shows that the peptide10 is having negative maxima at 200 nm and 235 nm. The peptide 14 is having negative maxima 215 nm and 245 nm. The secondary structure of peptide 10 shows 19% α-helix, 40% β-beta sheets, 39% aperiodic where as peptide 14 shows 0% α-helix, 73% β-sheets, 27% aperiodic structures respectively.

The multifunctional bioactive peptides obtained from α-casein was studied for their secondary structure using NMR. The proton nuclear magnetic resonance spectra of the 4.15 μM peptide 10 and the 4.65 μM peptide 14 were recorded at one dimension and two dimensions. The one-dimension ^{1}H NMR spectrum (figure 18) of the peptide 10 is broad and shows broadened peak line widths. The peptide broad peaks were not resolved even at higher temperatures. The proton resonance assignment was done on the basis of Wishart *et al.,* (1991). The final chemical shift assignments are shown in the table 12. The TOCSY and ROESY spectrum were indicative of a random

peptide. The intra residue and inter residue NOE of the amino acids show an extended conformation of the peptide. The peptide was studied in the DMSO as it solubility is a limiting factor in other polar inorganic solvents and biological buffers. The possibility of the DMSO (a hydrogen bonding solvent) (Mahalakshmi *et al.,* 2006) breaking the intermolecular hydrogen bonds is also not ruled out. The possibility of this peptide showing different conformations in CD could be due to induced structure in presence of various solvents.

The solution structure of peptide 14 was derived using nuclear magnetic resonance. The NMR spectrum was also recorded at one and two dimensions. The one-dimension NMR spectrum of peptide 14 is shown in the figure 19. The spectrum in presence of 90% water and 10% D_2O shows satisfactory dispersion of resonances and further structural characterization was done in this solvent. The proton resonance assignment was done on the basis of Wishart *et al.,* (1991). The final chemical shift assignments are shown in the table 13.The table shows the chemical shifts of the residues of peptide 14 as compared to trimethyl silane. The TOCSY cannot show 14 residues assignments, as there is a severe overlap in the resonances. ROESY also supports the hypothesis that the peptide is highly flexible and aperiodic due to the few NOE ‘s detected between residues. The structural studies by NMR proved that the two peptides are flexible and random. The presence of secondary structures as analyzed by CD can

be due to the ensemble of conformations. The possibility of peptides having conformations and changing the structure in milli time base due to the flexibility of the backbone is also not ruled out. The TOCSY, NOESY, ROESY and NOE are defined in list of abbreviations.

The *in silico* methods based on the sequence homology to other bioactive peptides was attempted to visualize these bioactive peptides. The modeling of the peptides was done using the software available from the bioinformatics center IMTECH, Chandigarh, India. The *pepstr* software was used to analyze the sequence of peptide and using the algorithms therein the peptide structure is deduced in form of PDB format coordinates (Kaur and Raghava, 2003). The peptide 10 and peptide 14 structural coordinates was converted in to 3D structure and shown in the figure 20. The peptide 10 is having α-helix and peptide 14 is having β-turns, which can be seen from the structure. All the studies on the casein peptides reveal the backbone flexibility and mobility, which will help the molecules to adopt the different conformations and probably the transition from one form to other form in the milli time base, which cannot be observed by NMR (Huq *et al.,* 2004).

From the above study we can conclude that the intestinal enzymes have a very major role in releasing peptides, which have got biological role. The hydrolysates and the purified peptides from

α-casein are found to possess multiple biological activities. The ability of peptides to show biological activity after synthesis supports the premise that the peptides may simultaneously exhibit all or few of the biological activities. The structural studies show that the peptides have flexible backbone conformation and can be induced to form different conformations in various environments.

The biological activities exhibited by peptides are influenced by a variety of external factors, which might limit or enhance their function. In order to tune up their operational stabilities they need to be stabilized with various cosolvents. The next chapter focuses on the structural and functional stability of α-casein and peptides in various cosolvents.

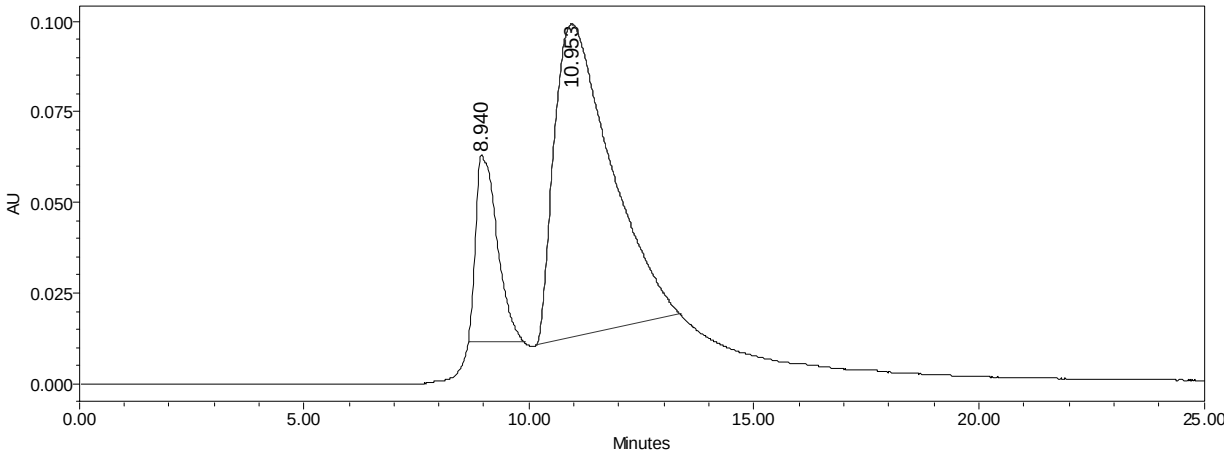

Fig.4. Gel filtration profile of isolated α-casein. The α-casein was checked for its purity on Shodex column, The 10 µl of protein was injected and isocratic elution performed at flow rate of 1.0 ml/min. The buffer used was 10 mM HEPES buffer, pH 6.8.

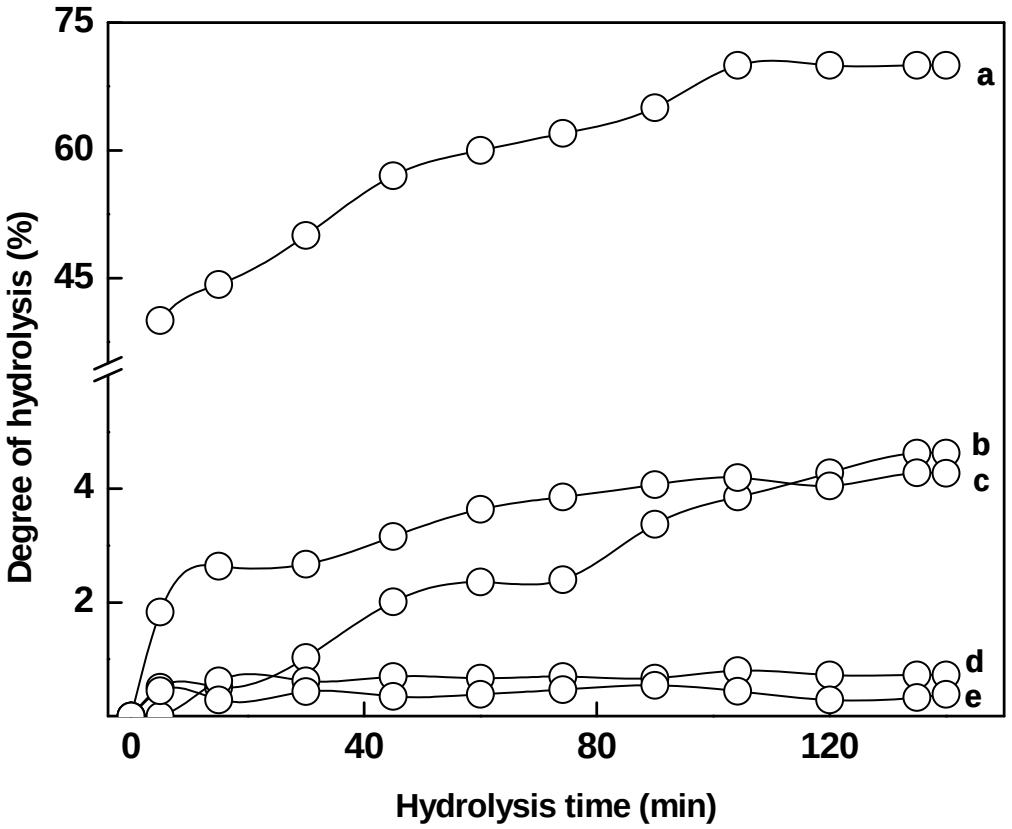

Fig.5. Comparison of degree of hydrolysis of different enzymatic hydrolysates of α-casein. The various enzymes used for hydrolysis are (a) fungal protease (b) bacterial protease (c) chymotrypsin (d) carboxypeptidase and (e) aminopeptidase.

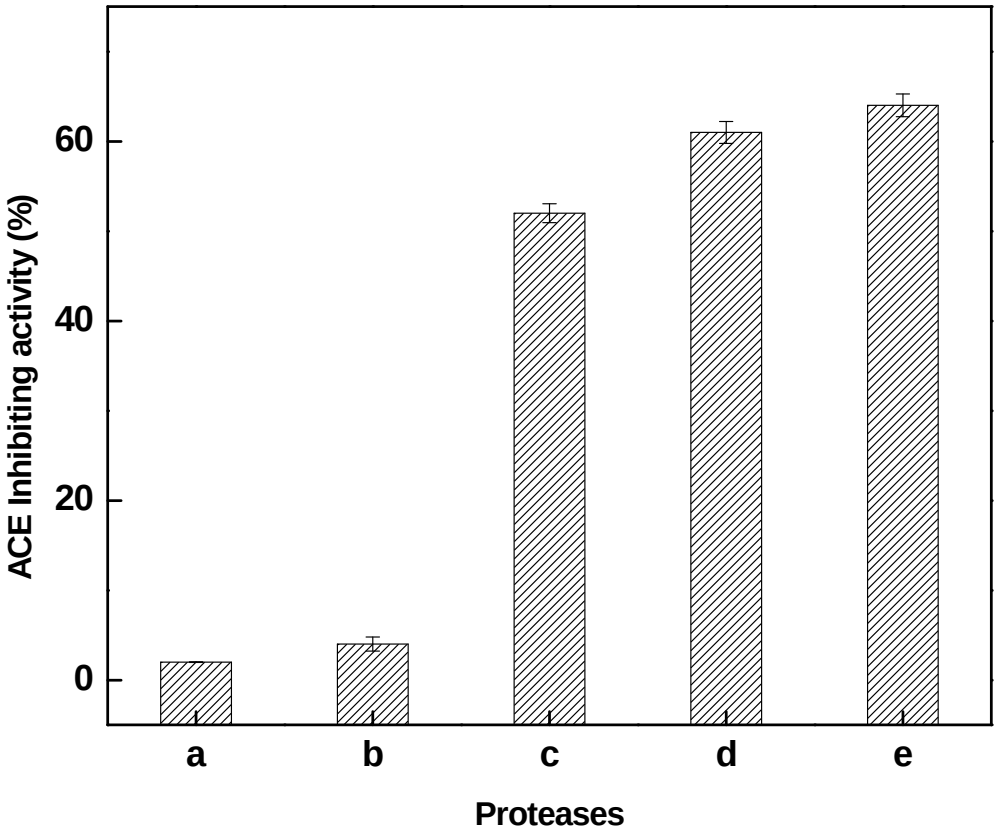

Fig.6. ACE Inhibitory activity of the hydrolysates obtained from α-casein by using various enzymes (a) aminopeptidase (b) carboxypeptidase (c) fungal protease (d) bacterial protease and (e) chymotrypsin

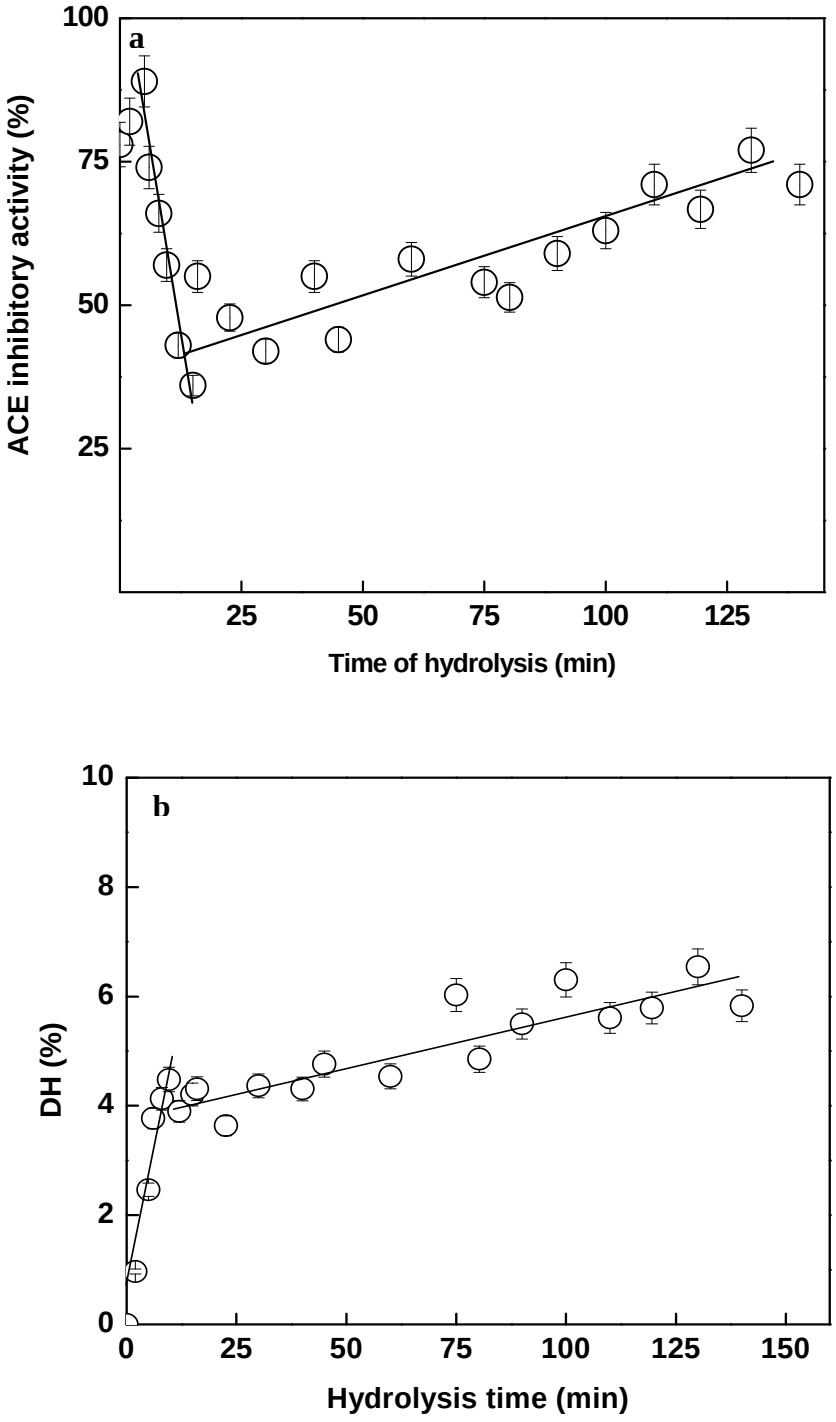

Fig.7. Comparison of ACE inhibitory activity and the DH of hydrolysates. (a) Release of ACE inhibitory peptides from α-casein at different time intervals and (b) The DH pattern of α-casein

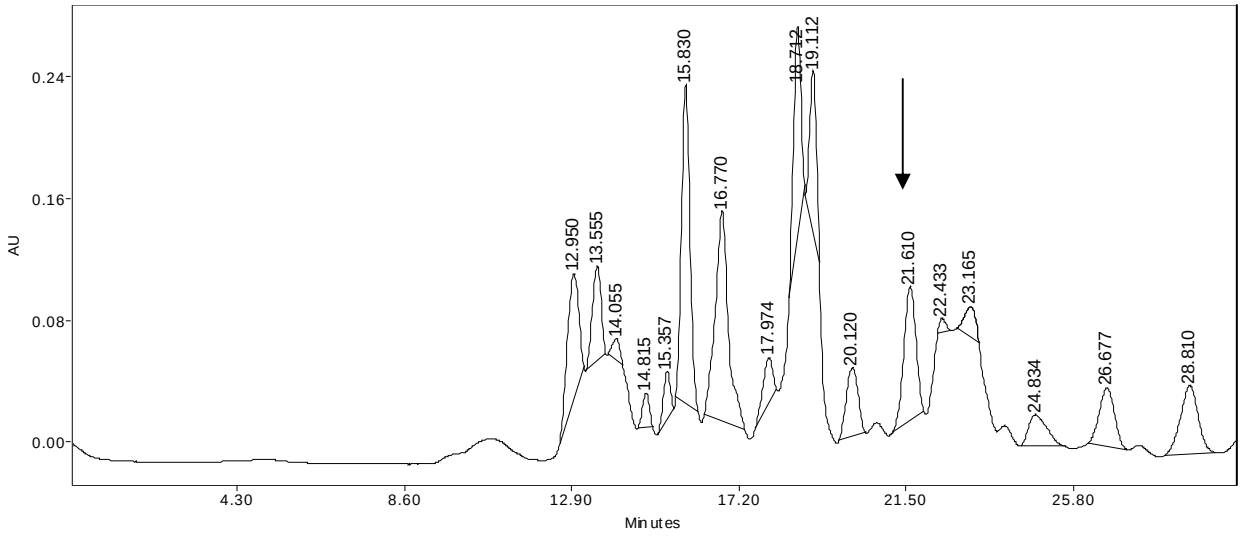

Fig.8. Size exclusion chromatography profile of chymotrypsin hydrolysates. The peptides were separated in 0.01 M morpholine TFA buffer pH 7.8. The flow rate was maintained at 1.0 ml/min. The arrow indicates the peak used for further separation

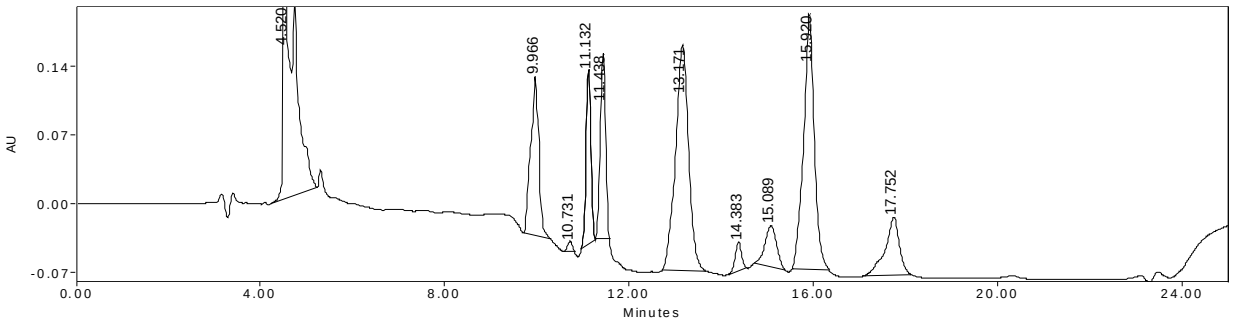

Fig.9. Reverse phase HPLC pattern of chymotrypsin hydrolysates. The peptides were separated by C_{18} Reverse phase column using binary gradient with a flow rate of 0.7 ml/min. The arrow indicates the peak used for further separation

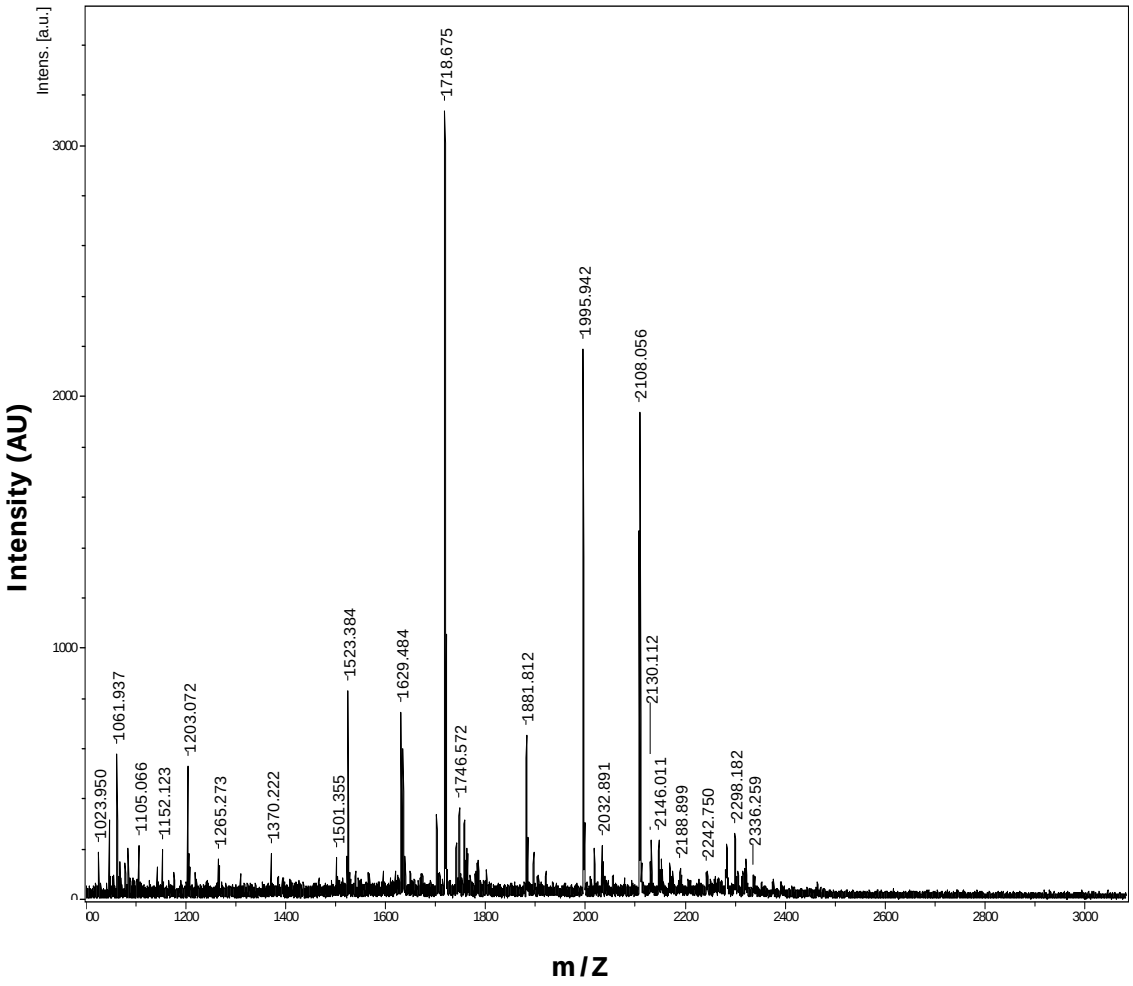

Fig.10. Peptide mass fingerprinting of crude peptides on MALDI-TOF. The peptides were loaded on to MALDI plate along with CHCA matrix.

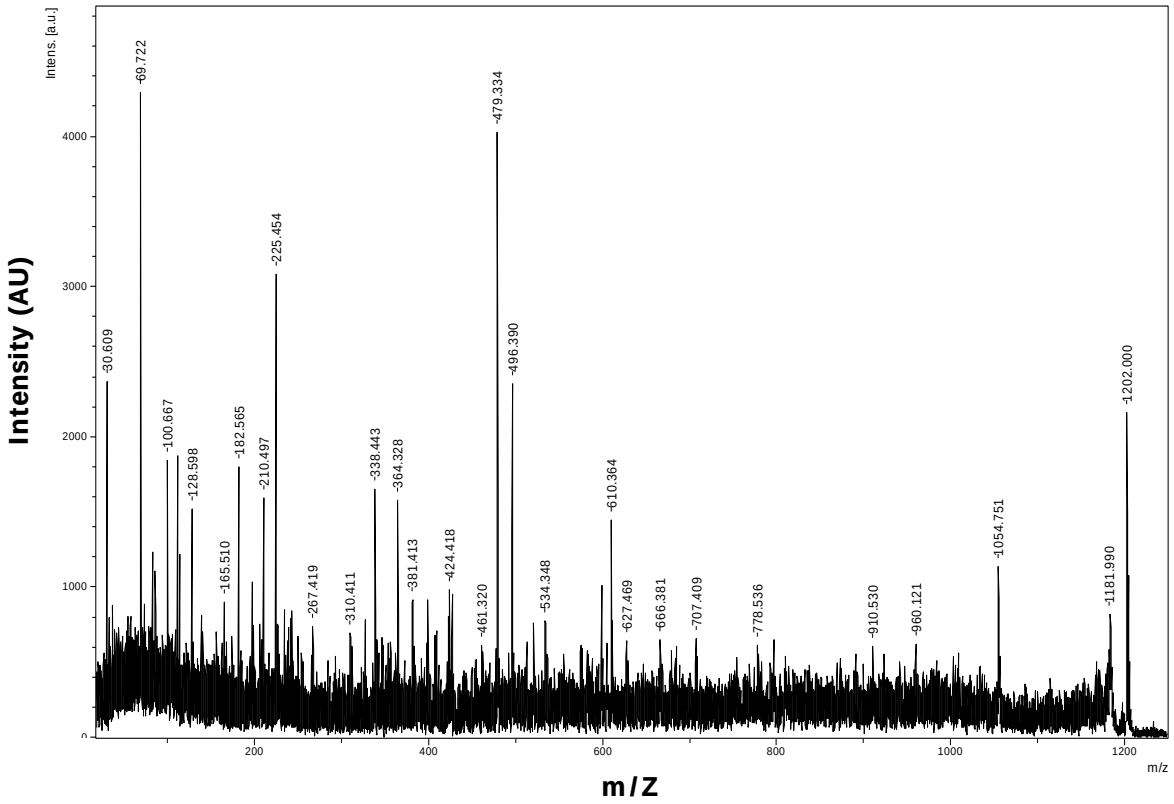

Fig.11. The MALDI TOF MS/MS pattern of the 1204 Da. peptide

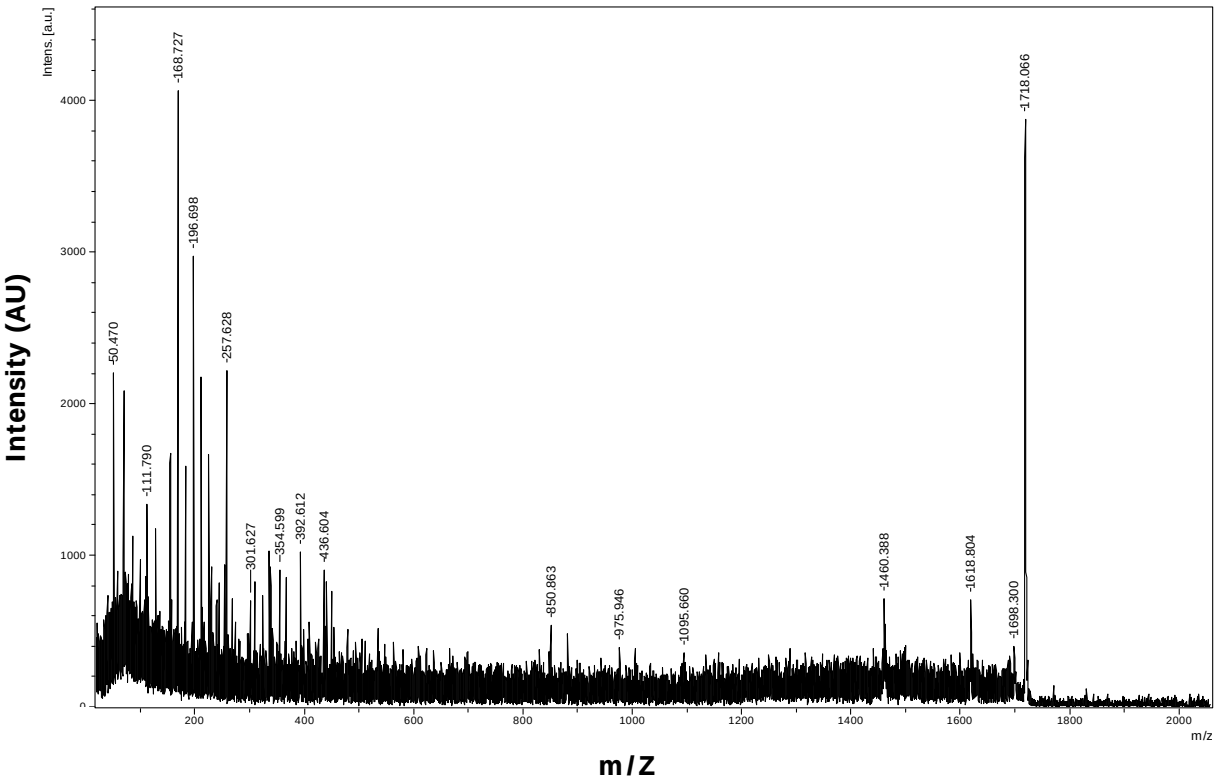

Fig.12. The MALDI TOF MS/MS pattern of the 1718 Da. peptide

1 MKFFIFTCLL AVALAKNTME HVSSSEESII SQETYKQEKN

2 MAINPSKENL CSTFCKEVVR NANEEEYSIG SSSEESAEVA

3 TEEVKITVDD KHY**QKALNEI NQF**YQKFPQY LQYLYQGPIV

4 LNPWDQVKRN AVPITPTLNR EQLSTSEENS KKTVDMESTE

5 VF**TKKTKLTE EEKNRL**NFLK KISQRYQKFA LPQYLKTVYQ

6 HQKAMKPWIQ PKTKVIPYVR YL

Fig.13. The α_{S2}-casein and the peptides sequences deduced using MALDI - TOF (in bold).

