# BIOTECHNOLOGICAL APPROACHES FOR ENHANCING FUNCTIONAL AND SURFACE PROPERTIES OF WHEY AND SEED PROTEINS AND TO STUDY THEIR INTERACTIONS

A THESIS SUBMITTED TO THE UNIVERSITY OF MYSORE,

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IN

BIOTECHNOLOGY

by

Anuradha S. Nambiar

Department of Protein Chemistry and Technology

Central Food Technological Research Institute

Mysore – 570020, India

May, 2008.

*.....Dedicated to*

*My Late Grandfather,*

*LTC Krishnan Nambiar*

# DECLARATION

*I hereby declare that the thesis entitled* **"BIOTECHNOLOGICAL APPROACHES FOR ENHANCING FUNCTIONAL AND SURFACE PROPERTIES OF WHEY AND SEED PROTEINS AND TO STUDY THEIR INTERACTIONS"** *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 of*** *Dr. V. Prakash* ***during the period of*** *2002-2007.*

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

**Date: 30th May, 2008**

**Place: Mysore.** Anuradha S.Nambiar

# CERTIFICATE

*I hereby certify that this Ph.D thesis* entitled "**BIOTECHNOLOGICAL APPROACHES FOR ENHANCING FUNCTIONAL AND SURFACE PROPERTIES OF WHEY AND SEED PROTEINS AND TO STUDY THEIR INTERACTIONS**" *for the degree of* **DOCTOR OF PHILOSOPHY** *in* **BIOTECHNOLOGY** *of the* **UNIVERSITY OF MYSORE, MYSORE**, *is the result of research work carried out by her in the* ***Department of Protein Chemistry and Technology, Central Food Technological Research Institute, Mysore, India*** *under my guidance and supervision during the period of* ***2002-2007****. This has not been submitted either partially or fully for any other degree or fellowship earlier.*

Date: 30th May, 2008

Place: Mysore.

**Dr.V.Prakash**

Guide & Supervisor
and
DIRECTOR,
Central Food Technological Research Institute,
Mysore. 570020.

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**Date: 30th May, 2008**

**Place: Mysore.** Anuradha S. Nambiar

CONTENTS

# *LIST OF ABBREVIATIONS*

| | |
|---|---|
| % | percent |
| ε | molar extinction coefficient ($M^{-1}$ $cm^{-1}$) |
| ΔA | difference in absorbance |
| α | alpha |
| β | beta |
| γ | gamma |
| κ | kappa |
| β-Lg | β-lactoglobulin |
| °C | degree centigrade |
| μg | microgram |
| μl | microliter |
| μM | micromolar |
| $\lambda_{max}$ | absorption maxima |
| A | absorbance |
| Å | angstrom |
| ANS | 8-anilino-1-naphthalene sulphonic acid |
| AU | arbitrary units |
| BSA | bovine serum albumin |
| cal | calorie |
| c | concentration of protein |

| | |
|---|---|
| CD | circular dichroism |
| cm | centimeter |
| CN | casein |
| cps | centipoise |
| DH | degree of hydrolysis |
| DTT | dithiothreitol |
| $E^{1\%}_{1cm, \lambda max}$ | absorption coefficient of 1% solution in 1 cm path length |
| EDTA | ethylene diamine tetra acetic acid, disodium salt |
| FAO | Food and Agricultural Organization |
| FI | fluorescence intensity |
| g | gram |
| g/g | gram/gram |
| h | hour(s) |
| HCl | hydrochloric acid |
| HPLC | high performance liquid chromatography |
| K | absolute temperature in kelvin |
| kDa | kilodalton |
| L | liter |
| m | meter |
| M | molar concentration |
| mg | milligram |
| min | minutes |
| mol | mole |

| | |
|---|---|
| MRW | mean residue weight |
| N | normality |
| NaCl | sodium chloride |
| NaOH | sodium hydroxide |
| NBS | N- bromosuccinimide |
| ND | not detected or not determined |
| NEM | N-ethylmaleimide |
| ng | nanogram |
| PAGE | polyacrylamide gel electrophoresis |
| pH | negative logarithm of hydrogen ion concentration |
| pI | isoelectric point |
| rpm | rotations per minute |
| s | second |
| SD | standard deviation |
| SDS-PAGE | sodium dodecyl sulphate-polyacrylamide gel electrophoresis |
| SEM | scanning electron microscope |
| SH | thiol group |
| TCA | trichloroacetic acid |
| TEA | triethylamine |
| TEMED | N, N, N′, N′, -tetramethyl ethylene diamine |
| Tgase | transglutaminase |
| $T_m$ | thermal denaturation temperature |

| | |
|---|---|
| TNBS | trinitro benzene sulphonic acid |
| Tris | tris (hydroxy methyl) amino methane |
| Trp | tryptophan |
| Tyr | tyrosine |
| UV | ultraviolet |
| V | volts |
| v/v | volume /volume |
| $\overline{V}_{app}$ | apparent partial specific volume |
| $\overline{V}$ | apparent partial specific volume extrapolated to zero protein concentration |
| w/v | weight /volume |
| $\rho_0$ | density of solvent buffer (g/ml) |
| $\rho_p$ | density of protein solution (g/ml) |
| $\xi_3$ | preferential interaction parameter of protein |
| $\phi_2^\circ$ | isomolal partial specific volume extrapolated zero protein concentration |
| $\phi'^\circ_2$ | isopotential partial specific volume extrapolated to zero protein concentration |
| $\delta g_3 / \delta g_2$ | preferential interaction parameter (g/g) |
| $\delta m_3/\delta m_2$ | preferential interaction parameter (mol/mol) |

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# 1 . Introduction

Proteins are the major functional molecules of life which serve many a functions like therapeutic agents, catalysts and as functional ingredients in food. Protein is a critical component of all the cells and is involved in practically every function of the body. Many proteins are enzymes and are vital to metabolism. Proteins have structural and mechanical functions and are also essential in animals' diet, since animals cannot synthesize all amino acids they need. Proteins are structural components, which play a major role in the texture of foods through their functionality. Suitability depends upon the molecular size and structure of the protein which affects the functionality. Proteins exhibit wide range of functional properties because of their varied structure and their interactions with other components like lipids, carbohydrates, water, ions and other proteins (Zayas, 1997). Functionality of proteins is studied, so that that may be effectively utilized in various food products with wider applications.

As the development of designer and nutritious food continues to increase in scope, the requirement for food proteins with well defined functional performance will become of paramount importance. This presents a unique opportunity to the dairy industry. As there is a readily available source of milk and its protein and whey from milk which is the by-product of dairy industry (Ostojic *et al*., 2005). Whey proteins improve emulsification, gelation and water binding properties of food. Their isolates are used in infant foods (Damodaran and Paraf, 1997).

Milk is a complex biological fluid, the composition and physical characteristics of which vary from species to species (Wouters *et al*., 2006). The major constituent of milk is water and contains varying quantities of lipids, proteins and carbohydrates synthesized in the mammary glands and also contain smaller quantities of minerals and other fat-soluble and water-soluble components derived from blood plasma. Milk proteins are the rich source of

biologically active peptides. It is an important source of minerals in particular calcium, phosphorus, magnesium, potassium, zinc and a rich source of all essential amino acids, which makes it a fairly complete food (Renner, 1983).

## 1.1. Composition of milk

The average content of protein in bovine milk is about 3.4-3.5% (Renner, 1983). Milk proteins are composed of mainly caseins and whey proteins (Table 1). Caseins are the major constituents of milk and account for 80% of milk proteins. Casein may be defined as the protein precipitated by acidification of the defatted milk to pH 4.6. The proteins that remain after casein has been removed from the defatted milk are known as whey proteins and they represent 20% of total milk proteins. Casein is a heterogeneous protein mixture formed of several proteins like α-casein, β-casein and κ-casein of relatively low molecular weight (29,000-23,000 Da). α-Casein, the major calcium sensitive milk protein that contains about 1% phosphorus and does not have carbohydrate. This protein accounts to about 43.5% of the total caseins. β-Casein accounts to 24.1% of the total caseins. κ-Casein accounts to 10.7% and is poor in phosphorus. The major constituents of whey proteins are α-lactalbumin, β-lactoglobulin, serum albumins, proteose-peptones and other minor proteins including lactoferrin, lysozyme and a number of enzymes. The physicochemical properties of whey proteins are shown in Table 2. Milk contains a large number of enzymes. Milk lipase is a mixture of several enzymes and is capable of hydrolyzing many types of fats. The other enzymes present in milk are esterase, alkaline phosphatases, xanthine oxidase, protease, amylase, aldolase, lactase etc (Bryon and Arnold, 1965; Wouters *et al*., 2006).

### *1.1.1. Lactoferrin*

An iron containing glycoprotein is a non-enzymatic antioxidant present in whey fraction of milk. This protein is a single polypeptide chain with two binding sites for ferric ions. It consists of 689 amino acids while

human lactoferrin has 691 residues. It occurs in very low concentration in milk. Lactoferrin exhibits a range of biological activities like bacteriostastic and bactericidal effects against a range of microbes, including those responsible for gastro intestinal infections (Dionysius *et al*., 1993). Areas of application of this protein include speciality dietary food formulations, natural preservatives and pharmaceuticals to list a few.

### *1.1.2. Immunoglobulins*

These include 2.2% of the total milk proteins**.** All of these molecules have a similar basic structure having two light chains with a molecular weight of 20,000-25,000 Da and two heavy chains of molecular weight of 50,000-70,000 Da respectively (Renner, 1983).

### *1.1.3. α-Lactalbumin*

The second most prevalent protein after β-Lg in bovine milk whey is α-lactalbumin, which constitutes about 3.7% of the total milk proteins, which comprises about 20-25% of the total whey proteins. This protein molecule consists of a single polypeptide chain with 123 amino acids of molecular weight 14,200 Da. This protein molecule has four disulfide linkages and no phosphate groups. This protein contains a wide variety of amino acids including readily available essential amino acids and branched chain amino acids also (Renner, 1983).

### *1.1.4. Lactoperoxidase*

Whey contains many types of enzymes. Among these, lactoperoxidase is the most abundant enzyme in milk. This enzyme is a heme protein having an iron content of 0.07%. This enzyme catalyzes peroxidation of thiocyanates and halides, which generate products which are very effective against a range of pathogenic bacteria (Kussendrager and Hooijdonk, 2000).

### *1.1.5. Glycomacropeptide*

This is a protein present in whey in the range of 10-15% of the total whey proteins. It is produced by the action of chymosin on casein during the production of cheese. One of the few naturally occurring proteins that lacks phenylalanine making it safe source of protein for individuals with phenylketonuria (Brody, 2000).

### *1.1.6. Bovine serum albumin*

This protein has a molecular weight of 66,000 Da and contains no phosphorous. BSA has 17 disulfide linkages and a free sulfydryl group (Renner, 1983). BSA is not synthesized in mammary gland, but secreted into the milk by passive leakage from blood stream. This molecule has specific binding sites for hydrophobic molecules and acts as a carrier of various hydrophobic molecules. This is a large protein that makes upto 0.9% of the total milk proteins. This protein is a source of essential amino acids.

### *1.1.7. Proteoses and Peptones*

This fraction of milk protein has been defined as the protein, which remains in solution after milk has been heated at 95°C for 20 min, and then acidified to pH 4.7. This fraction could be divided into four major components and minor components that accounts to 3.3% of the total milk proteins. Some of these are derived from proteolysis of β-casein and their concentration in milk can be expected to increase with time.

In addition to proteins, whey is a source of other value added dairy ingredients including lactose, lipids (sphingolipids, conjugated linoleic acid) and minerals such as calcium. Lactose serves a source of energy, acts as a probiotic, has a relatively low glycemic index and a precursor of other biomolecules like lactulose and lactobionic acid. Lactose thus has pharmaceutical quality, and is used in instant infant food and baked goods.

Concentration of lipid is low in milk but the bioactivities of sphingomyelin, which is a phospholipid and conjugated linolenic acid, are potential health promoting components. Calcium helps in protection against major chronic diseases (Miller *et al*., 2000).

Whey is the largest by-product of the dairy industry. It is obtained during the manufacture of cheese, casein, paneer, channa and shrikhand. Presence of several nutritionally important constituents having excellent functional characteristics, which opens opportunities for a wide range of applications of whey in food industries (Lakkis and Villota, 1992).

The high cost of disposal and the need to reduce environmental pollution have prompted considerable efforts world wide to increase the use of whey. Because of the potential economic and nutritional advantages, much of the effort has been directed towards the recovery of proteins from whey. In addition to their favorable nutritional attributes, whey proteins possess unique functional characteristics (Havea *et al*., 2004).

The primary function of dietary proteins is to supply the body adequately with indispensable amino acids. Many whey proteins are claimed to possess physiological properties. Bovine whey proteins contain several metal binding proteins, immunoglobulin, growth hormones and other hormones (Walzem *et al*., 2002). Most of the functions of whey proteins are related to immune or digestive systems. Whey protein has shown to act as an antioxidant, antihypertensive, antitumour, hypolipidemic, antiviral, antibacterial and a good chelating agent. The primary mechanism by which whey is thought to exert its effects is by intracellular conversion of amino acid cysteine to glutathione, a potent intracellular antioxidant (Marshall, 2004).

Milk proteins are currently the main source of a range of biologically active peptides, even though other animal and plant proteins contain

potential bioactive sequences (Wu and Ding, 2002). These peptides, which are encrypted within the sequence of the parent proteins, can be released by enzymatic proteolysis (Gobetti *et al*., 2002). Once produced, the bioactive peptides may act in the body as regulatory compounds with hormone like activity. Concentrates of these peptides are potential health enhancing nutraceuticals.

## *1.2. β-Lactoglobulin - The major whey protein*

β-Lg is the principle whey protein in bovine, ovine and caprine milk, a similar protein is present in the milk of several bovine species but is absent from human and rodent milk. β-Lg is capable of binding hydrophobic molecules including retinol and may function *in vivo* to protect retinol against oxidation and transport it from stomach to the small intestine (Kontopidis *et al*., 2004).

β-Lg being the major whey protein dominates the functional properties of all whey protein preparations. Solubility greatly depends on pH and ionic strength (Zayas, 1997). The protein is subject to a number of changes in quaternary structures with changes in pH and temperature. β-Lg is a dimer and both subunits are tightly bound to each other mainly by hydrophobic interactions. At higher temperatures dimer dissociates into monomers. Various genetic variants of β-Lg are present in milk (Pieter *et al*., 2006) such as β-Lg A, β-Lg B, β-Lg AB etc. The frequency of occurrence of β-Lg A is 19%, β-Lg AB is 51% and β-Lg B is 30%.

## 1.3. Functional properties

Functionality is defined as any property of a food/ food ingredient, which affects its utilization. For proteins, large number of functional properties could be ascribed. Functional properties of proteins make it a good ingredient for desirable characteristics in food. Several factors like structure of

proteins (native or denatured) pH, temperature and ionic concentration affect the functional attributes of the proteins (Kinsella, 1982). Solubility of a protein in a given solvent is always the good indicator for enhanced functional attributes. A protein manifests its functionality by interacting with other components in food system. Protein structure is highly dependent of its environment.

### *1.3.1. Water absorption*

Amphiphilic proteins are principally surface-active agents (Kinsella, 1981). The water absorption capacity is an important functional property of a protein. The water binding capacity of proteins includes all types of hydration of water that remains loosely associated with the protein (Kuntz and Kauzmann, 1974). The extent of protein hydration correlates strongly with the content of polar and charged amino acid residues. Water holding capacity determines the rheology of many food products. Most conventional food products contain more than 50% water and sometimes up to 95%. During processing proteins are subjected to different environmental conditions following extraction and purification. Such chemical and physical treatments affect the functionality of proteins overall. Protein composition and conformation have significant effects on water holding capacity. The interactions of water with proteins are very important both to the structure of the proteins and to their behavior in food systems (Chou and Morr, 1979).

### *1.3.2. Fat absorption*

Many important properties of foods involve the interaction of proteins with lipids. The surface properties of the proteins play an important role in fat absorption. The possibility of changing functional properties by blending proteins has been examined on a limited scale (Kinsella, 1976).

### *1.3.3. Gelation*

Gelation is an important functionality of food proteins (Matsumura and Mori, 1996), since many foods are made of protein gels. This phenomenon can be defined as protein aggregation in which the attractive and repulsive forces are finely balanced and a well ordered tertiary network or matrix capable of holding water is formed. Gels act as a medium for holding water, flavour etc. A gel is an intermediate state between a protein solution and a protein precipitate formed from a combination of protein-protein and protein-solvent interactions. Protein–protein interactions are very important for a protein to form a gel. The interactions could involve calcium or other ionic bridges, hydrophobic interactions disulfide bonds or other forces. Temperature affects the gel formation in a variety of ways. The protein concentration determines the likelihood of gel formation and also the characteristics of the gel formed.

### *1.3.4. Viscosity*

An important functional attribute of a protein is viscosity and is very essential for maintaining the physical stability of emulsion (Schenz and Morr, 1996). Processing induced changes such as polymerization, aggregation and hydrolysis affect the viscosity of proteins. Molecular weight, hydrophobicity and protein conformation also affect the viscosity of the protein solution. Viscosity can be related to the volume occupied by solute compared to volume occupied by solvent. As a protein occupies more volume in solution, it has a greater chance of interacting with other molecules and for there to be a resistance to flow.

### *1.3.5. Enzymatic hydrolysis*

Enzymatic hydrolysis is used to modify the functional properties of proteins (Johnson, 1970; Radha *et al*, 2008). Partial hydrolysis of novel proteins improves their solubility and foaming properties (Hermansson, 1974). It has

been demonstrated that by controlled hydrolysis, it is possible to achieve desired improvement in protein functionality.

Enzymatically hydrolysed protein possesses several functional properties such as low viscosity, increased whipping ability, high solubility that makes them suitable for use in many food products. Protein solubility is an important criterion for improved functional properties (Vojdani, 1996). Enzymatic hydrolysis of proteins must be carried out under controlled conditions for desirable functional properties (Alder-Nissen, 1982). Limited proteolysis can release structural domains of proteins that retain tertiary structure but have decreased stability and thus increased flexibility.

Proteolytic enzymes have been reported to improve functional properties of proteins from cottonseed (Arzu *et al*., 1972; Rahma and Rao, 1983a,b), soybean seed (Roozen and Pilnik, 1973), and rapeseed (Hermansson *et al*., 1974). Enzymatic hydrolysis enhanced the functional attributes of quinoa proteins (Aluko and Monu, 2003) and also of lupin proteins (Stark *et al*., 2004). Protein digestibility by a proteolytic enzyme affects the nutritional quality of a protein. The rate of proteolysis depends upon the conformation of the proteins. Changes in the protein conformation, alters the number of accessible peptide bonds, and the rate of proteolysis (Mihalyi, 1978). At neutral pH, β-Lg exits as a dimer, and slowly dissociates into monomers due to strong repulsive forces below pH 3.5 (McKenzie, 1971).

### *1.3.6. Foaming capacity*

Proteins are polymers of amino acids with hydrophilic and hydrophobic side chains. This amphipathic character of the protein is responsible for their adsorption at interface in foam formation. To form foam, protein adsorbs rapidly during the transient stage of foam formation. Proteins can be modified to a limited extent to enhance foaming properties. Enzymatic hydrolysis reduces molecular weight and increases the flexibility of the

proteins, which facilitates formation of interfacial membrane and foam production (Alder-Nissen, 1982). Therefore, flexible protein structure facilitates to better foam formation than compact structure. Studies by Mannheim and Cheryan (1992); Mahajan and Dua (1998); Van der *et al*., (2002) have shown that foaming ability increased after enzymatic hydrolysis. However excessive hydrolysis and reduction of molecular size beyond a critical stage impair the capacity of the hydrolysate to form cohesive interfacial membranes with the rigidity necessary for the formation of stable foam.