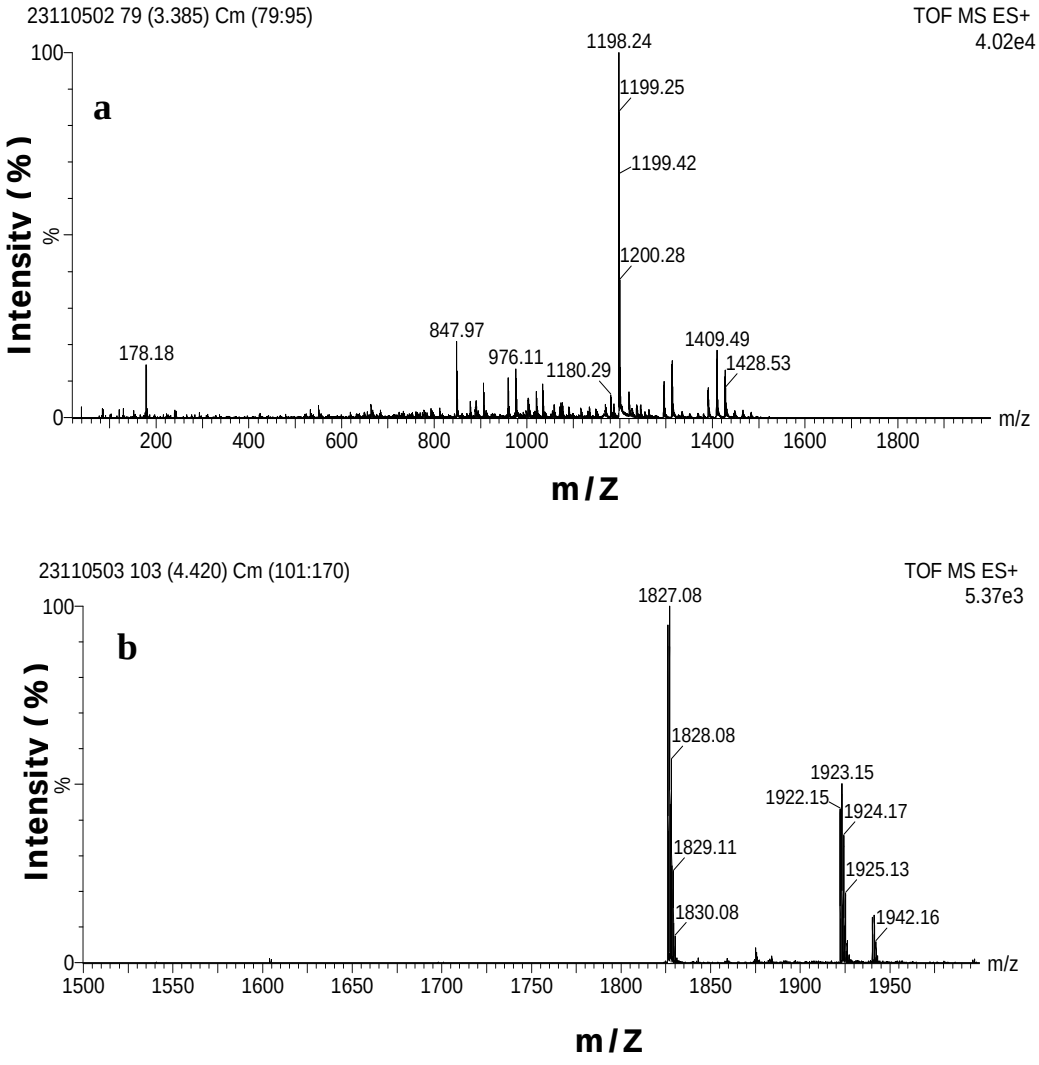

Fig.14. Electro spray ionization – mass spectra of the two synthesized peptides. (a) peptide 10 and (b) peptide 14

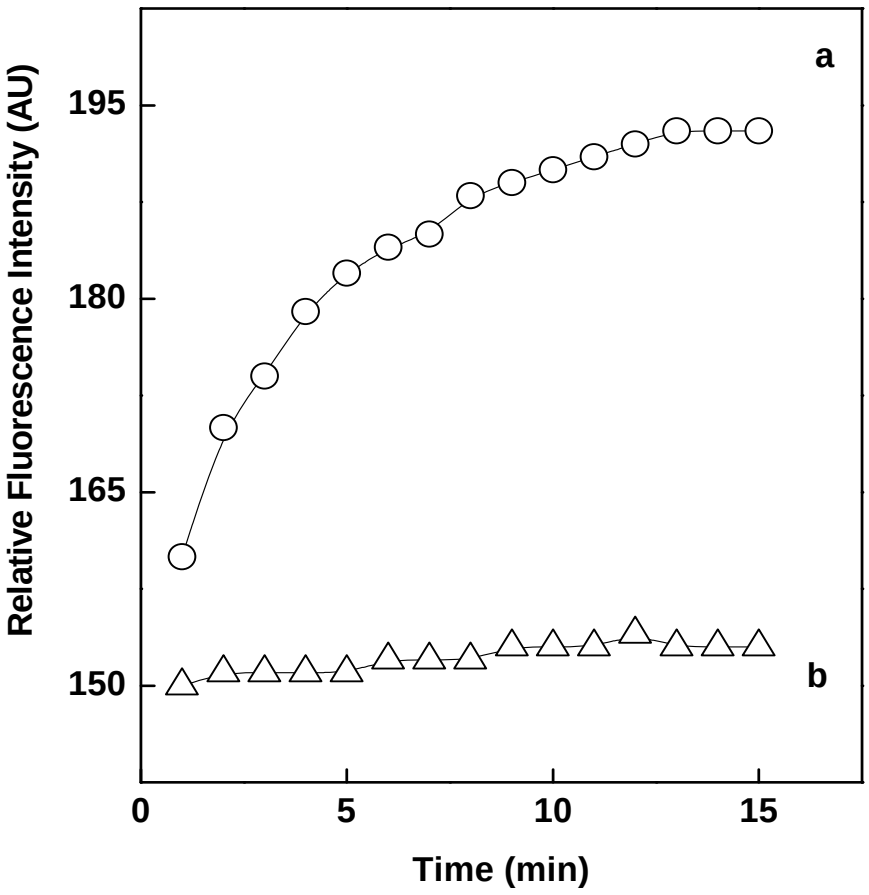

Fig.15. Studies on membrane polarization of *B. Cereus* in presence of (a) peptide 10 and (b) peptide 14.

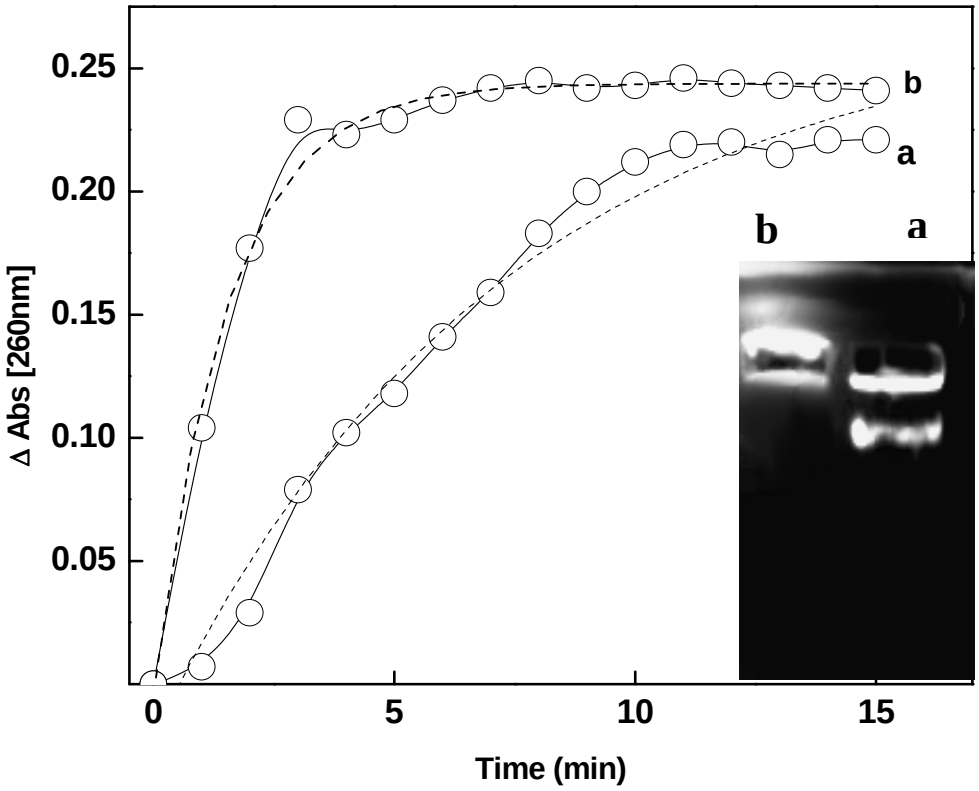

Fig.16. The binding of peptide 14 to DNA as studied by time kinetics mode on a UV spectrophotometer. (a) Binding of peptide 14 to *B.cereus* genomic DNA. and (b) Binding of peptide 14 to Calf thymus DNA. The dashed line indicates the first order exponential fit of the data.

Inset: Gel shift mobility assay of peptide interaction with *Bacillus cereus* DNA as studied on 0.8% agarose gel electrophoresis (a) control DNA and (b) DNA with peptide 14.

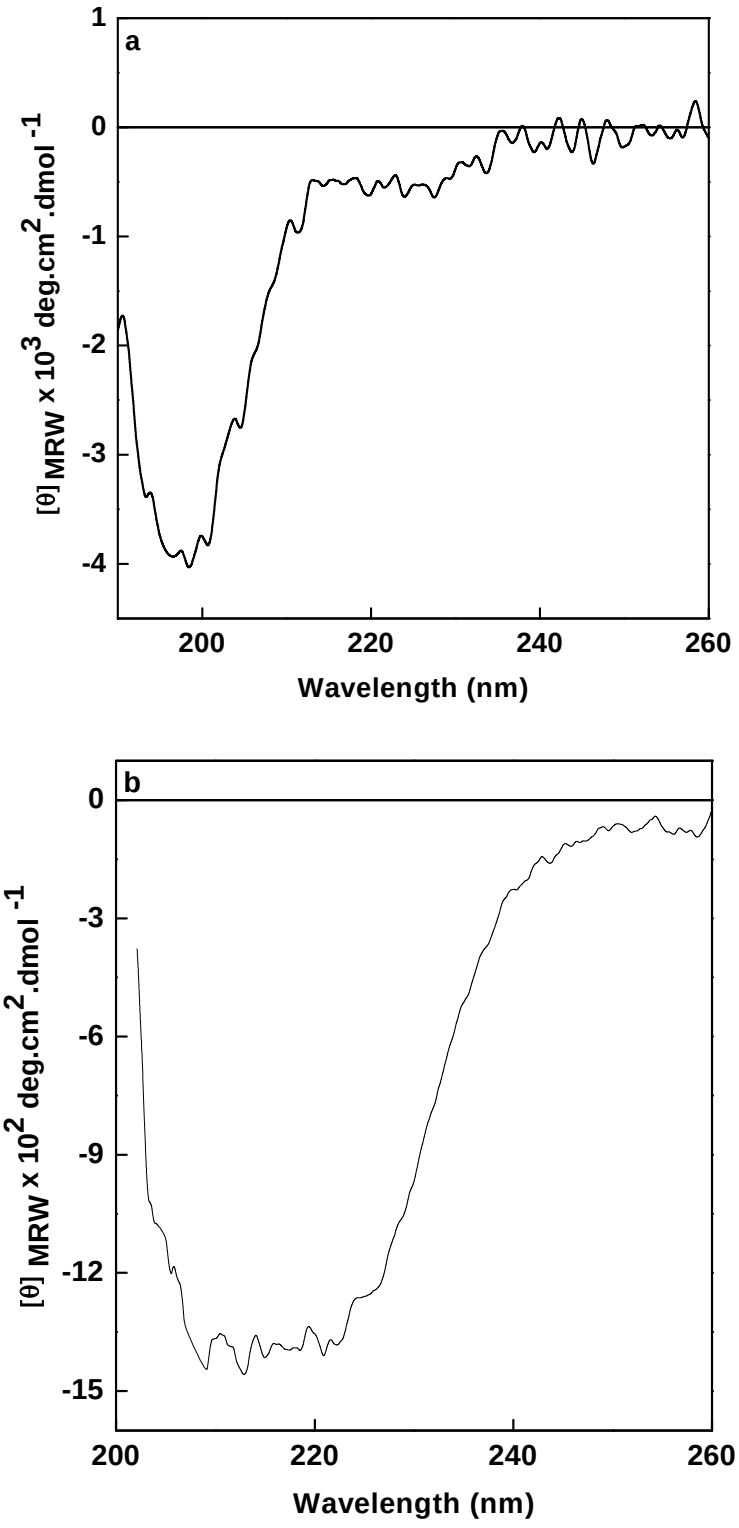

Fig.17. Far UV-CD Spectra of peptides. The peptides were scanned from 190-260 nm with a concentration of 0.3 mg/ml. (a) peptide 10 and (b) peptide 14

Table 4

The aminoacid composition of α-casein

Amino acids	Gram (%)[a]	Gram (%)[b]
Asp + Asn[c]	7.6	9
Glu + Gln[d]	17.9	21
Ser	7.4	4
Gly	2.5	1.5
His	1.8	1.2
Arg	2.8	2.2
Thr	5.1	4.8
Ala	5.3	2.9
Pro	6.2	3.9
Tyr	5.1	8.5
Val	6.5	5.9
Met	2.5	2.8
Iso	5.5	5.9
Lue	8.8	8.1
Phe	3.9	5
Lys	9.2	11.6
Typ[e]	0.9	0.9
Cys[f]	0.9	0.8

[a] *calculated based on sequence*
[b] *calculated by amino acid analysis*
[d] *Aspargine and Glutamine are identified as aspartic acid and glutamic acid.*
[e] *Tryptophan was estimated by N-bromosuccinamide.*
[f] *Cysteines are estimated as cysteic acid.*

Table 5
The biological activity of hydrolysates

Sl.	Biological Activity	Standard	IC_{50} [a]	IC_{50} [b]
1.	ACE Inhibitory activity	Ramipril	0.1 mg/ml	0.025 mg/ml
2.	Antioxidant activity (DPPH)	L - ascorbic acid	1.25 mg/ml	0.175 mg/ml
3.	PEP Inhibitory activity (PEP)	Z-prolyl prolinal	1.3 mg/ml	0.165 mg/ml

[a] *IC_{50} values for hydrolysates*
[b] *IC_{50} values for standards*

Table 6
The antibacterial activities of hydrolysates

Concentration (mg/ml)	Growth rate ($\Delta OD_{600\ nm}$) gen/h (±SD)	
	hydrolysates	lactoferrin
Bacillus cereus		
Control	2.4 (± 0.2)	2.4 (± 0.1)
0.75	1.2 (± 0.1)	1.2 (± 0.1)
1.5	2.4 (± 0.2)	1.8 (± 0.2)
3 .0	1.8 (± 0.1)	1.8 (± 0.1)
Escherichia coli		
Control	0.96 (± 0.03)	1.2 (± 0.1)
0.75	0.45 (± 0.02)	0.72 (± 0.02)
1.5	1.14 (± 0.1)	0.9 (± 0.01)
3 .0	1.14 (± 0.1)	1.02 (± 0.1)
Lactobacillus acidophilus		
Control	0.04 (± 0.02)	0.04 (± 0.01)
0.75	2.7 (± 0.2)	0.04 (± 0.01)
1.5	ND	0.04 (± 0.01)
3 .0	ND	0.04 (± 0.02)

Student's t-test ($p<0.05$) significant.
Lactoferrin is a standard anti microbial protein from bovine milk

Table 7
Biological Activity of synthesized peptides

Sl. No.	Assay	Peptide 10 (nM)	Peptide 14 (nM)	Standard	IC_{50} (nM)
1.	ACE inhibition assay	20	37	Ramipril	60
2.	Antioxidant activity (DPPH)	50	6	L - Ascorbic acid	150
3.	PEP Inhibitory activity (PEP)	5.8	36	Z- prolyl prolinal	500
4.	Aurora Kinase inhibition activity (Anti tumor activity)	0.01	ND[b]	Hesperadin[a]	250
5.	Mineral binding activity (Zn^{2+})	NAD[c]	NAD	Bacitracin	1

[a] *Taken from Nicholas and Taylor, 2004*
[b] *ND – not detected*
[c] *NAD- no activity detected*

Table 8
Antibacterial activities of Peptide 10

Peptide (mg/ml)	**1.5**	**2**	**2.8**	**3**	**3.6**	**4**
Initial population (CFU/ml)	**Population survived (CFU/ml)**					
B.cereus						
2×10^4	5	116	28	48	40	0
2×10^5	102	1200	1042	24	600	0
2×10^6	720	TNTC	TNTC	480	TNTC	720
E.coli						
2×10^4	848	TNTC	TNTC	TNTC	TNTC	0
2×10^5	TNTC	TNTC	TNTC	TNTC	TNTC	0
2×10^6	TNTC	TNTC	TNTC	TNTC	TNTC	0
S.aureus						
2×10^4	960	704	180	3.36	280	0
2×10^5	1782	TNTC	800	92	176	0
2×10^6	1952	TNTC	TNTC	336	TNTC	TNTC
L.monocytogenes						
2×10^4	0	TNTC	TNTC	TNTC	TNTC	0
2×10^5	TNTC	TNTC	TNTC	0	TNTC	0
2×10^6	TNTC	TNTC	TNTC	2624	TNTC	TNTC

Student's t-test ($p<0.05$) significant.
'0' indicates none of the population survived after incubation.
TNTC-too numerous to count

Table 9
Antibacterial activities of Peptide 14

Peptide (mg/ml)	**1.5**	**2**	**2.8**	**3**	**3.6**	**4**
Initial population (CFU/ml)	**Population survived (CFU/ml)**					
B.cereus						
2×10^4	60	50	46	40	10	20
2×10^5	560	880	456	328	260	360
2×10^6	TNTC	TNTC	TNTC	160	TNTC	712
E.coli						
2×10^4	TNTC	TNTC	8	TNTC	TNTC	TNTC
2×10^5	TNTC	TNTC	TNTC	TNTC	TNTC	TNTC
2×10^6	TNTC	TNTC	TNTC	TNTC	TNTC	TNTC
S.aureus						
2×10^4	TNTC	560	0	5	288	3
2×10^5	TNTC	TNTC	80	100	3840	7
2×10^6	TNTC	0	344	TNTC	TNTC	80
L.monocytogenes						
2×10^4	ND	128	8	ND	0	ND
2×10^5	ND	TNTC	40	ND	2	ND
2×10^6	ND	TNTC	0	ND	0	272

Student's t-test ($p<0.05$) significant.
'0' indicates none of the population survived after incubation.
TNTC-too numerous to count

Table 10
Antibacterial activities of bacitracin

Bacitracin (mg/ml)	**1.5**	**2**	**2.8**	**3**	**3.6**	**4**
Initial population (CFU/ml)	**Population survived (CFU/ml)**					
B.cereus						
2×10^4	6	19	328	7	TNTC	0
2×10^5	64	TNTC	0	40	88	0
2×10^6	0	16	12	0	120	0
E.coli						
2×10^4	TNTC	ND	ND	TNTC	ND	ND
2×10^5	TNTC	ND	ND	512	ND	ND
2×10^6	108800	ND	ND	800	ND	ND
S.aureus						
2×10^4	0	TNTC	0	0	10	TNTC
2×10^5	0	25	0	0	0	0
2×10^6	0	TNTC	12	0	0	780
L.monocytogenes						
2×10^4	0	0	0	0	0	TNTC
2×10^5	0	0	0	0	0	TNTC
2×10^6	0	0	0	0	0	TNTC

Student's t-test ($p<0.05$) significant.
'0' indicates none of the population survived after incubation.
TNTC - too numerous to count

Table 11
The antimicrobial activity of synthetic peptides

Organism name	Peptide 10 (MIC) μM	Peptide 14 (MIC) μM	Standard	Standard (MIC) μM
Bacteria				
B. cereus	0.87	2.99	Bacitracin	0.12
S. aureas	1.75	2.3	Bacitracin	0.12
L. monocytogenes	NAD	2.99	Bacitracin	0.12
H. pylori	0.083-0.25	NAD[a]	Amoxicillin	0.13
Fungi				
Aspergillus flavus	NAD[a]	NAD	Amphotericin B	0.004
Fusarium sp.	+	+	Amphotericin B	0.004
Pencillium sp.	NAD	NAD	Amphotericin B	0.004

[a] *No activity detected*
"+" Indicates activity but not determined quantitavely

Table 12
Chemical Shift Indexes (PPM) for peptide10 recorded at 25°C in DMSO-d_6.

Residue	NH	αH	βH	γH	Other H
Gln (Q)	7.95(8.41)	4.34 (4.32)	3.06 (2.13)	2.85 (2.38)	δNH_2 7.244
Lys (K)	7.51 (8.41)	4.02 (4.36)	1.91 (1.85)	1.75 (1.65)	δNH_2 2.144
					εNH_2 7.03
Ala (A)	8.041(8.25)	4.32(4.35)	1.216	-	˜
			(1.39)		
Leu (L)	7.98 (8.42)	4.31 (4.38)	1.66	1.49	δNH_3 0.855
					0.834
Asn (N)	8.223 (8.25)	4.61 (4.25)	2.81 (7.25)	-	˜
			2.93 (2.84)		
Glu (E)	8.25 (8.32)	4.53 (4.29)	2.82	2.93	˜
Ile (I)	8.06 (8.19)	4.18 (4.23)	1.67	1.38	1.07
					0.73
Asn (N)	8.50 (8.25)	4.56 (4.75)	2.76 (2.75)	˜	˜
			2.87 (2.83)		
Gln (Q)	2.80 (8.41)	4.30 (4.37)	2.82 (1.01)	3.0(2.13)	δNH_2 7.25
Phe (F)	7.992 (8.23)	4.57 (14.66)	2.51(3.22)	˜	2.6-7.34
			2.49(2.99)		3.5-2.26
					4-7.43

Standard chemical shift indexes as given in brackets

Table 13
Chemical Shift Indexes (ppm) for peptide 14 at 25°C and in 0.01 M phosphate buffer, pH 7.0

Residue	NH	αH	βH	γH	Other H
Thr (T)	6.9	3.95	3.95	1.05	
Lys (K)	7.2	3.2	1.6; 1.8		
Lys (K)	7.4	4.2	2.9;	1.52; 1.22	
Lys (K)					
Lys (K)	8.2	4.3	1.9; 1.5	0.87	δCH_2 0.6
Thr (T)	7.98	4.22	4.22	1.1	
Thr (T)	8.01	4.3	4.4	1.2	
Leu (L)	8.2	4.2	1.75; 1.5	1.54	
Leu (L)	8.3	4.1	1.8; 1.6		
					δCH_2 0.6
Arg (R)	8.2	4.2	2.2; 2.3	1.89	δCH_2 0.6
					NH *
Asp (N)	8.33	4.62	2.88; 2.62		
Glu (E)	8.23	4.31	2.14	1.97	
*Glu (E)					
*Glu (E)					

* *Some of the assignments for the peaks were not done due to overlap*

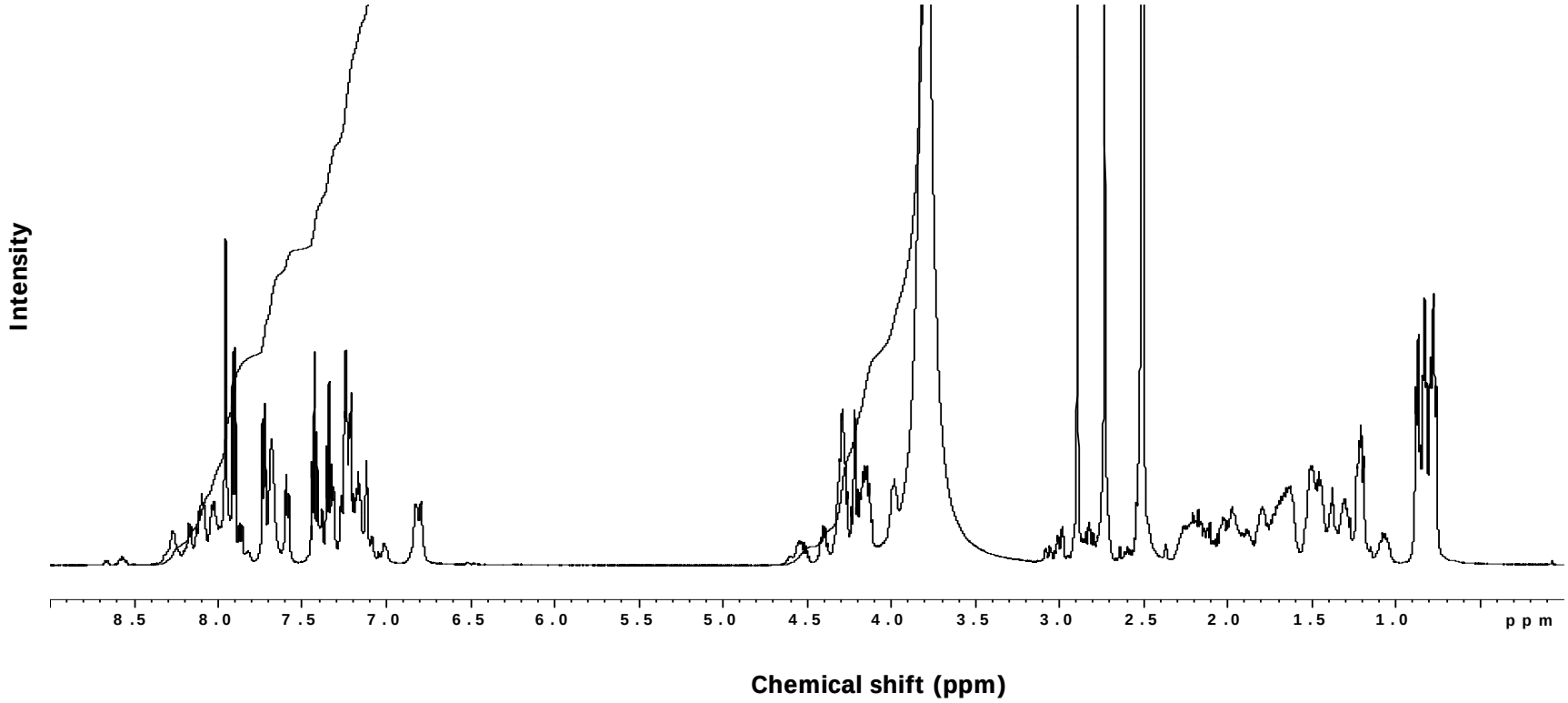

Fig.18. The NMR spectra of peptide 10. The one-dimensional spectrum was acquired with a peptide concentration of 4.15 μM in DMSO-d_6 at 25° C

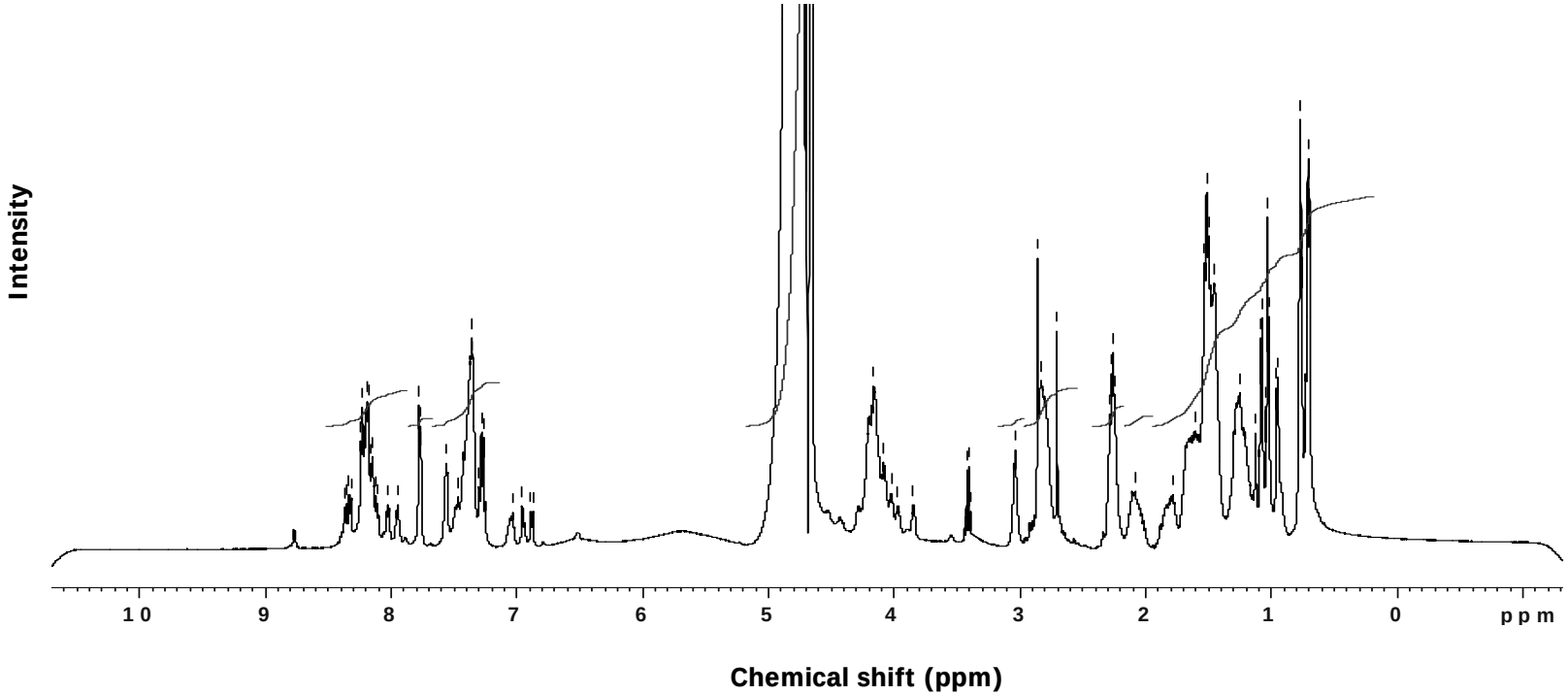

Fig.19. The 500 MHz NMR spectra of peptide 14. The one-dimensional spectrum was acquired with a peptide concentration of 4.65 μM in 0.01 phosphate buffer pH 7.0.

CHAPTER – II

EFFECT OF COSOLVENTS ON THE STABILIZATION PROCESS OF α-CASEIN AND ITS BIOLOGICALLY ACTIVE PEPTIDES

4.2. EFFECT OF COSOLVENTS 0N THE STABILIZATION PROCESS OF α-CASEIN AND ITS BIOLOGICALLY ACTIVE PEPTIDES

The stability of folded protein is known to be affected by a variety of substances that act at high concentrations. These substances stabilize the native structure of protein. The range of such additives includes substrates, specific ligands, glycerol, sugars and polyethylene glycols. Several studies have shown that stability of protein in aqueous solution is increased by addition of sugars (Back *et al.,* 1970; Lee and Timasheff 1981; Arakawa and Timasheff, 1982).

Low molecular weight additives exert stabilizing effects by inducing preferential hydration of proteins, i.e., the additive tends to be excluded from the surface of the protein molecule. Polyhydric alcohols and sugars have been used for many years as stabilizing agents for the maintenance of biological activity of macromolecules. Gerlsma (1970) have shown that polyhydric alcohols and sugars increase the transition temperature of proteins, and this effect is due to decrease in the hydrogen bond rupturing potency. The preferential interaction arises from the increase in the surface tension of the solvent water, as is the case with polyhydric alcohols.

Polyhydric compounds such as glycerol and sorbitol can stabilize the native conformations of many proteins and enzymes. The intrinsic

conformational stability of the protein molecule itself is not increased but its unfolding is greatly retarded by the presence of stabilizing additives. It has been postulated that glycerol and pressure act similarly on decreasing internal free volume and increasing protein rigidity. Studies on the pressure-induced denaturation of proteins show that the glycerol stabilizes proteins by means of preferential hydration (Oliveira *et al.,* 1994). In contrast, Gekko and Timasheff (1981) used volumetric measurements of protein-glycerol solution and attributed the glycerol effect on the apparent specific volume of proteins to the modification of hydration layer, with no effect on the protein interior.

Sucrose stabilizes proteins by preferential hydration. Sucrose does not induce a conformational change in the proteins. Sucrose is preferentially excluded from the protein domain, increasing the free energy of the system. The exclusion of sucrose from the protein domain seems to be related to the higher cohesive force of the sucrose water solvent system since all the experimental observations can be correlated with the effect of sucrose on the surface tension of water (Lee and Timasheff, 1981).

The disaccharide trehalose (α-D-glucopyranosyl . α-D-glucopyranoside) effectively prevents protein denaturation at high temperatures. Singer and Lindquist, (1998) showed that trehalose acts *in vivo* to suppress the aggregation of denatured proteins. The addition

of cosolvents to aqueous solutions of proteins is known to modify both thermodynamic and dynamic properties of the proteins in a variety of ways (Timasheff, 2002).

Protein stabilizing action of sorbitol is driven by stronger exclusion from the unfolded protein than from the native structure. However there has been no exact mechanism proven responsible for such a stabilizing effect of the sugars and polyols. This is because different proteins are stabilized to different extent by these polyols and sugars. Moreover, the same sugar and polyols elicit different stabilizing effects in different proteins (Kaushik and Bhat, 1998).

There are no references available for the structure-stability relationships of α-casein with respect to cosolvents. The stability of α-casein in presence of cosolvents like sorbitol, sucrose, glycerol and trehalose has not been studied. The cosolvents-protein interaction study would help us to understand the mechanism of stabilization of the macromolecules. The changes occurring in the protein due to the cosolvents is also elucidated by spectroscopic and other biophysical techniques.

4.2.1. Effect of cosolvents in stabilization of α-casein

α-Casein was studied for its structural stability in presence of various cosolvents like sucrose, sorbitol, glycerol, and trehalose. The

effect of different cosolvents on the microenvironment of aromatic amino acids and the tertiary structure of the protein was studied using fluorescence and UV difference spectra. The effect of cosolvents on the microenvironment of tryptophan residues and other aromatic amino acids has been monitored by intrinsic fluorescence measurements. It will be evident from the fluorescence spectra whether the tryptophan groups of α-casein are experiencing either a more polar or nonpolar environment in presence of cosolvents. Thus, fluorescence spectra was used to the study the effect of cosolvents on α-casein, as they show differences in presence and absence of cosolvents in the microenvironment of the aromatic amino acids and the tertiary structure of the α-casein. The difference spectra can be useful in studying the changes in the bulk solvent as the perturbation in the spectra can be related directly due to the cosolvents.

The fluorescence emission spectra of α-casein in presence of different concentrations of sorbitol at 25°C were measured. In the figure 21, the fluorescence emission spectrum of α-casein in sorbitol was monitored from 300- 400 nm, when excited at 278 nm. There is no significant change in the fluorescence intensity as a function of sorbitol concentration.

The effect of sorbitol concentration on fluorescence emission maximum of α-casein is shown as figure 22. There are no significant

changes observed in the emission maximum as a function of sorbitol concentration.

The effect of glycerol as a function of concentration on fluorescence spectra of α-casein is studied and shown in figure 23. The fluorescence intensity increased from 225 to 330 as a function of glycerol concentration. The maximum change was observed at 40% glycerol concentration.

The shift in the fluorescence emission maximum is shown in figure 24. There are no significant changes in the emission maximum were observed up to 30% glycerol where as 40% glycerol shows a minute perturbations as well as shift in the emissions maximum (Gekko and Timasheff, 1981). This study shows, at higher concentrations of glycerol (above 30%) the microenvironment of aromatic amino acids shifts to non-polar environment. From this we can conclude that protein structure is stabilized in presence of 10-30% concentration of glycerol.

The effect of different concentrations of trehalose and sucrose on α-casein was studied. α-Casein in presence of sucrose and trehalose was experiencing changes in the tertiary structure as a function of cosolvent concentration. The figure 25 and figure 27 shows the relative fluorescence intensity of α-casein in presence of various

concentrations of the sugars. Sucrose and trehalose was found to be perturbing the proteins microenvironment to the same extent. The 40% trehalose and sucrose shows maximum changes in the intensity.

The shifts in the fluorescence emission maximum of the α-casein at 40% concentrations were observed for sucrose and trehalose (figure 26 and figure 28). This shift in the emission maximum is relatively less in sucrose compared to trehalose which experiences a blue shift in the emission maximum from 344 nm to 340 nm.

The difference spectra of α-casein in presence of 5 - 40% sucrose concentrations show perturbations in the aromatic amino acids at 281 nm. The positive perturbation experienced by the protein in presence of sucrose was shown in the figure 29. These changes in the sucrose could be due to the increase in the compaction of the molecule. The molecule hydrodynamic volume may be reducing due to the exclusion of sucrose from the surface of the α-casein.

The difference spectra of α-casein in presence of 5% to 40% glycerol concentration were studied and show perturbations in the aromatic aminoacids at 281 nm. These positive perturbations experienced by the protein in presence of glycerol as shown in the figure 30. These changes in the glycerol could be due to the increase in the structural order of the molecule.

Studies on the difference spectra of α-casein at different concentrations of sorbitol and trehalose were studied. The difference spectrums have not shown any significant changes in the spectral region monitored.

Cosolvents induced perturbations of aromatic amino acids of protein was well documented in the literature (Rajeshwara and Prakash, 1994; Muralidhar and Prakash, 1995). α-Casein in presence of cosolvents shows minor perturbations in the structure as evidenced by the fluorescence spectroscopy. These studies show that the changes in structure are minor and the molecules are rigid in presence of cosolvents. We can conclude from the fluorescence studies the cosolvents induce stabilization through water mediated marginal structural alterations. The process of stabilization could be due to the interaction of water with the amino acids and water has a major role in the stabilization. The stabilization could be due to the enthalpy-entropy compensation and the thermodynamic state of the protein (Eftink *et al.,* 1983).

Several studies have shown that stability of proteins in aqueous solution may be increased by addition of sugars and polyhydric alcohols (Back *et al.*, 1970; Arakawa and Timasheff, 1982). The stabilizing effect of the cosolvents on proteins is due to the preferential hydration (Lee and Timasheff, 1981). The stabilizing effect of these

additives has been attributed to their effect on water structure, which may enhance hydrophobic interactions in the protein (Back *et al.,* 1970). According to their theory, sugars and polyols are preferentially excluded from the domain of the protein, which leads to unfavorable interactions between protein and the solvent because of ordering of water structure around the protein molecule. These unfavorable interactions raise the free energy of the system, but more so for the unfolded state of the protein because of large 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 folded and unfolded states of protein.

In case of sugars the stabilizing effect seems to be related to surface tension of water. Lee and Timasheff (1981) and Arakawa and Timasheff (1982), suggested that the major factor in preferential hydration is the free energy required to form a cavity in the solvent, for accommodating the protein molecule. Since for unfolded protein such a cavity would be larger, the free energy would increase and thereby, stability of the folded state is enhanced relative to unfolded state. In case of glycerol, which also induces preferential hydration and thus the stability of protein it actually lowers the surface tension of water (Gekko and Timasheff, 1981; Gekko and Morikawa, 1981). Glycerol

seems to be excluded from the protein surface by the solvophobic effect (Gekko and Timasheff, 1981).

4.2.2. Effect of cosolvents in stabilization of bioactive peptides

The chapter focuses on the stabilization of the peptides in presence of cosolvents. Biologically active peptides are widely used as therapeutic drugs. In order to have efficient stability and retaining the biological activity of peptides during the operation and storage of the peptides, the peptides are stabilized by a wide variety of cosolvents. These cosolvents increase the structural stability, which in turn increases their operational performances for variety of activities.

Stability of drugs in various formulations is the latest field of activity in cosolvent engineering. The excipients like sugars and polyols are added to the peptides to screen for their enhanced biological activity. These chemical chaperones help the peptide backbone chains to fold in proper way. Cosolvents like trifluoroethanol (TFE) help peptides to be protected from the bulk environment by forming a coating (Roccatano, *et al.,* 2002). The coating displaces the water thereby remove alternative hydrogen bonding partners and provide a low dielectric constant that favors intra peptide hydrogen bonds. TFE induces stability by interacting only weakly with nonpolar residues and the hydrophobic residues remain intact. Walker *et al.,* (2003) has

shown that the peptides can be stabilized by the addition of small motifs at the ends. Structural stability studies by Kamberi *et al.,* (2005) on brain natriuretic peptide [hBNP (1-32)] and human parathyroid hormone [hPTH (1-34)] in presence of sucrose, shows retarded aggregation, oxidation and deamidation of the peptides. Additives like sucrose and poloxamer 407 have been shown to limit the rate of deamidation of the Asn residue in a model peptide with sequence, Val-Tyr-Pro-Asn-Gly-Ala (VYPNGA) (Stratton *et al.,* 2001). Susanne *et al.,* (2004) have shown that in presence of sucrose, there was a reversal of aggregation and met oxidation of recombinant factor VIIa (rFVIIa) peptide, in turn enhances the thermal stability.

In the present chapter, the two peptides QKALNEINQF, TKKTKLTEEEKNRL (from now on referred as peptide 10 and peptide 14 depending on their number of amino acids) from α_{S2}-casein f(94-103); α_{S2}-casein f(163-176); respectively are synthesized and their activities were analyzed in presence of cosolvents like sucrose, trehalose, glycerol, and sorbitol. The peptides were stabilized for their biological activities like ACE inhibition and antioxidant activity.

To study the stability of peptides in cosolvents the peptide 10 was dissolved in 20% sucrose, sorbitol, glycerol, and trehalose. The peptide ACEI activity was studied in presence and absence of cosolvents. The peptide in presence of sucrose, sorbitol, trehalose and

glycerol shows 54%, 46%, 12% and 18% ACEI activity respectively (figure 31). Peptide 10 shows more inhibition in presence of sucrose and sorbitol whereas in presence of trehalose and glycerol, peptide 10 was having less inhibition compared to control (absence of cosolvents). From this study, sucrose and sorbitol are good stabilizers for peptide 10 biological activities. The decrease in the stability follows the order

Sucrose > Sorbitol > Control > Glycerol

Effect of cosolvents was also studied at higher temperatures. The peptide 10 was incubated for one hour at 50°C in presence of 20% sucrose, sorbitol, glycerol and trehalose their effect on ACE inhibition was studied (figure 32). The peptide 10 in the absence of cosolvents is completely devoid of activity after incubation at 50°C, whereas in presence of sucrose, sorbitol, trehalose and glycerol shows 66%, 48%, 58% and 52% ACEI activity respectively. The study shows that the peptide 10 in presence of 20% sucrose is the showing maximum inhibition, which is equivalent to the percentage inhibition of ramipril, a known inhibitor of ACE. The inhibition of ACE enzyme by peptide 10 at higher temperature shows the following trend

Sucrose > Trehalose > Glycerol > Sorbitol

The peptide 10 was also studied for its antioxidant activity in presence of 20% sucrose, sorbitol trehalose and glycerol (figure 33). The peptide 10 in presence of glycerol shows maximum antioxidant activity of 41% whereas 20% sucrose, trehalose and sorbitol show 18% 15% and 42% respectively. The glycerol was found to be more effective in case of polyols.

The peptide 10 was also studied for its antioxidant activity in presence of 20% sucrose, sorbitol, trehalose and glycerol at 50°C (figure 34). The peptide 10 in presence of 20% sucrose shows 13% antioxidant activity, whereas 20% glycerol and trehalose showed similar antioxidant activity of 12% and 11% respectively. The sucrose was found to be more effective in case of sugars where as in case of polyols there is no change in the antioxidant activity.

Similar experiments were also carried out with peptide 14. The 20% sorbitol was found to be more effective compared to any other sugars or polyols used in case of ACEI activity. Sorbitol was also found to be effective stabilizer of peptide 14 in case of antioxidant activity (figure 35 and 36).

The antioxidant activity of peptide 14 in presence of different cosolvents at 20% (W/V) concentration was studied at 50°C and shown

as figure 37. The sugars were less effective in case of peptide 14 compared to polyols like sorbitol and glycerol.

From these studies, the enhanced biological activity and stability of peptide 10 in presence sucrose could be due to the compaction of the molecule. The reasons could also be due to the prevention of oxidation of Asn residues, present in the peptide (Stratton *et al.,* 2001). The peptide sequence shows QKALNEINQF two Asn residues, which may be protected by the sucrose and will lead to enhance stability at higher temperatures. Sucrose causes the peptide to be more compact there by preventing the amino acids from exposure to bulk solvent (Kendrick *et al.*, 1997; Kemberi *et al.*, 2005).

The effect of sorbitol in stabilizing proteins could be due to the preferential hydration and simultaneously exclusion of sorbitol from the bulk solvent (Back *et al.*, 1970).

From the above study we can conclude that the structural stability of the α-casein was found to be maximum in presence of sorbitol among the cosolvents investigated. These structural changes are minute and are insignificant. The caseins are rheomorphic, flexible, disordered and stable at very high temperatures. Due to its flexibility, randomness and often with structural changes it is performing all the various biological activities.

The two peptides QKALNEINQF and TKKTKLTEEEKNRL are synthesized and their biological activities were analyzed in presence of cosolvents. The peptide 10 shows increase in the ACE inhibitory activity in presence of 20% sucrose where as the peptide 14 was stabilized in presence of 20% sorbitol. Peptide 10 is showing more antioxidant activity in presence of 20% glycerol where as peptide 14 in presence of 20% sucrose. The presence of cosolvents has increased the activity of peptides at higher temperatures. This shows that the peptides are thermostable for the activities tested at higher temperatures. The presence of cosolvents might have induced the secondary and tertiary structures in the peptides, which in turn has perhaps enhanced their biological activities. The activity of peptides in presence of sucrose could be due to the compaction of the peptides. The stabilizing effect of polyols could be due to preferential hydration phenomenon.

In conclusion cosolvents could be used to increase stability of proteins and thermostability of biologically active peptides. Cosolvents prevent the permanent inactivation of proteins/peptides subjected to adverse conditions. This might be of special interest to biotechnologists who wish to reutilize proteins/peptides after industrial processes. In addition, study of interaction of proteins/peptides in presence of cosolvents has recently led to consideration of their use as therapy in protein unfolding diseases.

In the next chapter an attempt has been made to understand the effect of metal ions like zinc on the structural changes on α-casein. These structural studies changes can throw light on the interaction and the mechanism, which can be helpful in addressing together the effect of cosolvents and metal ions on α-casein.

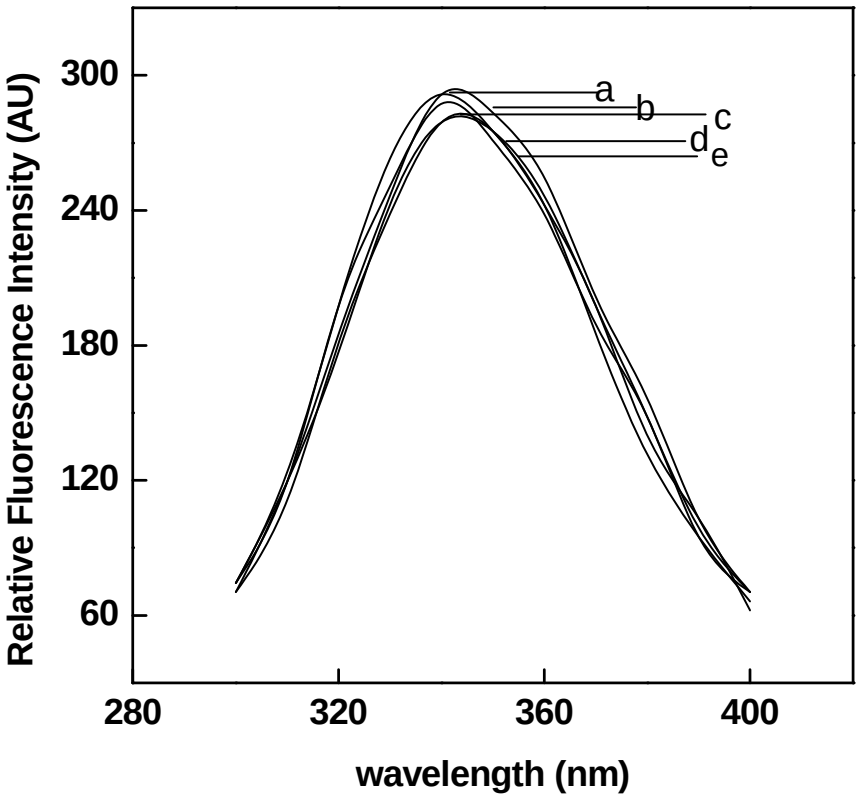

Fig.21. Fluorescence emission spectra of α-casein in presence of different concentrations of sorbitol (a) control in buffer (b) 10% sorbitol (c) 20% sorbitol (d) 30% sorbitol and (e) 40% sorbitol

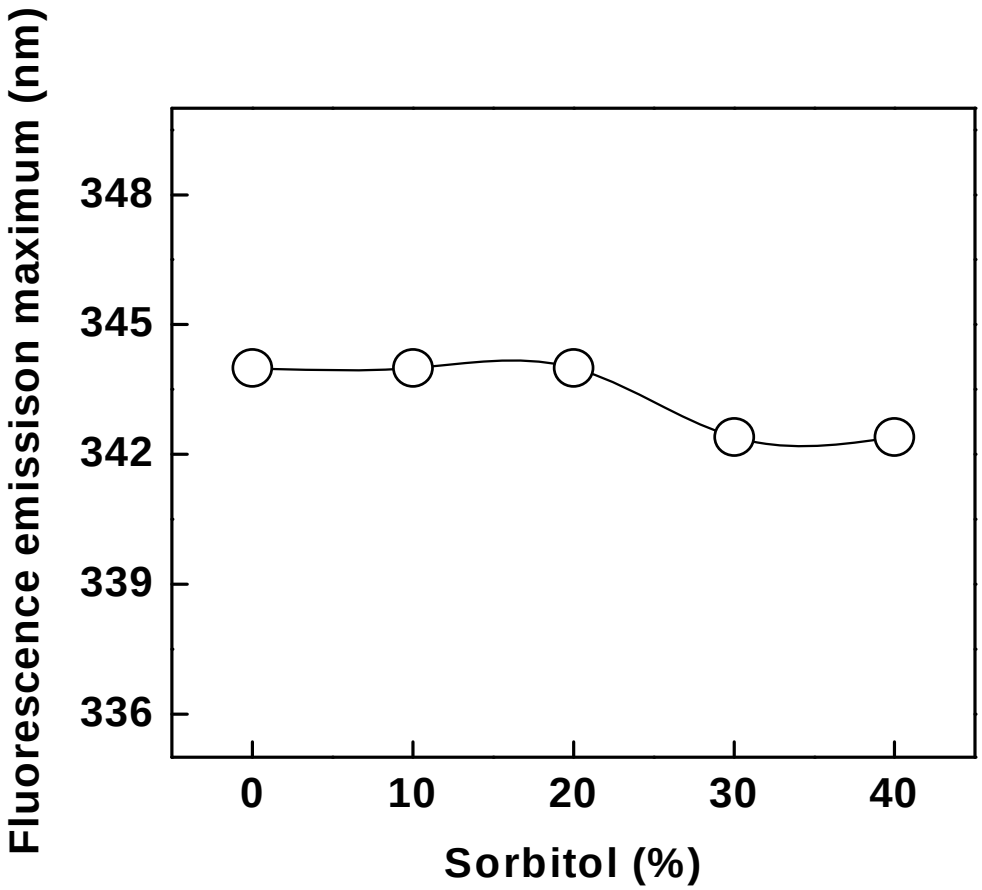

Fig.22. Effect of different concentrations of sorbitol on the fluorescence emission maximum of α-casein.

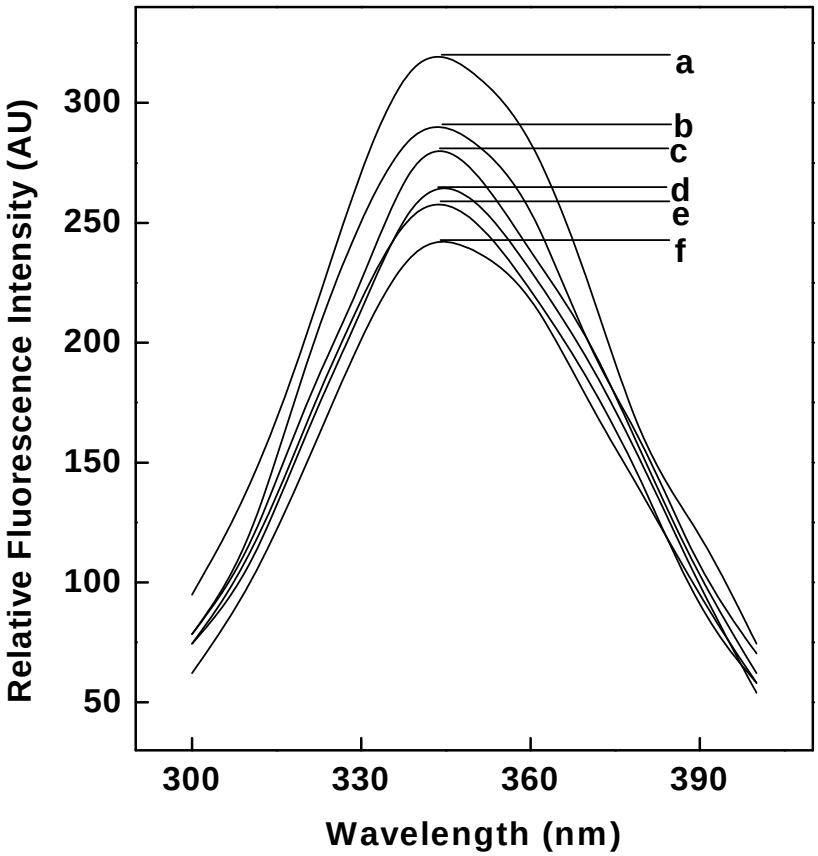

Fig.23. Fluorescence emission spectra of α-casein in presence of different concentrations of glycerol (a) 40% glycerol (b) 30% glycerol (c) 20% glycerol (d) 10% glycerol (e) 5% glycerol and (f) control in buffer.

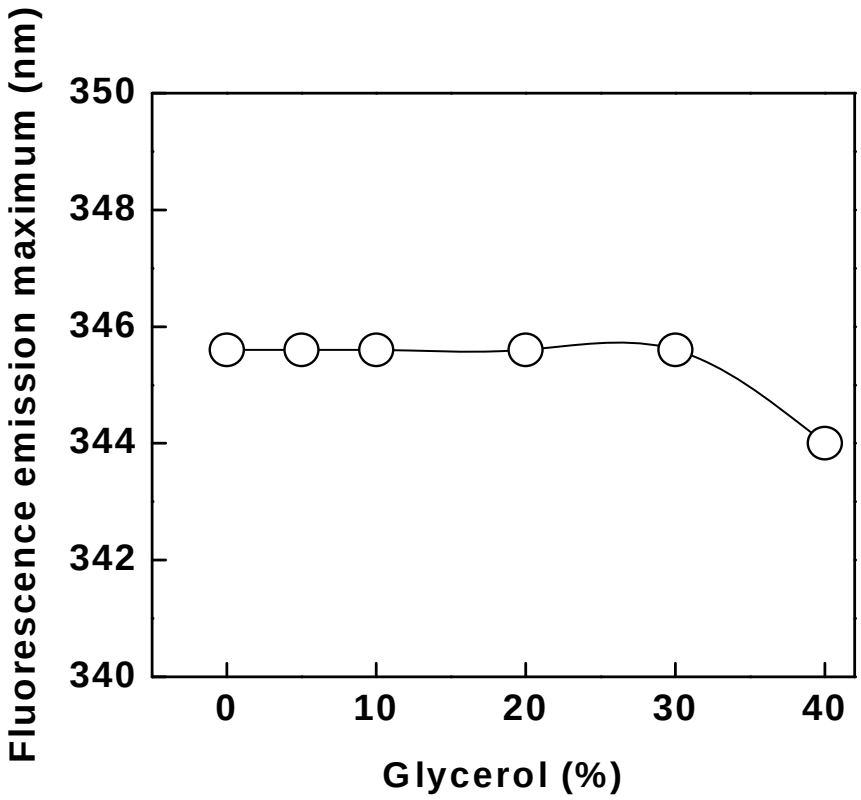

Fig.24. Effect of different concentrations of glycerol on the fluorescence emission maximum of α-casein.