### *1.3.7. Emulsion*

A molecule that contains a moiety that is soluble in water and another moiety soluble in non polar solvents are called amphiphilic molecules. Proteins minimize their energy by folding into structures of low energy. These structures are formed when polar groups interact with water the maximum and the interaction of non polar groups is minimized. Solubility plays a major role in emulsifying activity of proteins (Kinsella *et al*., 1985). The ability to form an emulsion is the primary functional attribute of a protein. An emulsion is defined as a dispersion or suspension of two immiscible liquids. Margarines and butter are one of the best examples of water in oil emulsions (Hill, 1996). The stability of the emulsion depends on the balance of the forces associated with the interface of the oil and water. Proteins which are partially denatured generally exhibit good emulsifying properties (Kato and Nakai, 1980). To stabilize an emulsion, hydrophobic domain of the protein should be ideally oriented towards the oil phase. Proteins are often included in emulsion to aid in the formation and increase in the stability of emulsion.

## 1.4. Stabilization of proteins by cosolvents

Protein stability is the balancing between intra molecular interaction of protein functional groups and their interaction with solvent molecules.

Sugars/polyhydric alcohols have lower dielectric constants than water. Thus electrostatic interactions should be stronger in these solutions than in water. However this contribution to the stabilizing effect must be relatively small (Taravati *et al*., 2007). The main phenomenon of stabilization of globular protein by cosolvents is by preferential hydration of the protein by raising the surface tension of water or by increasing solvophobicity (Gekko and Timasheff, 1981).

Hydrophobic interactions are generally considered to be a major single factor in stabilizing three dimensional structure of the protein. The effects of sugars on hydrophobic interactions and consequently on the thermal stability should depend upon how they affect the structure of water. Hydrophobic interactions between hydrophobic groups are more in sucrose solution than in water alone. Deamidation of asparagine residues is of relatively more important and has been proposed to be the principle cause of irreversible denaturation. A protein may be stabilized against deamidation through interaction with cofactors (Wright, 1991).

The dominant mechanism by which sugars and polyhydric alcohols stabilize proteins against heat denaturation is through their effect on the structure of protein, which in turn determines the strength of hydrophobic interactions (Gekko, 1981). Stabilization achieved by additives is simpler and less expensive, than the stabilization resulting from protein engineering. Different proteins are known to interact with cosolvents in diverse ways and their interactions depend on physicochemical properties of the proteins.

A number of naturally occurring organic molecules, such as sugars and polyhydric alcohols mainly, stabilize the native conformation of proteins and prevent the denaturation. Most of these small molecules are sugars, polyhydric alcohols or amino acids. Addition of sugars leads to viscosity changes, which affects the conformational stability of globular proteins

(McClements, 2001). Preferential interaction of proteins with this mixed cosolvent system may lead to either hydration or solvation of the protein molecule. Interaction between protein surfaces and cosolvent molecule leads to redistribution of cosolvent around the protein molecules. Presence of cosolvents modifies the hydrogen bonding potential of the amino acid residues, which in turn increases or decreases the overall hydrogen bonding potential of neighbouring polar residues. These substances stabilize protein structure and are strongly excluded from the protein structure without interacting with the protein (Arakawa and Timasheff, 1985). Stabilizing phenomenon is a complex one and there is no single mechanism responsible for stabilization but a multitude of stabilizing interactions besides preferential exclusion phenomenon.

### *1.4.1. Thermal stabilization*

Under unfavorable environmental conditions such as extreme temperature, high salt concentration or desiccation, osmolytes accumulate in the cells and protect the proteins against denaturation and loss of function. These osmolytes exhibit protective effect, which involves their ability to alter the viscosity and the water activity of the medium (Patricia and Penna, 2005). The main factor that is involved in the stabilization of protein molecule is hydrophobic interactions. It was found that the hydrophobic interactions are more in presence of sugars and other polyols. This explains the enhanced stability of proteins at higher temperature (Fersht, 1977).

The capacity of cosolvents to form hydrogen bonds plays the most important role in the stabilization of protein molecules. The low hydrophobicity of cosolvents ensures that the solvation shell around the exposed non-polar groups of a protein remains intact at relatively high temperature leading to protein stabilization (Pedro *et al*., 1993; Gekko and Timasheff, 1981). The protective effect is proportional to the cosolvent concentration and also its molecular weight. The protective effect of the

cosolvent is proportional to the molecular size and the number of hydroxyl groups present in the cosolvent molecule (Gekko and Timasheff, 1981). The ability of cosolvent to alter the physico-chemical properties of water, especially the interfacial tension of the protein-water interface, plays a very significant role in stabilization of proteins.

## 1.5. Interaction studies

Functionality of a protein is largely affected by circumstances. The behaviour in model system is different form that in real food products, because of interaction of proteins with other components.

Thermal stability of food proteins explains their resistance to aggregation in response to heating. Gelation induced by heat is a requirement for food industries where products like sausages and cheese are manufactured. Substitute proteins need study of interaction with other proteins in the system and also their thermal stability.

The utility of the proteins is affected by its interactions with other proteins. Thus studies on these interactions form an important topic from the point of view of commercial application in food industry and pharmaceuticals. This is applicable to proteins with utilization in food industry where it is expected that there will be other components like proteins during various stages of processing (Mauron, 1990).

Although effects of protein interactions have been observed in food products, little has been done to facilitate understanding of basic functional attributes and structure-function relationship. Whey proteins are comparatively inexpensive source of functional proteins. It would be interesting to study functional attributes with whey proteins in presence of additive with other proteins especially seed proteins. Thermal stability is an

important attribute in heat processed foods. In combination of proteins the functionality is altered (Aryana *et al*; 2002). Functional properties of proteins are known to be enhanced with protein-protein interactions. One way to improve protein-protein interaction is to have mixture of proteins with opposite charges. Therefore a mixture with other proteins may improve functionality.

### *1.5.1. Effect of heat on milk proteins*

Casein micelles are remarkably stable at temperature up to 140°C. In contrast, whey proteins are relatively heat labile and denaturation occurs at 80°C. Denaturation is accompanied by extensive breaking and randomization of hydrogen and disulfide bonds. β-Lg is more heat labile, as a consequence of its one free sulfydryl group, which permits the initiation of autocatalytic disulfide exchange reactions (Renner, 1983). Reports also indicate interaction of β-Lg with BSA through thiol oxidation reaction (Matsudomi *et al*., 1994).

Heating of whey protein fraction leads to polymer formation. Controlled aggregation of whey protein is utilized to increase the viscosity and improve the palatability of various food products. Whey protein polymerization offers new means to control texture and stability. Therefore, it opens new ways for formulated foods especially designed for health conscious consumers (Britten, 2002).

## 1.6. Enzymatic modification of proteins

### *1.6.1. Polymerization*

Many proteins lack appropriate functional properties for specific applications. So proteins are modified for improving functional attributes through covalent crosslinking (Singh, 1991). Chemical modification of proteins has been generally used to enhance the functional attributes, but concerns about safety have limited its use in food products. Enzymes have

been the best option for enhancement of functional attributes with various applications in food industry. Whey proteins could be used for the manufacture of food products with improved texture and overall quality. A better control of polymerization is essential to optimize the use of whey proteins.

The enzyme Tgases are widespread in animal tissues and is involved in the polymerization of proteins (Elvan, 2006). The enzyme is employed to enhance the functional attributes of proteins. These Tgases are from commercial sources, (Ajinomoto Company Inc, Tokyo, Japan), which are isolated from *Streptoverticillium sps.*

Experiments performed aiming at crosslinking of food proteins with the help of Tgases (Zhu *et al*., 1995) have shown that several industrial food proteins such as casein and soy proteins are good substrates for mammalian Tgases. Crosslinking of these proteins with these enzymes improved their functionality (Nio *et al*., 1986 and Schorsch, 2000). Another development that pushed the application of Tgase in processed foods forward was the isolation of a bacterial Tgase from *Streptoverticillium* sps. Modification of a number of crosslinks in proteins can alter the functional attributes without altering the nutritional quality (Feeney and Whitaker, 1988).

## 1.7. Seed proteins

Oil seeds have been a rich source of proteins with good functional properties (Khalil *et al*., 1985). Oil seeds are generally regarded as prime source of protein for human consumption. These oil seeds have appreciable protein content which find potential use in varied food application. Oil seeds contain very high quality protein, which are also unique in their composition and have many beneficial properties. The sesame seed proteins are rich in aromatic and sulfur containing amino acids like metheonine and cysteine, which are limiting in other staple food grains like pulses and cereals (Nath

and Giri, 1957b). Nutrition from vegetable sources of proteins has been through cereals and with pulses. Legumes have been used as a source of protein. But due to the antinutritional factors in the legumes like protease inhibitors, alternative source that is, oil seeds find varied application in food. For plant proteins to be useful in food application, they should possess desirable functionality. However the industrial applications of food proteins are limited because proteins are generally unstable to heating, organic solvents and proteolytic attack. Therefore if proteins are converted into stable form their application would be broadened.

### *1.7.1. Soybean (Glycine max)*

Soybean proteins in their various forms have properties that make them useful in food systems. Methionine is the first limiting amino acid in soybean proteins. The functional properties are generally attributed to the proteins. An important chemical property of soybean proteins is their amino acid composition, which determines nutritional value of proteins. But these proteins are high in lysine and thus are more useful in food supplements (Wolf and Tamura, 1969). The properties of soybean proteins are markedly affected by pH, salt concentration, temperature and storage conditions of the seeds and the proteins. The molecular weight of soybean proteins vary between 302,000 and 350,000 Da. The secondary structure was investigated and the protein was found to have low α-helix content and a fair amount of β-structures and aperiodic structures.

11S protein fraction constitute one third of the soybean total seed proteins. An important property is that this protein forms disulfide-crosslinked polymers, which contribute to insolubility of soybean proteins. The polymers formed by disulfide linkages cause turbidity.

Soybean proteins give desirable functional properties to the food products, when it is added to a variety of foods. Soybean possesses unique

property for whipping or aerating agents. They are used functionally in the manufacture of many confectionaries. How an ingredient exerts its function in a given food system, may be studied by incorporating it into the food formulations and then analyze it for the functional properties. The results obtained in the complex system will perhaps fill the gaps between simple model systems and actual food products. Soybean proteins give desirable functional properties to finished products when added in a variety of foods. Soybean proteins possess a suitable property for whipping and aerating agents which makes then useful in the manufacture of confection, whipped toppings, desserts, ice creams etc.

### *1.7.2. Sesame seed proteins (Sesamum indicum)*

Sesame seed *(Sesamum imdicum L.*) contains nearly 25% protein and the defatted meal contains about 50% protein. Except for oxalates in the hull, there are no known toxic antinutritional or colouring principles in sesame. These proteins coagulate upon heating and are insoluble in water unless dissolved in high concentration of salt concentration (Prakash and Rao, 1986).

Major protein fraction of sesame seed is α-globulin (Jones and Gersdorff, 1927; Nath and Giri, 1957b), which constitutes about 65% of the total proteins. Therefore, this protein plays a major role in the sesame products. This is a multimeric protein with 12 subunits and the physicochemical properties are well studied (Prakash, 1985). This protein is very stable and soluble at very high salt concentration (Guerra and Park, 1975; Prakash and Rao, 1986). The stability of α-globulin is enhanced by hydrophobic interactions. α-Globulin and other proteins are an important source of essential amino acids (Nath and Giri, 1957a; Prakash and Rao, 1986). Sucrose and other polyelectrolyte have shown to prevent heat coagulation of α-globulin. The endothermic nature of aggregation process has been considered to arise from hydrophobic interaction of aliphatic side chains of subunits. This protein contains about 5% α-helix, 25% of β-sheets and

remaining aperiodic structures. With a good degree of conformational freedom which results in rapid conformational changes with changes in the environment which are relatively mild. The other fractions of sesame protein constitute about 30% of the total proteins, are β-,γ-, and δ-globulins.

The seeds of sesame contain proteins rich in aromatic amino acids and sulfur containing amino acids, which are limiting in other staple foods. This can form important components in the diets for human and livestock consumption. Therefore it is essential to study these proteins to elucidate their behavior in different systems so that their application is maximized.

The applicability of proteins is affected by their interactions with other food components such as other proteins, carbohydrates and fat in the system. These studies about their interactions are applicable to proteins and their application in food industry where it interacts with other food components like carbohydrates and fats and even other complex proteins. Therefore the application of proteins depends on their physicochemical characteristics of the major protein fractions.

## 1.8. Structural stability of proteins

Stability of proteins depends on various factors like presence of salt bridges, hydrogen bonding, and binding with ligands, hydrophobic interactions and disulfide bridges, which play a major role in stabilization of proteins. There are several methods available to stabilize proteins such as immobilization, chemical and enzymatic modification, addition of additives and crosslinking (Tyagi and Gupta, 1998). The presence of crosslinks greatly adds to the stability of the native structure and makes it resistant to unfolding.

### *1.8.1. Role of disulfide bonds in protein stability*

Cysteine, which is an essential amino acid, contains a highly reactive thiol (SH) group. Disulfide bond formation between cysteine residues is a common occurrence in proteins (Thornton, 1981), which form disulfide bonds in presence of oxidizing environment. These bonds generally contribute to the stability of the native proteins. It is often found that removing one or many disulfides frequently leaves the folded conformation intact (PriceCarter *et al*., 1998).

The mechanism of disulfide bond formation in uncertain *in vivo*, and probably involves thiol-disulfide exchange between cysteine residues. The number of free cysteines and disulfide linkages are determined from the difference between sulfhydryl yields obtained before and after conversion of all SS to SH group (Haschemeyer and Haschemeyer, 1973). Disulfide linkages play a vital role in the stabilization of tertiary structure of proteins. Normally disulfide bonds in proteins are buried in the interior of the molecule and remain out of contact with the bulk solvent. Disulfide bond reduction of cysteine results in loss of conformational stability (Pace *et al*., 1988).

## 1.9. Applications of whey proteins

The increasing awareness of nutrition, health and quality food consciousness among consumers have accelerated keen competition in the market and compelled the food industry to search for novel food formulations. There are certain vital ingredients, which impart specific functionalities to food products, and also retain the nutritional quality of foodstuffs in order to sell their products profitably. In this context, the food industry has come to realize that milk proteins in general and whey proteins in particular have potential to improve the quality of food products. Whey protein ingredients are one of the most complete sources of proteins and essential amino acids available. Whey proteins possess excellent functional properties and high nutritive and important biological properties. Whey

proteins are therefore used in a wide range of food applications not only because of their nutritional values but also functional properties.

Lactoferrin is an important whey protein with varied biological functions. Studies on lactoferrin have demonstrated its ability to activate natural killer (NK) cells and neutrophils, induce colony stimulating factor activity, and enhance macrophage cytotoxicity (Nishiya and Horwita, 1982). α-Lactalbumin can chelate heavy metals. It reduces oxidative stress because of its iron chelating properties (Ha and Zemel, 2003). Whey has been recently utilized as a healthy dietary supplement to reduce hypertension. Antihypertensive peptides have been isolated from primary sequence of β-Lg (Mullally *et al*., 1996). Whey peptides are found to have significant angiotensin-I converting enzyme inhibitory activity. β-Lg has been described as a cholesterol-lowering effect by inhibiting cholesterol solubility in the intestine (Nagaoka, 1996).

*Thus whey protein is a rich source of essential amino acids. These proteins also possess excellent functional properties, which could be enhanced by hydrolysis using various enzymes. So enhancement of the functional attributes is the focal point of this study. β-Lg is the major whey protein, which has excellent functional attributes. This protein is used in combination with other proteins like seed proteins for better functional attributes like solubility, foaming capacity etc. Interaction studies of these proteins therefore become important. Hence keeping in view of the above points, the major whey protein, β-Lg was isolated and characterized. Interaction studies of this protein with 11S protein fraction from soybean and sesame seed were studied to improve the functional and surface properties. The thermal and structural stability of β-Lg were also studied in presence of various cosolvents.*

## SCOPE AND OBJECTIVES

Bovine milk proteins, that is, casein and whey proteins can be regarded as a comprehensive source of essential amino acids in a normal diet. About 80% of bovine milk proteins consist of casein (2.7 g/100 g milk), which is a phosphate containing protein and classified as α, β and κ-caseins. Whey proteins constitute about 20% of bovine milk proteins (0.6 g/100 g milk). The whey protein fraction consists of α-lactalbumin, β-lactoglobulin, bovine serum albumin, immunoglobulins, lactoferrin, proteose-peptone fraction and also a large number of peptides. Whey proteins provide one of the richest sources of amino acid, having the best biological value, and the optimum protein efficiency ratio (PER). Whey proteins have been known to have a biological value superior to that of other naturally occurring proteins. These proteins are well digestible and easily absorbable.

β-Lg, the major whey protein is a single polypeptide chain with a molecular weight of 18,200 Da. This protein exists as a dimer at physiological pH. The biological and functional properties are very well defined. β-Lg has an excellent heat set gelation property that can be immediately applied in formulation of foods. Its solubility at very low pH makes it useful as an active agent in protein fortified beverages. Bioactive peptides liberated from this protein could be used as dietary supplements for pharmaceutical preparations. There are few reports available on this aspect.

Whey protein has good foaming capacity. Attempts have been made to combine egg white proteins with milk proteins. Enzymes like Tgases have been used to form complexes of β-Lg with soybean and sesame proteins. Native whey proteins have excellent functional properties and can be used as a substitute for egg white. Legumes and cereal proteins have complimentary nutritional qualities. Legume seeds have a deficiency of metheonine and cereal proteins such as corn protein have lower proportion of lysine. Several

new acceptable food products from commercial types of dry beans supplemented with milk proteins and could lead to development for optimizing amino acid balance in the final food product. Blending calculated amounts of complimentary plant or milk proteins are done to produce a more satisfactory essential amino acid balance. Plant proteins are deficient in metheonine. Blending of plant proteins with milk proteins to enhance nutritional quality of proteins is not uncommon. Digestibility of plant proteins is lower than high quality animal proteins. Improved digestibility may be imparted when blends of required ingredients are appropriately mixed and heat processed. Blending of β-Lg with vegetable proteins requires a deeper understanding of protein-protein interaction studies, which would be the focal point of this study. It has been reported that the solubility differs with protein-protein interaction. Studies on the interaction of β-Lg with vegetable proteins are therefore very important.

Food proteins are hydrolysed for the improvement of nutritional and functional properties, removal of odour, flavour and anti-nutritive components. Milk and soybean proteins have been commonly used for better digestibility and utility. Soybean protein hydrolysate is usually used in combination with other protein hydrolysate. Soybean protein isolate is added to increase water-holding capacity, to increase viscosity and for the stabilization of protein based emulsion.

Interaction of cosolvents with β-Lg may lead to changes in the stability of the protein. So the mechanism of stabilization of the protein in these cosolvents can be useful in the understanding the structural stability in more detail. The preferential interaction parameters can give an insight into the extent of the interaction of these cosolvents with the protein. The data on the thermal stability gives a proper insight into understanding the structural stability of β-Lg at higher temperature. So structural stability of β-Lg as a

function of glycerol, sorbitol and sucrose forms one of the major objectives of this study.

Few basic studies on β-Lg like, interaction with cosolvents, fluorescence and thermal denaturation studies have also been carried out to understand the mechanism of the above mentioned phenomenon. The results obtained with these experiments would indicate various conditions for the structural and thermal stabilization of the β-Lg in presence of various cosolvents like glycerol, sorbitol and glycerol. The data obtained with protein – protein interaction would throw insight into the enhancement of the various functional attributes of the proteins through this interaction.

***Specific objectives of the present study can be summarized as follows:***

(a) *Isolation and characterization of β-Lactoglobulin from bovine milk whey.*

β-Lg was isolated from bovine milk and characterized for various physico-chemical properties. Whey as such was hydrolysed and evaluated for functional properties like foaming and emulsifying activity.

(b) *Protein-protein interactions of β-Lactoglobulin with 11S fraction of soybean and 11S fraction of sesame seed.*

The interaction of β-Lg with 11S protein fraction of soybean and 11S protein fraction of sesame seed at higher temperature was evaluated by turbidity and solubility studies.The functional properties like foaming and emulsifying activity of the protein mixture of β-Lg with 11S protein fraction of soybean and 11S protein fraction of sesame seed were also evaluated.

(c) *Effect of Transglutaminase on protein-protein interaction of β-Lactoglobulin with 11S fraction of soybean and 11S fraction of sesame seed.*

The effect of transglutaminse on the interaction of β-Lg with 11S protein fraction of soybean and 11S protein fraction of sesame seed

was studied. The foaming capacity and emulsifying activity and heat capacity of the enzyme treated proteins were also studied.

(d) *Structural stability of β-Lactoglobulin as a function of cosolvents.*

The stability of β-Lg in presence of various cosolvents like sucrose, sorbitol and glycerol were studied using densitymeter technique. The structural stability of β-Lg in presence of these cosolvents were also studied by CD experiments. The thermal stability of the protein was evaluated in presence of these cosolvents to correlate structure stability of the proteins in the presence of various cosolvents.

Hence these above objectives of the present study and the data generated would give more insight into the understanding the mechanism of structural stabilization of β-Lg in presence of cosolvents. The study of interaction between β-Lg and the 11S protein fraction of soybean and sesame seeds would be helpful in the enhancement of the functional attributes and understanding of protein-protein interactions in general.

# 2. Materials & Methods

## 2.1. Chemicals

α-Chymotrypsin from bovine pancrease (C-4129), denatured hemoglobin (H-2625), azocasein (A-2765), coomassie brilliant blue G-250 (B 5133), , 8-anilino-1-naphthalene sulphonic acid (A-1028), glycerol (G-5516), sorbitol (S-1876), sucrose (S-7903), urea (U-1250), N-bromosuccinimide (B-9252), Tris hydroxymethyl amino methane (T-4661), leucine ( L-8912), L-Tyrosine (T-1020), dithiothreitol (D-0632), N-ethylmaleimide (E-3876), trinitro benzene sulfonic acid (P-2297), ovalbumin (A-5503), trypsin inhibitor (T-9003), β-mercaptoethanol (M-7154), bovine serum albumin (A-2153) and pepsin (EC 3.4.23.1) were purchased from Sigma Chemical Co., St. Louis, MO, USA. Glycine, sodium dodecyl sulfate, sodium chloride, trichloroacetic acid, sodium hydroxide, petroleum ether, n-hexane, acrylamide, bis-acrylamide, acetic acid, dibasic and monobasic sodium phosphate, hydrochloric acid, sulfuric acid and methanol were from Qualigens Fine Chemicals, Mumbai, India. Ammonium sulfate, calcium chloride, copper sulfate, ferrous sulfate were purchased from E. Merck (India) Ltd, Mumbai, India. N, N, N', N', tetramethyl ethylene diamine and ammonium persulfate, bromophenol blue were from Research Organics Inc., Cleveland, Ohio, USA. Sodium azide of AR Grade procured from Loba Chemie Indo Austranal Co., Mumbai, India. Spectrum dialysis tubings, parafilm were from Thomas Scientific Co., Philadelphia, and USA. Transglutaminase (EC 2.3.2.13) was courtesy from Ajinomoto Company Inc, Tokyo, Japan (the commercial sample contained 99% maltodextrin and 1% enzyme). Sephadex G-75 was procured from Pharmacia Fine Chemicals, Sweden. All the chemicals were of analytical grade. Quartz triple distilled water was used for the preparation of all the reagents and in all experiments.

## 2.2. Methods

### *2.2.1. Preparation of whey*

Fresh milk was collected from a local breed of cow (*Bos taurus*). Milk was defatted by centrifugation of the milk (7500 x g for 30 min, 4°C) in a Kubota centrifuge (Kubota High – Speed refrigerated Centrifuge, Kubota Corporation, Tokyo, Japan). Whey was prepared from this defatted bovine milk according to the procedure of Fox *et al.,* (1967). The pH of the milk was adjusted to 4.6 with 0.1 N HCl with continuous stirring for 30 min at 25°C. The precipitate was removed by centrifugation (8000 x g for 30 min, 4°C). The supernatant was collected and lyophilized. The lyophilized sample was stored at 4°C and used for further experiments.

### *2.2.2. Preparation of defatted sesame and soy flour*

The sesame seeds *(Sesamum indicum* L.) and soybean seeds (*Glycine max L.*) were purchased from local market. The seeds were cleaned and graded in a grading machine to remove stones and impurities. Water was sprayed on the seeds to raise moisture level to 2%. Conditioned seeds were dried in an electrically heated roaster at 50-55°C. The dehulling was done by passing through a plate type mill (Model A453, Chandra Manufacturing Co., Chennai, India) with an attached blower. The dehulled seeds were equilibrated at 20% moisture and passed through flaking machine (Model J#6725, Kvarnmaskiner, Malmo, Sweden) maintaining a drum clearance of 0.3-0.5 mm to obtain flakes of 0.3 mm thickness and dried to 5% moisture level. Dried flakes were defatted and repeated extractions with n-hexane, vacuum dried to remove solvent and ground and passed through 60 microns mesh sieve. Defatted flakes were dried and powdered in a quadrumat mill (Brabender, Quadrumat Senior, Duisburg, Germany). Fat was estimated. The extraction with solvent was repeated till the fat content reached less than 1% as confirmed by standard method of fat estimation (AOAC, 2005). The defatted seeds were milled and the flour sieved with a mesh size of

60 microns and stored at 4°C and used for the isolation of 11S protein fractions.

## 2.3. Isolation of proteins

### *2.3.1. Isolation of β-Lactoglobulin*

β-Lg was isolated from defatted bovine milk in accordance with the procedure of Fox *et al.,* (1967) as shown in Figure 1. A local breed of cow was selected and each time milk was collected freshly. To 500 ml of whey 15 g of TCA was added with stirring and kept for 30 min. The solution was centrifuged at 8000 x g for 30 min at 4°C. The supernatant was saturated with 0-60% ammonium sulfate with stirring at 4°C and kept for precipitation for 1 h. The precipitate was separated by centrifugation at 8000 x g for 30 min at 4°C. The precipitate was dissolved in buffer and dialyzed against triple distilled water for 24 h at 4°C. The dialyzed sample was lyophilized and stored at 4°C. The yield of β-Lg from whey was calculated and found to be approximately 1.2 g/litre of whey. The homogeneity of the isolated β-Lg was established by SDS-PAGE and amino acid composition, which was found to have 99% correlation with the literature values.

### *2.3.2. Isolation of α-Globulin*

α-Globulin was isolated from defatted flour of sesame seeds (*Sesamum indicum L.)* (Figure 2), according to the method of Prakash and Nandi (1978). The total protein of the defatted sesame seed flour was extracted in 0.06 M phosphate buffer of pH 7.5 containing 1 M NaCl using flour to buffer ratio of 1:10 and stirred for 1 h and centrifuged at room temperature and 6000 x g for 30 min. The supernatant obtained by centrifugation was diluted with distilled water in the ratio of 1: 5.5 times with water and allowed to stand for 2 hr. The solution containing precipitated α-globulin was centrifuged at 6000 x g for 10 min. The precipitate thus obtained was redissolved in the extraction buffer and diluted with distilled water in the ratio of 1:1.5 times and allowed to

stand for 2 hr for and reprecipiation. This process of precipitation and centrifugation was repeated twice. α-Globulin thus obtained was dissolved in water and dialyzed against same buffer for 24 h using dialysis membrane having a molecular weight cut off of 6000-8000 Da. Purity of the isolated protein was checked by Native and SDS–PAGE and it was found to be pure and homogenous. The homogeneous protein was dialyzed against triple distilled water and lyophilized to obtain a pure protein without salt, stored at 4°C and used for further experiments.

### *2.3.3. Isolation of Glycinin*

Glycinin was isolated from defatted soy flour using 0.03 M Tris HCl buffer pH 8.0 containing 0.01 M mercaptoethanol as the extraction buffer (Figure 3). The pH of the whole buffer extract was adjusted to 6.4 using 0.1 N HCl with continuous stirring at 25°C for 30 min. The extract was centrifuged at 8000 x g for 30 min at 4°C. The precipitate obtained was the 11S protein fraction. The isolated protein fraction was dissolved in the same buffer of pH 8.0 and lyophilized and stored at 4°C for further use (Thanh and Shibasakhi, 1976).

## 2.4. Spectrophotometric methods

### *2.4.1. Ultraviolet absorption spectroscopy*

Ultraviolet absorption spectra of protein solutions were recorded with Shimadzu UV 1601 (Shimadzu, Japan) double beam spectrophotometer in the range of 200–350 nm using quartz cuvettes of path length 1 cm. $E^{1\%}_{1cm,}$ at 280nm of β-Lg is 9.5 (Fox *et al.*, 1967). The $E^{1\%}_{1cm}$, at 280nm of glycinin is 8.1 (Badley, *et al.*, 1975) and that of α-globulin is 10.8 (Prakash and Nandi, 1978).

### *2.4.2. Fluorescence emission spectroscopy*

Protein solution of concentration 2.7 x $10^{-6}$ M containing different cosolvents of varying concentration were used for fluorescence measurements using Shimadzu Spectrofluorimeter (model RF-5000) at 25°C. The protein solution was excited at 280 nm and emission spectra were recorded from 300-400 nm. Corrections were applied both for inner filter effect for protein concentration and solvent effect (Brand and Witholt, 1967).

### *2.4.3. UV-Circular dichroic spectroscopy*

Far UV Circular dichroic spectra measurements were performed at 25°C in a Jasco J-810 Spectropolarimeter equipped with a Xenon arc lamp. The far UV-CD measurements were recorded from 200–260 nm and near UV-CD was measured from 300–350 nm with 1 mm path length in a quartz cuvettes, using a β-Lg concentration of 0.30 mg/ml (5.2 x $10^{-6}$ M) in 0.02 M phosphate buffer of pH 7.9 (Alder *et al*., 1973) using a slit width of 1 nm. The protein solution was dialyzed against various concentrations of cosolvents (10% to 40%) for 12 h at 4°C and centrifuged at 6000 x g for 30 min before recording the CD spectra. The rotations were converted to molar ellipticity values, with a mean residue weight of 113 calculated from the amino acid composition of β-Lg (Boguslawa, 2006). The molar ellipticity values were obtained at 1 nm interval using the equation (Alder *et al*., 1973).

$$(\theta)_{MRW} = \frac{(\theta)_{obs} \times MRW}{10 \times d \times C} \qquad \text{----------} \quad (1)$$

where $(\theta)_{MRW}$ is ellipticity, d is path length in cm, C is protein concentration in g/ml and MRW is mean residue weight of the protein. The analyses of the data for the secondary structural elements were calculated using the method described by Chen and Yang (1971) and Yang *et al*., (1986).

### *2.4.4. Measurement of apparent thermal denaturation temperature*

Thermal denaturation profile of β-Lg in presence and absence of different concentration of cosolvents were measured using Cary-100 Bio-UV-Visible spectrophotometer from Varian INC (Australia). A protein concentration of 0.8–1.0 mg/ml was equilibrated with respective concentration of cosolvents for 24 h. The thermal denaturation profile of the protein was monitored at 287 nm in the temperature range of 25-90°C by 1°C increment in temperature per min. Apparent thermal transition temperature and other thermodynamic parameters were calculated according to the standard procedure (Arakawa *et al.*, 1990).The fraction unfolded was calculated using the formula:

$$F_D = (A_T - A_N) / (A_D - A_N) \quad \text{----------} \quad (3)$$

$F_D$ is the fraction of the protein unfolded. $A_N$ is the absorbance of the protein sample at 25°C. $A_D$ is the absorbance of the fully denatured protein sample. $A_T$ is the absorbance at different temperature from 25-90°C. The temperature at which $F_D$ is 0.5 is taken as the apparent melting temperature ($T_m$) of the protein.

## 2.5. Partial specific volume of proteins in cosolvents

For the partial specific volume studies, β-Lg was dissolved in triple glass distilled and deionized water and dialyzed against the same for 36 h at 8°C. The proteins were centrifuged at 6000 x g for 30 min and the clear supernatant was lyophilized. Apparent Partial Specific Volume (Φ) of β-Lg was determined by measuring densities of protein solutions in presence of cosolvents at 20 ± 0.05°C (Lee *et al.*, 1979).

The densities of the solvents and of the protein solutions were determined on a precision density meter DMA-55 (Anton paar, Gratz,

Austria). The apparent partial specific volume 'Φ', was calculated from the obtained density data using the following equation (Prakash, 1982).

$$\Phi = 1/\rho_0 [1 - (\rho - \rho_0 / C)] \quad \text{----------} \quad (4)$$

Where, C is the protein concentration, ρ is the density of the protein solution and $\rho_0$ is density of the solvent (g/ml). The densities of the protein solutions were measured at 20 ± 0.05°C at conditions such that the molality of the solvent composition and the chemical potential are kept in turn, identical in the solvent and in the protein solution. Here components 1, 2 and 3 are water, protein and cosolvent respectively (Stockmayer, 1950; Scatchard, 1946). The apparent partial specific volumes of the protein, in isomolal and isopotential conditions, were measured as a function of different protein concentrations. The observed values were extrapolated to zero protein concentration to obtain partial specific volumes both in isomolal ($\phi_2^\circ$), and isopotential values ($\phi_2'^\circ$) respectively.

### *2.5.1. Preferential interactions*

The preferential interaction parameter ($\xi_3$) (g/g) of protein, in three-component system containing cosolvent can be calculated from the following equation (Casassa and Eisenberg, 1964).

$$\xi_3 = (\delta g_3 / \delta g_2)_{T \mu_1 \mu_3} = 1/\rho_1 \; (\phi_2^\circ - \phi_2'^\circ) / 1 - \overline{V}_3 \rho_3) \quad \text{---------} \quad (5)$$

where $\rho_3$ is density of the third component (cosolvent), $\rho_i$ are the densities of the solvent /protein solutions and T is the thermodynamic temperature, $g_i$ is the concentration of component i, in grams per gram of solvent, and μ is its chemical potential, and $\overline{V}_3$ is the partial specific volume of the component 3.

The preferential interaction parameter in mole/mole basis is calculated from the following equation (Arakawa and Timasheff, 1984).

$$(\delta m_3/\delta m_2)_{T\,\mu_1\,\mu_3} = M_2/M_3\,(\delta g_3/\delta g_2)_{T\,\mu_1\,\mu_3} \quad \text{---------} \quad (6)$$

where, $m_i$ is the molal concentration of component i, where $M_2$ is the molecular weight of the protein and $M_3$ is the molecular weight of the cosolvent.

The values of 'ϕ' were plotted as a function of protein concentration and the value extrapolated to zero concentration gives the partial specific volume of the protein, $\overline{V}$. Preferential interaction measurements were done in isomolal and isopotential conditions. In isomolal condition ($\phi_2^\circ$) the concentration of diffusible component is kept identical where as in isopotential condition ($\phi'^\circ_2$) the chemical potential of the component 3, is kept constant between the protein solution and the solvent. The isopotential condition could be achieved by dialyzing the protein solution against the cosolvent solution.

## *2.6.* Chemical analysis

### *2.6.1. Moisture*

The moisture content of whey was determined according to the oven method described by AOAC (2005). 10 g of the sample was accurately weighed into the clean dry petri dish and dried at 100°C for 5 h, cooled in a dessicator and weighed till the constant weights were obtained. Moisture content was expressed as g/100 g sample.

$$\% \text{ Moisture} = \frac{100\,(w_1\text{-}w_2)}{(w_1\text{-}w_2)} \quad \text{----------} \quad (7)$$

where, $w_1$ is the weight of petri dish and sample before drying and $w_2$ is the weight of petri dish and dried sample.

### *2.6.2. Total lipids*

The crude lipid content of whey was determined by Soxhlet extraction method. About 1 g of the whey was accurately weighed into a dry thimble and extracted using petroleum ether (60-80°C boiling range) as solvent for 10-12 h. Extraction was carried out by heating the soxhlet unit at 60°C on a thermostatically controlled water bath. The fat extracted was collected in a previously weighed dry flat bottom flask and separated from the solvent by evaporating over hot water bath. The flask was dried in an oven at 80-100°C and cooled till the constant weight was achieved. The fat content was expressed as g/100 g sample (AOAC, 2005).

$$\text{Crude fat (\%)} = \frac{\text{Final weight} - \text{Initial weight}}{\text{Sample weight}} \times 100 \quad \text{----------} \quad (8)$$

### *2.6.3. Total ash*

The total ash was determined according to the procedure described in AOAC (2005). 2-5 g of sample was accurately weighed into cleaned dried, weighed tarred silica crucible ($W_2$). The initial ashing was carried out over a low flame to char the sample. The crucible was then transferred to muffle furnace maintained at 500-550°C to get the white ash. The crucible was then cooled until a constant weight ($W_1$) was achieved (AOAC, 2005).

$$\text{Ash content (\%)} = \frac{W_2 - W_1}{\text{Weight of the sample}} \times 100 \quad \text{----------} \quad (9)$$

### *2.6.4. Total nitrogen*

The total nitrogen was determined by Kjeldhal method described in AOAC (2005). 1 g of the sample was weighed in to the Kjeldhal flask. About

0.5 g of digestion mixture was added and digested with 20 ml of concentrated sulphuric acid, until organic matter was oxidized and greenish blue digest was obtained. The digest was cooled and made up to 50 ml with distilled water. An aliquot of 5 ml sample was taken for steam distillation in the Gerhardt distillation unit with 20 ml of 40% sodium hydroxide solution. The liberated ammonia was trapped in 5 ml of 2% borate containing 0.01 ml of mixed indicator (0.033% (w/v) methyl red and 0.167% (w/v) bromocresol green) and titrated against N/70 HCl. Oven dried analytical grade ammonium sulfate was used as the standard for determining the acid factor. Total protein was calculated from the nitrogen content of the sample protein, calculated using the factor of 6.38 and expressed as percentage protein in the sample.

## 2.7. Electrophoretic methods

### *2.7.1 Native polyacrylamide gel electrophoresis (Native-PAGE)*

Non–denaturing PAGE was carried out using 1.5 mm slab gels in a discontinuous buffer system (Laemmli, 1970). The gel buffer used was 0.125 M Tris, 0.3 M glycine of pH 8.3. The protein samples (40-50 μg) were mixed with the sample buffer (0.125 M Tris-HCl buffer, 20% glycerol and 0.02% bromophenol blue). After an initial pre run for 30 min, the samples were loaded on to the wells and run at 100 volts till the moving front touch the boundary. The gel was stained with 0.1% coommassie brilliant R-250 prepared in 25% methanol and 10% acetic acid and destained in 25% methanol and 10% acetic acid.

### *2.7.2. Sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE)*

SDS-PAGE was carried out using discontinuous buffer system as described by (Laemmli, 1970). Electrophoresis was carried out with the

proteins extracted in buffer (0.125 M Tris-HCl buffer, 4% SDS, 2% 2-mercaptoethanol, 20% glycerol and 0.02% bromophenol blue). A discontinuous gel of acrylamide concentration T% = 10% and C% = 2.5% was used (T% is the concentration of total monomer present in the solution and C% is the concentration of crosslinker bis acrylamide, to the total monomer concentration). For polymerization of the gel, TEMED was used as an initiator and ammonium per sulfate as catalyst. The protein samples were mixed with the sample buffer (0.125 M Tris-HCl buffer, 4% SDS, 2% 2-mercaptoethanol, 20% glycerol and 0.02% bromophenol blue) in the ratio of 1:1 and boiled in boiling water bath for 5 min, cooled and centrifuged. The centrifuged samples were loaded to the wells and run under constant voltage of 100 A. After the run the gel was stained in 0.1% coommassie brilliant blue R-250 dissolved in 10% acetic acid and 25% methanol and destained. The destaining was done in 10% acetic acid and 25% methanol.