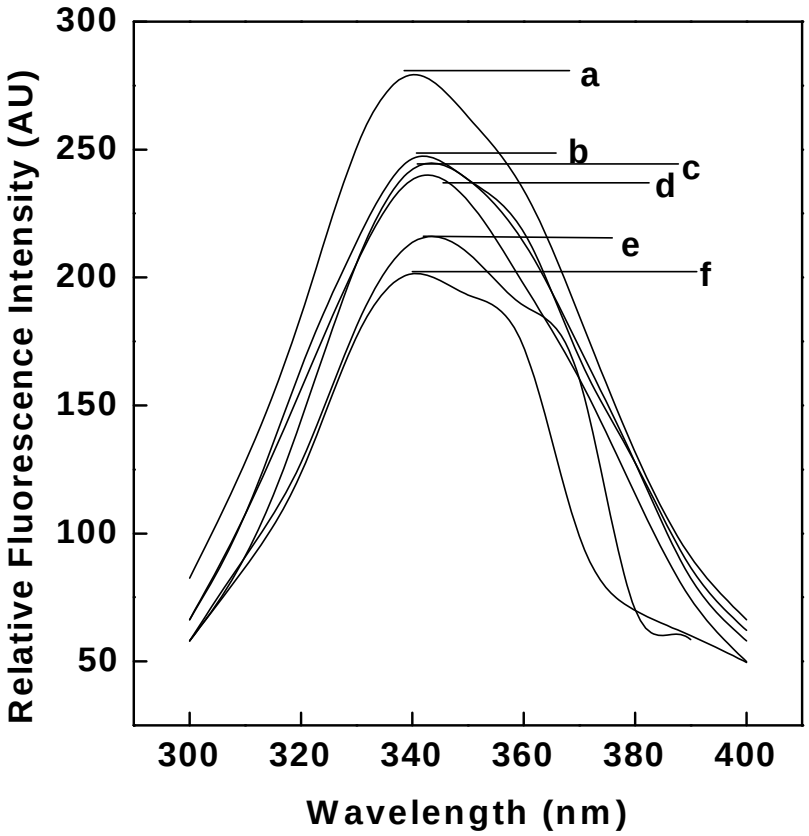

Fig.25. Fluorescence emission spectra of α-casein in presence of different concentrations of sucrose (a) 40% sucrose (b) 30% sucrose (c) 20% sucrose (d) 10% sucrose (e) 5% sucrose and (f) control in buffer

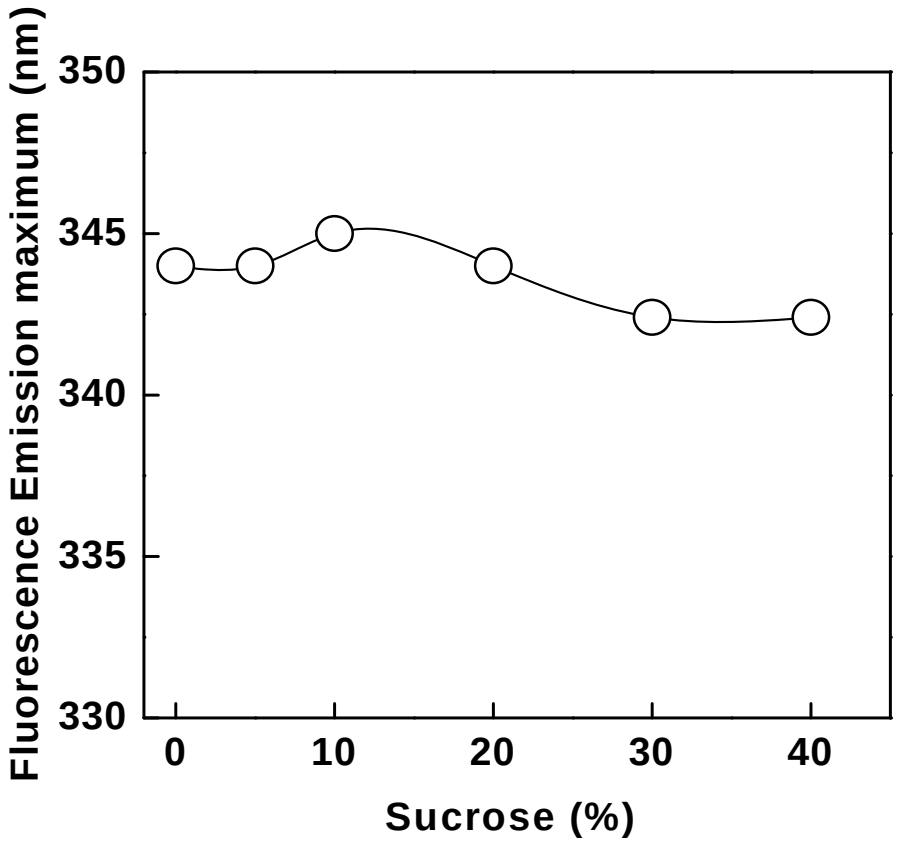

Fig.26. Effect of different concentrations of sucrose on the fluorescence emission maximum of α-casein

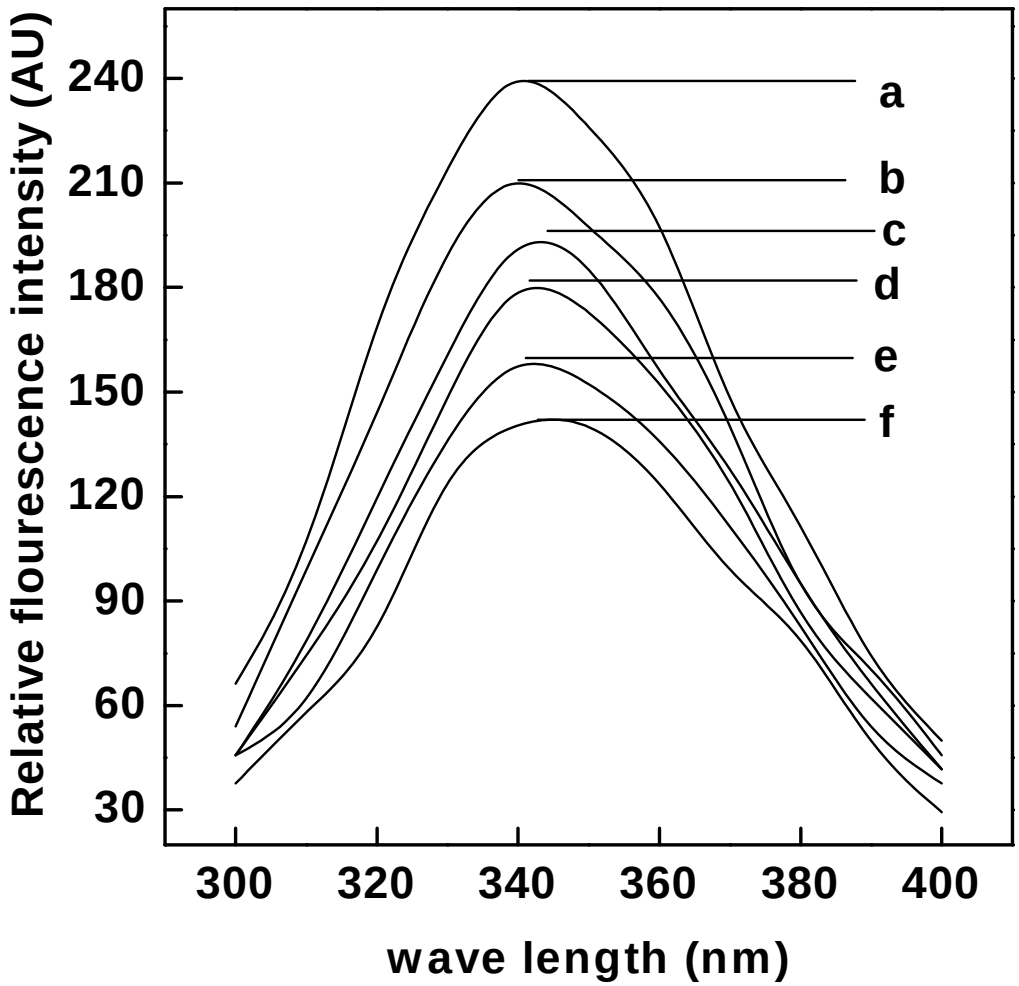

Fig.27. Fluorescence emission spectra of α-casein in presence of different concentrations of trehalose (a) 40% trehalose (b) 30% trehalose (c) 20% trehalose (d) 10% trehalose (e) 5% trehalose and (f) control in buffer.

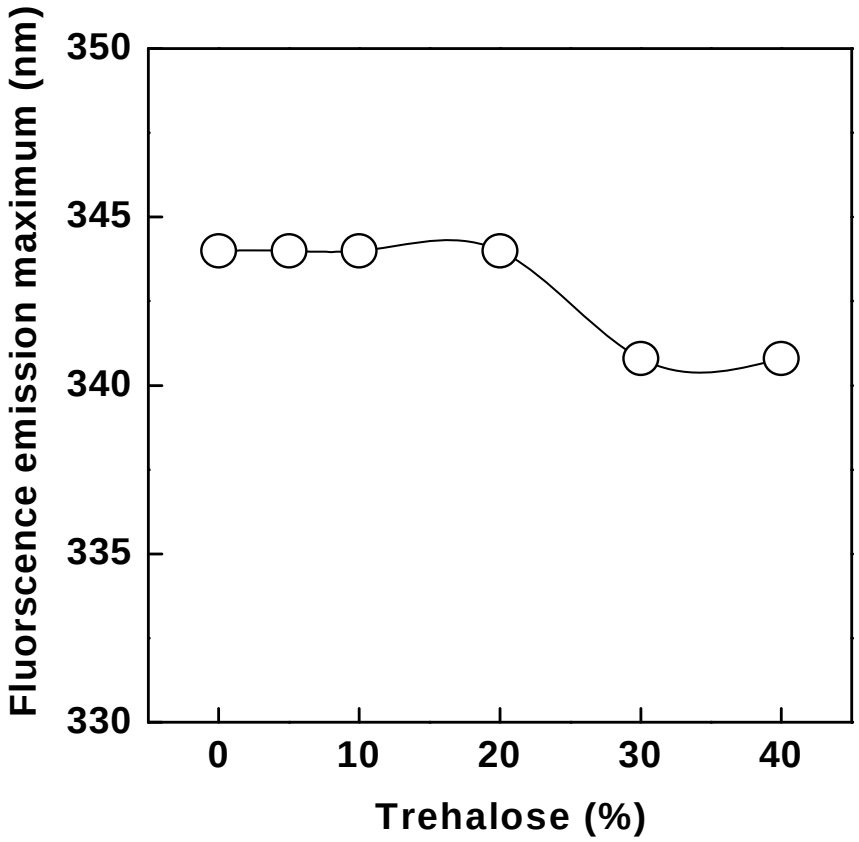

Fig.28. Effect of different concentrations of trehalose on the fluorescence emission maximum of α-casein

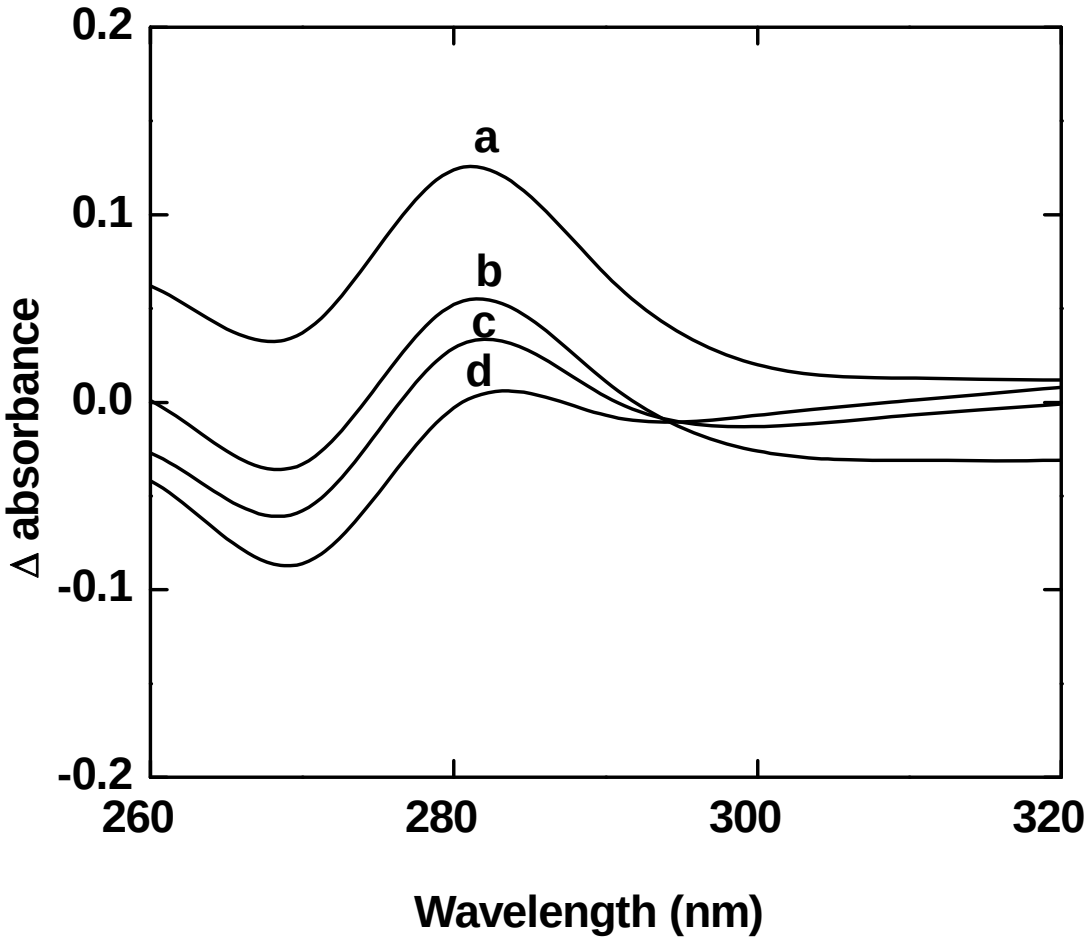

Fig.29. UV-difference spectra of α-casein at different concentrations of sucrose (a) 5% sucrose (b) 10% sucrose (c) 30% sucrose and (d) 40% sucrose. The absorbance was monitored using the protein concentration of 2.2×10^{-4} M.

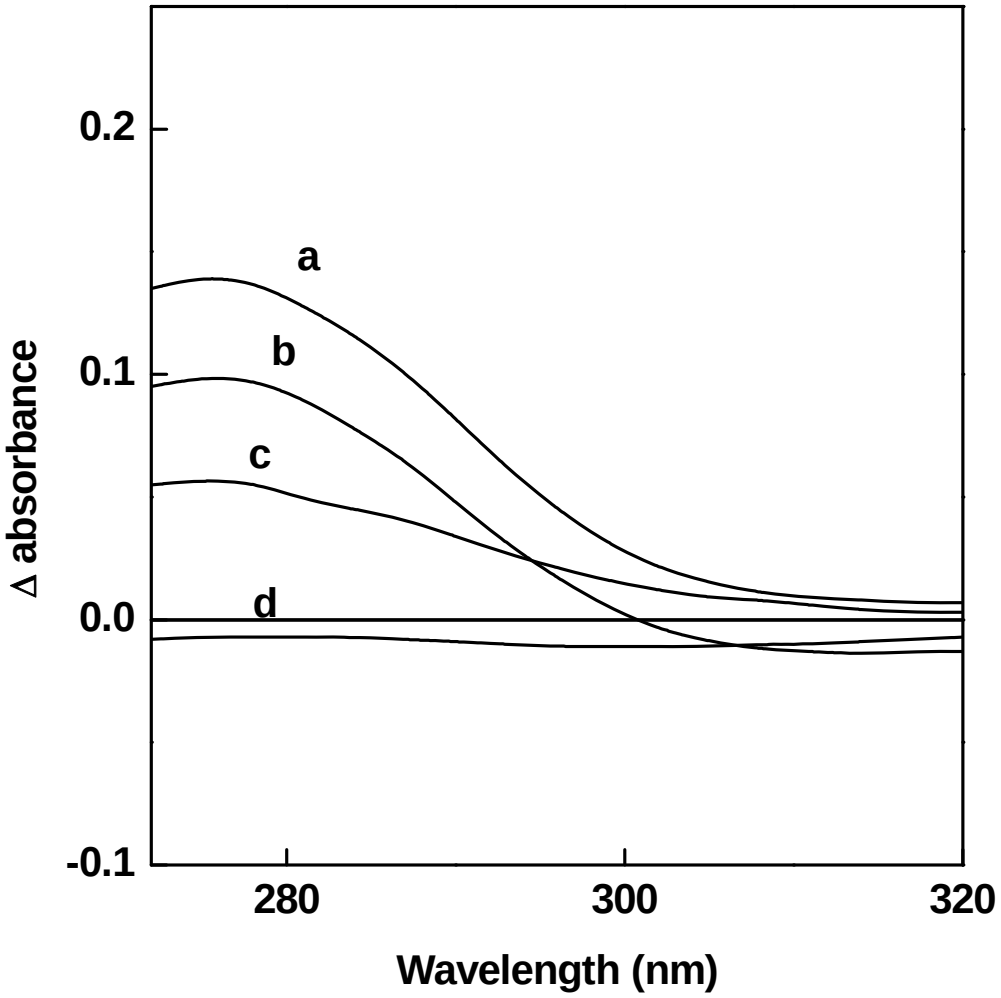

Fig.30. UV-difference spectra of α-casein at different concentrations of glycerol (a) 10% glycerol (b) 20% glycerol (c) 30% glycerol and (d) 40% glycerol. The absorbance was monitored using the protein concentration of 2.2×10^{-4} M.

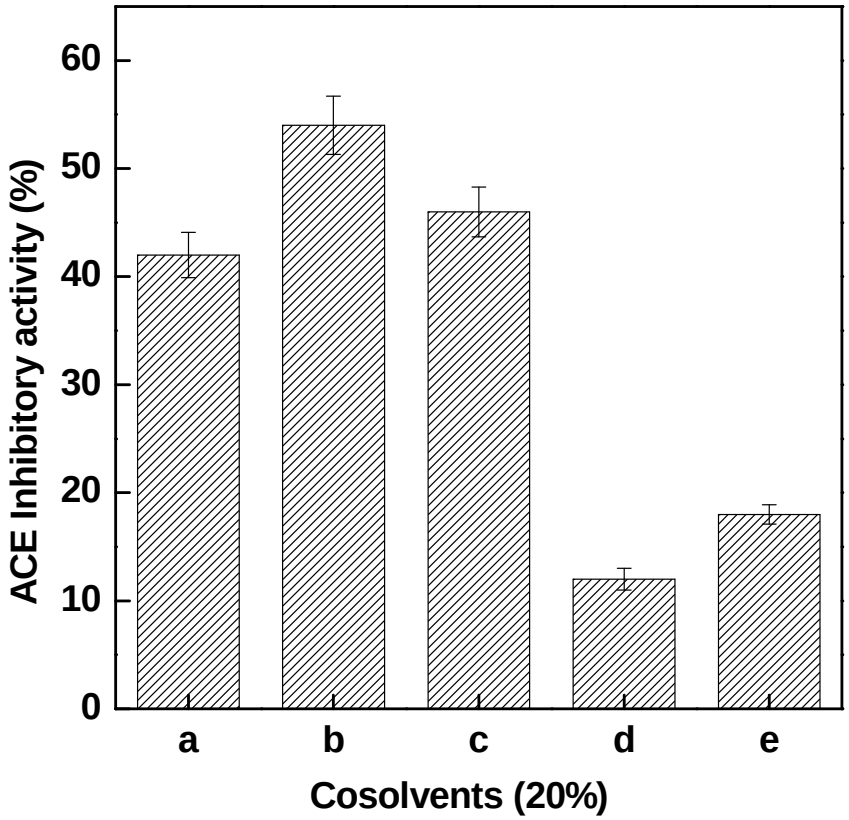

Fig.31. The ACE inhibitory activity of peptide 10 in presence of different cosolvents at 20% (W/V) concentration at 25°C. (a) control in buffer (b) 20% sucrose (c) 20% sorbitol (d) 20% trehalose and (e) 20% glycerol

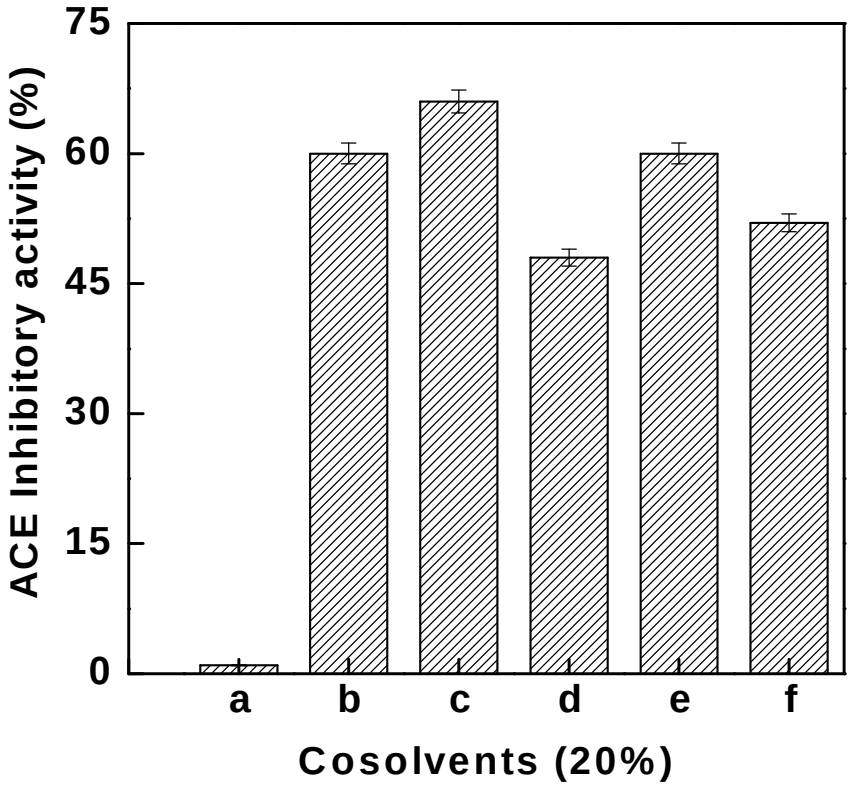

Fig.32. The ACE inhibitory activity of peptide 10 at 50°C in presence of different cosolvents at 20% (W/V) concentration. (a) Control in buffer (b) Ramipril in buffer as standard (c) 20% sucrose (d) 20% sorbitol (e) 20% trehalose and (f) 20% glycerol. The ACE and peptide were incubated in presence of cosolvents for 30 min.

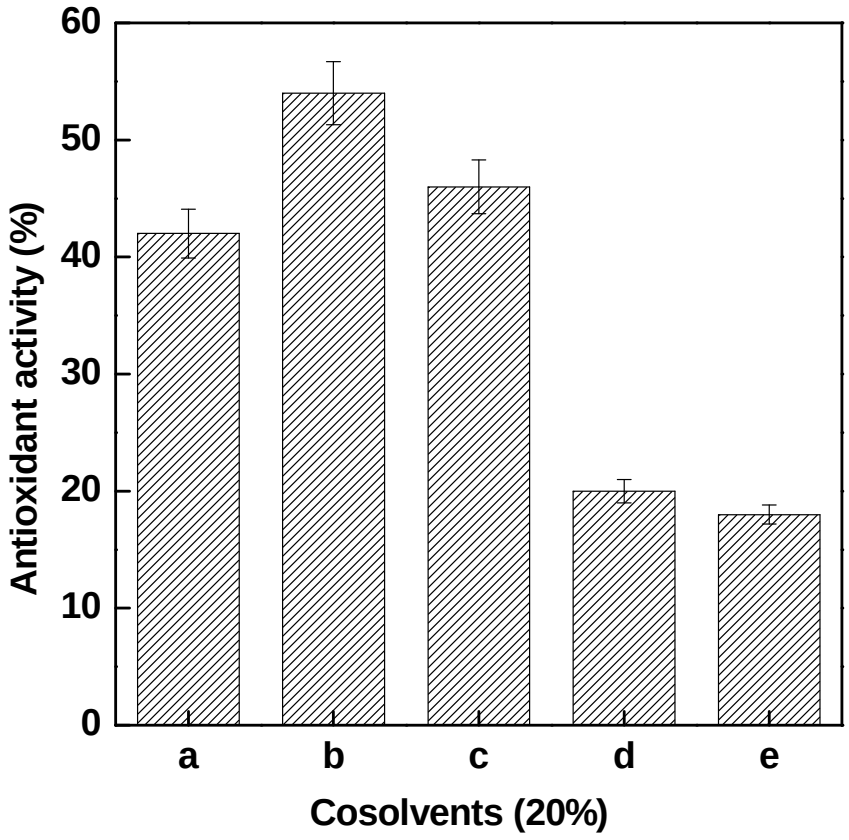

Fig.33. The antioxidant activity of peptide 10 in presence of different cosolvents at 20% (W/V) concentration at 25°C. (a) Control in buffer (b) 20% glycerol (c) 20% sorbitol (d) 20% sucrose and (e) 20% trehalose.

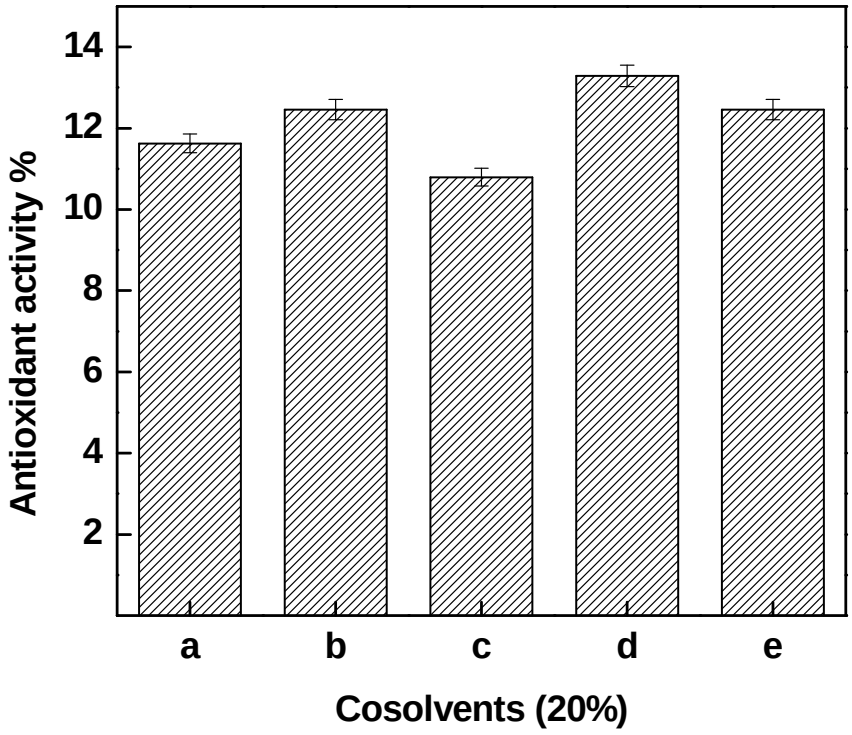

Fig.34. The antioxidant activity of peptide 10 at 50°C in presence of different cosolvents at 20% (W/V) concentration. (a) Control in buffer (b) 20% glycerol (c) 20% sorbitol (d) 20% sucrose and (e) 20% trehalose.

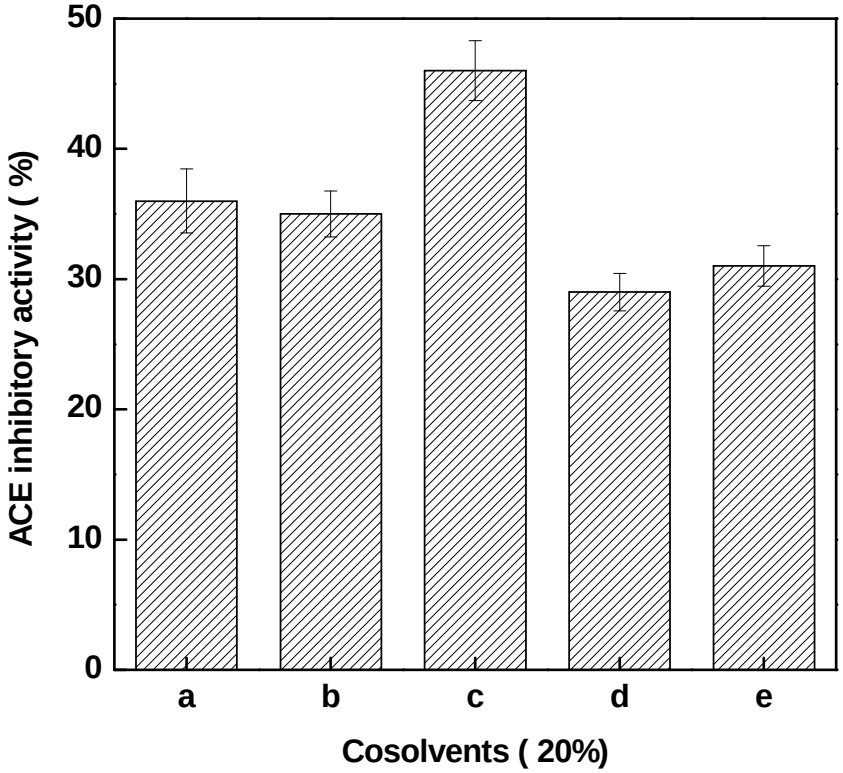

Fig.35. The ACE inhibitory activity of peptide 14 in presence of different cosolvents at 20% (W/V) concentration at 25°C. (a) Control in buffer (b) 20% glycerol (c) 20% sorbitol (d) 20% sucrose and (e) 20% trehalose.

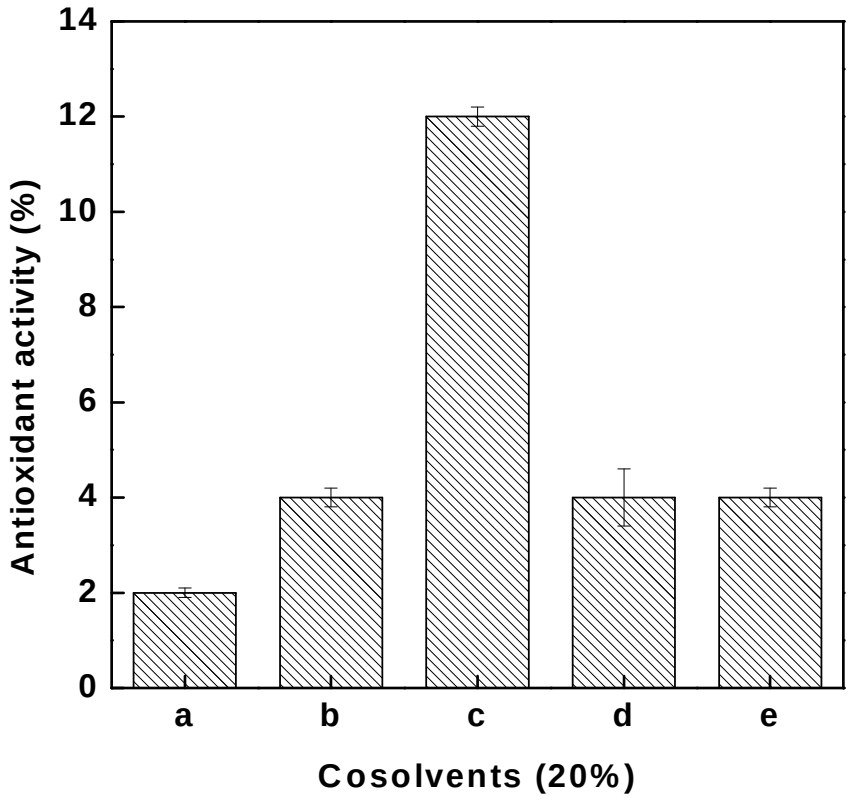

Fig.36. The antioxidant activity of peptide 14 in presence of different cosolvents at 20% (W/V) concentration at 25°C. (a) Control in buffer (b) 20% sucrose (c) 20% sorbitol (d) 20% glycerol and (e) 20% trehalose.

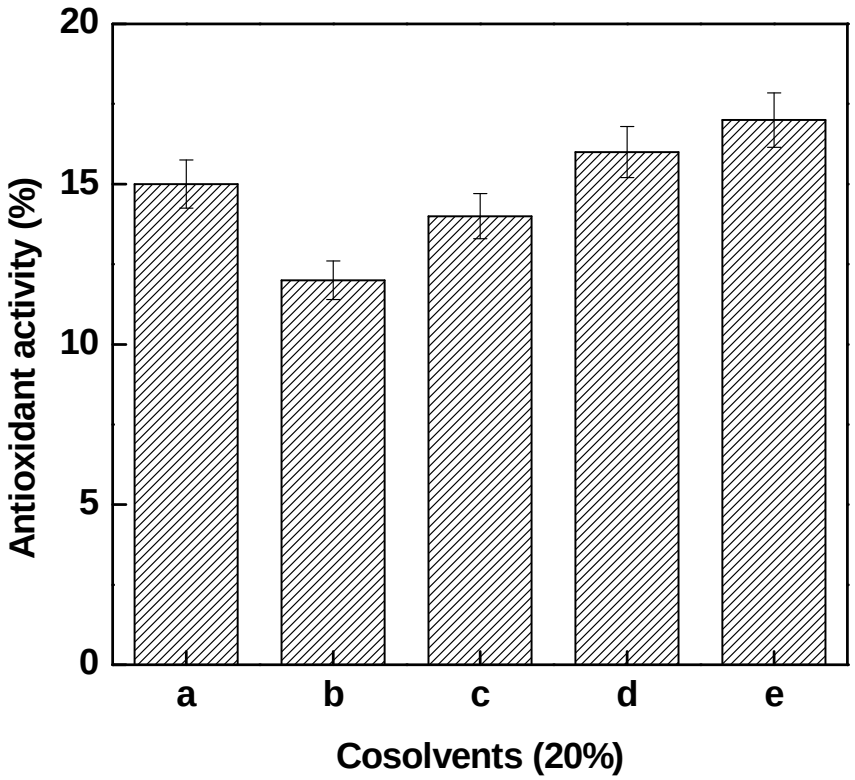

Fig.37. The antioxidant activity of peptide 14 at 50°C in presence of different cosolvents at 20% (W/V) concentration. (a) Control in buffer (b) 20% sucrose (c) 20% trehalose (d) 20% sorbitol and (e) 20% glycerol. The peptide was incubated in presence of cosolvents for 30 min.