### *2.7.3. Isoelectric focusing*

Isoelectric focusing was done on 1mm thick 5%T, 3%C polyacrylamide gels containing 10% glycerol and pharmalyte pH 3-10. The gels were placed on the isoelectric focusing apparatus (Multiphor II, electrophoresis unit, Amersham Biosciences, UK) and connected to appropriate electrodes. 1 M NaOH is used the cathode buffer and 1 M $H_3PO_4$ is used as the anode buffer. The gel was focused for 1 hr at 1500 V, 200 mA and 30 W. The focused gel was fixed in 10% TCA for 1 h and washed with distilled water and equilibrated for 30 min in aqueous solution of 25% methanol, 5% acetic acid. The gel was then stained for 10-20 min in 0.1% coommassie blue G-250 in aqueous solution containing 25% methanol, 5% acetic acid). Destaining of the gel was done in 25% methanol, 5% acetic acid, until background becomes clear. The destained gel was preserved after dipping in solution containing 5% glycerol, 25% methanol for 1 hr. Broad range (pH 3-10) protein markers were used for isoelectric pH determination.

1. pI – 4.35 – Soybean trypsin inhibitor; 2. pI – 5.20 – β-Lg A; 3. pI – 5.85 – Bovine carbonic anhydrase B; 4. pI – 6.55 – human carbonic anhydrase B; 5. pI – 6.85 – Myoglobin acidic band; 6. pI – 7.35 – myoglobin –basic band; 7. pI – 8.15 – lentil lectin-acidic band; 8. pI – 8.65 – lentil lectin-basic band; 9. pI – 9.30 – trypsinogen.

## 2.8. Amino acid analysis

Amino acid analysis of the proteins was performed according to the method of Bidlingmeyer et al., (1984) using Waters Associates Pico-Tag ™ Amino acid system. An aliquot of the purified protein (5 mg) of β-Lg and whey were dried under vacuum and 1 ml of HCl was added. β-Lg and whey were hydrolysed in the oven at 110°C for 48 h.

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

The HPLC system (Water Associates) consisted of two water M 6000A solvent delivery system and an M 441 fixes wavelength detector (254 nm) controlled with an M 660 solvent programmer. The temperature was maintained at 38 ± 1°C with column heater. The samples were injected in volumes ranging from 5-50 µl using a model U6 k injector. The column was an application specific Pico-Tag column (150x3.9 mm). The solvent system consisted of (1) Solvent A: mixture of an aqueous buffer (0.14 M sodium acetate containing 0.5 ml/L TEA, pH 6.4): Acetonitrile (94:6) and (2) solvent

B: 60% Acetonitrile in water. The gradient run for the separation at the flow rate of 1 ml/min consisted of: 100% A and 0% B (initial) 54% A and 46% B (10 min), 0% A and 100% B (11 min), 0% A and 100% B (13 min) 100% A and 0% B (14 min) and 100% A and 0% B (25 min).

## 2.9. Degree of hydrolysis

The degree of hydrolysis was determined by TNBS method. The TNBS reaction was carried out as follows: 0.250 ml of the sample containing in between $2.5x10^{-4}$ and $2.5x10^{-3}$ aminoequi/L, is mixed in a test tube with 2 ml of phosphate buffer at pH 8.2. 2 ml of TNBS solution was added mixed and placed in water bath at 50°C for 60 min. During incubation, the test tubes and the water bath were covered with aluminium foil because the TNBS gives colour with the exposure to light even in absence of protein. After 60 min, 4 ml of 0.1 N HCl is added at 25°C to terminate the reaction. Absorbance was recorded after 30 min against water at 340 nm. The reaction on the blank and the standard solution was carried out by replacing the sample with 1% SDS and $1.5x10^{-3}$ M L-Leucine in 1% SDS. The absorbances of the blank and the standard were determined as the averages of three individual determinations (Alder-Nissen, 1979).

### *2.9.1. Hydrolysis of β-Lg with α-chymotrypsin*

β-Lg was hydrolysed using α-chymotrypsin using enzyme to protein ratio of 1:100 at 37°C, pH 7.9 in 0.02 M phosphate buffer. The samples were withdrawn at a time interval of 30 min and checked for degree of hydrolysis. The degree of hydrolysis was calculated for the protein dissolved in 20% cosolvent concentration. The effect of cosolvent on the degree of hydrolysis was studied using same method (Singh and Creamer, 1993).

### *2.9.2. Hydrolysis of β-Lg with pepsin*

β-Lg was hydrolysed with pepsin using enzyme to protein ratio of 1:30. β-Lgwas dissolved in 0.5 M Tris-HCl buffer. The pH of the protein solution was adjusted to 2. The reaction was carried out at 37°C. Samples were removed at intervals of 30 min and the enzyme was inactivated by adjusting the pH to 7 and analyzed by PAGE (El -Zahar *et al.*, 2005).

### *2.9.3. Hydrolysis of β-Lg with fungal protease*

β-Lg was hydrolysed with fungal protease using enzyme to protein ratio of 1:100. β-Lgwas dissolved in 0.02 M phosphate buffer, in pH 7.9. The reaction was carried out at 45°C. Samples were removed at intervals of 30 min and the enzyme was inactivated by boiling the protein with sample buffer and analyzed by PAGE (Martinez *et al.*, 2007).

## 2.10. Estimation of SS and SH groups

20 mg of the each of β-Lg, glycinin and α-globulin were dissolved in 2 ml guanidium hydrochloride in Tris-Glycine (TG) buffer (pH 8) and centrifuged. 0.5 ml of the protein was diluted to 1 ml with guanidium hydrochloride buffer. To this, 4 ml of urea Gu-HCl buffer was added. To this, 0.05 ml Ellman's reagent was added and absorbance was read at 412 nm. From the absorbance values the number of SH groups were calculated. To 1 ml protein, 50 µl mercaptoethanol and 4 ml of urea guanidium buffer was added and incubated for 2 h at 25°C. To this, 10 ml of 12% TCA was added and incubated for 1.5 h and centrifuged at 8000 x g at 25°C for 30 min. Precipitate was suspended in 10 ml of TCA. This experiment was repeated thrice and the error bar was 1%. The precipitate was dissolved in 10 ml urea TG buffer. 50 µl of Ellman reagent was added to this and absorbance was read at 412 nm. From this the number of SS groups was calculated (Beveridge *et al.*, 1974).

## 2.11. Measurement of transglutaminase activity

Enzyme activity of Tgase was measured by the colorimetric hydroxamate procedure (Folk and Cole, 1966) using 30 mM (benzyloxycarbinyl)-L-glutaminylglycine, 1 mM EDTA, 5 mM $CaCl_2$ and 0.1 M hydrozylamine in 0.1 M Tris acetate, pH 6 and 37°C. A unit of activity was defined as the amount of enzyme that catalyzes the formation of 1 μmol of hydroxamate/min.

### *2.11.1. Enzymatic reaction*

Solutions containing 10 mg/ml of protein (β-Lg and 11S protein fractions of soybean and sesame seed) were prepared in the Tris acetate buffer of pH 7.9. To these solutions enzyme was added at the concentration of 5 mg/ml of the commercial product. Reaction temperature was maintained at 37°C for 2 h. Samples were withdrawn at particular time intervals and reaction stopped by freezing the samples immediately. This enzyme does not need calcium for its activity. Tgase exchanges a wide variety of primary amines and ammonia at the carboxamide groups of glutamine residues. As a result of crosslinking high molecular weight protein complexes are formed.

## 2.12. Interaction studies

### *2.12.1. Effect of temperature on interaction*

β-Lg and 11S protein fraction of soy individually (in molar concentrations of $1x10^{-4}$ M) and in combinations were heated (50-90°C) for 10 min. The samples were then cooled in ice bath immediately after heating. Turbidity curves were established after cooling and by reading the absorbance at 660 nm at 25°C (Peng and Nielsen, 1986). The same procedure was used with different molar concentrations of 11S protein fraction of sesame seed. The two proteins were also heated from 50-90°C for 10 min in presence of 1 mM N-ethylmaleimide that is used as a disulfide linkage

blocker. To study the effect of dithiothreitol on the complexation, the proteins were heated as above in presence of 20 mM dithiothreitol. The experiments were repeated twice.

### *2.12.2. Effect of temperature on solubility*

The precipitates obtained by heating the protein samples were removed by centrifugation at 8,000 x g and at 4°C. The supernatant was collected and the absorbance was read at 280 nm and the solubility curve was plotted as a function of temperature (Peng and Nielsen, 1986)

## 2.12.3. Functional properties

### *2.12.3.1. Foaming capacity*

25 ml of different molar concentrations of β-Lg and 11S protein fractions of soybean and sesame seed individually and in various combinations in their respective buffers (β-Lg and 11S protein fraction of soybean were dissolved in 0.02 M phosphate buffer of pH 7.9, 11S protein fraction of sesame seed was dissolved in 0.06 M phosphate buffer containing 1 M NaCl) were whipped using a homogenizer (Miccra, D-8, Milian Labware, Gahanna, Ohio, USA), at a speed of 10,500 x g, for 1 min and at atmospheric pressure at 25°C. The whipped solution was poured immediately into a measuring cylinder and foam volume was measured (Poole *et al*., 1984).

### *2.12.3.2. Emulsifying activity*

25 ml of different molar concentrations of β-Lg and 11S protein fractions of soybean and sesame seed were dissolved in respective buffers, individually and in combinations were whipped with a homogenizer (Miccra, Ohio, USA) at a speed of 10,500 rpm with 8 ml of refined sunflower oil at atmospheric pressure and at 25°C for 1 min. 50 µl of the sample was

withdrawn and dissolved in 5 ml of 0.1% SDS. The absorbance was read at 500 nm and plotted against respective absorbance (Pearce and Kinsella, 1978).

### *2.12.3.3. Water absorption capacity*

The water absorption of whey was determined according to the procedure of Sosulski (1962). 1 g of the whey protein was taken in to the centrifuge tube, which was previously weighed. To this 10 ml of the distilled water was added mixed well with the help of cyclomixer. The tubes were allowed to stand for 30 min with gentle stirring. After 30 min the suspension was centrifuged at 6000 x g for 30 min and supernatant discarded. The tubes were dried in hot air oven at 50°C for 30 min and were weighed. The difference in the weights gave the amount of water absorbed by the material. Water absorption capacity was expressed as the amount of water absorbed by 100 g of the protein.

### *2.12.3.4. Fat absorption capacity*

The fat absorption of whey was determined according to the procedure of Sosulski (1962). 1 g of the whey was taken in to the centrifuge tube, which was previously weighed. 10 ml of the oil was added and mixed with the help of cyclomixer and allowed to stand for 30 min. After 30 min, the suspension was centrifuged at 6000 x g for 30 min. The supernatant oil was collected and discarded. Centrifuge tube was weighed and the difference in the two weights gave the amount of oil absorbed by the material. Fat absorption capacity was expressed as the amount of oil absorbed by 100 g of the protein.

## 2.13. Determination of gel strength

Gels were obtained using different concentrations of protein in a boiling water bath. Gel strength was determined using TAxT2i Texture Analyzer of Stable Micro systems, England. A load cell of 5 kg was used for the analysis. Compression platens were used to measure compression/

recovery of the gel formed. The maximum force reading is taken as the gel strength (Singh and Balange, 2005).

## 2.14. Scanning electron microscopy

SEM studies of the freeze-dried protein samples were carried out using LEO 435VP, Cambridge model surface Scanning Electron Microscope (Poloron SEM, Cambridge, UK). Before loading the samples into the system, the samples were coated with gold using Poloron SEM coating system E-5000. Average coating time was 2-3 min. Thickness of coating was 200-300 nm, which was calculated using the formula given by Hoshi and Yamauchi (1983),

$$T = 7.5\ I \times t \qquad \text{----------} \quad (10)$$

where, I is current in mA; t is the time in min and T is Thickness in Å. The coated samples were loaded to the system and the image viewed under 20 KV potential using 35 mm Picoh camera.

## 2.15. High pressure liquid chromatography

HPLC was done using a binary HPLC pump Waters 1525, Waters Associate Pvt. Ltd., Milford, USA. The column used was SHODEX-PROTEIN SHODEX- Column 803; (Shoko America Inc, Colorado, Springs Co, 80906, USA); 8 mm x 300 mm, the exclusion limits were 1.5 x $10^5$ Da and 6 x $10^5$ Da (Rodrigo and Milena, 2005). Buffer is used as the eluent for all the runs.

### *2.15.1. Determination of the complex formed by β-Lg and 11S protein fractions of soybean*

β-Lg and 11S protein fractions of soybean dissolved in 0.03 M Tris HCl buffer of pH 8.0 was mixed in molar ratios (1 x $10^{-4}$ M) of 1:1 and heated at 70°C in a water bath for 10 min. The heated samples were then centrifuged at 8000 x g at 4°C for 30 min. 20 µl sample was injected into the column (the

column used was SHODEX-PROTEIN 803; 8 mm x 300 mm, the exclusion limits was 1.5 x $10^5$ Da ). The proteins were eluted with 0.03 M Tris HCl buffer of pH 8.0.

### *2.15.2. Determination of the complex formed by Tgase*

Solutions containing 10 mg/ml of protein (β-Lg and 11S protein fractions of soybean and sesame seed) were prepared in the Tris acetate buffer of pH 7.9. To these solutions, Tgase was added at the concentration of 5 mg/ml of the commercial product. Reaction temperature was maintained at 37°C for 2 h. Reaction was stopped by immediately freezing the samples withdrawn at time interval of 30 min. 20 µl sample was injected into the column (The column used was SHODEX-PROTEIN 803; 8 mm x 300 mm, the exclusion limit was 1.5 x $10^5$ Da). The proteins were eluted with 0.1 M Tris acetate buffer of pH 7.9.

## 2.16. Gel filtration chromatography

Gel filtration of β-Lg was carried out using Sephadex G-75 packed into a glass column (1.2 x 100 cm). Gel column was equilibrated with 0.02 M phosphate of pH 7.9 before loading the protein. A known amount of the protein in mg/ml was loaded to the column. Elution was carried out with 0.02 M phosphate buffer of pH 7.9. Protein fractions were collected using fraction collector (Redifrac, Amersham Pharmacia). The protein content of the collected fractions was monitored by the absorbance recorded at 280 nm using spectrophotometer (Shimadzu, UV-Mini, 1240). Absorbance values were plotted against the elution volume to get the gel filtration profile of the isolated protein. Void volume of the column was determined with blue dextran.

## 2.17. Protein estimation by Folin Ciocaltue's phenol reagent

The protein content was measured according to the procedure of Lowry *et al*., (1951). Reagent A: 2% $Na_2CO_3$ in 0.1 N NaOH; Reagent B: 0.5% $CuSO_4.5H_2O$ in 1% potassium tartarate; Reagent C: Alkaline copper solution. Mix 50 ml of reagent A with 1 ml of reagent B. Reagent D: diluted Folins reagent. To a sample of 5–100 μg of protein in 0.2 ml of buffer, 5 ml of reagent C is added. Mixed well and allowed to stand for 10 min. To this 0.5 ml of reagent D is added very rapidly and mixed within seconds of adding the reagent. After 30 min the absorbance was recorded at 660 nm. Standard graph was plotted against known protein concentration of BSA versus absorbance.

## 2.18. Estimation of tryptophan

Tryptophan content of β-Lg was estimated by spectrophotometric titration method using N-bromosuccinimide (NBS) in Cintra - 5 double beam Spectrophotometer. Concentration of protein used was 1 mg/ml. The concentration of NBS was 5 mM. Addition of NBS results in the oxidation of indole chromophore of tryptophan, which has a strong absorption at 280 nm, to oxindole a much weaker chromophore at this wavelength. The addition of NBS was continued until no further decrease in absorbance ignoring the small decrease expected from dilution. The number of tryptophan residues/mole of protein (N) was calculated by using the equation (Spande and Witkop, 1967),

$$N = \frac{\Delta A \times 1.31 \times MW}{A_i \times a_f \times 5500} \qquad \text{---------- (11)}$$

where, ΔA is the corrected absorbance decrease at 280 nm, $A_i$ is the initial absorbance of the protein at its absorption maxima, $a_f$ is the absorptivity

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

## 2.19. Measurement of pH

The pH of the solution and buffers were measured using Control Dynamics pH meter (CD Instruments, Bangalore, India). The pH was measured at 27°C. The pH meter was calibrated with standard pH buffers from Merck, India, before every measurement.

## 2.20. Measurement of apparent viscosity

Protein was dissolved in 0.02 M phosphate buffer, pH 7.9 at various protein concentration and stirred for 1 hr at 27°C. Viscosity was measured using rheometer (Rheology International Shannon Ltd, Shannon, Ireland, R:I: M-3 Model). Spindle number 2 was used at a speed of 50 rpm and rotated for 1 min in the protein solution. Apparent viscosity is measured as percentage torque versus time in sec and expressed in units of centipoises (Quinn and Beuchat, 1975). Mean of triplicate values were calculated.

## 1. *Isolation and characterization of β-Lactoglobulin from bovine milk whey*

Foams are biphasic colloidal systems with a continuous liquid phase and dispersed gas bubble phase. Some food proteins are capable of forming good foams and their capacity to form and stabilize foams depends on the type of protein, degree of denaturation, pH and whipping methods. It has been reported that limited proteolysis may improve foaming capacity (Chobert *et al*., 1988). Rapeseed protein hydrolysates showed higher foaming capacity and whippability than the native proteins (Chobert *et al*., 1988). Hydrolysates with increased degree of hydrolysis are capable of foaming but reduces the foam stability as a result of reduction in peptide size.

The most frequently used emulsion is oil-in-water emulsion. It is reported that the emulsifying properties are improved by limited hydrolysis, owing to exposure of hydrophobic amino acid residues, which interact with oil, and the hydrophilic amino acid residues interact with water (Javier *et al*., 2000). Many enzymes could be used to modify functional properties. The difference in functionality of proteins results from difference in the structural properties. Enzymatic modification affects conformation of the protein molecule and consequently their physicochemical properties. Hydrolysis of protein results mainly the breakdown of peptide bonds, increase in number of functional groups to ionize and also leads to the exposure of hydrophobic sites. A certain minimum molecular weight of the peptides formed is therefore a necessary criterion for good foaming and emulsifying activity.

Proteins are hydrolysed to improve water solubility. Production of enzymatic protein hydrolysates has undergone considerable development in recent years. Protein hydrolysates with low degree of hydrolysis (less than 10%), with better functional properties than the native proteins like water and oil absorption, foaming capacity or emulsifying activity are used as food ingredients (Panyam and Kilara, 1996). Protein hydrolysates with variable

degree of hydrolysis are used as flavoring agents in soups, sauces and meat products (Weir, 1986). Protein hydrolysates are used as protein supplements or in special diets such as in the production of hypoallergenic foods (Frokjaer, 1994).

The heat induced protein precipitation constitutes a serious constraint in the manufacture of various whey products, when thermal processing is carried out under conditions that enhance protein aggregation. Seed proteins like sesame and soybean proteins are also incorporated into various products for enhanced functional and nutritional values. Retention of the protein solubility in many food systems is for maintaining the nutritional qualities of the final product (Bernal and Jelen, 1985).

β-Lactoglobulin is reported to be present in many, but not in all mammalian species, human and lagomorph milks being the notable examples. β-Lg belongs to lipocalin family of proteins-which presents diverse functions. β-Lg is a small molecule, soluble in dilute salt solution, as befits a globulin with 162 amino acids that folded up into an eight-stranded antiparallel β-barrel with a three turn α-helix on the outer surface and a ninth β-strand flanking the first strand (Kontopidis *et al*., 2004) as shown in Figure 4.

β-Lg exists as a dimer at normal physiological temperature and pH and dissociates slowly into monomers with elevated temperature and lower pH. The critical changes involve exposure of sulphydryl group to the protein surface that becomes available for intermolecular interactions (Thomas *et al*., 2004). Heat treatment of the protein results in considerable loss of solubility due to considerable intermolecular interaction resulting in aggregation and precipitation. The heat-denatured proteins interact via irreversible thiol-disulfide interchange reactions to form protein aggregates.

With reference to the above-mentioned literature, work was carried out on the isolation of β-Lg, the major whey protein to 99% purity. Whey was hydrolysed in a view to enhance the functionality and also nutritional properties. Therefore, due to the nutritional and functional attributes, the combination of β-Lg and seed proteins could be an appropriate approach towards a balanced protein rich food.

## 1.1. *Characterization of whey*

Whey proteins were separated from the defatted milk and characterized for further experiments. Solubility and the native structure of the proteins are important determinants for the functional properties of the whey proteins. Therefore the solubility of whey proteins was determined as a function of pH (Figure 5). The solubility of whey was 70% in the pH range 4-5 and almost fully soluble in other pH ranges. Result shows that the minimum solubility of whey is in the pH range 4-5, which is the isoelectric pH of most of the whey proteins. All the further experiments were done in phosphate buffer of pH 7.9, since the solubility of whey proteins is pH dependent.

Whey contains 3.1 ± 0.1% moisture, 5.4 ± 0.2% of ash, 2 ± 0.3% carbohydrates in the form of glucose and 57 ± 1% protein (Table 3). Whey had a high content of ash, which shows that whey is a rich source of minerals. Fat content of whey is 1.5 ± 0.1%. Whey has good foaming capacity of 140 ml/g, which makes it a good candidate for beverage industries.

The amino acid composition of whey was determined as shown in Table 4. The whey proteins are rich in leucine and lysine. The high lysine content is noteworthy, as it is a limiting amino acid in many other proteins. It has low content of aromatic amino acids like tyrosine and phenylalanine. Such a property could make this protein a good source in special diets for patients with an impaired capacity to metabolize these amino acids

(Hambraeus, 1982). It is also a rich source of acidic amino acids like aspartic acid and glutamic acids and also a good source of essential amino acids. Hydrophobic nature of the proteins, which has been associated with foaming and emulsification results from the proportion of nonpolar amino acid side chains. The ionic nature of proteins, which influences the solubility and water binding, resulted from the suitable proportion of amino acids with basic and acidic amino acid side chains on the protein surface. The amino acid content of a protein largely determines the physicochemical properties of the proteins, which in turn determine the functional properties (MacRitchie, 1992). Whey has all the essential amino acids in higher concentrations compared to various vegetable sources, such as soybean, corn and wheat gluten (Walzem *et al*., 2002). It also has high concentration of branched chain amino acids, which are important factors in tissue growth and repair. With a high content of sulfur rich amino acids, immune system is enhanced through intracellular conversion of these amino acids to glutathione.

Whey proteins have good fat absorption capacity, solubility, viscosity and gelation (Table 5). Whey has good fat absorption capacity of 2.7 ± 0.25 g/g comparable to the standard soybean protein concentrate. These functionalities of whey proteins have wide application in food industry. The literature values for soy protein concentrate shows that it has a fat absorption capacity of 2.6 ± 0.1 g/g (Boyarioglu, 2006). The water absorption capacity was reported as 2.7 ± 0.2 g/g (Wong and Kilts (2003). The water absorption of whey was not detectable since the whey protein concentrate was soluble in water and the water layer did not separate out to measure the volume of water absorbed. So the functionalities of whey proteins are comparable with soy protein concentrate which is taken as the standard protein for functional properties.

One of the important functional attributes of whey is heat set gelation. The gelation increases with increasing concentration of whey (Table 6). 12% of

whey protein is the minimum concentration required for the formation of gel. Below this concentration there was no formation of gel. The soy protein concentrate gels at a minimum concentration of 8%, which is taken as the standard protein (Boyarioglu, 2006). Adhesiveness indicates the stickiness of the gel. Negative value indicates the force on the probe whereas; gel strength is the force on the gel, which decreases with increased gelation. Adhesiveness of the gel is concentration dependent. Whey protein has good gelling capacity when compared to soy protein concentrate.

Protein gels can be formed by addition of salts, changes in pH, addition of enzymes or by application of heat. Whey protein gels are obtained by application of heat. Gelation is a two step process; initial thermal unfolding (denaturation) of the protein and subsequently aggregation. Many factors affect the formation and properties of the gel. Protein concentration determines both the likelihood of gel formation and also the characteristics of the gel that forms. This is because when the protein concentration is too low to form a protein network. As the protein concentration increases, intermolecular crosslinks increase and gelation occurs. Further increase in protein concentration, change the strength and texture of the gel and water holding capacity.



## *1.2. Isolation and characterization of β-Lg*

β-Lg was isolated from bovine milk whey and characterized for further experiments. Gel filtration of the isolated protein was done by HPLC with shodex-150 column. The protein was eluted with phosphate buffer of 0.02 M and a pH of 7.9. HPLC chromatogram showed a single peak, which eluted at 11.4 min (Figure 6). The purity of the isolated protein was further checked by SDS-PAGE. The bands obtained in SDS-PAGE corresponded to 18.2 KDa compared to the β-Lg obtained from commercial source (Figure 7).

Gel filtration pattern of the isolated protein showed a single peak, which eluted at 50 ml on a Sephadex G-75 column (Figure 8).

The isoelectric pH of the isolated β-Lg was determined (Figure 9a). The isolated protein showed bands corresponding to marker bands at isoelectric pH of 5.2. The isoelectric pH of the protein is at pH 5.2, where the protein is least soluble. Hence all the further experiments with the isolated protein were done in phosphate buffer of 0.02 M and pH 7.9. The solubility of the isolated protein was determined in different molarities of NaCl. Solubility was not affected by the concentration of sodium chloride. Therefore solubility of protein was determined as a function of pH. The minimum solubility was at pH range of 4–5 as shown in Figure 9b.

The amino acid composition of the isolated protein was determined and compared with the commercially available β-Lg (Table 7). The isolated protein had 98% correlation with the standard protein. The protein is rich in leucine and lysine. β-Lg has two tryptophan residues as determined by NBS method. The two tryptophan residues are at the position 19 and 61 ($Trp^{19}$, $Trp^{61}$) of the protein molecule (Sava *et al*., 2005). Out of the five cysteine residues, four of them were reported to form the disulfide linkages ($Cys^{106}$ - $Cys^{119}$) and ($Cys^{66}$ - $Cys^{160}$) and has one free thiol group in position 121. In native β-Lg, the free thiol group is masked in the hydrophobic interior of the protein. The reactivity can be increased by protein unfolding induced by heat (Sava *et al*., 2005).

Intrinsic fluorescence emission spectrum of the isolated β-Lg was recorded over a range of 300 – 420 nm after exciting the protein molecule at 280 nm. The fluorescence spectrum of the isolated protein showed emission maxima at 334.5 nm (Figure 10). The intrinsic fluorescence is dominated by tryptophan emission. β-Lg has two tryptophan residues and it was shown that Trp19 is the major fluorophore in native β-Lg (Cho *et al*., 1994).

The secondary structure of β-Lg was followed by UV-CD spectra. The near UV-CD spectrum of native β-Lg (Figure 11a) shows very intense negative bands, the typical feature of native folded conformations. It displays negative bands at 294 nm, 285 nm, 277 nm, 268 nm, and 260 nm attributed to the transition of aromatic residues (Hong and Creamer, 2002). Trp residues are responsible for the ellipticities at 294 nm and 285 nm. Ellipticities at 277 nm, 268 nm and 260 nm are ascribed to tyrosine (Matsuura and Manning, 1994), with a contribution from sulfur containing amino acids of the protein below 270 nm. The circular dichroic spectra of β-Lg indicated that the protein is rich in β-structures (Figure 11b). The secondary structural content of β-Lg was calculated and the values were 24% of α-helix, 50% of β-structure and 26% aperiodic structure, respectively.

## *1.3. Functional properties of whey protein hydrolysate*

Whey protein was hydrolysed using papain and fungal protease in a view to improve the functional properties like foaming capacity, emulsifying activity and solubility. Fungal protease is a mixture of endo and exo peptidases for catalyzing the break down of a wide range of peptide bonds. The degree of hydrolysis was 60%, which was attained within 200 min (Figure 12). The foaming capacity of the control protein was 11 ± 1 ml was increased to 14 ± 1 ml when hydrolysed with fungal protease (Table 8). But the emulsion activity increased by 10% in the initial 30 min of hydrolysis and then decreased by 32% as the hydrolysis progressed to 150 min (Table 9), in accordance with earlier reports of sodium caseinate, which showed decreased emulsion activity on hydrolysis with bacillus *Subtilis protease* (Slattery and FitzGerald, 1998). As the degree of hydrolysis increased, emulsion capacity decreased. It is suggested that in order to maintain good emulsifying property, molecular weight of the peptide should not be less than 5000 Da (Kilara and Panyam, 2003). The decreased emulsifying activity may be due to

decreased surface hydrophobicity and substantially decreased activity of the hydrolysate to interact with oil-water interface.

There was not much change in foaming capacity when hydrolysed with papain (Table 10). On the other hand, hydrolysis with papain improved the emulsifying activity of whey by nearly 12% (Table 11). Papain has broad specificity, which allows it to more extensively hydrolyse proteins. Chen *et al*., (1993) and Monaco *et al*., (1987) have suggested that the β-barrel domain is the hydrophobic region of β-Lg, which binds the small hydrophobic molecules such as retinol. Hence this domain fragment showed excellent surface activity (Huang *et al*., 1996).

Similarly β-Lg was hydrolysed using pepsin and fungal protease. The hydrolysed β-Lg was analysed for the extend of hydrolysis on the PAGE. The protein was found to be resistant to the enzyme pepsin. In Figure 13 is shown the native PAGE of β-Lg hydrolysed with pepsin. There were no changes in the native PAGE in both the native protein and hydrolysed protein, where the protein treated with pepsin showed bands, which correspond to the native protein even after 2 h of hydrolysis. But the protein was hydrolysed completely with fungal protease (Figure 14). The protein band corresponding to the native protein completely disappeared after the protein was hydrolysed for 2 h. β-Lg is very stable at acidic pH and acid stability of β-Lg could be due to increased internal hydrogen bonding between either two titrated carboxyl groups or one amide and one carboxyl group. Thus the resistance of native β-Lg to peptic digestibility may reflect its stable conformation at acidic pH. Pepsin has specificity for peptide bonds adjacent to tryptophan, tyrosine, phenylalanine, leucine and isoleucine (Fersht, 1977). The resistance of native β-Lg to peptic digestibility indicates that these groups are not accessible to this enzyme in the given conditions.

*The major whey protein β-Lg was isolated from bovine milk whey and characterized. Nutritive value of a protein is related to its amino acid composition and*

*its hydrolysable character. Whey proteins, in particular β-Lg, has high content of essential amino acids. Hydrolysis of whey by various enzymes improved the functional attributes like foaming capacity and emulsifying activity. Whey protein hydrolysates are used in many food formulations. β-Lg was resistant to pepsin hydrolysis but was fully hydrolysed with fungal protease. These have a role in the biotechnological approaches for ensuring better hydrolysed proteins available for new food formulations.*

## 2. Protein-Protein interactions of β-Lactoglobulin with 11S fraction of soybean and 11S fraction of sesame seed

Soybean seed proteins are composed of two major components, β-conglycinin and glycinin. β-conglycinin is a trimeric protein composed of three kinds of subunits. The major soybean protein (Glycinin/11S Globulin) is a hexameric protein, which consists of six acidic and six basic polypeptide components (Prakash and Rao, 1986, Kitamura and Shibasakhi, 1975). An important chemical property of soybean proteins is their unique amino acid composition, which determines high nutritional value of proteins. These proteins are rich in lysine (Wolf and Tamura, 1969). 11S protein fraction makes up one third of the total soybean proteins and it accounts for most of this fraction. Glycinin contains a higher concentration of sulfur containing amino acids than the other protein subunits. The insolubility of soybean proteins mainly at higher temperature is because of interactions of disulfide linkages, which results in polymer formation. Solubility of the proteins is essential if the desired functional properties are to be achieved. A soluble product is also easier to formulate into finished products. Heat treatment makes the proteins insoluble, which inturn affects the functional properties. Soybean protein isolates had been in use in the formulation of coffee creamers, whipped toppings and infant formulas to replace milk proteins. Presently there is renewed interest in the study of soybean protein functionality in part, caused by the health claims related to the consumption of products containing soybean proteins (Kolar *et al*., 1979).

How an ingredient exerts its function in a given food system, may be studied by incorporating the various ingredients into the food formulations to produce food products. The results obtained in the complex system would be appropriate in model systems and formulation of food products using them.

Major protein fraction of sesame seed is α-globulin (Jones and Gersdorff, 1927; Nath and Giri, 1957b), which constitutes about 65% of the total proteins. It is a multimeric protein with 12 subunits with 6 basic and 6 acidic subunits. The physicochemical properties of α-globulin are well studied (Prakash, 1985; Lakshmi *et al*., 1985). This protein is soluble only at very high salt concentration (Guerra and Park, 1975; Prakash and Rao, 1986). α-Globulin from sesame seed and glycinin from soybean are an important source of essential amino acids (Nath and Giri, 1957a).

The utility of proteins in food formulations is affected by its interactions with other components in the food system. The studies on their interactions form an important topic from the point of utility in commercial applications. Protein interactions, which enhance the physicochemical characteristics, always find various applications in food processing industries.

The functional properties of soybean and milk proteins have been studied mainly from the product development prospective. Little research has been reported on the complex formed between the two proteins (Lee *et al*., 1990). Not much work is reported on the interaction of whey proteins and seed proteins at higher temperatures at which processing of various foods are carried out. A limited number of studies are available so far on the interactions of seed proteins in mixed protein systems. Little research has been undergone on the characterization of the complex formed between seed proteins like soybean and sesame and milk proteins during processing of foods at higher temperatures. In mixed seed and milk proteins systems various types of aggregates may be formed during heating, however the mechanism of formation of such aggregates are quiet unclear (Roesch and Corredig, 2005).

A better understanding of the formation of aggregates between the proteins is needed to optimize the texture and stability of food products containing these protein aggregates. The mechanism of aggregate formation

and the types of interactions need to be further investigated. The understanding of interaction between milk proteins and seed proteins may facilitate design of wide range of novel functional ingredients with enhanced functionality. In addition, a better knowledge of the stabilization and aggregation processes in milk is potential avenues to create new milk based products with novel textures and other organoleptic properties (Johnston *et al*., 1992; Morgan *et al*., 2005). The formation of soluble heat induced complex of soybean protein and β-Lg may not only depend on the temperature but also on the concentration of proteins available for interactions.

In the present work, we have studied the aggregation behaviour of a mixed system of major protein fractions of seeds (α-globulin from sesame seed and glycinin from soybean seed) and whey protein (β-Lg) and the formation of soluble and insoluble complexes in response to various physico-chemical parameters. The use of whey proteins and the major seed proteins in the formulations of protein-based foods is often attractive for both processing performance and nutritional quality.

## *2.1. Isolation of 11S protein fractions from soybean and sesame seed*

The major protein fractions from two different seeds (α-globulin from sesame seed and glycinin from soybean seed) were isolated and checked for homogeneity. HPLC profile of the isolated 11S protein fraction of sesame seed showed a single peak, which eluted at 10.2 min (Figure 15). Glycinin from soybean seeds also showed a single peak with a retention time of 10.1 min when eluted in 0.03 M Tris–HCl buffer, pH 8.0 (Figure 16). SDS-PAGE profile of α-globulin showed two bands corresponding to acidic and basic subunits (Figure 17). Both glycinin and α-globulin are heterogenous oligomeric protein

with a molecular mass ranging from 340–375 KDa and consists of both acidic and basic subunits.

## 2.2. *Effect of temperature on the interaction of β-Lg and 11S protein fractions*

The effect of heating on the turbidity of β-Lg, 11S protein fraction of soybean (concentration of $1\times10^{-4}$ M) and the mixture of β-Lg and 11S protein fraction of soybean in various molar ratios are shown in the Figure 18. The figure clearly shows that β-Lg remained almost clear throughout the temperature range from 50-95°C. On the other hand, the turbidity of 11S protein fraction of soybean appeared at 65°C and increased with increasing temperature. The turbidity of the mixture of β-Lg and 11S protein fraction of soybean reduced when compared to the turbidity of 11S protein fraction heated without the incorporation of β-Lg. These results indicate that interaction occurred between these two proteins at temperature of 60°C and above. The data clearly shows that the presence of β-Lg interfered with the precipitation of 11S protein fraction of soybean. The turbidity reduced when the molar ratios of β-Lg in the mixture was increased. The turbidity was less when the molar ratio between β-Lg and 11S protein fraction of soybean was 15:1 when compared to a molar ratio of 10:1.

Based on the above results, interaction occurred between the two proteins in the temperature range of 65–95°C. Similarly the effect of heating on the turbidity of β-Lg, 11S protein fraction of sesame seeds (concentration of $1\times10^{-4}$ M) and the mixture of β-Lg and 11S protein fraction of sesame seeds in various molar ratios are shown in Figure 19. The turbidity was high for both β-Lg and 11S protein fractions of sesame when heated above 60°C. Precipitation was high in case of β-Lg, 11S protein fraction of sesame seed and in combination at various molar ratios. The precipitation behaviour of β-Lg was different in this case, where the protein precipitated when heated above

60°C. β-Lg did not precipitate when heated at higher temperature in phosphate buffer of low ionic strength. But the presence of sodium chloride in buffer induced the coagulation of the protein at higher temperature. The high salt concentration of the buffer in which 11S protein fraction of sesame seed was dissolved induced precipitation of β-Lg. So both the proteins precipitated at higher temperature.

The effect of temperature on the solubility of β-Lg, 11S protein fraction of soybean seeds (concentration of $1\times10^{-4}$ M) and the mixture of β-Lg and 11S protein fraction of soybean seeds in various molar ratios were also determined (Figure 20). The solubility of β-Lg remained high throughout the temperature range of 50-95°C. 11S protein fraction of soybean showed minimum solubility above 60°C and remained insoluble throughout the temperature range. Solubility was high when β-Lg and 11S protein fractions of soybean were heated in various molar ratios. The solubility increased with the increase in the molar ratio of β-Lg. The solubility was high when β-Lg and 11S protein fractions of soybean was heated in molar ratios of 15:1 when compared to the solubility, when the proteins were in the molar ratio of 10:1.

Thus solubility data implies that interactions occur between β-Lg and 11S protein fractions between these temperatures ranges. In case of β-Lg and 11S protein fraction of soybean mixture, the solubility shifted towards the solubility curve of β-Lg where maximum solubility was observed when β-Lg and 11S protein fraction of soybean are heated together. However in case of β-Lg and 11S protein fraction of sesame seed, both the proteins indicated minimum solubility when heated in the temperature ranges as shown in Figure 21. The solubility was minimum even when β-Lg and 11S protein fraction of sesame seed were heated in various molar concentrations. The presence of β-Lg did not affect the solubility of 11S protein fraction of sesame

seed. Thus it can be concluded that interaction occurred between β-Lg and 11S protein fractions from both soybean and sesame seeds.

Heating of soybean protein with milk proteins form large size aggregates as mentioned by Roesch *et al.*, (2004). This thermal aggregation presumably involves predominantly of hydrophobic interactions between the 11S protein fraction and also between β-Lg and 11S protein fractions as reported earlier by Catsimpoolas *et al.*, (1970); Takagi *et al.*, (1979); Nemethy *et al.*, (1963). Soluble complex formation between soybean and whey proteins was reported earlier (Roesch and Corredig, 2005). Complex formation between 11S protein fraction and 13S globulin of soybean through disulfide linkages was also reported by Mori *et al.*, (1979). Hydrophobic interactions at higher temperature and thiol-disulfide exchange reaction are the two possible mechanisms responsible for the aggregation of unfolded β-Lg. Formation of intra and intermolecular disulfide bonds is shown to be the main factor responsible for the irreversible aggregation of β-Lg (Tatiana *et al.*, 1998).

Hence based on the above results described, it could be concluded that β-Lg form disulfide linkages with 11S protein fraction of soybean at higher temperatures which makes the protein more soluble at higher temperature. But the interaction of β-Lg with 11S protein fraction of sesame seed did not improve the solubility of these proteins at higher temperature.