CHAPTER –III

INTERACTION OF ZINC WITH α-CASEIN

4.3. INTERACTION OF ZINC WITH α-CASEIN

Zn(II) is an essential micronutrient and has a role in neonatal growth and immunity. The major consequences of Zn(II) deficiency were shown to be growth retardation, retarded sexual maturation, skin lesions, iron deficiency anemia and neuromodulatory effect (Watt and Hooper, 2003). Zn(II) requirement for cell-mediated immunity is also well-established (Pekarek *et al.,* 1979). Despite the apparent nutritional significance of the Zn(II), Zn(II)-casein interaction has received little attention.

The binding of Zn(II) with α-casein was studied by equilibrium dialysis. The binding isotherm obtained by equilibrium dialysis, shows saturation of binding of Zn(II) at a concentration of 2×10^{-3} M. From the Scatchard plot, the points can be resolved in to two straight lines, implying the presence of at least two binding sites for Zn(II) on α-casein as shown in the figure 38. The number of binding sites are found to be n_1 5 ± 1 and n_2 12 ± 2 with an association constants of ka_1 0.2 ± 0.03 × 10^{6} M^{-1} and ka_2 2.7 ± 0.3 × 10^{6} M^{-1} respectively. Free energy change ΔG° calculated from the association constants are approximately –31 kJ/mol and –38 kJ/mol respectively.

Two different binding sites one with 5 ± 1.0 moles and low binding constant of ka_1 0.2 ± 0.03 × 10^{6} M^{-1} and other site with 12 ±

2.0 moles and a high binding constant ka_2 2.7 $\pm$ 0.3 $\times$ 10^6 M^{-1} were found to be present on the α-casein. Studies of Harzer and Kauer (1982) had shown that serine monophosphates are responsible for binding to Zn(II) in α-casein and dephosphorylation of the serine monophosphates in α-casein with dephophorylases shows no binding of Zn(II). Further studies by Singh *et al.,* (1989) have shown that the dephosporylation has not prevented the binding of Zn(II) to α-casein. From these studies it is evident Zn(II) has many sites on α-casein.

The effect of Zn(II) binding on the fluorescence spectra of α-casein was studied as a function of Zn(II) concentration. The Zn(II) was titrated against α-casein by fixing the fluorescence excitation and emission at 287 nm and 341 nm respectively. Quenching of fluorescence was observed with a red shift from 341 - 344 nm. The quenching occurred with 35% quenching of total fluorescence intensity. The data was analyzed using Stern - Volmer equations (figure 39), and shows an association constant of 1.2 ± 0.3 $\times$ 10^6 M^{-1}. The Free energy change ΔG° calculated from the association constant is approximately –33 kJ/mol.

The quenching of intrinsic fluorescence by Zn(II) at 287 nm could be due to the perturbations happening to the tertiary structure of the molecule upon binding of Zn(II). It is evident from this data α-casein–Zn(II) complex has spectral activity, as the Zn(II) alone is

known to be spectroscopically silent due to the completely filled electronic configuration. These changes are may be due to the binding of Zn(II) to serine monophosphates (Harzer and Kauer, 1982), and probably due to the binding of Zn(II) other amino acid residues like His, Glu, Asp and Cys. α-Casein has 19 Ser, 8 His, 50 Glu, and 11 Asp residues (Hunt *et al.,* 1999). These amino acids are interdispered in the entire length of the molecule along with aromatic amino acids (22 Tyr, 14 Phe, 4 Trp) such that, any changes happening to these Zn(II) binding amino acids will lead to changes in the microenvironment of aromatic amino acids and subsequent changes in the collective absorbance region of 287 nm (Berg and Shi, 1996; Wong *et al.,* 1996) Studies by Veprintsev *et al.,* (1996) on binding of heavy metals ions like Hg(II) and Pb(II) to α-lactalbumin have shown that binding of heavy metals quenches the tryptophan fluorescence. From the studies of Alaimo *et al.,* (1999b) α-casein interactions are driven by electrostatic and hydrophobic amino acids.

UV- difference spectra of α-casein at different concentrations of Zn(II) shows positive difference spectra, with intensity peaks at 290 nm and 268 nm (figure 40). These intensity peaks increase by increasing the concentration of Zn(II). This is due to the continuous perturbations in the molecule happening around the aromatic residues. The red shift from 277 nm to 290 nm is seen with increase in concentration of Zn(II). Changes in the intensity of the shift are more

pronounced with increase in the concentration of zinc. The red shift may be due to the presence of ionizable carboxyl group near indole chromophore of aromatic amino acid residues. The intensity of the peaks near 268 nm may be due to the presence of ionizable amino group in the proximity of the phenylalanine as described by Donovan, (1973) and Leach and Scheraga, (1960) in their earlier studies on several proteins.

The changes in the ultraviolet difference spectra at 290 nm and 268 nm can be due to the perturbation of the tryptophanyl and phenylalanyl residues being exposed from a lower dielectric constant region to higher dielectric constant region. Such changes in the migration of the differential spectral peaks been explained in several proteins studies by Glazer and Smith, (1960) and Amici *et al.,* (2002) due to charge - charge interaction changes as a result of interaction of zinc with α-casein.

The secondary structural analysis of the macromolecule ligand complex in α-helix, β-structure and aperiodic structures by far-UV CD spectra shows not so significant changes in the secondary structure at lower concentration of zinc (1×10^{-6} M) and at higher concentration of zinc (1×10^{-5} M) there is significant changes in the β-structure content with a concomitant decrease in aperiodic structure (table 14). The near UV CD changes in both α-casein and α-casein Zn(II) complex shows perturbations of the aromatic residues of α-casein as a result of Zn(II) binding (figure 42).

In order to understand the complexity of zinc binding to the α- casein molecule at higher temperature both the control protein in HEPES buffer at pH 6.8 and the protein complexed with Zn(II) at 1×10^{-5} M is subjected to temperature gradient incubation during the peltier data generation of the secondary structure using the CD spectrometer. Table 15 summarizes the secondary structural data of α-casein and α-casein – Zn (II) complex. From the table, it can be seen that there is marginal decrease in the β - structure with a significant change in aperiodicity when α- casein is compared for its secondary structural indices at 25°C and 85°C. It is clear that α-casein is fairly resistant to structural changes as a result of extended exposure to higher temperature as seen in CD measurements. However when the same protein is complexed with Zn(II) at 1×10^{-5} M concentration at 25°C as compared to the same concentration (1×10^{-5} M) of Zn(II) at 85°C there is a enhancement of nearly 7 % α - helix and nearly 17% decrease in β - structure and 10% increase in aperiodic structure. Similar studies were reported by Jones *et al.,* (2004) and Lippard and Berg, (1994) on the interaction of Cu (II) and Zn(II) with other proteins and similar study with other zinc binding proteins reported by Matsubara *et al.,* (2003) and Hoagland *et al.,* (2001) have shown that such results are not unusual.

Figure 43 shows the IR spectra of α-casein and α-casein – Zn (II) complex recorded at 25°C.

The role of such differential effect of Zn(II) on the surface properties of α-casein is interesting to probe. The hydrophobic residues on the surface are very sensitive to the tertiary structural changes derived from specific and non-specific protein interactions. This is true with a third component such as ANS and has been very successful as a hydrophobic fluorophore to monitor such changes. ANS hydrophobic probe is used to understand the surface hydrophobicity of the molecule before and after binding of Zn(II). As shown in the figure 44, the binding of ANS was studied by titrating ANS with α-casein and α-casein -Zn(II) complex. The changes in the relative fluorescence intensity as a result of binding of ANS to native α-casein and α-casein-Zn(II) complex, was monitored. The addition of ANS increases the fluorescence intensity. The binding of ANS was more to α-casein–Zn(II) complex than to native casein. There is an increase in the surface hydrophobicity of α-casein–Zn(II) complex compared to native α-casein. The binding shows changes in the hydrophobic core of the molecule. This was evident from the studies of Fitzgerald and Swaiswood, (1989) on binding of ANS to α-Lactalbumin. Cox *et al.,* (2000) has shown similar results with carbonic anhydrase where in the surface hydrophobicity increases up on binding to metal ions.

The reversibility studies were studied by dialyzing the α-casein -Zn(II) complex against EDTA. The EDTA is a chelating agent and chelates the unbound or the loosely bound Zn(II). The α-casein -Zn(II)

complex was dialysized against the EDTA. EDTA chelation of the bound zinc (5 mg/L) shows, 3 mg/L, gets chelated by EDTA, which shows different sites on α-casein. The reversibility studies shows that the α-casein has two different binding sites. This is also reported by the Singh *et al.,* (1989) on the dialysis experiments carried out on α_{S1}-casein–Zn(II) complex.

The rate of formation of α-casein -Zn(II) complex was studied by slow and rapid kinetics. The slow kinetics followed by using UV-visible spectrophotometer (figure 45) at 287 nm shows increase in the absorption after addition of ligand follows the pseudo-first order kinetics with rate constant of 40 s^{-1}.

The kinetics of binding of Zn(II) to α-casein was studied by stopped flow spectrophotometer. The stopped flow spectrophotometer rates were 36 and 37 s^{-1} respectively at 1:70 and 1:7 ratio of protein: ligand studied. The rate of reaction follows pseudo-first order kinetics at the different concentrations of protein and ligand studied (Twine *et al.,* 2003). Binding of Zn(II) to ligands in proteins is known to proceed relatively rapidly. In other terms, Zn(II) proteins interactions are kinetically faster and stronger. This behavior of Zn(II) is due to the non-redox activity, borderline acidity and completely filled *d*-shells in Zn(II) (Eigen, 1963).

The hydrolysis of proteins by proteolytic enzymes results in deciphering the structural details. The α-casein and α-casein -Zn(II) complex was hydrolyzed by chymotrypsin and fungal protease under optimum conditions. The hydrolytic products obtained were estimated by *O*-phthalaldehyde (OPA) method. The figure 46 shows the graphical representation of hydrolysis of α-casein with chymotrypsin and fungal protease as function of time. The chymotrypsin and fungal protease hydrolyze α-casein -Zn(II) complex with 1.6 ± 5% and 70 ± 5% degree of hydrolysis as compared to α-casein with 1.2 ± 5% and 60 ± 5% respectively.

The hydrolysis of the α-casein and α-casein–Zn(II) complex with chymotrypsin and fungal protease enzymes shows that degree of hydrolysis is more with α-casein –Zn(II) complex than to α-casein. The increase in the hydrolytic pattern, shows that the increased digestibility of the molecule. The molecular conformational changes could be one of the reasons for the increase in the digestibility, as Zn(II) in general inhibits the chymotrypsin activity (Farrell Jr. *et al.,* 1999; Permyakov *et al.,* 1991). Binding of Zn(II) to α-lactalbumin was shown to increase the hydrolysis of the complex by chymotrypsin, when compared to native protein (Vallee and Auld, 1993). Permyakov *et al.,* (1991) have shown that strong Zn(II) binding increases its susceptibility to trypsin and chymotrypsin digestion. From these studies, binding of Zn(II) to α-casein is stronger and stable. The

increase in the hydrolytic pattern with fungal protease establishes the fact that Zn(II) binding increases the digestibility of the complex.

The above-mentioned work on Zn(II) interaction with α-casein were in agreement with the studies of Permyakov *et al.,* (2003) on recoverin where the interaction of Zn(II) with recoverin, increases its alpha-helical content, hydrophobic surface area, and environmental mobility/polarity of its tryptophan residues.

From these studies one can understand the mechanism of Zn(II) interaction with α-casein. Zn(II) binds to the phosphoserines and this reaction is fast and but has a weak binding affinity. Further addition of Zn(II) will result in binding of Zn(II) to other amino acids present in α-casein, with resultant increase in the surface hydrophobicity/increased polarity of the aromatic amino acids and secondary structure. Alternatively zinc binding brings in changes in the hydrophobic core which increase the affinity of Zn(II) binding to amino acids. The amino acid residues at the binding site may originate from a short linear sequence in a polypeptide chain or may be assembled from distant parts of the polypeptide chain. In the latter case, it is the secondary and tertiary structures, which bring together and maintain the necessary residues in appropriate geometry.

The interaction of zinc with α-casein has shown the various structural changes in the molecule. The ability of the zinc to stabilize the structure and brings in changes in other structural parameters can be useful in understanding the zinc proteins interaction and in general metal proteins interactions. This study can be utilized to as a model for studying other proteins interacting with zinc. In the next chapter a detailed study was undertaken to study the interaction of lead with α-casein to understand whether zinc and lead can bring in structural stability to the same molecule or otherwise.

Table 14

Secondary structure of α-casein in 0.01M HEPES buffer pH 6.8 at 25^0 C and in presence of various concentrations of Zn(II)

	Secondary structure (%)		
Concentration of Zn(II)	**α-Helix**	**β-Structure**	**Aperiodic**
Control (buffer[a])	5 ± 1	51 ± 2	44 ± 2
1 x 10^{-6} M	3 ± 1	48 ± 2	50 ± 3
1 x 10^{-5} M	10 ± 2	57 ± 3	33 ± 2

[a] *0.01 M HEPES buffer pH 6.8*

Table 15

Effect of temperature on the secondary structures of α-casein and α-casein - Zn(II) complex

	Secondary structure (%)		
	α-Helix	**β-Structure**	**Aperiodic**
α-casein (in buffer [a]) at 25^0 C	5 ± 1	51 ± 2	44 ± 2
α-casein (in buffer [a]) at 85^0 C	5 ± 1	43 ± 2	52 ± 2
1 x 10^{-5} M Zn(II) at 25^0 C	10 ± 2	57 ± 3	33 ± 2
1 x 10^{-5} M Zn(II) at 85^0 C	17 ± 2	40 ± 3	43 ± 2

[a] *0.01M HEPES buffer pH 6.8*

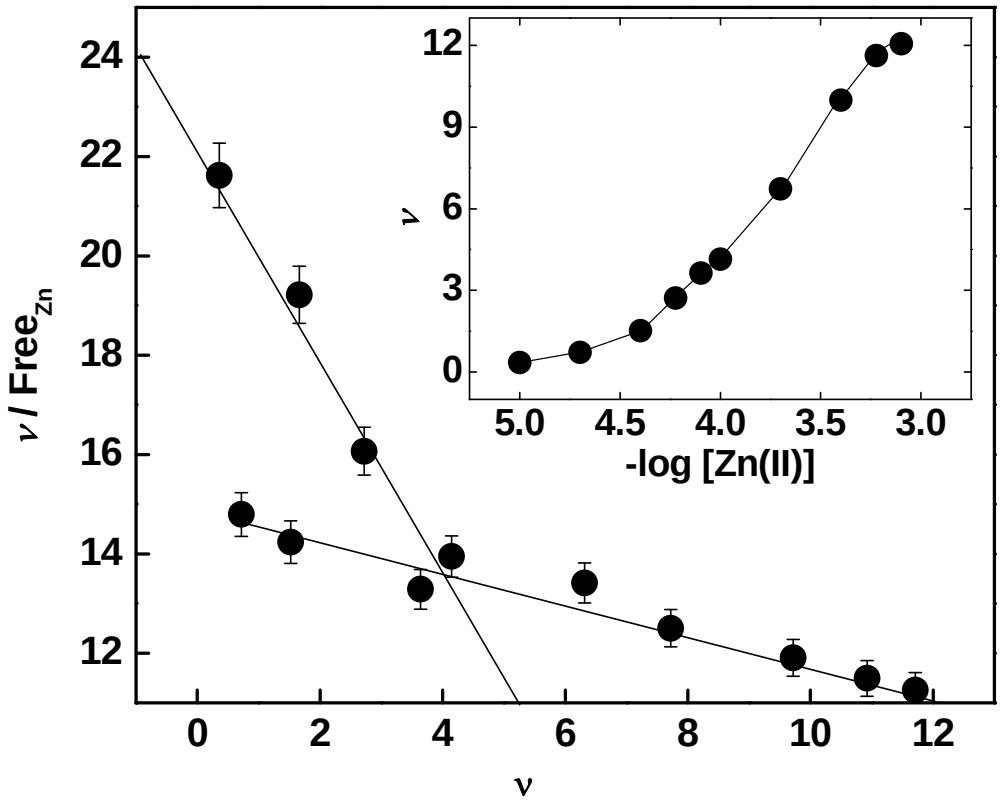

Fig.38. Scatchard plot of α-casein interaction with Zn(II) as studied by equilibrium dialysis. 5ml of α-casein (1.83×10^{-4} M) was dialyzed against 10 ml of Zn(II) (1×10^{-8} M – 1×10^{-6} M) in 0.01 M HEPES buffer pH 6.8 for 24 h at 25°C with shaking at 75 rpm. The concentrations of free Zn(II) in equilibrium were determined by flame atomic absorption spectroscopy at 213.9 nm. Inset shows a semi logarithm plot of ν (moles of ligand bound to protein) vs total [Zn(II)].

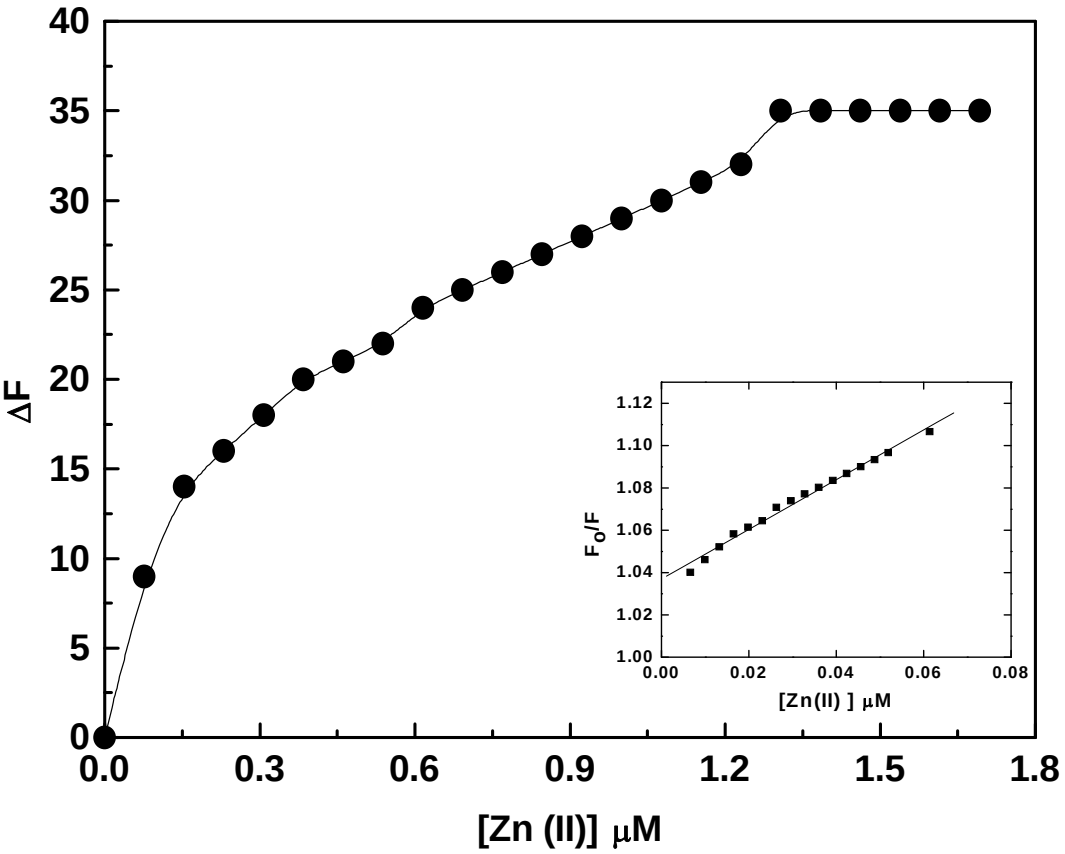

Fig.39. Primary plot of fluorescence % quench as a function of Zn(II) concentration. Inset shows the stern–Volmer plot of α-casein (0.01 M HEPES buffer at pH 6.8). Excitation wavelength and emission were at 287 nm and 300 - 400 nm respectively. Excitation and emission slit widths for both were 5 nm. A stock Zn(II) solution of 5×10^{-7} M was used. A protein concentration of 2.2×10^{-6} M was used. ΔF is the difference in fluorescence intensity.

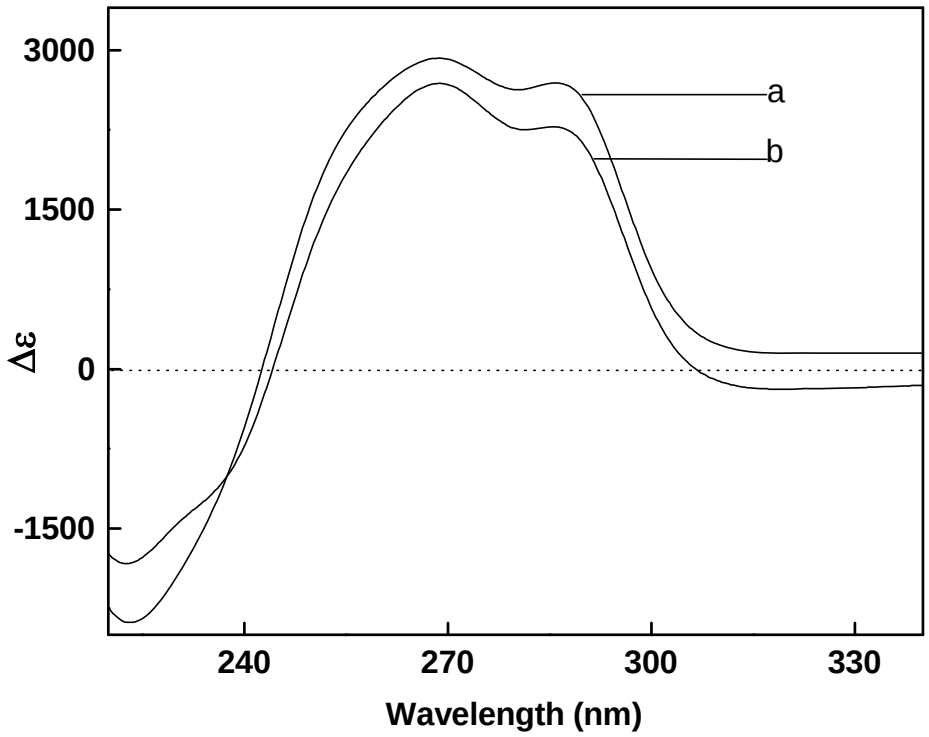

Fig.40. Effect of Zn(II) on UV difference spectra of α- casein at 25°C. A protein concentration of 1.8 x 10^{-4} M was used for all the measurements. The Zn(II) concentration of (0.01 M HEPES Buffer pH 6.8) (a) 2×10^{-5} M (b) 1×10^{-4} M. where $\Delta\varepsilon$ is the difference in molar extinction coefficient obtained from $\Delta A/c \times l$.

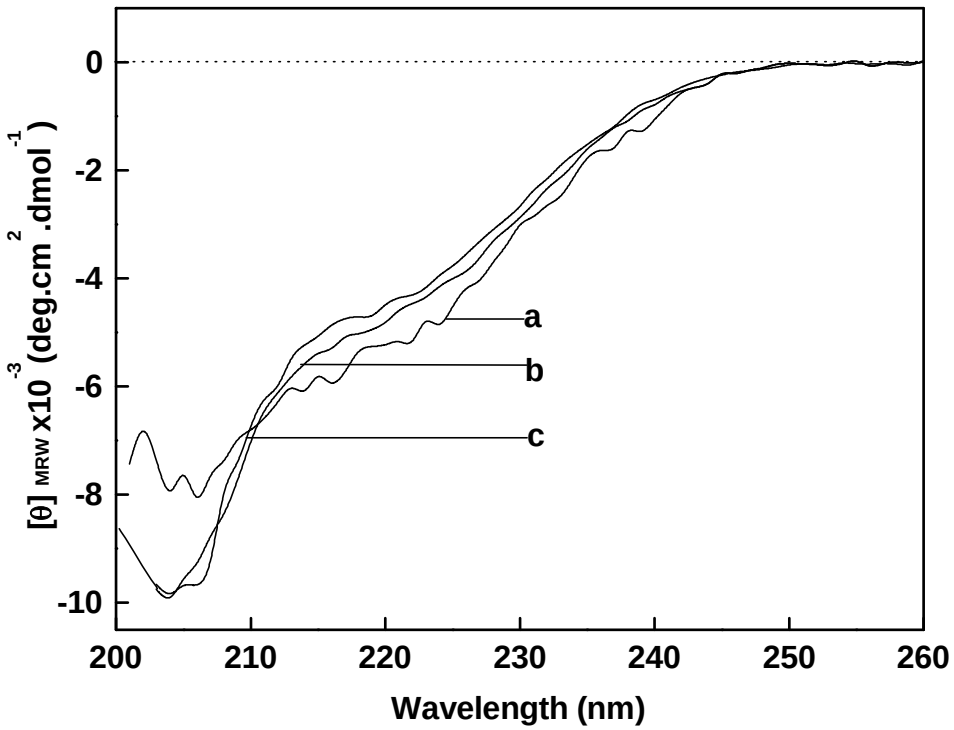

Fig.41. Effect of Zn(II) on the far UV-CD spectra of α-casein. (a) 8×10^{-5} M Zn(II) (b) 1 x 10^{-6} M Zn(II) and (c) control in buffer. A protein concentration 1×10^{-5} M was used for all measurements.

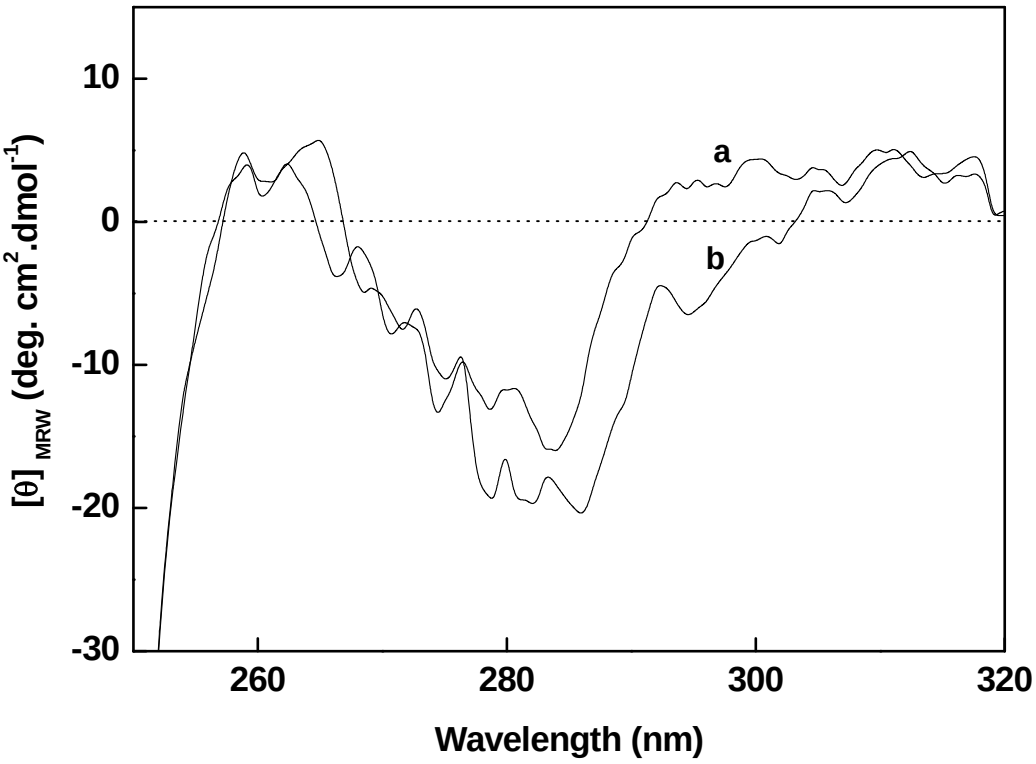

Fig.42. Effect of Zn(II) on the near UV CD spectra of α-casein in 0.01M HEPES buffer pH 6.8. (a) Control in buffer and (b) 1x 10^{-4} M Zn(II). A protein concentration of 3.6 × 10^{-5} M was used.

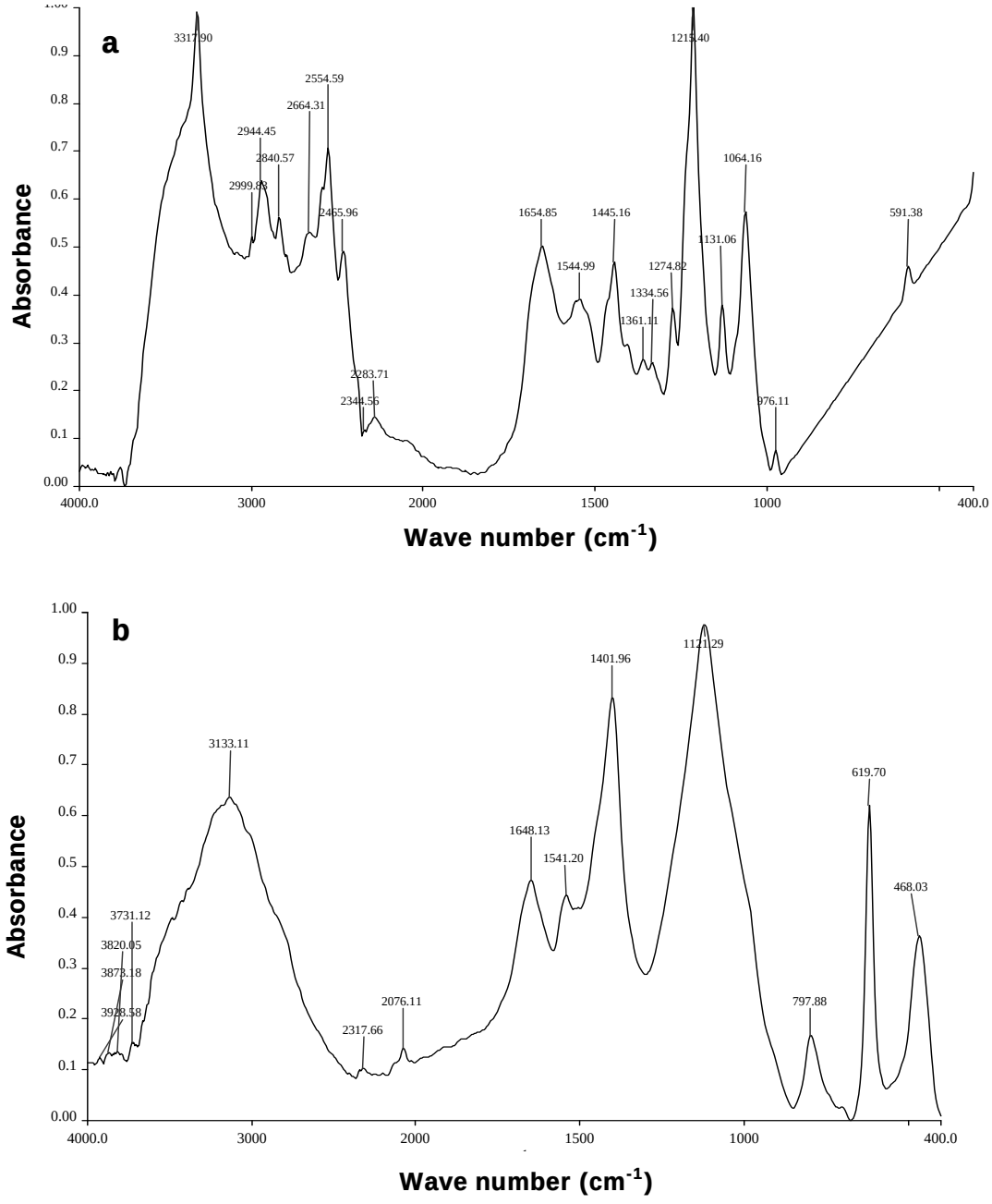

Fig.43. Effect of Zn(II) on Fourier transform-infra red spectra of (a) α-casein and (b) α-casein–Zn(II) 0.001 M as scanned from 400 to 4,000 cm^{-1} in KBr. A protein concentration 4.2 $\times 10^{-4}$ M was used for all measurements.

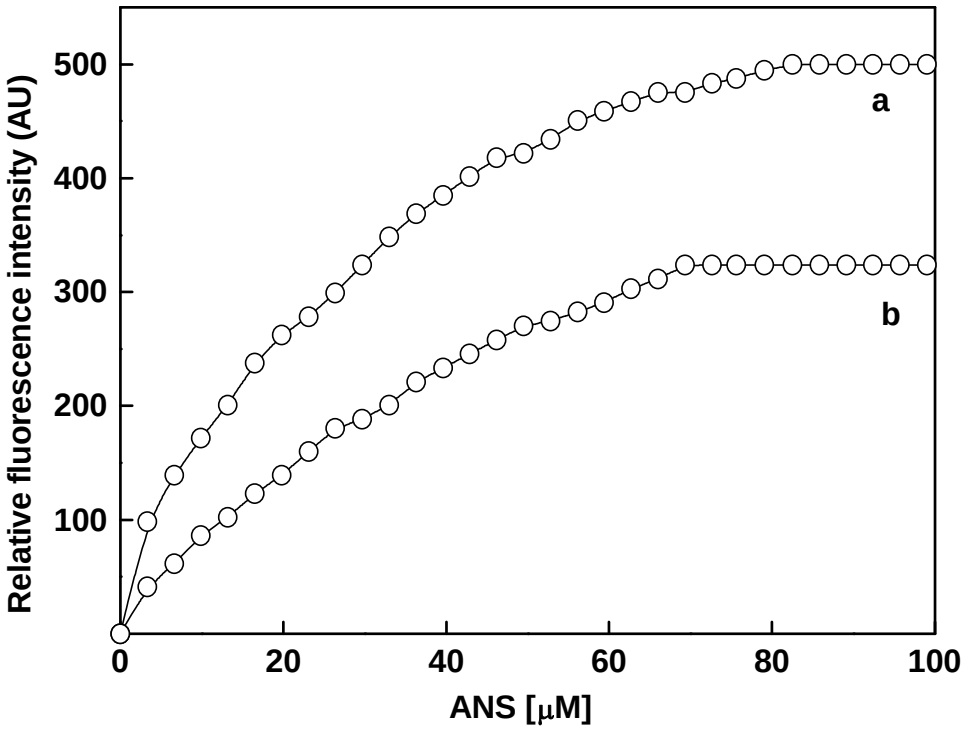

Fig.44. Effect of Zn(II) on surface hydrophobicity of α-casein using ANS at 25°C. The protein was excited at 375 nm and emission was followed at 400-600 nm. A protein concentration of 2.2×10^{-6} M and a stock of 1mM ANS was used for all the measurements. (a) α-Casein Zn(II) and (b) α-Casein.

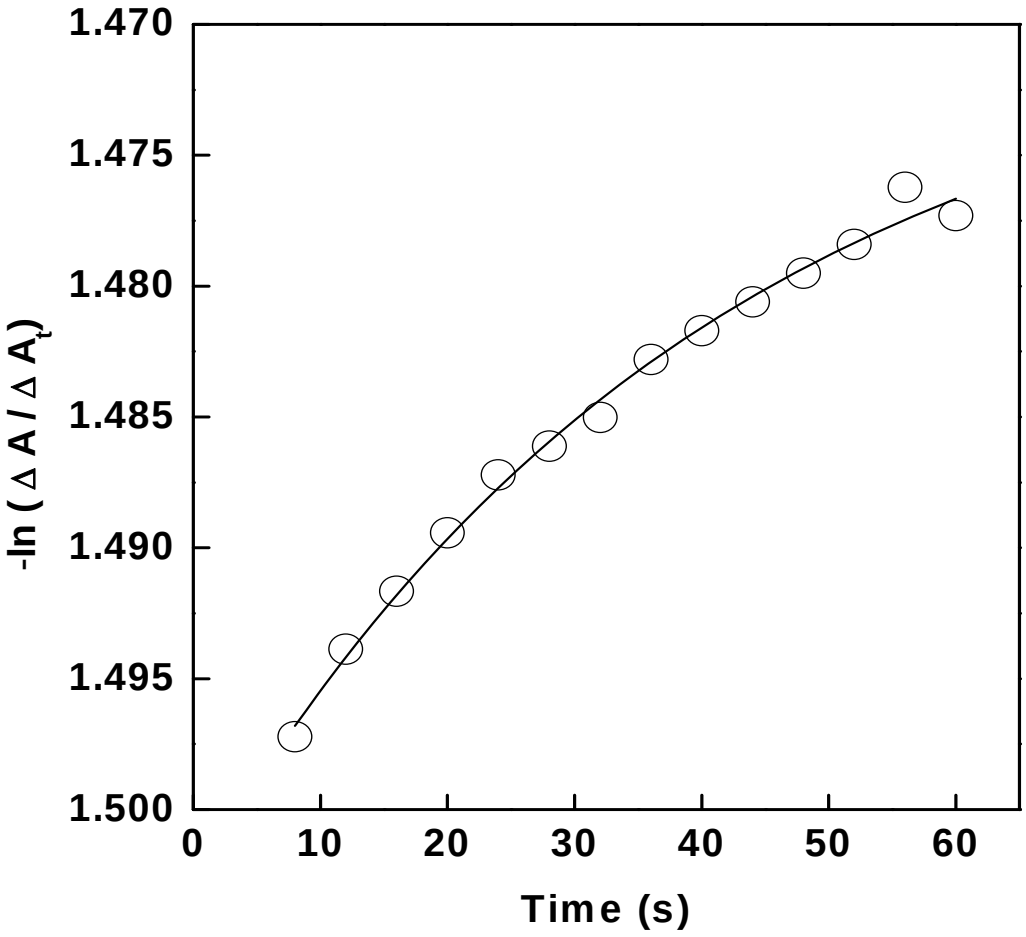

Fig.45. Slow kinetics of interaction of α-casein with Zn(II) as followed on a UV-Visible spectrophotometer at 287 nm. The reaction is followed up to 60 s at 25°C. The Zn(II) concentration of 1×10^{-5} M and α-casein concentration of 2×10^{-5} M was used for the experiments.

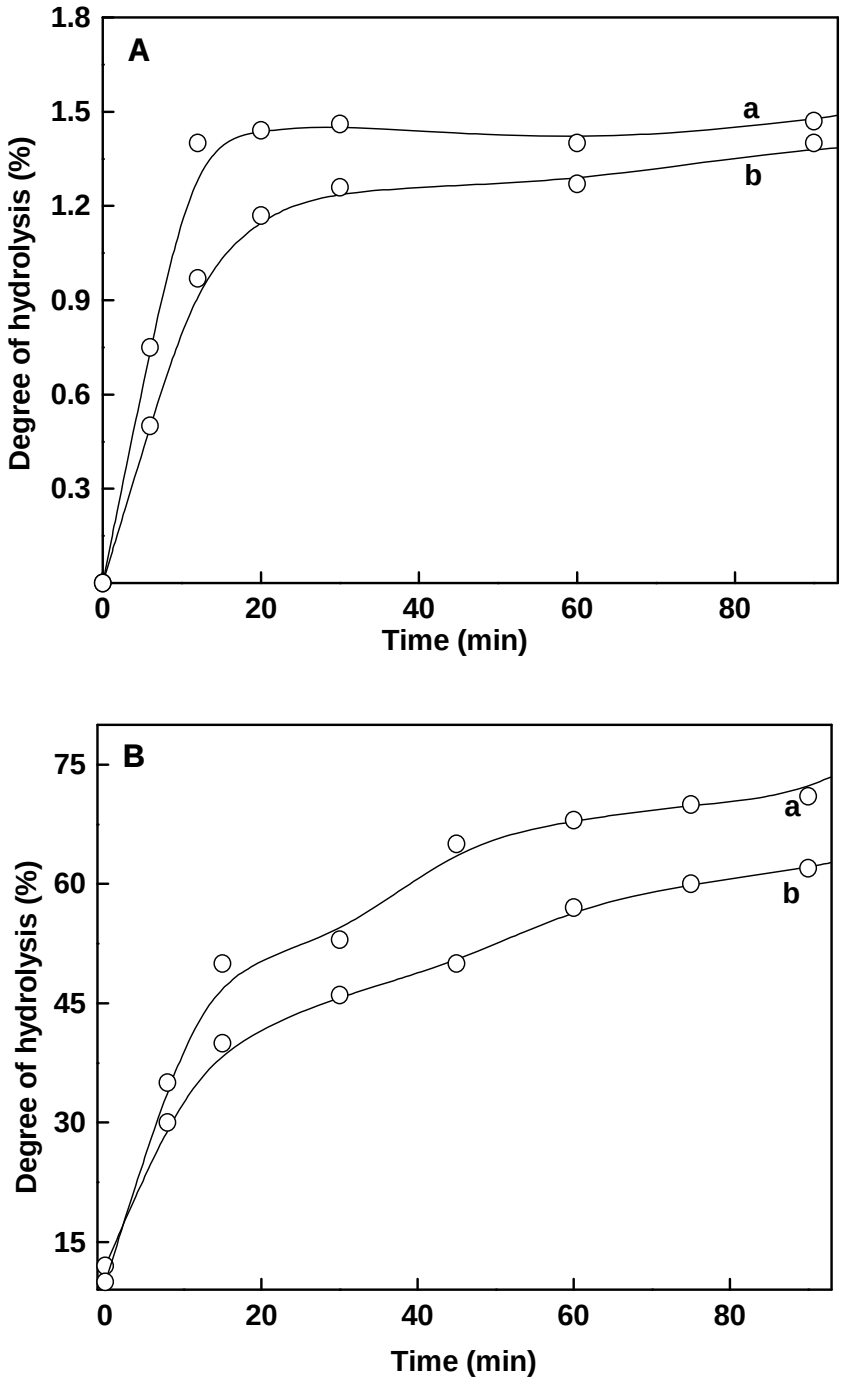

Fig.46.

(A) Effect of Zn(II) on the hydrolysis of α-casein by chymotrypsin at 37°C. (a) Zn(II) treated α-casein and (b) control α-casein.

(B) Effect of Zn(II) on the hydrolysis of α-casein by fungal Protease at 45°C. (a) Zn(II) treated α-casein and (b) control α-casein.

A protein concentration of 1×10^{-5} M was used for the above experiments.

CHAPTER IV

INTERACTION OF LEAD WITH α-CASEIN

4.4. INTERACTION OF LEAD WITH α-CASEIN

Interaction of Pb(II) with proteins and enzymes has been extensively studied. The polar specificity of lead, large ionic radius and relatively weak binding to oxygen and nitrogen ligands makes it a good inorganic probe to study active sites of macromolecules (Mailer *et al.,* 1982). The Pb(II), unlike Cu (II) and Fe(II), exists as a stable divalent ion and does undergo redox reactions. Biochemical properties of Pb(II) allows it to interact with a diverse array of ligands. Lead is placed as a borderline group with group characteristics like universal ligand preference, high toxicity, charge, size and electronegativity. Lead shows clear preference towards sulfhydryl groups, which is characteristic of borderline metals. Thus, Pb(II) reacts primarily with the following bases possessing nucleophilic ligands: sulfhydryl, amine, phosphate, and carboxylic groups. It is evident from the binding constants for various ligands that thiolate group binds Pb(II) with highest affinity (Goering, 1993; Godwin, 2001). This study has been taken up to elucidate the role of lead nitrate on the structure and function of α-casein isolated from bovine milk.

The binding of Pb(II) with α-casein was studied by spectrophotometric titration. Addition of increasing amounts of lead quenched the absorbance of α-casein up to 17%. Binding isotherm shows saturation of α-casein at Pb(II) concentration of 20 μM (Figure

47). From the Scatchard plot, the points gave a straight line, suggesting two binding sites for Pb(II) on α-casein. The maximum number of binding sites is found to be 2.0 ± 0.05. The association constant calculated from the slope is 2.3 (± 0.2) × 10^5 M^{-1}. Free energy change ΔG° calculated from the association constant is approximately -30.8 kJ/mol. Studies of Mannino and Bianco (1988) have shown that whole casein binds 0.28 moles of Pb(II) by continuous flow potentiometric stripping analysis with k_a of 10^5. Veprintsev *et al.,* (1996) have shown that Pb(II) binds to α-Lactalbumin with an association constant of 10^5. Ayranci and Duman (2004) has shown that the bovine serum albumin binds to Pb(II) with an association constant of 10^5. Goering, (1993) has shown that the approximate stability constant of complexes of Pb(II) with thiol groups has an association constant of 10^5. From the above, we can postulate that the association constants for Pb(II)- α-casein interactions are may be due to binding of Pb(II) with cysteines (Kagi and Hapke, 1984). The two binding sites present on α-casein could be due to two cysteines present in the α-casein as shown by the amino acid composition of α-casein (Wong *et al*., 1996).

The equilibrium dialysis of α-casein at different concentrations of Pb(II) was studied and the representative Scatchard plot is shown in the figure 48. The Scatchard plot shows the presence of 2.0 ± 0.05

binding sites in α-casein. The slope of the line passing through the data points, show the association constant k_a 2.3 (± 0.2) × 10^5 M^{-1}.

Far- UV circular dichroic studies show that, there are no significant changes in the secondary structure of α-casein as a result of Pb(II) binding (figure 49). The far – UV circular dichroic spectra shows no significant changes in the secondary structure of the protein over a ten fold change in Pb(II) concentration (table 16). Above 1 × 10^{-4} M concentration of Pb(II) there is micro precipitation of α-casein due to perhaps aggregation of the protein in presence of Pb(II) and hence measurements could not be made. It has been reported recently that Pb(II) induces conformational changes and brings about disorderliness in proteins as reported by Payne *et al.*, (1999). This can be due to Pb(II) binding induced entropically driven energy dynamics destabilization of protein. The increase in the molar ellipticity values in the region of 205 nm may throw same light on such structural changes in the Pb(II)–protein complex. Studies by Payne *et al.*, (1999) have shown that in the case of few peptides the binding of Pb(II) destabilizes the folding. Studies by Whittington *et al.*, (2005) shows that the poly(L-

proline) II helix conformation has a negative band around 205 nm. Our results indicated binding of Pb(II) increases negative ellipticity at 205 nm, which may be due to the formation of the poly (L-proline) II helix conformation.

Raman spectrum provides an extremely valuable tool for studying the metal-protein interactions. The Raman spectral measurements for the cysteine sulfhydryl (SH) group in a typical protein generates a Raman bands in the region of 2500 - 2600 cm^{-1}, a region devoid of any interference from any other fundamental mode of vibration of the protein (Stephen *et al.,* 2001). Supporting evidence was also obtained from the studies on Raman spectra, which revealed significant changes in α-casein upon binding Pb(II). Raman spectrum of α-casein and α-casein–Pb(II) complex from 1200-1800 cm^{-1} is shown in the figure 50. There is a reduction in the amide I (1619 cm^{-1} and 1581 cm^{-1}) region in α-casein after binding Pb(II). These results can be correlated with CD data, where it shows decrease in the β-structures. α-Casein Pb(II) complex shows bands for amide III at 1251 cm^{-1}, and a new band at 1328 cm^{-1}. The band centered at 1251 cm^{-1} is due to the increase in aperiodicity in the α-casein upon binding to Pb(II). The new band centered at 1328 cm^{-1} could be due to the formation of PPII helix in the molecule. Syme *et al.*, (2002) have shown that the caseins have a strong band for poly (L-proline) II helix in the region of 1315-1325 cm^{-1}.

From this we postulate, that Pb(II) induces formation of poly (L-proline) II helix in α-casein. Studies by Payne *et al.*, (1999) on peptides and Stephen *et al.*, (2005) on proteins showed changes in Far-UV CD spectrum at 205 nm, can also be correlated due to the binding of Pb(II) to cysteines in proteins and peptides. Significant changes in the region of S-H groups were also seen in the Raman spectra. The two free cysteines (Cys^{36} and Cys^{40}) of α-casein generate a complex band around 2546 cm^{-1}, which in case of α-casein -Pb(II) complex get shifted to 2524 cm^{-1} (figure 51). The shift is due to the influence of metal ions in the vibrational mode of functional groups to which they are attached. The metal ligand bonds usually appear at relatively low frequency because metal ions are greater in mass (Xing *et al.*, 2003; Te, 1952). From this data, we interpret that Pb(II) binds to sulfhydryl groups of cysteines. The Infra red spectroscopy of α-casein and α-casein- Pb(II) complex shows clear perturbations in α-casein upon binding of lead (figure 52). There is also clear disappearance of the S-H band pertaining to 2554 cm^{-1} upon binding of lead to α-casein

The propensity to form poly (L-proline) II helix in natively unfolded proteins is reported by Syme *et al.,* (2002) during the studies on Raman optical activity of caseins, synucleins and tau proteins. Caseins are also grouped under natively unfolded proteins and suggested that the poly (L-proline) II helix conformation helps them to

be flexible, extended and rheomorphic, which is essential for their native function. Binding of Pb(II) to α-casein increases the poly (L-proline) II helix in α-casein. The explanation for these observations that a protein forms poly (L-proline) II helix is to maximize the peptide backbone favorable interaction with solvent (Rucker and Creamer, 2002). Pb(II) has to orient the backbone to poly (L-proline) II helix in α-casein, so as to fill the coordination number by interacting with water or buffer.

Surface hydrophobicity studies by ANS on α-casein and α-casein–Pb(II) complex shows changes in α-casein upon binding to Pb(II). The binding of Pb(II) to α-casein shows an increase in the surface hydrophobicity index (55%). The ANS binds more to the surface of α-casein-Pb(II) complex than to native protein indicating exposure of hydrophobic amino acids to the bulk solvents (figure 53). The surface hydrophobicity changes are due to the orientation of molecule to accommodate bulky Pb(II) ions in best fit geometric conformations. Pb(II) tends to assume high coordination numbers (four or six) and is unlikely to bind four ligands in a perfectly tetrahedral orientation. Pb(II) coordination is probably completed by water or buffer (Shimoni-Livny *et al.,* 1998). In order to complete Pb(II) geometric coordination it has to bind with thiols and also with water or buffer so as form the correct coordination site. From the

above data we infer that α-casein orients itself in a conformation, which brings about binding of Pb(II).

The rate of formation of α-casein-Pb(II) complex was studied by slow kinetics. The slow kinetics followed by using UV-visible spectrophotometer (figure 54) at 250 nm shows increase in the absorption after addition of ligand follows the pseudo-first order kinetics. The kinetics of the formation of α-casein-Pb(II) complex proceeds up to 60 s with a rate of 21.77 s^{-1}.

The reaction kinetics of Pb(II) binding to α-casein was followed by stopped flow spectrophotometer. The spectrum follows the pseudo-first order rate of 25 s^{-1} at 250 nm (Twine *et al.,* 2003). The kinetics was monitored up to 1 sec and the rates were analyzed at different concentrations as shown in table 17. The kinetics shows that binding of Pb(II) to thiolates is faster (figure 55). Kinetics was also followed at 277 nm in order to check for the molecular changes and the reaction rate was found to be significant with 70 s^{-1}. The data shows simultaneous fluctuations/perturbation/ reorientation of casein molecule upon Pb(II) binding.

The time resolved kinetic spectra was monitored for the α-casein and α-casein Pb(II) complex in the range of 200 – 400 nm which shows no changes in α-casein but α-casein Pb(II) complex

shows formation of significant LMCT bands with maxima at 330 and 360 nm in the time resolved spectrum (figure 56). The appearance of peaks starts from 25 ms reaches maximum at 125 ms. After 125 ms no further change in absorbance and appearance of peaks was obaserved indicating, absorbance change covers the whole process of formation of complex. This shows the binding is rapid and takes place in milli time base (Payne *et al.*, 1999; Xing *et al.*, 2003). Studies by Busenlehner *et al.*, (2001) on absorption spectroscopy and spectral behavior of Pb(II) on p1258 CadC has shown similar results for absorption bands at 240 nm and 350 nm which were due to the binding of Pb(II) to three or four thiolate ligands. They had shown by various techniques that Pb(II) binds to thiolates in CadC. Studies by Payne *et al.*, (1999) on Pb(II) peptide complexes has shown that characteristic two prominent Pb(II)- thoiolate LMCT bands positioned at 310 nm and 260 nm, which is due to the contributions of two sulphur and two nitrogens ligands present in the peptides.

Studies on interaction of α-casein Pb(II) complex were studied by using iodoacetamide, a thiol modifier. Iodoacetamide binds to free sulphur thiolates and blocks them from further interactions. The rapid kinetic rate of the α-casein Pb(II) complexes shows 25 s^{-1}. The addition of Pb(II) to α-casein iodoacetamide complex reduces the kinetic rate to 0.2 s^{-1}. There is a 125 folds decrease in the rate of iodoacetamide-α-casein- Pb(II) complex and is due to the blocking of

sulphur/thiolates present in the α-casein, which in turn are not available for interacting with Pb(II) (Blouin *et al.,* 2002).

In order to evaluate the availability of free cysteines in binding, studies were also carried out by modification of α-casein with AMS. The figure 57 shows RF profile (with non-reducing SDS-PAGE in inset) where in the modified protein migrated at a substantially higher molecular mass than unmodified protein. AMS enhances the molecular weight of protein and each conjugated AMS moiety increases the molecular mass by 490 kDa. From the above studies we can conclude that the free cysteines are present in α-casein (Abajain *et al.,* 2004).

In the present model system the α-casein used is composed of α_{S1}-casein and α_{S2}-casein fractions in the ratio of 4:1. The sequence information shows presence of two cysteines in α_{S2}-casein, at Cys^{36} and Cys^{40} residues (Farrell *et al*., 2004). α_{S1}-casein is devoid of cysteines. These cysteines are possible sites of interaction with lead. The other ligands can be oxygen, nitrogen and water, which may be contributing to fill the coordination geometry of Pb(II) (Payne *et al*., 1999).

In order to study the thermodynamic parameters and the temperature dependence of the binding, the kinetic rates at different temperatures are taken in stopped flow spectrophotometer and plotted

as van't Hoff plot. From the figure 58, the calculated slope (r-0.991) shows temperature dependence of the molecular association process. The slope gives an enthalpy of $\Delta H = -3.7 \pm 0.12$ kJ/mol. The entropy calculated from the enthalpy and temperature shows 115.8 J/mol/K. The change in Gibbs free energy calculated from enthalpy and entropy parameters is –30.8 kJ/mol. The association constant obtained from Gibbs free energy ($e^{-\Delta G/RT}$) is 1.9×10^5 M^{-1}. The underlying thermodynamic complex formation is a spontaneous/exergonic reaction of binding at measured temperatures. The formation of α-casein - Pb(II) complex proceeds via release of energy (Moreno and Jimenez, 1999). The thermodynamic signature shows a considerable amount of change in the enthalpy and entropy during the formation of α-casein - Pb(II) complex which explains the involvement of hydrophobic and hydrophilic interactions in the formation of the complex.

The α-casein Pb(II) interaction is a high affinity interaction. The Pb(II) probably binds to sulphur/thiolates present in the α-casein. The interaction increases Poly (L-proline) II helix conformation/randomness in the molecule. The Pb(II) induces tertiary structure changes in α-casein. The kinetics shows a very fast rate of formation of complex. The interaction of Pb(II) with α-casein shows temperature dependence with simultaneous perturbation in microenvironment of aromatic amino acids.

The interaction of lead with proteins is not explored completely. There are a host of factors involved in lead protein interactions. All factors playing a role in the interaction of lead as well as other metals, with bioligands must be taken into account when elucidating the mechanism of lead toxicity. The various factors involved in lead-protein interactions will require a more comprehensive experimental approach to further understand these interactions and with relation to lead toxicity.

Table 16

Secondary structure of α-casein in 0.02 M Tris-HCl buffer pH 6.8 at 25^0 C and in presence of various concentrations of Pb(II)

	Secondary structure (%)		
Concentration of Pb(II)	**α-Helix**	**β - Structure**	**Aperiodic**
Control (in buffer[a])	5 ± 1	51 ± 2	44 ± 2
9.5×10^{-6} M	4 ± 1	47 ± 3	49 ± 3
9.5×10^{-5} M	3 ± 1	47 ± 3	50 ± 2

[a] *0.02 M Tris-HCl buffer pH 6.8.*

Table 17

Pseudo-first order kinetic rates for the binding of Pb(II) with α-casein as studied on stopped-flow spectrophotometer at 250 nm at 25°C

Sl. No.	Concentrations of Pb(II) (M)	Rate (s^{-1})
1	1.8×10^{-8}	23.08
2	9×10^{-8}	30.40
3	1.8×10^{-7}	27.6
4	9×10^{-7}	22.7
5	1.8×10^{-6}	28.59

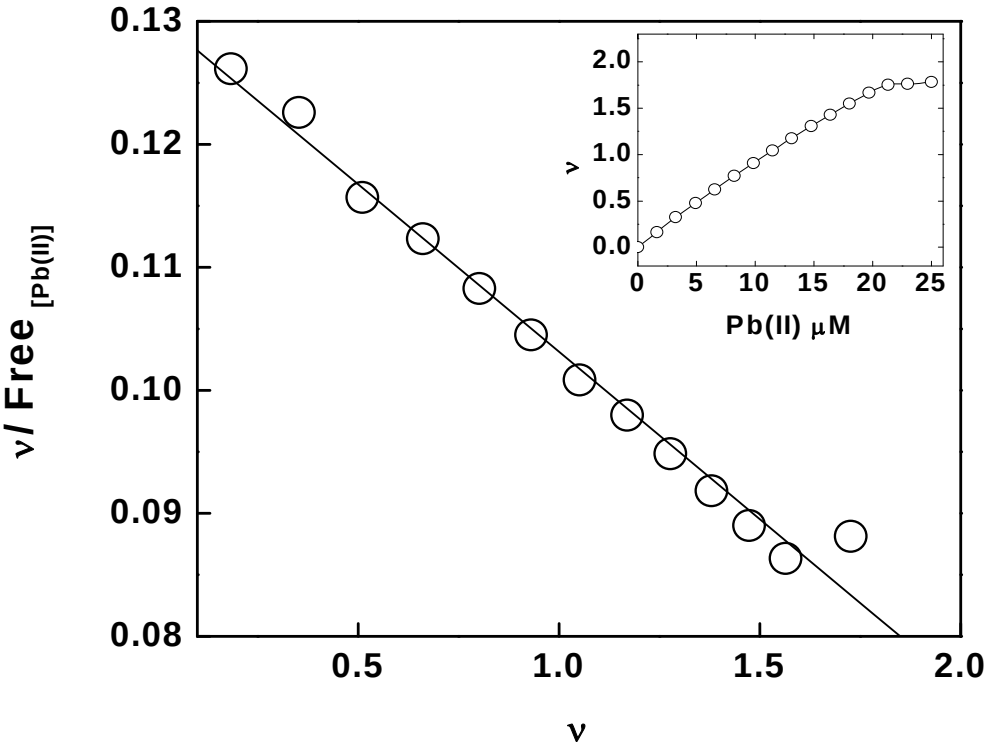

Fig.47. Scatchard plot analysis of α-casein interaction with Pb(II) as monitored by spectroscopic titration at 277 nm. α-Casein (4×10^{-5}M) was titrated with 10 μl of Pb(II) (1.0×10^{-4} M) in 0.02 M tris-HCl buffer pH 6.8 at 25°C. Inset shows binding isotherm of α-casein with Pb(II) and ν is moles of ligand bound to protein.