## *2.3. Effect of N-ethylmaleimide on the interaction of β-Lg and 11S protein fractions*

To understand the role played by disulfide linkages in the interaction, β-Lg with 11S protein fractions of soybean and sesame seeds (concentration of $1\times10^{-4}$ M) were heated in the presence of NEM. NEM is a disulfide blocker. Soybean protein fractions heated in presence of NEM showed a reduction in

precipitation (Figure 22). This result showed that most of the aggregate formation in 11S protein of soybean is via disulfide linkages. The turbidity β-Lg did not show any change in the presence of NEM. But heating of various molar ratios of β-Lg and 11S protein fraction of soybean reduced the precipitation of 11S protein fraction of soybean. The data clearly shows that heating soybean 11S protein fraction in the presence of β-Lg resulted in the formation of soluble complex via disulfide linkages, which reduced the precipitation of 11S protein fraction of soybean at higher temperatures. The number of SH and SS groups of β-Lg and 11S protein fraction of soybean and sesame seed were determined by DTNB method and is shown in Table 12. The high content of thiol groups in β-Lg (1 mol/mol), which is comparable with the literature value, where β-Lg is shown to have 1 mole/mole of thiol groups (Fernandez Diez *et al.*,1964) may be responsible for the intermolecular interaction with glycinin, which had a thiol content of 16 mol/mol through disulfide interchange reactions. The literature value for glycinin (Hoshi and Yamauchi, 1983) shows that it has 14 mol/mol of thiol groups which could be compared with the experimental results obtained.

Similarly β-Lg and 11S protein fraction of sesame seed were heated in presence of NEM (Figure 23). As shown in the figure turbidity did not show any variations in the presence of NEM in both β-Lg and 11S protein fraction of sesame seed. The proteins when heated in combination of various molar ratios also did not show any change in the precipitation behaviour and confirms the fact that most of the aggregate formation in sesame is mostly by non-covalent interactions. The data clearly shows that the high salt concentration (1 M) in the buffer used favored hydrophobic interactions, which is also enhanced by the higher temperatures (Yamamoto *et al*., 1973).

The ratio of the two proteins in the mixture is very important in determining the type of protein aggregates, which formed during heating. When the molar ratio of β-Lg was increased, the aggregation of 11S protein

fraction of soybean was significantly reduced. Precipitation was less, whereas increasing the molar concentration of 11S protein fraction of soybean on the other hand enhances turbidity. Concentration of β-Lg is very critical in deciding the type of aggregate formed when the two proteins are heated. Temperature and the concentration of the proteins use are the two major factors, which determine the aggregate formation.

## *2.4. Effect of dithiothreitol on the interaction of β-Lg and 11S protein fraction*

To improve the reactivity of the functional thiol groups, β-Lg and 11S protein fractions of soybean were heated in presence of DTT (Figure 24). As shown in the figure the aggregation of 11S protein fraction of soybean was reduced in the presence β-Lg as shown by the reduction in the light scattering when β-Lg and 11S protein fraction of soybean (concentration of $1\times10^{-4}$ M) at 70°C. DTT cleaves disulfide linkages enabling the reduction of disulfide bonds partially unfolding the globular proteins and increases the interaction with other proteins. DTT reduced intermolecular disulfide bonds that maintain the globular structure of the protein. The protein molecules are partially denatured and held together by non-covalent interaction. At higher temperatures the protein molecules unfold completely to expose the buried reactive thiol groups to form intermolecular cross linkages as shown in other proteins by Dunkerly and Zadow, (1984).

Figure 25 shows the elution profile of the soluble complex formed when β-Lg and 11S fraction soybean protein were treated at high temperatures. The soluble complex had high molecular weight protein component also in it that is the β-Lg and 11S protein conjugate. SEM studies (Figure 26) showed that the control samples before heat treatment had a homogeneous structure. But the mixed system at higher temperature showed the formation of large particulate aggregates that formed a protein network.

Free sulfhydryl groups increased upon heat denaturation in many proteins (Pofahl, 1967), which would enhance the complex formation. Most of the sulfhydryl groups are buried inside the glycinin molecule in the native state (Catsimpoolas *et al*., 1970). At temperatures above 70°C the protein dissociates into subunits. These dissociated subunits have a tendency to interact and form insoluble aggregates (Peng *et al*., 1982). It has been reported that the interaction of glycinin and myosin under various conditions inhibits their aggregation above 90°C (Peng *et al*., 1982).

In native β-Lg, the free thiol group is not available on the protein surface. This free thiol group is therefore not available for intermolecular interaction reactions through disulfide reactions (Bryant and McClements, 1998). Heat induced changes of β-Lg solutions between 68–83°C causes protein unfolding. The possible exposure of functional thiol groups is more pronounced at higher temperatures (Dannenberg and Kessler, 1988; Manderson *et al*., 1999). Several changes in structure were observed as induced by heat due to changes in secondary and tertiary structures driven by the hydrophobic interactions and intermolecular interactions (Vetri and Mihtello, 2005). Above 80°C, the formation of disulfide bonds is favored as indicated in other proteins by Mohanan *et al*., (1995).

It is generally recognized that the first effect of heat is the reversible dissociation of the native β-Lg dimer into the monomers (McKenzie, 1971; Mulvihill and Donovan, 1987). The second change is the partial unfolding of the β-Lg monomer with a loss of helical structure (Qi *et al*., 1997), allowing free sulfydryl group on Cys 121 to interact with Cys 106 - Cys 119 disulfide bond and presumably to reversibly create a Cys 106 - Cys 121 disulfide bond and a free thiol containing Cys 119 (Croguennec *et al*., 2003). This may be the activated monomer postulated by McKenzie, (1971); Mulvihill and Donovan, (1987) as the starting point for aggregation leading to larger polymers with other disulfide bond containing proteins.

## *2.5. Functional properties of the mixture of β-Lg and 11S protein fractions*

The functional properties of the protein mixtures were analyzed. β-Lg and 11S protein fractions of soybean and sesame seed were blended in different ratios to evaluate the functional properties like foaming and emulsifying activity in the blended mixture. 11S protein fraction of soybean showed increased foaming capacity (Table 13a) with increase in the concentration of the protein. The foam volume of 18 mg/ml of glycinin was 5 ± 2 ml which increased to 25 ± 2 ml at a protein concentration of 90 mg/ml. Similar increase in foam volume was also showed by β-Lg. The foam volume of β-Lg and 11S protein fraction of soybean in various molar ratios however decreased slightly. For the blended mixture the foam volume was 13 ± 2 ml. Emulsifying activities for β-Lg and 11S protein fraction of soybean and in combinations of various molar ratios was also evaluated (Table 13b). Emulsifying activity was high both for the proteins and in the combination of proteins in various ratios by weight.

11S protein fraction of sesame seed showed increased foaming capacity (Table 14a) with increase in the concentration of the protein. The foam volume of 6 mg/ml of α-globulin was 5 ± 2 ml which increased to 20 ± 2 ml at a protein concentration of 60 mg/ml. Similar increase in foam volume was also showed by β-Lg. The foam volume of β-Lg and 11S protein fraction of sesame seed in various molar ratios also showed a foaming capacity of 30 ± 2 ml. Emulsifying activity for β-Lg and 11S protein fraction of sesame seed and in combinations of various molar ratios was also evaluated (Table 14b). β-Lg showed high emulsifying activity. But 11S protein fraction of sesame seed showed reduced emulsifying activity. The high salt concentration reduced the emulsifying activity of 11S protein fraction of sesame seed. The emulsifying activity was however enhanced when proteins β-Lg and 11S protein fraction of sesame seed were blended in various molar ratios. The order of processing, temperature, protein composition and concentration are

fundamental in determining the physicochemical properties of mixed systems. These physicochemical properties can be changed with interactions with other components like proteins and salts in the mixed system (Manion and Corredig, 2006).

*Thus interactions between two proteins not only depend on the temperature but on various other factors like concentration of the proteins, ionic strength of the buffer and other components like reducing agents. Interaction of β-Lg and 11S protein fraction of soybean showed that the two proteins interact to form soluble complex only above 65 ℃. Thus even though the amount of complex formed either in the case of β-Lg and glycinin or β-Lg and α-globulin is very less, the presence of the two proteins alters the functional and protein-protein complexation process. Since soluble protein exists in large part of the bulk solvent the presence of these has a charge shielding effect on the other proteins and in one case enhances functional attributes and in other case, perhaps decreases the functional attributes. This depends on the nature of the protein molecules and its surface properties and has a major role in driving the protein–protein interactions through energetically driven hydrophobic interactions as one of the processes.*

## 3. *Effect of Transglutaminase on protein-protein interaction of β-Lactoglobuin with 11S fraction of soybean and 11S fraction of sesame seed*

To be competitive in food ingredient markets the functionality of whey proteins must be continuously improved and designed for specific uses. Chemical and physical methods are commonly used for the modification of protein functionality. Temperature is very effective in altering the functionality of proteins. The enzymatic method of crosslinking of proteins can be easily controlled for specific functional attributes. Furthermore, drastic developments in the area of biotechnology had made available a wide range of enzymes suitable for the modifying proteins for enhanced functional properties. More recently, Tgases including mammalian and microbial origin have been employed for the protein modification (Motoki and Seguro, 1998; Aboumahmoud and Savello, 1990). The covalent crosslinking of proteins catalyzed by Tgases can cause dramatic changes in the size, conformation, stability and other properties of the proteins. The enzymes have been used for modifying the functionalities of various proteins (Truong *et al*., 2004).

Protein crosslinking plays an important role in determining the functional properties of food proteins. Manipulation of the number and nature of protein crosslinks during food processing offers a means by which the food industry can manipulate the functional properties of food, often without changing the nutritional qualities (Tanimoto & Kinsella, 1988). Food proteins are often denatured during processing and there is a need to understand the protein both as a biological entity with a predetermined function and as a randomly coiled polymer. One type of chemical reaction that has major consequences for the protein function in either native or denatured states is protein crosslinking. It is therefore protein crosslinking can have profound effects on the functional properties of food proteins. Food processing often involves high temperature, extremes of pH, particularly alkaline and oxidizing conditions, results in the introduction of protein

crosslinks producing substantial changes in the structure of proteins and the final product. The formation of this crosslink does not reduce the nutritional quality of the food, as the lysine residue remains available for digestion.

Tgase catalyzes the acyl transfer reaction of γ-carboxyl groups of glutamine residues to several acceptors like ε-amino groups of lysine in proteins (Soares *et al*., 2004). It is widely distributed in most animal tissues and body fluids and is involved in several biological functions. ε-N- (γ- glutaminyl) lysine crosslink can also be produced by severe heating (Motoki and Seguro, 1998).

The production of microbially derived enzymes proved pivotal in paving the way for many industrial applications (Chiya *et al*., 2001). Commercial preparation of microbial Tgases is calcium independent therefore it has wide application since proteins from soybean precipitate even in the presence of traces of calcium. Many reports are available in which Tgases are used for improving the functional properties of proteins (DeJong and Koppelman, 2002). In all the cases Tgases are reported to improve firmness, elasticity, water holding capacity and heat stability of proteins (Kuraichi, 1997). This enzyme is tried in many dairy products. The quality of soybean products has also been benefited from the introduction crosslinking by Tgases (Soeda, 2003).



## *3.1. Polymerization of β-Lg and 11S protein fraction by Tgase*

Polymerization reaction of β-Lg and 11S protein fraction of soybean and sesame were carried out with Tgase in 0.03 M Tris buffer of pH 8.0. The reaction temperature was maintained at 37°C. A protein to enzyme ratio of 10:1 was used for the enzymatic reaction. Polymers formed by the Tgase induced crosslinking were evident from the immobile protein polymer bands at gel origin on Native PAGE (Figure 27). These bands are indicative of high molecular species formed from crosslinking reaction formed of either β-Lg or

11S protein fraction of soybean or between β-Lg and 11S protein fraction of soybean. Original protein bands for the 11S protein fraction of soybean fully disappeared and only polymer band is visible. But the Native PAGE shows that unpolymerized protein bands of β-Lg also remained after the enzymatic reaction that showed that all β-Lg molecules did not participate in the polymerization reaction. The extent of crosslinking is demonstrated by disappearance of bands and accumulation of immobile protein polymers at the gel origin. This polymerization is also evident from the SDS-PAGE (Figure 28) of 11S protein fraction of soybean, which shows that all the basic and acidic subunits of 11S protein fraction of soybean disappeared as the reaction time increases.

Figure 29 shows the HPLC profile of 11S protein fractions of sesame crosslinked with Tgase. The HPLC profile shows both high molecular proteins formed from crosslinking and also low molecular proteins. Even though the amount of complex formed is not very high, it clearly emphasizes the point that the tendency of protein to aggregate in presence of Tgase can be used for its complexation. This reaction produces different kinds of complexes of the proteins of β-Lg and 11S protein fractions either alone or with each other. Figure 30 shows the HPLC profile of the 11S protein fractions of soybean crosslinked. Extensive crosslinking of protein is obtained incase of 11S protein fraction of soybean, which is not seen in 11S protein fraction of sesame seed. Figure 31 shows the HPLC profile of β-Lg and 11S protein fraction of sesame seed crosslinked by Tgase. But not all 11S protein fraction of sesame participated in the polymerization reaction (Figure 31). Only intermolecular crosslinking would increase molecular weight. The high molecular weight bands in electrophoresis and peaks in HPLC indicate the formation of perhaps protein polymers through intermolecular crosslinking. The extent of polymerization increased with increased incubation with Tgase.

## *3.2. Functional properties of the polymerized β-Lg and 11S protein fraction*

Furthermore, the functional attributes (foaming capacity and emulsifying activity) of β-Lg and 11S protein fraction of sesame seed and soybean protein crosslinked by Tgase were also evaluated. Foaming capacity and emulsifying activity of β-Lg and 11S protein fraction of soybean is shown in Table 15. As evident from the results, foaming capacity was less in case of polymerized proteins. The unpolymerized protein had a foaming capacity of 25 ± 2 ml at 20 mg/ml concentration of protein, where as Tgase treated protein had a foaming capacity of 20 ± 2 ml at 20 mg/ml concentration. On the other hand emulsifying activity was enhanced by the polymerization reaction to a limited extent. The emulsifying activity for the untreated protein was 0.58 ± 0.02 at 20 mg/ml concentration of protein and the treated protein had 0.72 ± 0.02 at 20 mg/ml concentration of protein. The improvement in emulsifying activity can be attributed to the branched nature of the polymers in accordance with the results of Mingxia and Srinivasan, (1999) as in the case of Tgase catalyzed polymerization of β-casein.

The foaming capacity and emulsifying activity of Tgase treated β-Lg and 11S protein fraction of sesame seed were also evaluated (Table 16). Both the treated and untreated proteins had a foaming capacity of 25 ± 2 ml at 20 mg/ml concentration of protein. Foaming capacity of the treated proteins did not improve with polymerization. But emulsifying activity was enhanced with the polymerization reaction. The untreated protein had an emulsifying activity of 0.56 ± 0.02 at 20 mg/ml concentration of protein which increased to 0.59 ± 0.02 for the polymerized protein. Foam formation is generally faster for more flexible random coiled structured proteins than for the tightly held structures (Halling, 1981; Damodaran, 1990). In addition increased hydrodynamic size (Damodaran, 1994), molecular weight (Mita, 1978) and neutralization of charged amino groups of lysine residues by crosslinking could improve foaming capacity.

The higher emulsion and foaming attributes of the treated protein could have been due to increased ability to form an interfacial film of protein. The large molecular size of protein formed due to crosslinking, is more resistant to denaturation during homogenization. Moreover reduced electrostatic repulsion as the result of decrease in the number of amino groups could have enhanced protein-protein interaction and therefore protein adsorption on the interface. Tgase catalyzed polymers of ovomucin and soybean proteins were also found to form better foam and emulsion forming ability than the native proteins (Rotini and Rickey, 1995). But in case of β-Lg and 11S protein fraction of soybean, extensive crosslinking reduced foaming capacity. Extensive crosslinking reduced foamability in case of sodium caseinate as reported by Flanagan and Fitzgerald, (2003). Reduced foam volume and foam stability of Tgase-catalyzed crosslinked sodium caseinate compared to non-crosslinked sodium caseinate were reported (Lorenzen, 2000).

The effect of heating on the aggregation of β-Lg and 11S protein fraction of soybean treated with Tgase were monitored (Figure 32). Untreated and treated β-Lg did not show turbidity even at 90°C. But 11S protein fraction of soybean showed aggregation at 50°C and above. Turbidity was observed when 11S protein fraction of soybean and β-Lg were heated at 50°C (Figure 32a). But the temperature was enhanced to 65°C (the temperature was increased at the rate of 1°C per min) when 11S protein fraction of soybean and β-Lg were treated with the enzyme Tgase (Figure 32b).

Similarly, in 11S protein fraction of sesame, turbidity was observed at 60°C for the untreated protein. The temperature extended to 70°C when the 11S protein fractions of sesame seed and β-Lg and were polymerized with Tgase as shown in Figure 33. Tgase modify food proteins by covalent crosslinking is reported to induce crosslinks in myofibrillar proteins like actin

and myosin (DeBacker *et al*., 1992; Kim *et al*., 1993; Akamittah and Ball, 1992; Tseng *et al*., 2002).

Proteins treated with Tgase form more compact structures which make it more heat stable. Similar reports have been reported where increase in the thermal stability of oat globulin has been attributed to the formation of aggregates with compact network (Ma and Harwalkar, 1988). Effects of heat treatment on proteins are important since heat often diminishes solubility (Kinsella, 1976). A single crosslink between Lys $82\beta_1$ and Lys $82\beta_2$ of metheamoglobin A increased denaturation temperature from 40.7°C to 57.1°C as reported by Yang and Olsen, (1988); Whitaker, (1988). Crosslinking of sodium caseinate with Tgase resulted in the lower turbidity at 140°C. This indicated that the crosslinked products were more heat stable than the unmodified sodium caseinate (Flanagan *et al*., 2003). Thus Tgase polymerization of β-Lg and 11S protein fraction of soybean and sesame seed enhanced the heat stability of the polymerized proteins.

Only a small amount of oligomerization is necessary for a significant enhancement of functional properties as shown by Sabin *et al.,* (2000), wherein, small amount of casein oligomerization is necessary for a significant enhancement of yogurt breaking strength.

The changes in the fluorescence intensity indicated changes in the microenvironment of the chromophores induced by the crosslinking either embedded between the surfaces or at the interface of the proteins. However it is difficult to predict the exact location of the chromophores either in the complex or between. The fluorescence intensity decreased for the polymerized protein without change in the emission maxima as shown in Figures 34a & 34b. The decrease in the fluorescence intensity reflects that the tryptophan residues may have been associating in more non polar region because of polymerization.

Rate of crosslinking by Tgases depends on the particular structure of the proteins acting as a substrate. Most efficient crosslinking occurs in proteins that contain glutamine residues in a flexible region of the protein or within the reverse turns (Dickinson, 1997). Globular proteins are poor substrates for the crosslinking reaction of Tgase. Denaturation increases their reactivity. Heating of β-Lg at 70°C increased the polymerization reaction (Figure 35). Heating induces conformational and structural alteration in proteins causing proximity changes in some of the lysyl or glutamyl residues. This enhanced intermolecular crosslinking.

Treating β-Lg with DTT also improves the polymerization reaction as shown in the Figure 36. These treatments of the proteins cause partial unfolding of the globular protein, which enhances the polymerization by exposing the buried amino acid residues. The results showed that disulfide reduction was necessary to make lysyl and glutamyl residues accessible to the active site of Tgase. Whey proteins are more susceptible to Tgase in presence of DTT.

*The protein polymers obtained from crosslinking of β-Lg and 11S protein fractions of seed proteins enhances the functional properties compared to unpolymerized proteins. The heat stability of the polymers was enhanced significantly than the unpolymerized proteins due to introduction of covalent bond induced by the polymerization reaction. The results indicate that the Tgase induced crosslinking of β-Lg and 11S protein fractions of seed proteins can be used to modify the heat stability of proteins. Therefore the limitations to the utilization of whey or seed proteins in products, which requires higher temperature in processing, can be overcome by polymerization reaction using Tgases. The resulting protein-protein interaction enhances the emulsifying activity, which makes these protein polymers useful in various food products like whipped toppings and creams. Thus Tgase could be used to improve the functional attributes of proteins with varied applications in food products.*

## 4. Structural stability of β-Lactoglobulin as a function of cosolvents

β-Lg is the major whey protein with wide applications in many industrial processes. During processing, this protein is subjected to extreme of temperature that results in the denaturation of the protein. Protein stability always plays an important role not only in its biological function but also in various commercial applications. When compared to physiological conditions, isolated proteins are usually subjected to adverse environmental conditions, which might lead to inactivation, denaturation and aggregation. So search on a technique to protect proteins against extreme conditions is always one of the major topics of protein science (Chi *et al*., 2003).

Stability of proteins could be enhanced by a variety of substances that maintains the native conformation of the protein. Sugars, sugar alcohols, polyethylene glycol and amino acids were reported to enhance the structural stability of the proteins (Timasheff, 1994). These substances are termed as cosolvents, which stabilize the proteins and therefore termed as thermodynamic boosters. Glycerol and other cosolvents have been shown to affect both structural and thermodynamic properties of proteins (Timasheff and Arakawa, 1990). Stabilizing effect of sucrose stems from preferential hydration of proteins in this medium, which in turn, may be related to the increase in free energy of cavity formation by the addition of sucrose to water (Arakawa and Timasheff, 1996).

Protein molecules in aqueous solution are surrounded by a hydration shell, which is composed of water molecules on the protein surface. If an organic molecule is present, the solvent molecule tends to displace the water molecules both in the hydration shell and in the interior of the protein thereby distorting the interactions responsible for the maintaining the native conformation of the proteins (Kita *et al*., 1994; Timasheff, 2002). The

mechanism by which these cosolvents stabilize the protein is either by preferential hydration or by preferential interaction.

Stabilization effects are due to solvent medium effects or due to the alteration of the water structure in the bulk solvent. This view explains why protein stabilization is generally enhanced with increasing concentration of cosolvents. Gekko and Timasheff, (1981) had proposed that the preferential hydration of protein in aqueous glycerol must be due to repulsive forces between glycerol and non polar regions located on the protein surface. Since glycerol is essentially hydrophilic may penetrate into the solvation sheath of protein. There seems to be a delicate balance between repulsion from non polar regions and attraction from polar regions of the protein surface by which the glycerol molecules are preferentially excluded form the protein surface.

The protective mechanism of cosolvents has traditionally been attributed to "preferential hydration", of the protein. From the results of hydrogen/deuterium (Bolen, 2001; Bolen and Baskakov, 2001) further suggested a mechanism of "solvophobic thermodynamic force", which indicated that the unfavorable interaction between cosolvents and the peptide backbone raised the free energy of the denatured state and as a result, protected the protein by shifting the equilibrium in favour of the native state (Bolen, 2001).

The fundamental observation in the stabilization of macromolecules in a three-component system has a role to play in the stabilization of enzyme and proteins (Radha *et al*., 1998; Rajendran *et al*., 1995; Rajeshwara and Prakash, 1994), especially in seed proteins. The behavior of certain proteins in polyols underlines a very fundamental concept of preferential exclusion and preferential interaction of such cosolvents with these macromolecules. In this section, the effect of cosolvents such as sucrose, glycerol and sorbitol on β-Lg

is elucidated. The effect of different concentration of cosolvents on the protein structure is determined by measurements on partial specific volume, from which the preferential interaction parameters were calculated. These results were further supported by fluorescence spectra, CD spectra and thermal denaturation curves. These results would therefore throw insight into the various molecular level interactions that ultimately leads to the stability of the native conformation of the protein molecule.

The interactions of β-Lg with various cosolvents were quantified by partial specific volume measurements. The partial specific volume of β-Lg was measured by precision densitimetric method as described under methods. The measurements were done under both isomolal and isopotential conditions. The concentration dependence of apparent partial specific volume of β-Lg in 0.02 M phosphate buffer pH 7.9 is shown in Figure 37. The extrapolated isomolal and isopotential values at zero protein concentration were 0.743 ± 0.001 ml/g and 0.744 ± 0.001 ml/g respectively. Similar plots of apparent partial specific volumes with various concentrations of β-Lg were obtained in three different cosolvents namely glycerol, sorbitol and sucrose.

In all the cases the apparent partial specific volume was found to be independent of protein concentration under both isomolal and isopotential conditions. The representative plot of apparent partial specific volume of β-Lg in 20% sucrose is shown in the Figure 38. The isomolal value of β-Lg in presence of 20% sucrose was 0.738 ± 0.001 ml/g and isopotential value was 0.798 ± 0.002 ml/g.

The preferential interaction parameters were calculated for these cosolvents from the partial specific volumes. In all the cases, preferential interaction parameter was negative suggesting preferential exclusion of the cosolvent from the surface of the protein molecule, in other words, the protein is preferentially hydrated. The value of preferential interaction was highest in case of 20% glycerol being -0.213 ± 0.002 g/g and the lowest being

-0.010 ± 0.001 g/g in 10% glycerol as shown (Table 17). In sorbitol, the value of preferential interaction was lowest at 10% sorbitol being –0.043 ± 0.001 g/g, and highest value of –0.140 ± 0.002 g/g at 40% sorbitol (Table 18). In sucrose the value was the least at 10% being –0.020 ± 0.002 g/g and highest at 30% being –0.190 ± 0.002 g/g (Table 19). Even in case of papain maximum hydration was observed in glycerol, where the interaction parameter was -0.165 g/g (Sathish *et al*., 2007), as also is the case of α-Lactalbumin, where the maximum degree of hydration was seen in 40% glycerol and the interaction parameter was -0.299 ± 0.006 g/g (GunaSekhar and Prakash, 2008).

Figure 39 shows the preferential interaction of β-Lg as a function of sucrose concentration. As can be seen preferential interaction reaches a maximum at 30% sucrose. The maximum preferential interaction in case of sorbitol is at 40% concentration (Figure 40). Glycerol provides maximum preferential interaction at 20% concentration (Figure 41). The results from density measurements show that the surface properties of proteins contribute to the extent of hydration by these cosolvents. It is also evident that the preferential interaction parameter differs with the various cosolvents used. The structure of cosolvent is a major criterion, which decides the extent of hydration of the protein molecule.

To study the effect of cosolvents on the extent of change in the microenvironment of tryptophan residues brought about by the preferential interaction has been monitored by intrinsic fluorescence measurements. The fluorescence emission spectrum of β-Lg in presence of glycerol slightly enhanced but with little shift in the emission maxima. As also is the case of sorbitol, where the intensity varied a little with the increase in concentration of sorbitol and no shift in the emission maxima was observed. But in case of sucrose, fluorescence intensity increased with increase in the concentration of sucrose concentration (Figure 42).

The fluorescence emission of β-Lg in presence of cosolvents, indicated changes in the emission intensity with various concentrations of cosolvents. This indicated a modification of microenvironment of tryptophan residues in the presence of cosolvents. But there is no shift in the emission maxima indicating no structural changes in the protein in presence of the above cosolvents.

Ananthanarayanan and Bigelow, (1969) have concluded that the perturbations of aromatic chromophores in cosolvents such as sucrose alter the absorbance of the aromatic chromophores, and have calculated the extent of such perturbations on UV-difference spectra. Model compounds, L-Tryptophanyl ethyl ester shows changes in the fluorescence emission spectra at 334 nm in the presence of cosolvents. So changes in the fluorescence intensity of β-Lg in presence of cosolvents were due to the perturbations of tryptophan moieties. Gavish *et al*., (1983) has shown that solvent viscosity has a greater effect on protein dynamics. Ansari *et al*., (1992) have showed that high viscosity is the dominant factor, which reduces the conformational changes in myoglobin. The difference in viscosity of sorbitol and glycerol causes the variation in the emission intensity of the protein.

The results indicated a change in the emission intensity with various concentrations of cosolvents. This indicated a modification of microenvironment of tryptophan residues in the presence of cosolvents. But there is no shift in the emission maxima indicating no structural changes of the protein in presence of cosolvents. Similar results were obtained in all the cosolvents used.

The degree of hydrolysis of β-Lg with α-chymotrypsin in phosphate buffer and in different cosolvents was calculated. As seen in the figure 43, the degree of hydrolysis of β-Lg was a maximum of 18% after 200 min of enzymatic reaction. The degree of hydrolysis of β-Lg in glycerol is shown in Figure 43. As seen in the Figure 43, the value decreased to 12% from a control

value of 18%. Figure 44 shows the degree of hydrolysis of β-Lg in presence of 20% sorbitol. The value for degree of hydrolysis of the protein in buffer was 18% which decreased to 12% in presence of sorbitol. In Figure 45, is shown the degree of hydrolysis β-Lg in presence of 20% sucrose. The protein in buffer which had a degree of hydrolysis of 18% was increased by 3% to improve the degree of hydrolysis to 21% in 20% sucrose solution.

Globular proteins undergo considerable volume fluctuations (Hans *et al*., 1979). Conformational fluctuations play an important role in the structural stability of the globular proteins. The results obtained with α-chymotrypsin hydrolysis of β-Lg in the presence of glycerol and sorbitol further supports the fact that in presence of cosolvents the hydrolysis is much lesser as compared to the control. Similar results were obtained when β-Lg was hydrolyzed by the protease in the presence of glycerol and sorbitol. But in presence of sucrose the degree of hydrolysis was enhanced. The results from fluorescence spectra in the presence of sucrose show some perturbations in the protein molecule. In the presence of sucrose the protein was found to be more prone for enzymatic hydrolysis.

An important aspect of milk protein is protease digestibility. Such a property has been used as a measure of the flexibility of proteins by assuming that the flexible proteins would be more easily digested by proteases. The high hydrophobic proteins such as BSA and β-Lg are susceptible to proteases. The hydrophobic proteins seem to cause an unstable conformation, which increases flexibility of proteins (Kato *et al*., 1985). Since flexible structure of proteins are more susceptible to proteases than rigid protein structures. The protease susceptibility method seems to be promising in detection of protein flexibility. Protein susceptibility was positively correlated to adiabatic compressibility, which shows large internal flexibility (Gekko and Yamagami, 1991). Internal cavities of proteins have been associated with conformational flexibility also (Alexander *at el*., 1986).

The conformation of β-Lg in presence of cosolvents were further evaluated by circular dichroism. The secondary structure values calculated from CD spectra of β-Lg in phosphate buffer are shown in the Table 20. As shown in the table the control protein had 24 ± 2% of α-helix, 50 ± 2% of β-structure and 26 ± 2% of aperiodic structure. The values did not show significant changes in the secondary structure data from the control values in the presence of sorbitol and glycerol as seen in the Table 20. These results show the protein did not show any structural modifications in the presence of various cosolvents used in this study. This clarifies the fact that the changes in the degree of hydrolysis of the protein and the changes obtained in the fluorescence spectra were due to the conformational fluctuations and not due to the structural changes in the molecule. The far UV spectra of β-Lg in glycerol did not show any changes (Figure 46). The results indicate that there were no significant changes in ellipticity values. Figure 47 shows the far UV spectra of β-Lg in sorbitol with no significant changes in the ellipticity curves. Figure 48 shows the far UV spectra of β-Lg in presence of sucrose. Minor changes in ellipticity values were shown in the far UV spectra were only due to the conformational fluctuations of the protein in presence of cosolvents, which do not affect the secondary structure of β-Lg.

The thermal stabilization of β-Lg by different cosolvents to different extents is evidenced by apparent thermal denaturation temperature measurements of the protein at 287 nm over the range of 25-95°C. Apparent thermal denaturation temperature is the melting temperature measured at a particular concentration of protein. If it is extrapolated as a function of concentration of protein to zero protein concentration, it is apparent $T_m$ .The apparent thermal denaturation curve of β-Lg in the presence of sucrose is shown in the Figure 49. The $T_m$ of protein found to be enhanced from 65°C to 71°C in presence of 40% sucrose where as in presence of 40% sorbitol it was increased to 68°C. The apparent thermal denaturation curve of β-Lg in the presence of sorbitol is shown in the Figure 50. The thermal transition

temperature shifted from 65°C to various higher levels being highest of 88°C in 10% glycerol. The apparent thermal denaturation curve of β-Lg in presence of glycerol is shown in Figure 51.

Maximum stabilization was observed with glycerol when compared to sorbitol or sucrose as shown in Table 21. These results suggest that the cosolvents exert a stabilizing effect on β-Lg at various concentrations. Preferential hydration restricts the thermal motions in the molecule. This is reflected by the increased thermal stability of β-Lg in cosolvents. From the partial specific volume, it is clear that the polyols induce ordered structuring of water around the protein molecules (Jencks, 1969). This additional hydration shell protects the protein from thermal denaturation. The extent of stabilization by the cosolvents depends on the nature of the cosolvent used. Sugars are found to act as stabilizers against thermal denaturation of proteins. This stabilization may be attributed to the strengthening of the hydrophobic interactions, which makes the protein more rigid and resist thermal unfolding (Ebel *et al*., 2000).

The stabilizing effect of sugars depends on the number and position of hydroxyl groups. Extensive hydrogen bonding of water in the hydration shell around the protein in presence of sugars is the major driving force towards stabilization. The unfavorable interaction of cosolvents with non polar amino acid residues results in the exclusion of cosolvents from protein surface. Thus glycerol stabilizes by strengthening hydrophobic interactions by overcoming the electrostatic interactions between charged residues (Rashid *et al*., 2006). Sorbitol increases the surface tension of water; on the other hand glycerol decreases surface tension of water. Solvophobic effects of glycerol are strong enough to offset the effect of decreased surface tension (Gekko and Timasheff, 1981). Compounds, which increase the surface tension of water, are known to be excluded from the protein surface. A large heat capacity of water in presence of polyols and sugars has also been attributed to stronger or more extensive hydrogen bonding between solute hydroxyl groups and water

molecules. The nature of both the protein and polyols are important in governing the thermal stability of the protein. An increase in surface tension of the medium in presence of polyols appears to be the dominant factor. A delicate balance of interactions between the surface hydrophobic, hydrophilic and charged residues with water and the ability of polyols to alter the physico chemical properties of water plays a significant role (Jai and Bhatt, 1998). Sugars affect the hydrogen bonded network around the protein molecule. So surface tension is not the only criteria for protein stabilization (Jai & Bhatt, 2006).

*From the above results, it is clear that the cosolvents glycerol, sorbitol and sucrose does alter the thermal stability of β-Lg. Thermal stability is dependent on the nature and the concentration of the cosolvent. In the case of β-Lg, stability is maximum in 20% glycerol, 40% sorbitol and in 30% sucrose. However at the two extremes of cosolvent concentrations used like 10% and 40%, the values many a times may be similar showing optimum concentration of cocolvent, where there is higher stability of the protein. This stability is attributed to the displacement of the water around protein from cosolvent domain which depends on the extent to which, particular cosolvent interacts with protein surface and which interacts with the residues on the side chain of the protein surface. With such suttle changes in the stability of the protein, data clearly indicates no structural changes in the protein molecule. This phenomenon of stability is the indirect solvent affect brought about by the changes in the water shell around protein by the effect of the cosolvent rather than direct effect either due to viscosity or other physical parameters of cosolvents. Applications are wide and has large ramification in biotechnological processes if one wants higher stability for β-Lg, in which case it can be achieved by selection of critical concentration of the cosolvent, based on such studies.*

## *Summary & Conclusions*

Nearly 80% of bovine milk proteins consist of caseins, a group of phosphate containing proteins named α, β and κ-caseins. Whey proteins constitute about 20% of bovine milk proteins (0.6 g/100 g milk). The whey protein fraction consists of α-lactalbumin, β-lactoglobulin, bovine serum albumin, immunoglobulin, lactoferrin and the proteose-peptone fraction.

β-Lg is the principal whey protein in bovine, ovine and caprine milk; a similar protein is present in the milk of several species but is absent from human and rodent milk. β-Lg is capable of binding hydrophobic molecules including retinol and may function *in vivo* to protect retinol against oxidation and to transport it through stomach to the small intestine.

β-Lg being the major whey protein dominates the properties of all whey protein preparations. Solubility greatly depends on pH and ionic strength. The protein is subject to a number of changes in quaternary structures with changes in pH and temperature. β-Lg constitutes about 60% of the total whey proteins. This protein exists as a dimer at the physiological pH and has a molecular weight of 18,400 Da for the monomer. In milk, this whey protein is present as dimer. Both molecules are tightly bound to each other mainly by hydrophobic interactions and so at higher temperatures dimer dissociates into monomers. Various genetic variants of β-Lg are present in milk.

In the present study, the major whey protein β-Lg was isolated from the bovine milk, purified to homogeneity and characterized. Enzymatic hydrolysis is used to modify the functional properties of proteins. Partial hydrolysis of novel proteins improves their solubility and foaming properties. It has been demonstrated that if the extent of hydrolysis is limited, it is possible to achieve improvement in functionality. The rate of proteolysis depends upon the surface property of the proteins. Changes in the

conformation, which in turn alters the number of accessible sites, alter the rate of proteolysis. Whey is hydrolysed by using various enzymes in a view to improve various functional attributes and the data discussed in detail.

Food processing often involves high temperatures, extremes of pH, and many a times alkaline and oxidizing conditions. Such conditions can result in the introduction of protein crosslinks producing substantial changes in the structure of proteins and therefore of the profile of final product is many a time reflected on such processes.

Protein crosslinking plays an important role in determining the functional properties of food proteins. Manipulaton of the number and nature of protein crosslinks during food processing offers a means by which the food industry can manipulate the functional properties of food, often without affecting the nutritional quality. More recently, Transglutaminase including mammalian and microbial origin is employed for the protein modification. The covalent crosslinking of proteins catalyzed by Tgases can cause changes in the size, conformation, stability and other properties of the proteins. Seed proteins like 11S protein fraction from soybean and sesame seed are rich in essential amino acids and had good functional properties. Crosslinking of β-Lg and 11S protein fraction from soybean and sesame seeds were worked out. Soybean seed proteins and sesame seed proteins generally are used in combinations with milk proteins for product development in food industry. Hence a better understanding about their interaction is one of the objectives of this study.

The fundamental observation of the stabilization of macromolecules in a three-component system has a great potential for stabilization of enzymes and proteins. The properties of proteins in cosolvents (polyols and sugars) underlines the very fundamental concept of preferential exclusion and/or preferential interaction of such cosolvents with these macromolecules. The thermal stability, conformation and aggregation of globular proteins depend

on the concentration and nature of cosolvents present in the surrounding aqueous phase.

The investigation is divided into following chapters:

(1) *Isolation and characterization of β-Lactoglobulin from bovine milk whey.*

(2) *Protein-protein interactions of β-Lactoglobulin with 11S fraction of soybean and 11S fraction of sesame seed.*

(3) *Effect of Transglutaminase on protein–protein interaction of β-Lg with 11S fraction of soybean and 11S fraction of sesame seed.*

(4) *Structural stability of β-Lactoglobulin as a function of cosolvents.*

The results obtained from above investigation are presented in the form of a thesis. Brief outlines of the highlights of results obtained in the present investigation are summarized below.