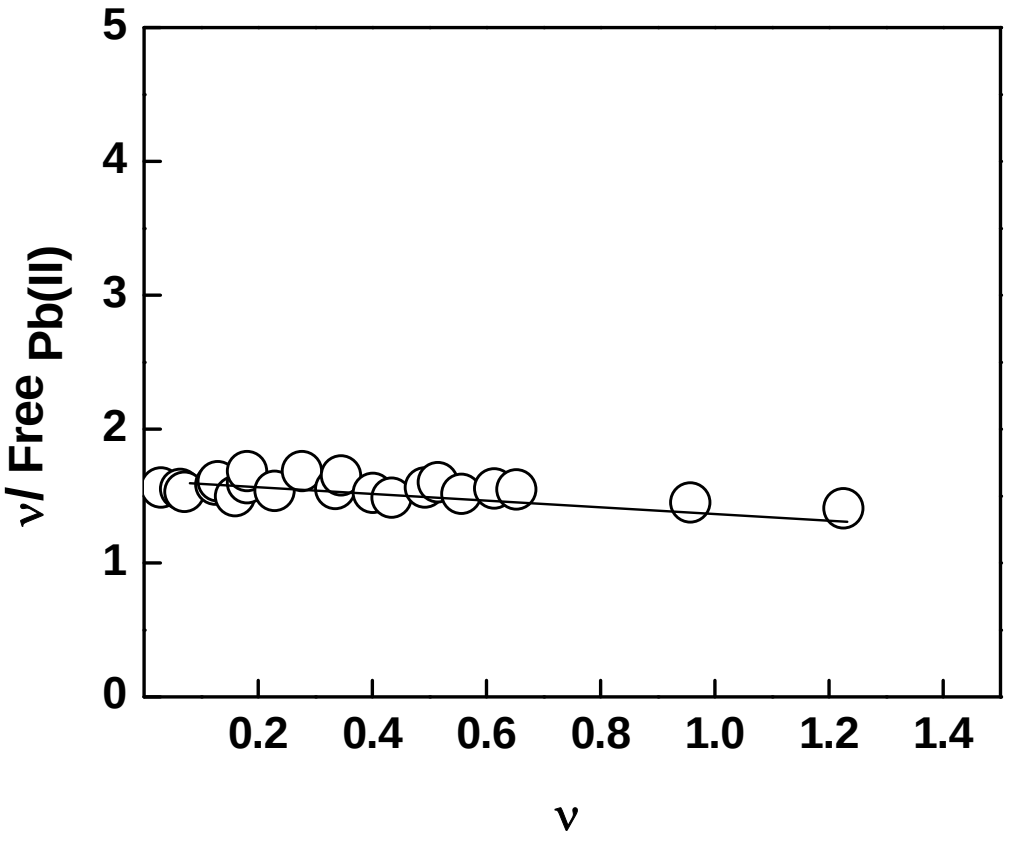

Fig.48. Scatchard plot analysis of α-casein interaction with Pb(II) as studied by equilibrium dialysis. Five-milliliter of α-casein (1.83×10^{-4} M) was dialyzed against 10 ml of Pb(II) (1.0×10^{-8} M to 1.0×10^{-4} M) in 0.02 M tris - HCl buffer pH 6.8 containing 0.17 M NaCl for 24 h at 25°C with shaking at 75 rpm for the entire period. The concentrations of free Pb(II) in equilibrium were determined by flame atomic absorption spectroscopy.

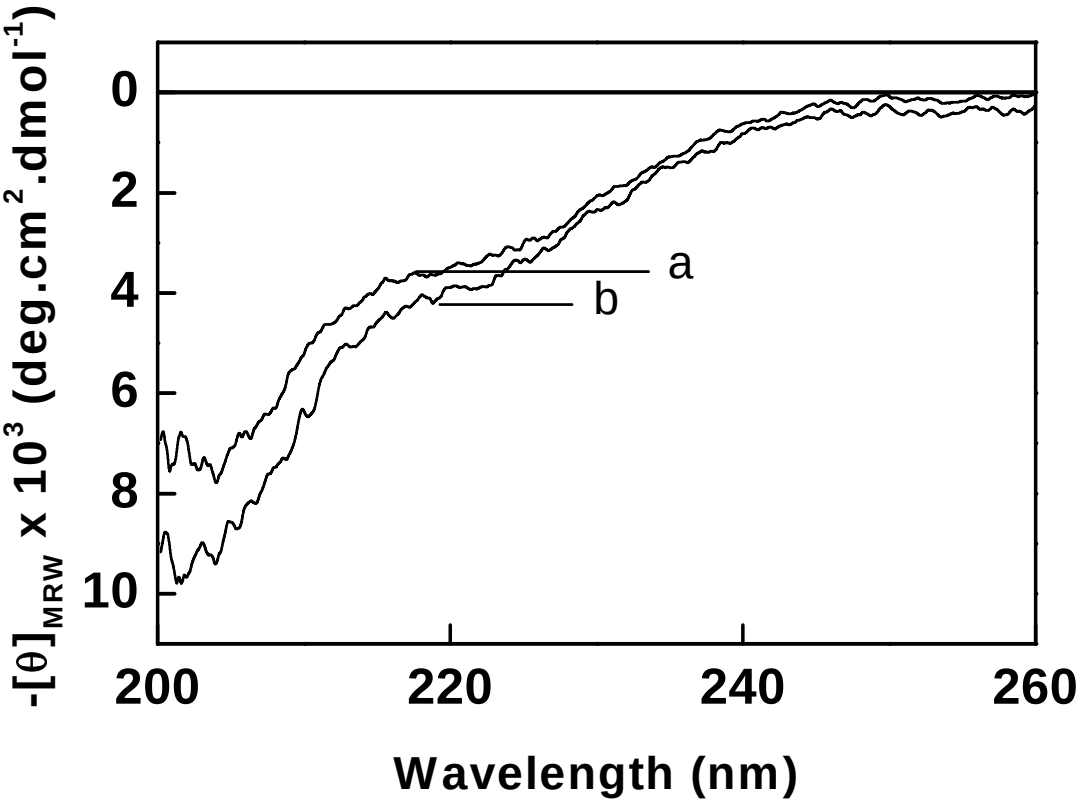

Fig.49. Effect of Pb(II) on the far UV - CD spectra of α-casein (a) α-casein in buffer (0.02 tris - HCl buffer, pH 6.8) and (b) 9.5×10^{-5} M Pb(II). The protein concentration of 1×10^{-5} M was used for all the experiments.

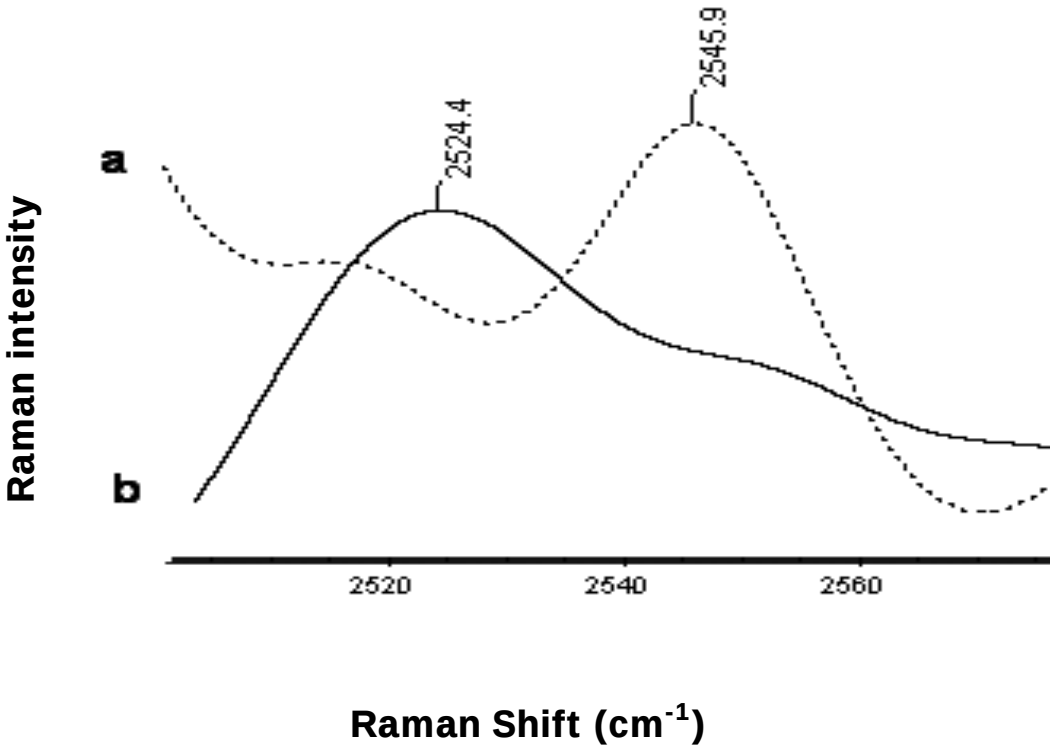

Fig.51. Raman Spectra of (a) α-casein in buffer and (b) α-casein Pb(II) complex as studied from 2500 - 2600 cm^{-1}. The α-casein concentration of 4.2×10^{-4} M was used for all the experiments. The Raman spectrum was recorded at excitation of 1064 nm laser with a laser power of 1.5W.

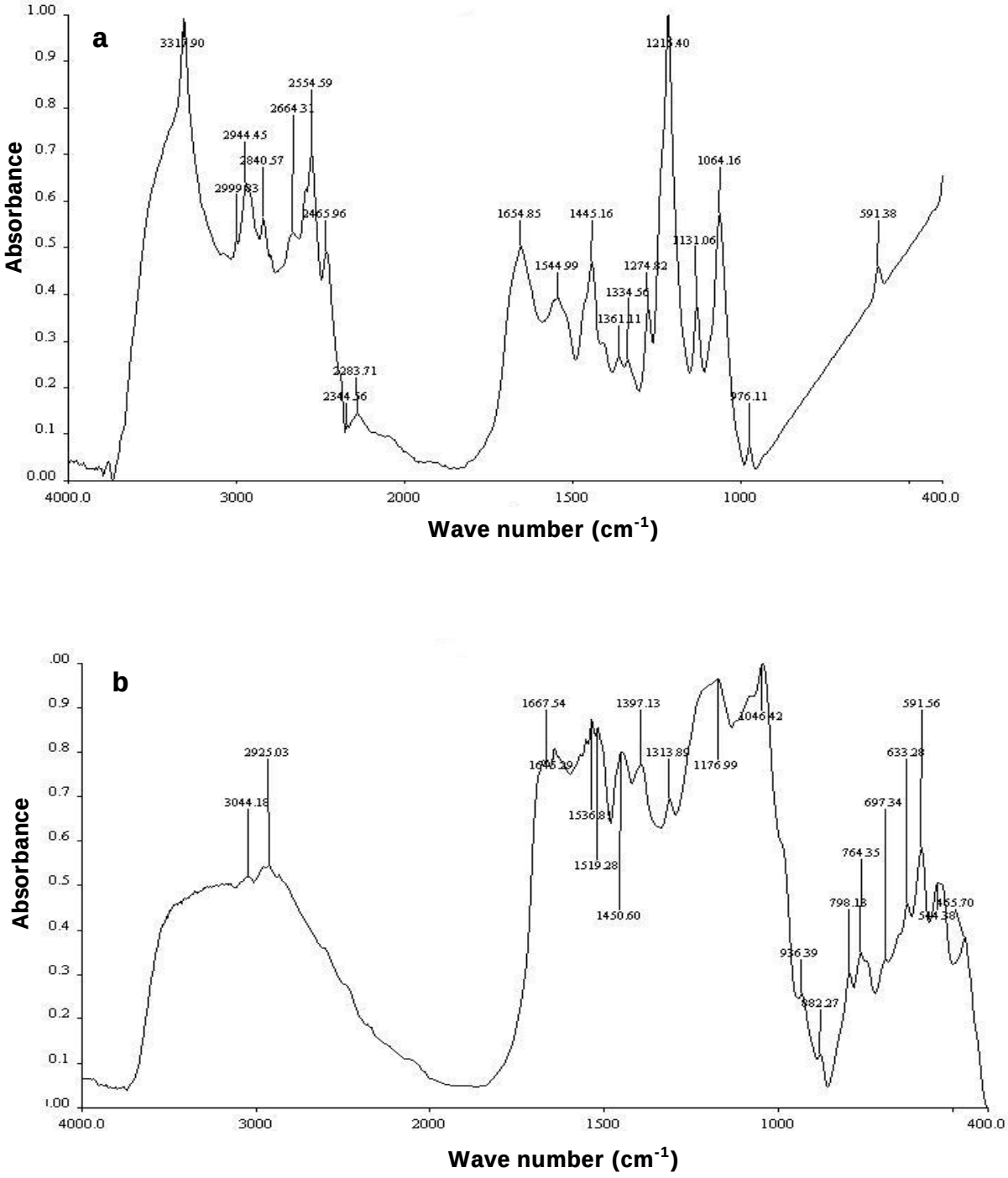

Fig.52. Effect of Pb(II) on Fourier transform-infra red spectra of (a) α-casein and (b) α-casein-Pb(II) complex as scanned from 400 to 4,000 cm^{-1} in KBr. A protein concentration of 4.2 x 10^{-4} M was used for all measurements.

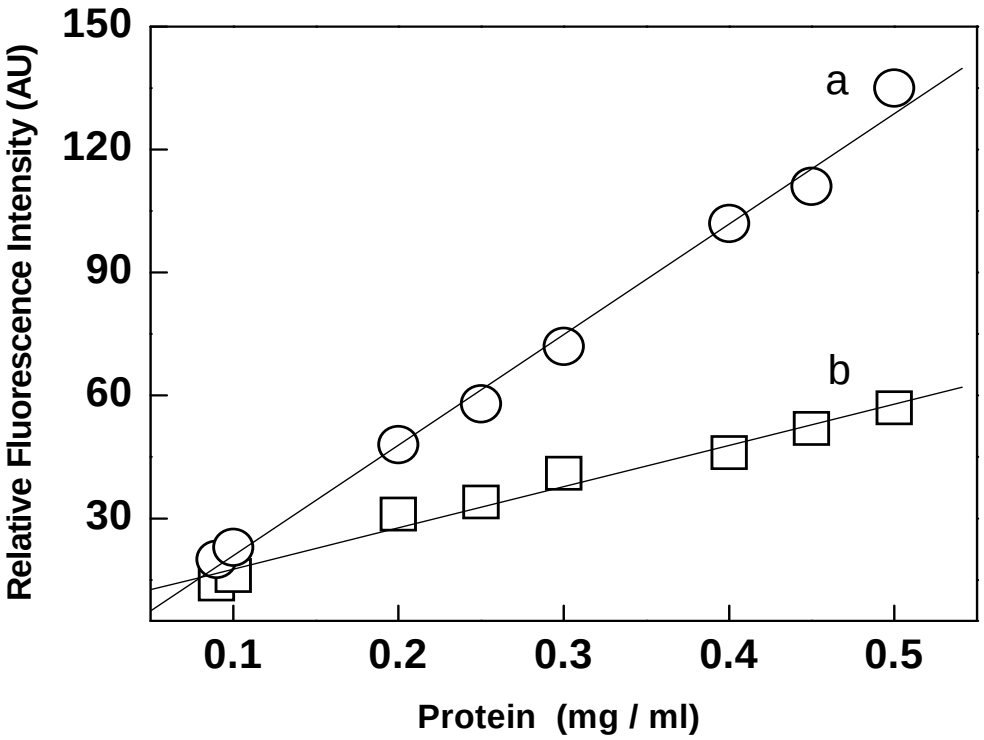

Fig.53. Fluorescence studies of surface hydrophobicity of (a) α-casein in buffer and (b) α-casein-Pb(II) complex as studied by ANS binding on Spectrofluorimeter The ANS stock solution of 10 mM was used. A protein concentration of 0.008-0.03 % was incubated with 10 μl ANS for 3 h and spectra recoded. The protein was excited at 375 nm and emission spectrum was recorded at 400-600 nm. The excitation and emission slit widths of 5 nm was used for the experiments.

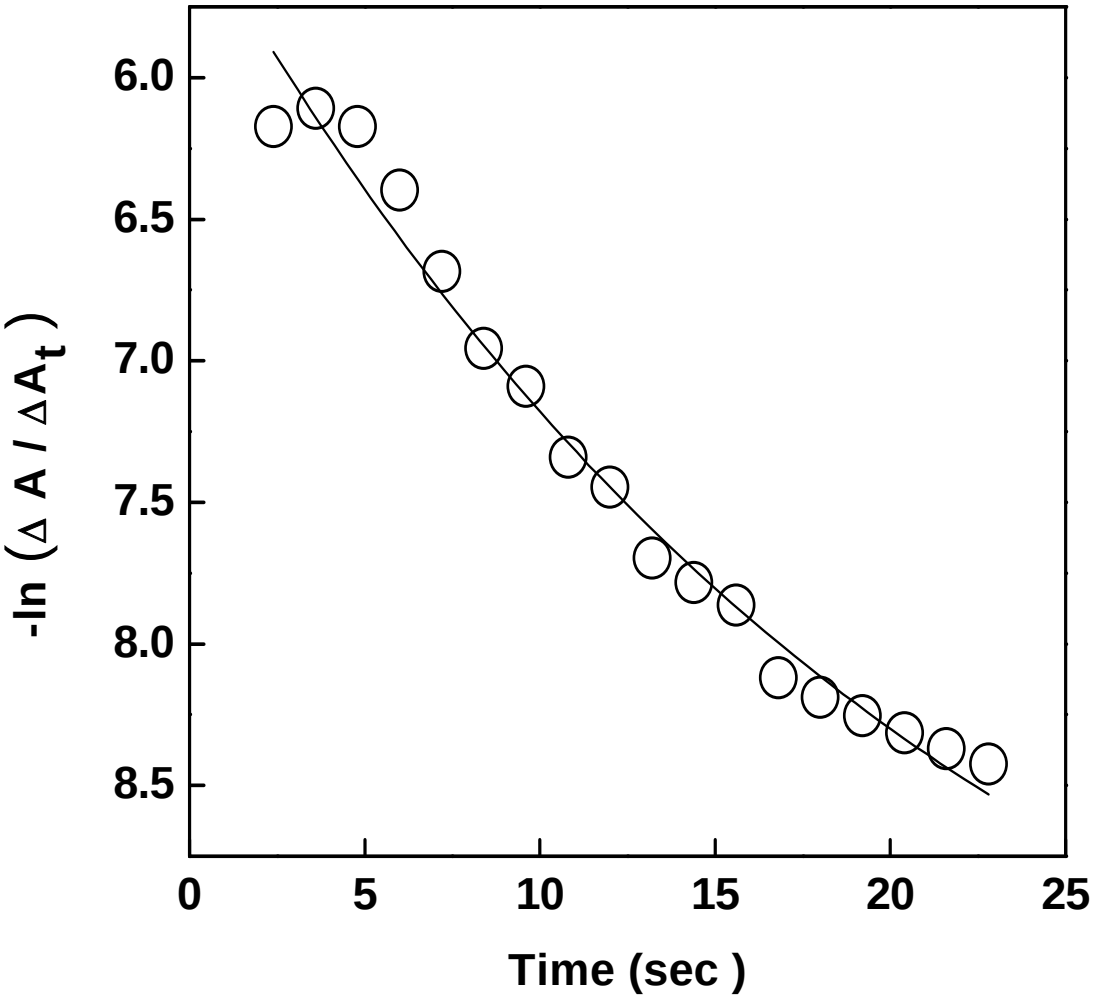

Fig.54. Slow kinetics of interaction of α-casein with Pb(II) as followed on a UV-visible spectrophotometer at 250 nm. The reaction is followed up to 25 s at 25°C. The Pb(II) concentration of 1x 10^{-5} M (0.02 M tris HCl, pH 6.8) and α-casein concentration of 1.8 x 10^{-5} M was used for the experiments. The slope of the curve gives the rate of the reaction.

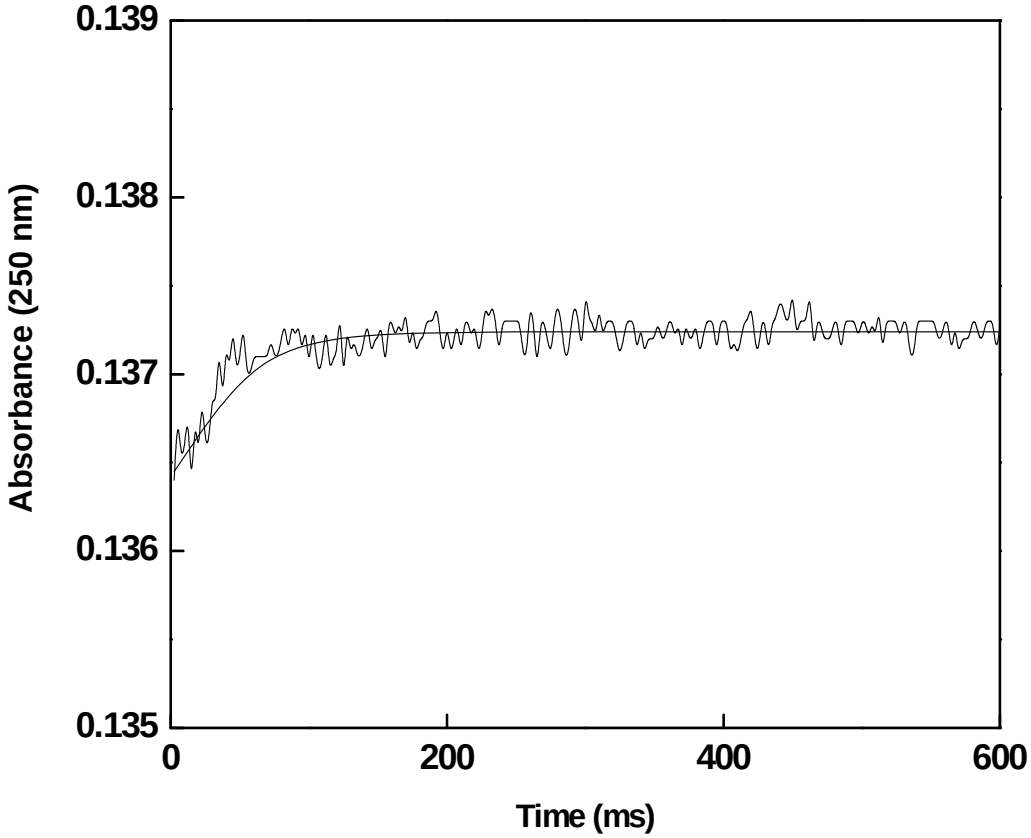

Fig.55. Fast kinetics of interaction of α-casein with Pb(II) as followed on a stopped flow spectrophotometer at 250 nm at 25^{o}C. The Pb(II) concentration of 1×10^{-4} M (0.02 M tris- HCl, pH 6.8) and α-casein concentration of 2×10^{-5} M was used for the experiments.

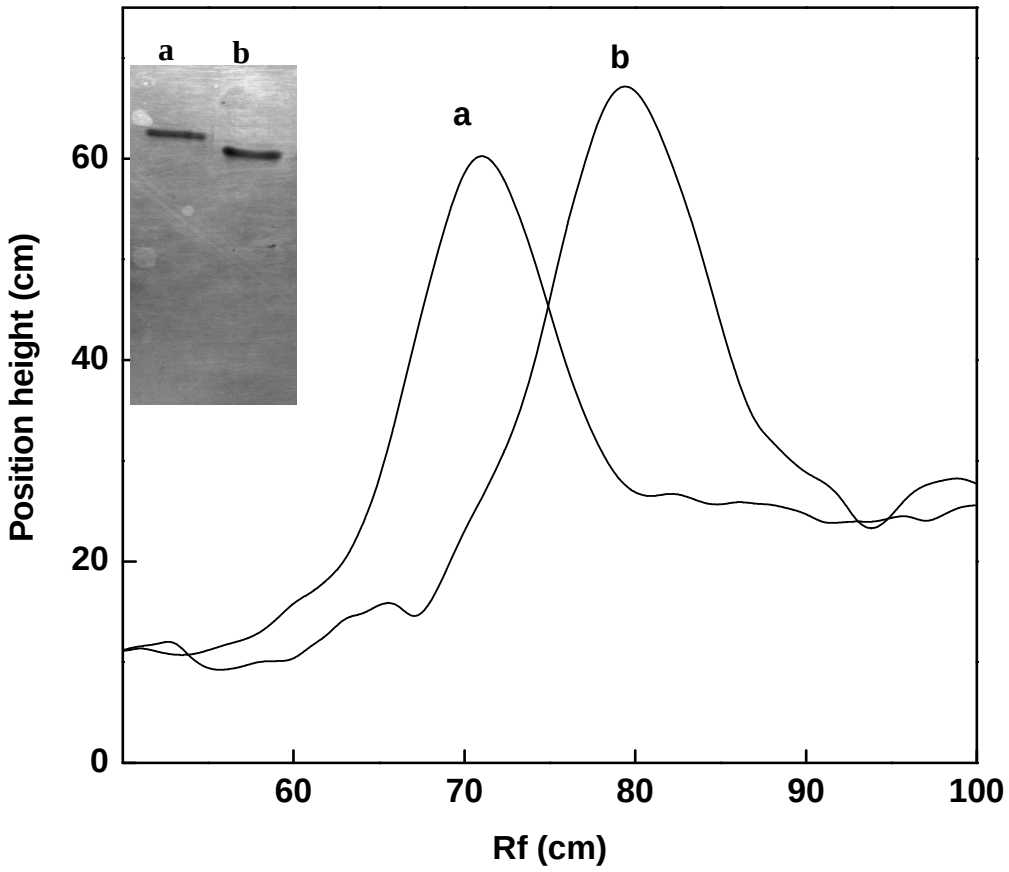

Fig.57. The AMS (4-acetamido-4'-maleimidyl stillbene-2, 2'-disulphonic acid) modification of free cysteines of α-casein. The relative mobility plot is generated using profile match program of the image acquisition software Syngene tools from Synoptics technologies, UK. Inset shows non-reducing 15 % SDS-PAGE, (a) native protein and (b) α-casein modified with AMS (addition of 980 Da).

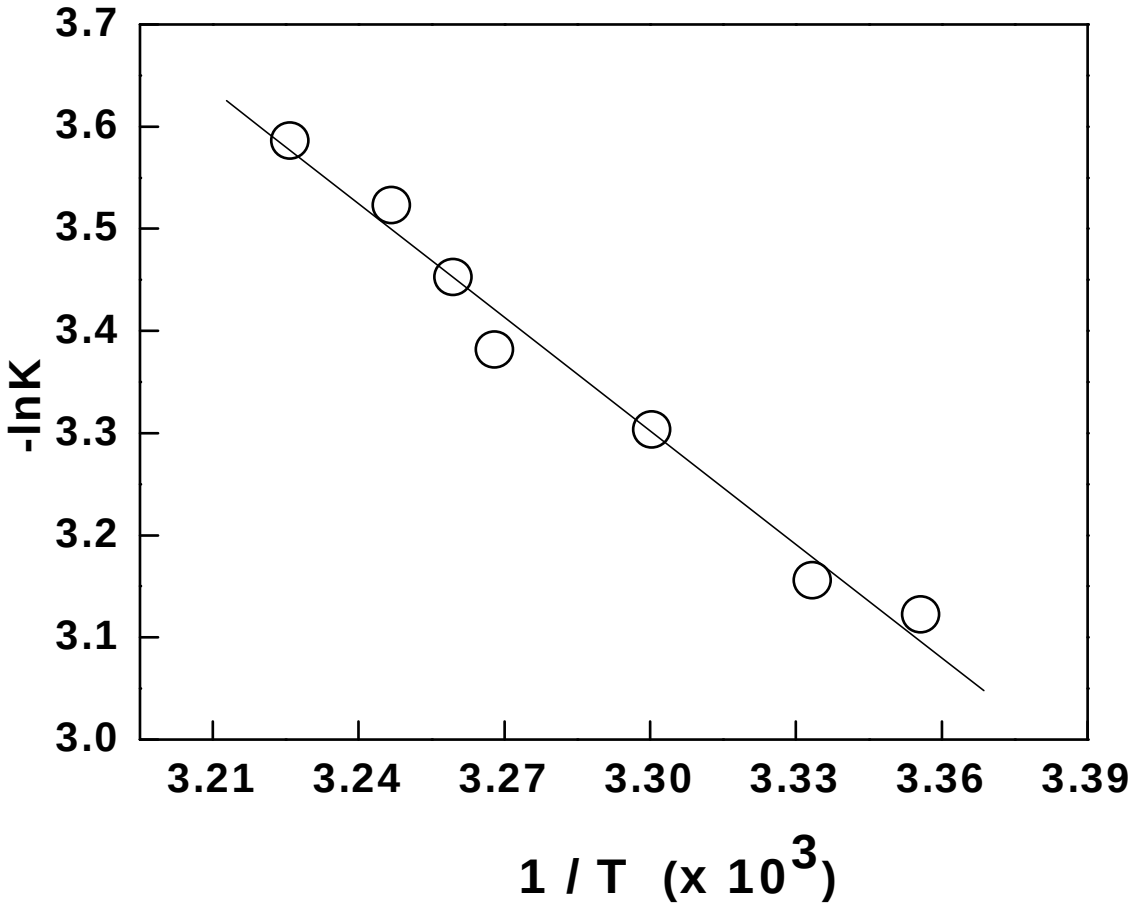

Fig.58. Effect of temperature on the reaction rates of Pb(II) interaction with α-casein. van't Hoff plot was constructed to show the thermodynamic parameters and reaction energetics of binding as studied on stopped flow spectrophotometer. The temperatures were in the range of 25 - 40°C.

SUMMARY
AND
CONCLUSIONS

Summary and Conclusions

α-Casein group makes up to 65% of the total casein and consists of α_{S1}- caseins, α_{S2}- caseins with a molecular weight of 27,300 Da. These two proteins are closely related and have common properties in their phosphorous content (1%), C and N terminal groups. Caseins are easily degradable proteins due to their random coil structure.

The present investigation entitled **"The interaction of cosolvents, proteolytic enzymes and selective metal ions with α-casein - the structure - stability relationship"** is divided into four chapters.

Chapter I describes isolation and characterization of biologically active peptides from α-casein. The study includes the hydrolysis of α-casein by various proteolytic enzymes and purification and structure – function studies of the biologically active peptides.

Chapter II describes effect of cosolvents on the stabilization process of α-casein and its biologically active peptides. The stabilization process involved the use of various biophysical techniques like fluorescence and UV difference spectra to monitor changes in α-

casein microenvironment. Effect of cosolvents on the biological activity of the peptides was also studied.

Chapter III describes interaction of zinc with α-casein. The study includes identifying number of binding sites by equilibrium dialysis and fluorescence titration. The changes in secondary structures were followed by CD and IR spectroscopy. The kinetics of formation of complex was monitored by stopped flow. The tertiary structural changes were studied by ANS surface hydrophobicity. The complex digestibility by proteolytic enzymes and reversibility were also studied.

Chapter IV describes interaction of lead with α-casein. This study involves identifying the number of binding sites by spectrophotometric titration and by equilibrium dialysis. Secondary structural changes were studied by CD, IR and Raman spectroscopy. Kinetics of the complex formation was monitored by stopped flow spectrophotometer. Effect of temperature on the kinetics was also analyzed. Finally chemical modification of the cysteines was studied which will assist in understanding the possible sites of interaction of the ligand to the protein.

Isolation, purification and characterization of α-casein from bovine milk was carried out and found to be 94% pure. The isolated α-casein was correlated with standard and found to be comparable.

Among the proteases aminopeptidase, carboxypeptidase, chymotrypsin, fungal protease, and bacterial protease employed for hydrolysis of α-casein, chymotryptic hydrolysates were found to be more active against angiotensin converting enzyme (ACE). The crude hydrolysates show ACE inhibitory activity, PEP-inhibitory activity, antioxidant capabilities, antimicrobial activities and mineral binding ability. The peptide shows growth inhibiting activity on pathogenic bacteria like *B. cereus* and *E. coli*, whereas they promote the growth of probiotic bacteria like *L. acidophilus*. Sequencing of peptides by MALDI-TOF reveals 2 peptides with molecular weights of 1204 and 1718 Da. and with sequences QKALNEINQF, TKKTKLTEEEKNRL from α_{S2}-casein f(94-103) and α_{S2}- casein f(163-176) respectively.

The peptides were synthesized on an automated solid phase synthesizer in F-moc mode. The ESI mass spectra of peptide show 98% purity. The synthesized peptides were also able show multifunctional activities like ACE inhibitory, antioxidant capabilities, PEP-inhibitory, antimicrobial, antitumor and anti-ulcer activities. Peptide 10 shows anti-ulcer activity whereas peptide 14 is devoid of the anti-ulcer activity, which could be due to the presence of hydrophobic residues in peptide 10. The peptides were not shown any antifungal activity, could be due to the thick cellulose, glucosamine and chitinaceous fungal cell wall. The peptides were found to be more potent compare to their respective standards. There is no

haemagglutination activity in peptides and they were found to be inactive on red blood cells. The mode of anti-microbial action of peptide 10 is due to depolarization of cell membrane whereas peptide 14 is due to its DNA-binding ability. The gel shift mobility test has proved that the aggregated peptide DNA complex retains in the well whereas the native DNA moves normally. Peptide 14 shows, binding to bacterial DNA and to calf thymus DNA. The binding to negatively charged DNA could be due to the presence of basic amino acid residues present in peptide 14.

The structural characterization of the peptides was monitored by CD and NMR. The structural studies by NMR show that the peptides are unstructured in solution. The presence of highly random and flexible structures in peptides is responsible for their multi-functional activities. The presence of biological activity with peptides could be due to the propensity to form structures in various environments. The peptides can be induced to form structures in various environments. The three dimensional theoretical modeling of peptides shows aperoidicity with little regular structure. α-Casein contains potential multiple functional peptides, which are due to the presence of overlapping sequences present hidden in the α-casein.

The structural studies of α-casein in presence of sucrose and sorbitol shows no changes in the structure, whereas the trehalose and

glycerol bring in minute and insignificant structural perturbations, which is due to the changes in the microenvironment of α-casein. Addition of cosolvents has not altered the structure of α-casein. The two peptides QKALNEINQF and TKKTKLTEEEKNRL from α_{S2}-casein f(94-103) and α_{S2}-casein f(163-176) are synthesized and their biological activities were analyzed in presence of cosolvents. The peptide 10 shows increase in the ACE inhibitory activity in presence of 20% sucrose where as the peptide 14 is stabilized in presence of 20% sorbitol. Peptide 10 is showing more antioxidant activity in presence of 20% glycerol where as peptide 14 in presence of 20% sucrose. The presence of cosolvents has increased the activity of peptides at higher temperatures. This shows that the peptides are thermostable for the activities tested at higher temperatures. The presence of cosolvents might have induced the secondary and tertiary structures in the peptides, which in turn enhanced their biological activities. The activity of peptides in presence of sucrose could be due to the compaction of the peptides. The stabilizing effect of polyols could be due to preferential hydration phenomenon.

Binding of zinc to α-casein was studied by equilibrium dialysis and shows two binding sites with 5.0 ± 1.0 and 12.0 ± 2.0 and association constants 0.2 ± 0.03 x 10^6 M^{-1} and 2.7 ± 0.3 x 10^6 M^{-1} respectively. The changes in the secondary structure as evident from the studies of far UV CD, shows 7.5 ± 3.0 % increase in α-helix and

10.0 ± 2.0 % increase in β-structures upon binding with zinc. Surface hydrophobicity studies of α-casein in presence of zinc show changes in the tertiary structure. The rapid kinetics of binding elucidates the pseudo-first order rate with a rate constant of 40 s^{-1}. The reversibility studies show zinc has a two binding sites on α-casein. The degree of hydrolysis of the complex shows enhanced digestibility. This interaction leads to formation of a stable complex, with α-caseins. The binding of zinc to two different sites was identified in our study, which may be phosphoserines, and histidines present in α-casein.

The interaction of lead with α-casein as studied by spectroscopic titration shows that Pb(II) has 2.0 ± 0.05 binding sites with an association constant (k_a) of 2.3 ± 0.2 × 10^5 M^{-1}. The far-UV CD spectra shows decrease in the β-structures by 16 ± 3% and increase in the aperiodicity by 15 ± 2% in presence of Pb (II). Raman spectra of α-casein – Pb(II) complex shows formation of aperiodic/ poly (L-proline) II structures, reduction in the amide I region, and perturbations in the sulfhydryl region of α-casein. Stopped flow studies on the reaction mechanism of Pb(II) α-casein complex follow pseudo-first order reaction with a rate of 25 s^{-1}. The time resolved spectra shows formation of peaks at 330 nm and 360 nm, with in 125 ms correlating to Pb(II) – thiolate, ligand to metal charge transfer bands in the UV spectral region. Thus interaction of Pb(II) with α-casein brings structural perturbations in the molecule. The studies on the kinetic

rates at different temperatures shows formation of Pb(II) α-casein complex is temperature dependent. Modification of cysteines with AMS and iodoacetamide shows absence of lead binding ability in α-casein. From the above studies we can conclude that lead binds to cysteines present in α-casein.

α-Casein and its biologically active peptides were studied for structure function and stability with the help of proteolytic enzymes, cosolvents and metal ions.

The effects of proteolytic enzymes have shown that hydrolysis of α-casein yields potential multi functional biologically active peptides. Crude peptides and their synthesized counter parts show multi-functional biological activities. Pancreatic enzymes containing exo- and endo- peptidases were not able to digest these peptides. Peptides are also found to be stable at high temperatures in presence of cosolvents. These peptides can be incorporated as a probiotics in functional foods. From the above studies we can formulate a future pharmaceutical or nutraceutical agents.

Studies on interaction of micronutrient zinc with α-casein have shown zinc forms stable complexes with α-casein, which can be incorporated in to milk and it's by-product fortification. The studies on the interaction of α-casein with toxic metal lead will helps to

understand the mechanisms of binding to milk proteins and can be utilized in devising ways to prevent such interactions.

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SYNOPSIS

OF

The Ph.D. Thesis Entitled

THE INTERACTION OF COSOLVENTS, PROTEOLYTIC ENZYMES AND SELECTIVE METAL IONS WITH α-CASEIN -THE STRUCTURE - STABILITY RELATIONSHIP

Submitted to the University of Mysore, Mysore

for the degree

of

Doctor of Philosophy

In

Biotechnology

By

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Title of the Ph.D. Thesis:

The Interaction of Cosolvents, Proteolytic Enzymes and Selective Metal Ions with α-Casein - The Structure - Stability Relationship

Caseins are the predominant milk proteins present in all mammalian species. They comprise approximately 80% of the total protein content in milk. They constitute a heterogeneous group of phospho-proteins present as stable calcium phosphate complexes termed micelles. The biological functions of the caseins are to provide the progeny with a source of phosphate and calcium for the mineralization process of calcified tissues, amino acids and biologically important peptides. The principal proteins in this group are α_{S1}-casein, α_{S2}-casein, β-casein, and κ-casein classified according to the homology of their primary structures.

In the present study, effect of cosolvents, proteolytic enzymes and metal ions on α-casein structure and function and stability was studied. Bovine milk α-casein constitutes 65% of total casein. α-Casein contains a mixture of α_{S1} casein and α_{S2} casein in the ratio of 4:1. This group consists of closely related phospho-proteins, with a molecular weight of 27,300 Da.

α-Casein is easily degradable protein due to its random coil structure. α-Casein are grouped under intrinsically unstructured proteins. The aperoidicity of the protein is due to the presence of 8.5% proline residues uniformly distributed in the polypeptide chain. The flexibility and randomness in the structure of α-casein, is a reason for the release of many potential

peptides with various biological activities, when hydrolyzed by proteolytic enzymes. The effect of proteolytic enzymes on the release of biologically activity peptides was studied. The part of the study is to screen for the novel multifunctional peptides from α-casein, which can be incorporated into nutraceutical and functional foods. The structure-function studies of these peptides will reveal their mechanism of biological activity and mode of action.

The interaction of cosolvents with proteins and peptides will help us to stabilize them for their optimum utilization. Effect of different cosolvents on the structural stability is investigated in order to understand the basic mechanism of stabilization of α-casein, as a result of microenvironment changes brought about by these cosolvents. Effect of different cosolvents on the functional stability is investigated to optimize their performance at extremes of temperatures. The biological activity of peptides was also studied in presence of various cosolvents for their functional optimization.

The studies on the interaction of essential and toxic metals ions with proteins will help us to understand binding mechanism. The present study is focused to address the question regarding the structure-function relationships of α-casein in presence of metal ions like zinc and lead.

With these objectives, the present investigation is undertaken and the results and conclusions are brought out in the form of a thesis entitled

"The interaction of cosolvents, proteolytic enzymes and selective metal ions with α-casein -the structure - stability relationship".

The investigation is divided into following chapters:

I. Isolation and characterization of biologically active peptides from α-casein.

II. Effect of cosolvents on the stabilization process of α-casein and its biologically active peptides.

III. Interaction of zinc with α-casein.

IV. Interaction of lead with α-casein.

The result 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 in to introduction, scope & objectives, materials & methods, summary & conclusions and references along with tables and figures.

Chapter I: Isolation, purification and characterization of α-casein from bovine milk were carried out and found to be 94 % pure. The amino acid composition of the isolated α-casein was correlated with standard (sequence

based) and found to be comparable. Among all the proteases, aminopeptidase, carboxypeptidase, fungal protease, bacterial protease and chymotrypsin employed for the hydrolysis of α-casein, chymotryptic hydrolysates were found to be possessing maximum angiotensin converting enzyme inhibitory activity (ACE). These chymotryptic hydrolysates were purified from the unhydrolysed protein and the hydrolysates show multiple biological activities like ACE inhibitory activity, Prolyl endopeptidase inhibitory activity, antioxidant capabilities, antimicrobial activities and mineral binding ability. The hydrolysates show growth inhibiting activity on pathogenic bacteria like *B. cereus* and *E. coli*, whereas they promote the growth of probiotic bacteria like *L. acidophilus*. The hydrolysates were purified by HPLC methods. The purified peptides were sequenced by MALDI-TOF and the pattern reveals two peptides with molecular weights of 1204 and 1718 Da. with sequences QKALNEINQF (peptide 10) and TKKTKLTEEEKNRL (peptide 14) from α_{S2}-casein f(94-103) α_{S2}-casein f(163-176) respectively.

The peptides were synthesized on an automated solid phase synthesizer using F-moc chemistry to confirm their biological activities in pure form. The ESI mass spectra of synthesized peptides show 98% purity. The purity of the synthesized peptides was also confirmed by amino terminal sequencing and by reverse phase HPLC. The synthesized peptide shows multifunctional activities like ACE inhibitory, antioxidant capabilities, PEP-inhibitory, antimicrobial, antitumor and anti-ulcer activities. The peptides were not inhibiting the fungal growth or production of fungal mycotoxins, which could

be due to presence of thick chitinaceous cell wall in fungi. There is no haemagglutination activity found in peptides. The peptides were found to be more potent compared with their respective standards. The antimicrobial activity of the synthesized peptides was studied in presence of *Bacilus cereus, Escherichia coli*, *Staphylococcus aureus* and *Listeria monocytogenes*. The peptides were showing growth inhibitory activity on two pathogenic organisms like *B. cereus* and *S. aureus* respectively. Further studies on the mechanism of anti-microbial action of peptides on *B.cereus* were studied. The antimicrobial activity of peptide 10 on *B.cereus* was due to depolarization of cell membrane whereas peptide 14 was due to its DNA-binding ability. The presence of basic amino acid residues in peptide 14 could be reason for the DNA binding activity, where as peptide 10 cell penetrating behavior could be due to its amphipathicity.

The characterization of the peptides was followed by CD and NMR. Modeling of the peptides was done using *pepstr* software. Three dimensional theoretical modeling of peptides shows aperoidicity with little regular structure. The presence of multifunctional biological activity in peptides could be due to the flexible backbone conformation and possibility of inducing structure in different environments.

Chapter II: Effect of cosolvents on α-casein and its bioactive peptides were studied in presence of sucrose, trehalose, sorbitol and glycerol. Sucrose and sorbitol are structure stabilizers with no changes in the tertiary structure

of α-casein, whereas the trehalose and glycerol at higher concentrations brings in minute structural perturbations in the microenvironment of α-casein.

The ACE inhibitory and antioxidant activity of synthesized peptides were studied in presence of 20% sucrose, sorbitol, trehalose and glycerol, respectively. The peptide 10 shows increase in the ACE inhibitory activity in presence of 20% sucrose where as peptide 14 is stabilized in presence of 20% sorbitol. The peptide 10 shows more antioxidant activity in presence of 20% glycerol where as peptide 14 shows in presence of 20% sucrose. The activity of peptides in presence of sucrose could be due to the micro alteration in the secondary structure of the peptide.

Cosolvents are used to increase stability of proteins and thermal stability of biologically active peptides. Cosolvents prevent the inactivation of proteins/peptides exposed to adverse conditions of temperatures and pH. In addition, study of interaction of proteins/peptides in presence of cosolvents helps in understanding the structure – function - thermodynamic energy of the peptides in solution.

Chapter III: Binding of zinc to α-casein was studied by equilibrium dialysis and shows two binding sites 5.0 ± 1.0 and 12.0 ± 2.0 with association constants (k_a) of 0.2 ± 0.03 x 10^6 M^{-1} and 2.7 ± 0.03 x 10^6 M^{-1} respectively. The fluorescence spectral changes as monitored by titration with zinc and α-casein shows an association constant of 1.2 ± 0.3 × 10^6 M^{-1}. The

secondary structural changes show not so significant changes at lower concentration of zinc (1 x 10^{-6} M) and at higher concentration of zinc (1 x 10^{-5} M) there is significant change in the β-structure content with concomitant decrease in aperiodic structure. The IR spectrum reveals formation of ordered structures in presence of zinc.

The surface hydrophobic studies by ANS show changes in the perturbation of the aromatic residues in the molecule. The UV difference spectrum shows perturbations in the α-casein due to the binding of zinc. The kinetics of binding as analyzed by stopped flow spectrophotometer and UV-visible spectrophotometer indicates the pseudo-first order rate of 40 s^{-1} and 37 s^{-1} respectively. The reversibility studies of zinc binding to α-casein shows the presence of perhaps two different affinity sites in α-casein for zinc. The hydrolysis of the complex and the native proteins by fungal protease and chymotrypsin respectively shows increased hydrolysis of the complex as compared to control. Such interaction of zinc leads to formation of a stable complex with α-casein. The ability of the zinc to stabilize the structure of casein and bring about changes in other structural parameters may pave way for better understanding of zinc-protein complex in general.

Chapter IV: The interaction of Pb(II) with α-casein is studied by spectrophotometric titration and shows at least 2.0 binding sites with an association constant (k_a) of 2.3 ± 0.2 × 10^5 M^{-1}. The equilibrium dialysis also confirms 2.0 binding sites for the protein with an association constant of

k_a 2.3 ± 0.2 × 10^5 M^{-1}. The far - UV circular dichroism spectra shows no significant changes in the secondary structure of the protein over a ten fold change in Pb(II) concentration. Raman spectra of α-casein – Pb(II) complex reveals formation of poly (L-proline) type II structures and reduction in the amide I region along with perturbations in the sulfhydryl region of α-casein which is also confirmed by IR spectrum.

Stopped flow kinetic studies reveals that the reaction mechanism of Pb(II) follows a pseudo-first order rate of 25 s^{-1}. The stopped flow time resolved spectra shows formation of peaks at 330 nm and 360 nm. The studies on the kinetic rates at different temperatures describes the formation of Pb(II)-α-casein complex is temperature dependent. Modification of cysteine residues with 4-acetamido-4'-maleilidyl stillbene-2,2'-disulphonic acid and iodoacetamide substantiates to the absence of lead binding ability of α-casein indicating that cysteine could be probably one of the Pb(II) binding sites as binding of lead also leads to perturbation of spectra of the aromatic amino acids.

Summary and conclusions depicts the salient features of the present investigation. The literature cited in the text is arranged in alphabetical order and the references, gives 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, India.

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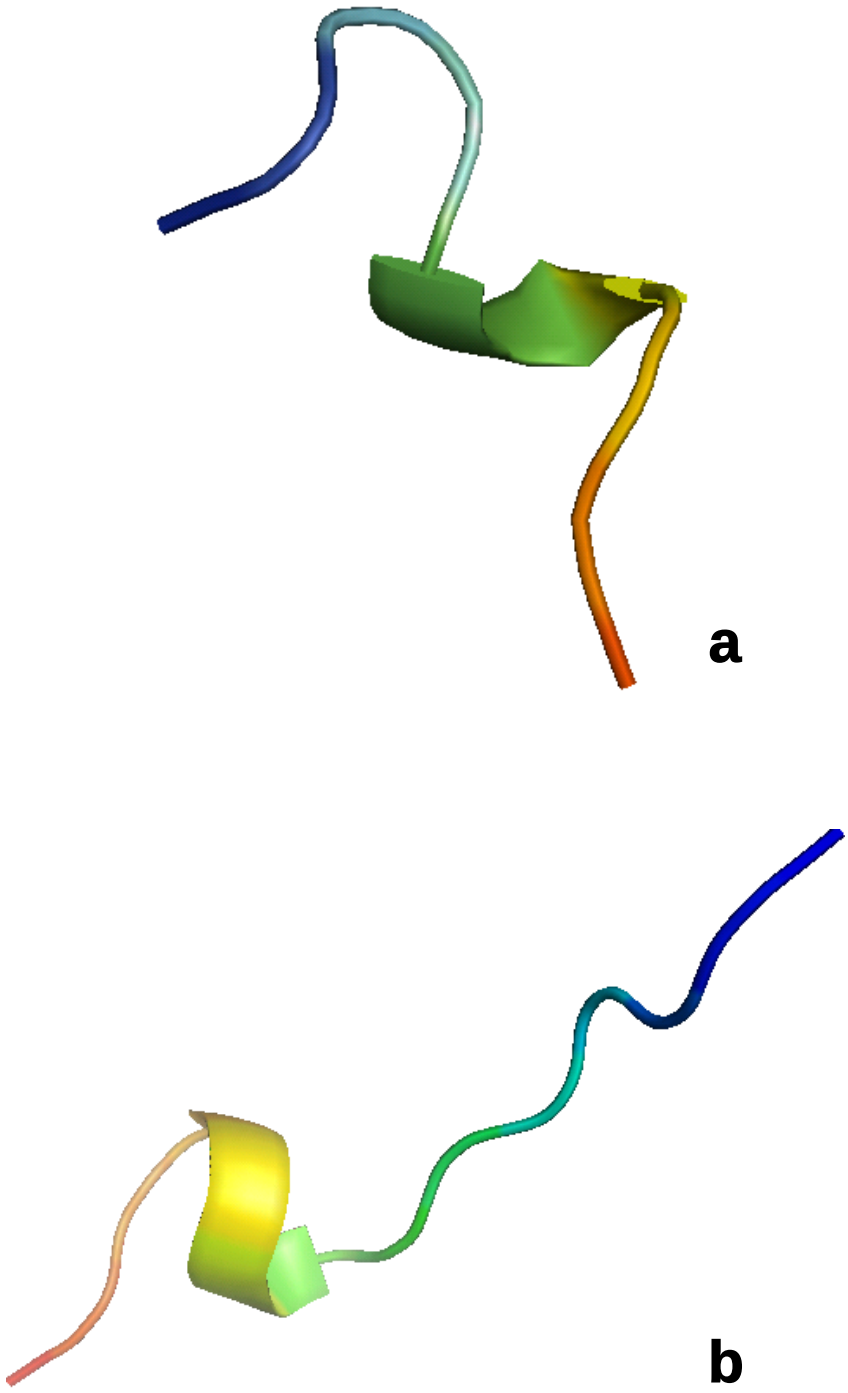

Fig.20. The three-dimensional model of peptides. The three dimensional view was generated with *Pymol* software using the coordinates of the peptide. The coordinates were obtained using *pepstr* software. (a) Peptide 10 (b) Peptide 14

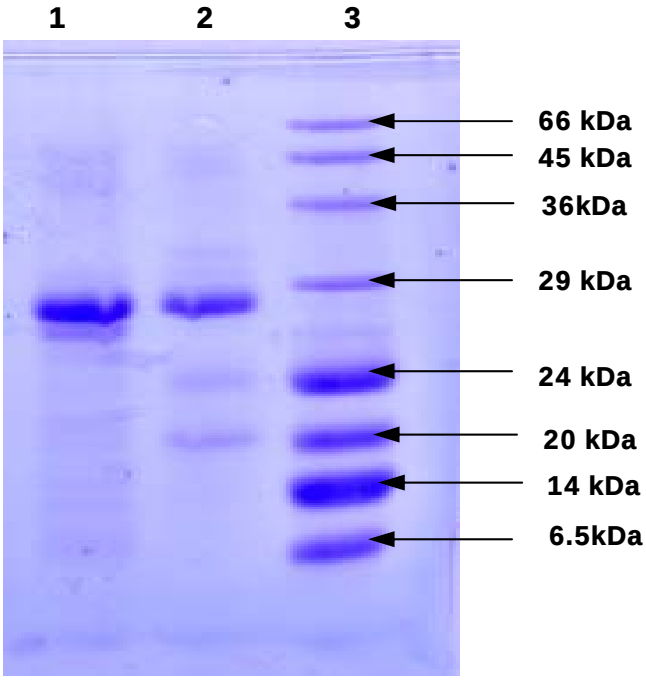

Fig.3. SDS - PAGE pattern of isolated α-casein from bovine milk. α-Casein was run on 12% SDS – PAGE. The lane 1 shows α-casein containing two bands α_{S1}-casein and α_{S2}-casein. The lane 2 shows total casein and lane 3 shows molecular weight markers BSA (66 kDa); ovalbumin (45 kDa); glyceraldehydes-3-phosphate dehydrogenase (36 kDa); carbonic anhydrase (29 kDa); trypsinogen (24 kDa); soybean trypsin inhibitor (20 kDa); α-lactalbumin (14 kDa); aprotinin (6.5 kDa).

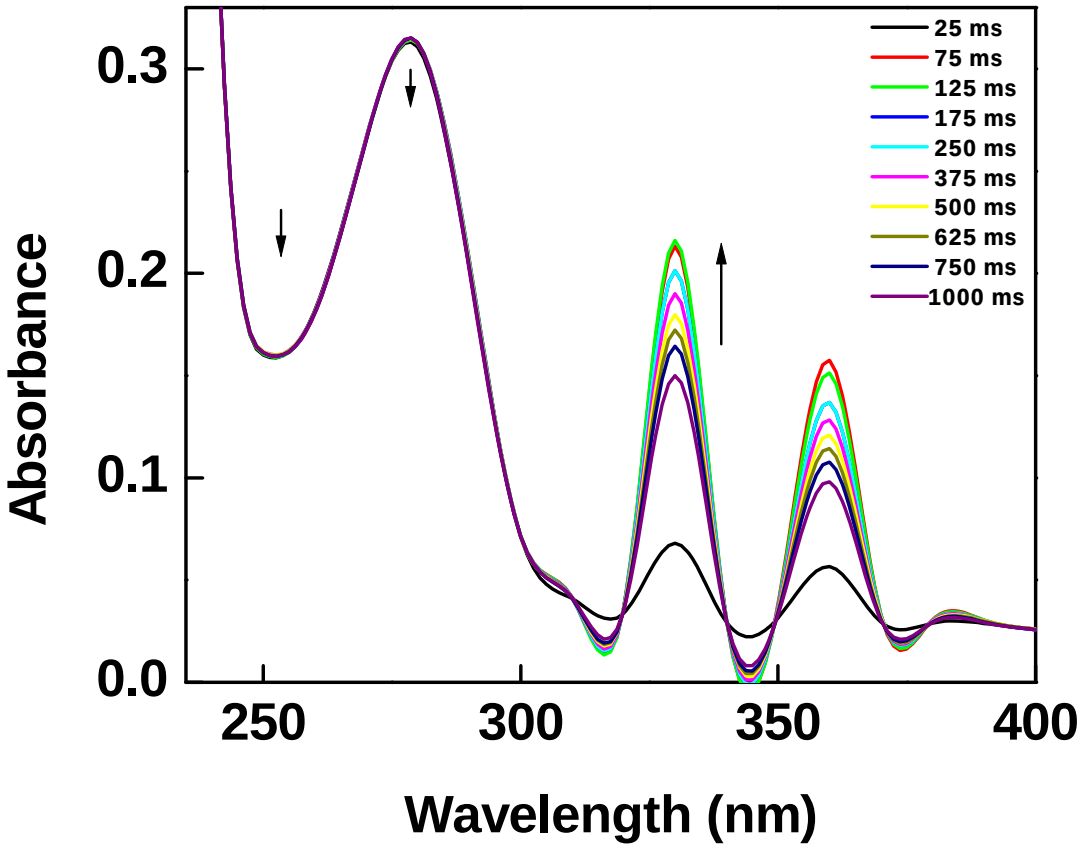

Fig.56. Time resolved spectra of α-casein interaction with Pb(II) as monitored on stopped flow spectrophotometer from 200 - 400 nm. The reaction is followed from 25 – 1000 ms at 25°C. The Pb(II) concentration of 1 x 10^{-4} M in 0.02 M tris-HCl buffer, pH 6.8 and α-casein concentration of 2 x 10^{-5} M was used for the experiment. The data was analyzed using *kinscan* program available with the instrument.

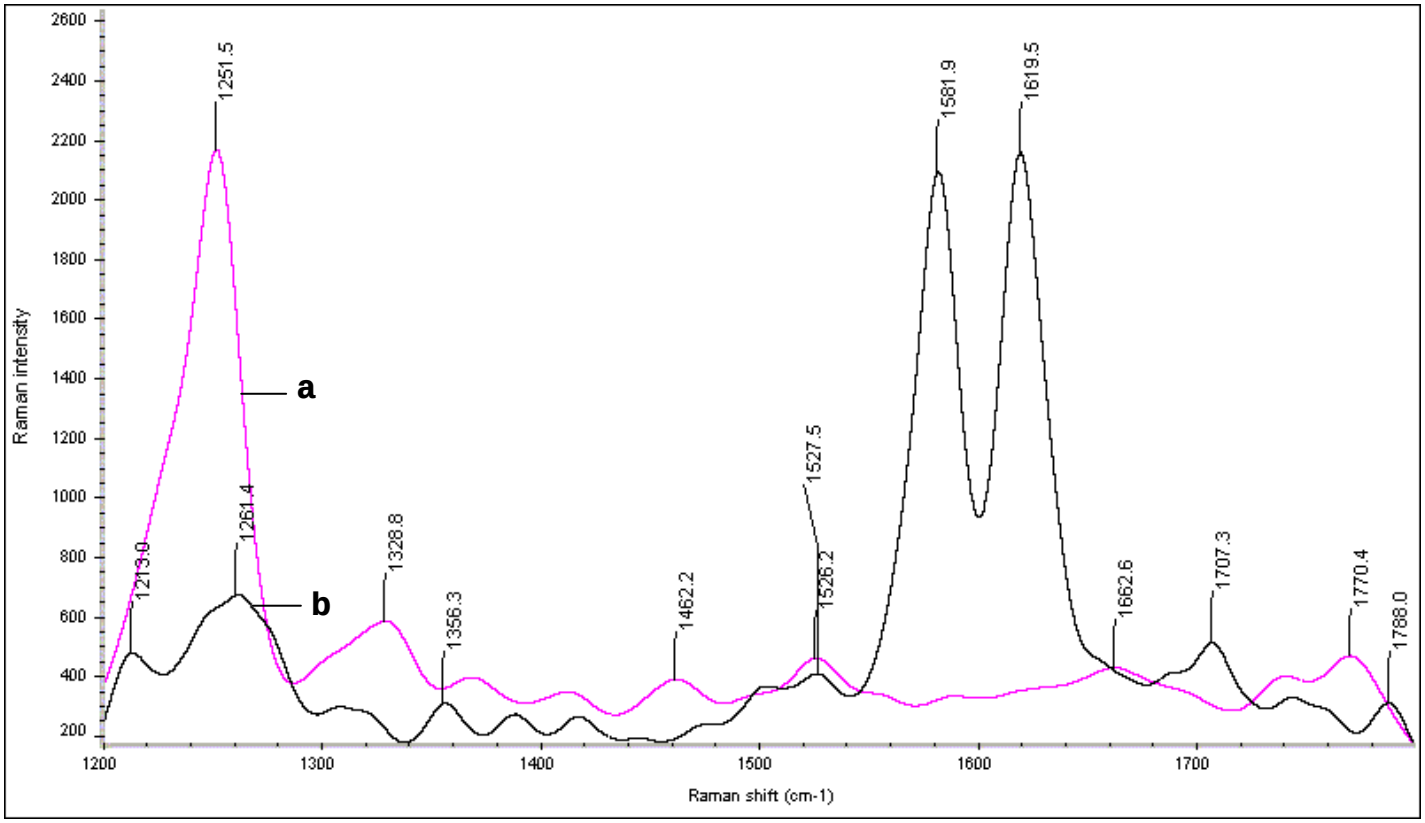

Fig.50. Raman Spectra of (a) α-casein Pb(II) complex and (b) α-casein in buffer as studied from 1200 - 1800 cm^{-1}. The α-casein concentration of 4.2×10^{-4} M was used for all the experiments. The Raman spectrum was recorded at excitation of 1064 nm laser with a laser power of 1.5 W.