**Chapter 1:** The major whey protein, β-Lactoglobulin was isolated from the bovine milk, purified to homogeneity and characterized. The purified protein was found to be homogenous as determined by Sodium Dodecyl Sulfate – Poly Acrylamide Gel Electrophoresis. The amino acid composition was done and had a correlation coefficient of 99% with the literature values. β-Lactoglobulin is a protein rich in β-sheets as calculated from the circular dichroic spectra. β-Lactoglobulin was hydrolysed with various enzymes in a view to enhance the functional attributes of as well as the total whey proteins. β-Lactoglobulin was hydrolysed by fungal protease and also found to be resistant to pepsin digestion. Whey as such was hydrolysed with enzymes like fungal protease and papain with a view to enhance the functional attributes like solubility, foaming capacity and emulsifying activity. Digestion with fungal protease enhanced the foaming capacity. The foaming capacity of the control protein was 11 ± 1 ml which increased to 14 ± 1 ml when

hydrolysed with fungal protease. The emulsion activity increased by 10% in the initial 30 min of hydrolysis and then decreased by 40% as the hydrolysis progressed. Hydrolysis with papain enhanced the emulsifying activity of the whey proteins by nearly 15%. Also the solubility of whey varied when digested with the enzymes. Fungal protease hydrolysis generated peptide with hydrophilic side chains, which improved the solubility of the hydrolysate. Hydrolysis with papain liberated peptide with hydrophobic side chains, which had lower solubility as compared to fungal protease hydrolysate.

**Chapter 2:** The chapter explains in detail the heat induced interaction of β-Lactoglobulin and 11S protein fractions of soybean and sesame seeds. Protein - protein interactions play a major role in the functional properties in food systems. Water, fat and salt in the system affect the functional properties of the food system to a greater extent. Protein - protein interaction between bovine β-Lactoglobulin and 11S protein fraction of soybean and sesame seeds were studied by turbidity, solubility tests and by evaluation of functional properties in the mixed systems. Turbidity and solubility studies showed that the proteins complexed at higher temperatures between through both disulfide linkages and also hydrophobic interactions. The self aggregation of 11S protein fraction of soybean was partially decreased by the presence of β-Lactoglobulin. But in the case of sesame, aggregation was more under similar conditions indicating that specific protein-protein interactions decide the ultimate aggregate size and stability based on the surface properties of the proteins used.

The effect of heating on the turbidity of β-Lactoglobulin, 11S protein fraction of soybean (concentration of $1\times10^{-4}$ M) and the mixture of β-Lactoglobulin and 11S protein fraction of soybean in various molar ratios were studied. On the other hand, the turbidity of 11S protein fraction of soybean appeared at 65°C and increased with increasing temperature. The

turbidity of the mixture of β-Lg and 11S protein fraction of soybean reduced when compared to the turbidity of 11S protein fraction heated without the incorporation of β-Lactoglobulin. These results indicated that interaction occurred between these two proteins at temperature of 60°C and above. The data clearly shows the presence of β-Lactoglobulin interfered with the precipitation of 11S protein fraction of soybean. The turbidity reduced when the molar ration of β-Lactoglobulin in the mixture was increased. The turbidity was less when the molar ratio between β-Lactoglobulin and 11S protein fraction of soybean was 15:1 when compared to a molar ratio of 10:1. Similarly the effect of heating on the turbidity of β-Lactoglobulin, 11S protein fraction of sesame seeds (concentration of $1\times10^{-4}$ M) and the mixture of β-Lactoglobulin and 11S protein fraction of sesame seeds in various molar ratios were also studied. The turbidity was high both in case of β-Lactoglobulin and 11S protein fractions of sesame when heated above 60°C. Precipitation was high in case of β-Lactoglobulin, 11S protein fraction of sesame seed and in combination at various molar ratios. The precipitation behaviour of β-Lactoglobulin was different in this case, where the protein precipitated when heated above 60°C. The high salt concentration of the buffer in which 11S protein fraction of sesame seed was dissolved induced the precipitation of β-Lactoglobulin.

**Chapter 3**: This chapter describes the effect of enzyme transglutaminase on protein-protein interaction of β-Lactoglobulin with 11S protein fraction of soybean and sesame seed. To be competitive in food ingredient markets the functionality of whey proteins must be continuously improved and designed for specific uses. Chemical and physical methods are commonly used. More recently, enzymes including mammalian and microbial transglutaminase have been employed. The covalent crosslinking of proteins catalyzed by transglutaminase can cause dramatic changes in the size, conformation, stability and other properties of the proteins. Crosslinking was attempted between β-Lactoglobulin and 11S protein fraction of soybean (glycinin) and

also with β-Lactoglobulin and 11S protein fraction of sesame seed (α-globulin) using microbial transglutaminase (crosslinking through acyl transferase reaction) and evaluated for the altered functional properties.

The complex formed was investigated by gel filtration chromatography and by electrophoresis. The changes in the fluorescence intensity indicated changes in the microenvironment of the chromophores induced by the crosslinking either embedded between the surfaces or at the interface. In both the cases of crosslinking of β-Lactoglobulin with glycinin and β-Lactoglobulin with α-globulin the emulsifying activity was enhanced by 36% and 50% respectively which is very significant. Foaming capacity did not improve significantly with crosslinking. Thermal stability of the proteins also improved as a result crosslinking. The effect of temperature on untreated and treated β-Lactoglobulin did not show turbidity even at 90°C. But 11S protein fraction of soybean showed aggregation at 50°C and above. Turbidity was observed when 11S protein fraction of soybean and β-Lactoglobulin were heated to 55°C. But the temperature was extended to 65°C when 11S protein fraction of soybean and β-Lactoglobulin were treated with the enzyme transglutaminase. Similarly, in 11S protein fraction of sesame, turbidity was observed at 60°C for the untreated protein. The transition temperature increased to 70°C when 11S protein fraction of sesame and β-Lactoglobulin were polymerized with transglutaminase.

**Chapter 4:** This chapter describes the effect of cosolvents on the structural stabilization of β-Lactoglobulin in the presence of sucrose, glycerol and sorbitol. Thermal stability in the presence of these cosolvents was also evaluated. Apparent partial specific volume of β-Lactoglobulin using 0.02 M phosphate buffer pH 7.9 was determined. Plots of apparent partial specific volumes versus β-Lactoglobulin concentrations were obtained in various concentrations of cosolvents. In all the cases the apparent partial specific volume was found to be independent of protein concentration under both

isomolal and isopotential conditions. The extrapolated values were calculated in all the cosolvent concentrations and results correlated.

The preferential interaction parameters calculated for these solvents from the partial specific volumes. In all the cases, preferential interaction ($\xi_3$) values were negative suggesting preferential exclusion of the cosolvent components from the domain of the protein molecule. The value of preferential interaction was highest in the case of 20% glycerol which was -0.213 ± 0.002 g/g and the lowest being -0.010 ± 0.001 g/g in 10% glycerol. In the case of both sucrose and sorbitol, preferential interaction values reached a higher value at 40% cosolvent concentration. On the other hand, in the case of glycerol, the preferential interaction parameter value increases to a maximum at 20% of cosolvent concentration and then gradually decreases with increase in cosolvent concentration.

The results obtained with α-chymotrypsin digestion of β-Lactoglobulin in the presence of glycerol and sorbitol further supports the fact that the cosolvent stabilizes the protein. The degree of hydrolysis in presence of glycerol and sorbitol decreased for the control value of 18% to 13% in presence of these cosolvents. But in presence of sucrose the degree of hydrolysis increased from a control value of 18% to 21% in presence of sucrose. The protein was hydrolysed more in presence of sucrose. The results from fluorescence spectra in the presence of sucrose show perturbations of the aromatic groups in the protein molecule which is expected. With glycerol and sorbitol there were no much significant changes in the fluorescence intensity. Far Ultra Violet-Circular dichroic spectra of β-Lactoglobulin in presence of these cosolvents shows that the cosolvents did not affect the secondary structure of the protein. These results indicate that these cosolvents stabilize the native structure of β-Lactoglobulin at the concentration of the cosolvents used and is cosolvent dependent.

# REFERENCES


Aboumahmoud, R., and Savello, P. (1990) Crosslinking of whey proteins by Transglutaminase. *J. Dairy Sci*. **73**, 256 – 263.

Adler, A.J., Greenfield, N.J., and Fasman, G.D. (1973) Circular dichroism and optical rotatory dispersion of proteins and polypeptides, *Methods Enzymol*. **27,** 675 - 735.

Akamittah, J.G., and Ball, H.R. (1992) Polymerization of crude actomyosin refined from mechanically deboned poultry meal. *J. Muscle Foods*. **3,** 1 - 14.

Alder-Nissen, J. (1979) Determination of degree of hydrolysis of food protein hydrolyzates by trinitrobenzoic acid. *J. Agric. Food Chem*. **27,** 1256 -1262.

Alder-Nissen, J. (1982) Limited enzymic degradation of proteins. A new approach in the industrial application of hydrolases. *J. Chem. Technol*. **32**, 138 - 156.

Alexander, A.R., Michel, I., and Barry, H. (1986) Internal cavities and buried waters in globular proteins. *Biochemistry*. **25,** 3619 - 3625.

Aluko, R.E., and Monu, E. (2003) Functional and bioactive properties of Quinoa seed protein hydrolyzate. *J. Food Sci*. **68**, 1254 - 1258.

Ananthanarayanan, V.S., and Bigelow, C.C. (1969) Unusual difference spectra of proteins containing tryptophan–I Studies with model compounds. *Biochemistry*. **8**, 3717 – 3723.

Ananthanarayanan, V.S., and Bigelow, C.C. (1969) Unusual difference spectra of proteins containing tryptophan–II Studies with model compounds. *Biochemistry*. **8**, 3723 - 3726.

Ansari, A., Jones, C.M., Henry, E.R., Hofrichter, J., and Eaton, W.A. (1992) The role of solvent viscosity in the dynamics of protein conformational changes. *Science*. **256**, 1796 -1798.

**AOAC., (2005) Official methods of analysis of AOAC international. 18th edition. Association of Official and Analytical Chemists international, Arlington, VA.**

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

Arakawa, T., and Timasheff, S.N. (1984) Protein stabilization and destabilization by guanidium salts. *Biochemistry*. **23**, 5924 - 5929.

Arakawa, T.**,** Bhat, R., and Timasheff, S.N. (1990) Why preferential hydration does not always stabilize the native structure of globular proteins. *Biochemistry*. **29**, 1924 - 1931.

Aryana, K.J., Haque, Z.Z., and Gerard, P.D. (2002) Influenze of whey protein concentrate on the functionality of egg white and bovine serum albumin. *Int. J. Food Sci. Technol*. **37**, 643 - 653.

Arzu, A., Mayorga, H., Gonzalez, J., and Rolz, C. (1972) Enzymatic hydrolysis of cottonseed protein. *J. Agric. Food Chem*. **20**, 805 - 809.

Ashutosh, T., and Bhatt, R. (2006) Stabilization of yeast Hexokinase A by polyol osmolytes: correlation with the physicochemical properties of aqueous solutions. *Biophys. Chem*. **124**, 90 - 99.

Badley, R.A., Atkinson, D., Hauser, H., Odani, D., Green, J.P., and Stubbs, H.M. (1975) The structure, physical and chemical properties of soybean protein glycinin. *Biochem. Biophys. Acta*. **412**, 214 - 217.

Bernal, V., and Jelen, P. (1985) Thermal stability of whey proteins. A calorimetric study. *J. Dairy Sci*. **68**, 2847 - 2852.

Beveridge, T., Toma, S.J., and Nakai, S. (1974) Determination of SH and SS-groups in some food proteins using Ellman's reagent. *J.Food Sci.* **39**, 49 - 51.

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

Boguslawa, C.M. (2006) 2DCOS and MCR-ALS as a combined tool of analysis of β-Lactoglobulin CD spectra. *J. Molecular Structure.* **799**, 211 - 220**.**

Bolen, D.W., and Baskakov, I.V. (2001) The osmophobic effect: Natural selection of a thermodynamic force in protein folding. *J. Mol. Biol*. **310**, 955 - 963.

Bolen, D.W. (2001) Protein stabilization of naturally occurring osmolytes. *Methods Mol. Biol*. **168**, 17- 36.

Boyarioglu, M.H. (2006) Soy ingredients in baking. *in Soy applications in food*. (Ed. Riaz, M.N.). pp 69 - 70, CRC Press, Taylor & Francis, Boca Raton, London.

Brand, L., and Witholt, B. (1967) Fluorescence measurements. *Methods Enzymol*. **11**, 776 - 845.

Britten, M. (2002) Functional properties of whey proteins. *J. Dairy Sci.* **85** (Suppl 1)**,** 51 - 54.

Brock, C. (1985) Life at high temperature. *Science*. **230,** 132 - 138.

Brody, E.P. (2000) Biological activities of bovine glycomacropeptide. *Bri. J. Nutrition*. **84**, S39 - S46.

Bryant, T.M., and McClements, D.J. (1998) Molecular basis of protein functionality with special consideration of cold-set gels derived from heat-denatured whey. *Trends Food Sci.Technol.* **9**, 143 -151.

Bryon, W., and Arnold, J. (1965) Fundamentals of dairy chemistry. pp. 813. AVI publishing company, Westport, Connecticut, USA.

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

Catsimpoolas, N., Funk, S.K., and Meyer, E.W. (1970) Thermal aggregation of glycinin subunits. *Cereal Chem*. **47**, 331 – 344.

Chen, S.X., Harding, C.C., and Swaisgood, H.E. (1993) Purification and characterization of β-structural domains of β-Lactolgobulin liberated by limited proteolysis. *J. Protein Chem*. **12**, 613 - 625.

Chen, Y.H., and Yang, J.T. (1971) A new approach to the calculation of secondary structures of globular proteins by optical rotatory dispersion and circular dichroism, *Biochem. Biophys. Res. Commun*. **44,** 1285 - 1291.

Chi, E.Y., Krishnan, S., Randolph, T.W., and Carpenter, J.F. (2003) Physical stability of proteins in aqueous solutions: mechanism and driving forces in non-native protein aggregation. *Pharm. Res*. **20**, 1325 - 1336.

Chiya, K., Katsutoshi, Y., and Yasuyuki, S. (2001) Transglutaminase: Its utilization in the food industry. *Food Rev. Int*. **17**, 221 – 346.

Cho, Y., Batt, C.A., and Sawyer, L. (1994) Probing the retinol binding site of bovine β-Lactoglobulin. *J. Biol. Chem*. **269**, 11102 - 11107.

Chobert, J.M., Sitohy, M.Z., and Whitaker, J.R. (1988) Solubility & emulsifying properties of caseins modified enzymatically by *Staphylococcus aureus* V8 protease. *J. Agri. Food Chem*. **36**, 220 - 224.

Chou, D., and Morr, C. (1979) Protein-water interactions and functional properties. *J.Am.Oil Chem.Soc*. **56**, 53 - 62.

Croguennec, T., Bouhallab, S., Molle, D., O'Kennedy, B.T., and Mehra, R. (2003) Stable monomeric intermediate with expose Cys- 119 is formed during heat denaturation of β-Lactolgobulin. *Biochem. Biophys. Res. Commun*. **301**, 465 - 471.

Damodaran, S. (1990) Interfaces, protein films, & foams. *Adv. Food Nutrition Res.* **34**, 1 - 79.

Damodaran, S. (1994) Structure function relationship of food proteins. in *Protein functionality in food systems*. (Eds. Hettiarachchy, N.S., Ziegler, G.R.). pp. 1 - 38, Marcel Dekker, Inc, New York, USA.

Damodaran, S., and Parag, A. (1997) Food proteins and their applications. Marcell Dekker, Inc. New York, Basel.

Dannenberg, F., and Kessler, H.G. (1988) Thermodynamic approach to kinetics of β-Lactoglobulin, denaturation of heated milk and sweet whey. *Milchwissenschaft* . **43**, 139 - 142.

DeJong, G.A.H., and Koppelman, S.J. (2002) Transglutaminase catalyzed reactions: Impact on Food applications. *J. Food Sci.* **67**, 2798 - 2806.

DeBacker, R.C., Traore, F., and Meunier, J.C. (1992) Polymerization of meat & soybean proteins by human placental $Ca^{2+}$- activated factor XIII. *J. Agric. Food Chem.* **40**, 2052 - 2056.

Dickinson, E. (1997) Enzymatic crosslinking as a tool for food colloid rheology control and interfacial stabilization. *Trends. Food Sci. Technol.* **8**, 334 - 339.

Dionysius, D.A., Grieve, P.A., and Milne, J.M. (1993) Forms of Lactoferrin: their antibacterial effect on enterotoxigenic E.coli. *J. Dairy sci*. **76**, 2597 - 2606.

Dunkerly, J.A., and Zadow, J.G. (1984) The effect of calcium and cysteine hydrochloride on the firmness of heat coagula formed from cheese whey protein concentrate. *Aus. J. Dairy Technol.* ***39***, 44 – 47.

Ebel, C., Eisenberg, H., and Ghirlando, R. (2000) Probing protein–sugar interactions. *Biophys. J*. **78**, 385 - 393.

Elvan, O. (2006) The use of trasnglutaminase in dairy products. *Int. J. Dairy Technol*. **59**, 1 - 7.

El-Zahar, K., Sitohy, M., Choiset, Y., Metro, F., Haertle, T., and Chobert, J.M. (2005) Peptic hydrolysis of ovine β-Lactoglobulin Exceptional susceptibility of native ovine β-Lactoglobulin to pepsinolysis. *Int.Dairy J*. **15**, 17-27.

Feeney, R.E., and Whitaker, J.R. (1988) Importance of crosslinking reactions in proteins. *Adv. Cereal Sci. Technol.* **9**, 21 - 46.

Fernandez Diez, M.J., Osuga, D.T., and Feeney, R.E. (1964) The sulphydryls of Avian Ovalbumins, Bovine β-Lactoglobulin, and Bovine Serum Albumin. *Arch. Biochem. Biophys*. **107**, 449-458.

Fersht, A.R. (1977) Enzyme structure and mechanism. W.H. Freeman, Reading, England.

Fersht, A.R (1999) Forces between molecules and binding energies. in *Structure and Mechanism in protein science*. pp. 324 - 347, *Freeman Co, New York*, USA.

Flanagan, J., and Fitzgerald, R.J. (2003) Functional properties of *Bacillus* proteinase hydrolyzates of sodium caseinate incubated with trasnglutaminase pre and post- hydrolysis. *Int. Dairy J*. **13**, 135 -143.

Flanagan, J., Gunning, Y., and Fitzgerald, R.J. (2003) Effect of crosslinking with transglutaminase on the heat stability and some functional characteristics of sodium caseinate. *Food Res. Int.* **36**, 267 - 274.

Folk, J.E., and Cole, P.W. (1966) Mechanism of action of guinea pig liver transglutaminase. I. Purification and properties of the enzyme: identification of a functional cysteine essential for activity. *J.Biol.Chem*. **241**, 5518 - 5525.

Fox, K.K., Holsinger, V.H., Posati, L.P., and Pallansch, M.J. (1967) Separation of β-Lactoglobulin from other milk proteins by trichloroacetic Acid. *J. Dairy Sci*. **50**, 1363 - 1367.

Frokjaer, S. (1994) Use of hydrolyzates for protein supplementation. *Food Technol.* **48**, 86 - 88.

Gavish, B., Gratton, E., and Hardy, C. (1983) Adiabatic compressibility of globular proteins, *Proc.Natl.Acad.Sci.,* USA. **80**, 750 - 754.

Gekko, K. (1981) Mechanism of polyol induced protein stabilization: Solubility of aminoacids and diglycine in aqueous polyol solutions. *J. Biochem*. **90**, 1633-1641.

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

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

Gekko, K., and Yamagami, K. (1991) Flexibility of food proteins as revealed by compressibility. *J. Agric. Food Chem*. **39**, 57 - 62.

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

Gobetti, M., Stepaniak, L., De Angelis, M., Corsetti, A., and Di Cagno, R. (2002) Latent bioactive peptides in milk proteins: Proteolytic activation and significance in dairy processing. *CRC Crit. Rev. Food Sci. Nutrition*. **42**, 223 – 239.

Guerra, M.J., and Park, Y.K. (1975) Extraction of sesame seed protein and determination of its molecular weight by SDS gel electrophoresis. *J. Am. Oil Chem. Soc.* **52**, 73 - 75.

Guna Sekhar, P.M., and Prakash, V. (2008) Interaction of selected cosolvents with bovine α-Lactalbumin. *Inter. J. Biol. Macromolecules*. **42**, 348-355.

Ha, E., and Zemel, M.B. (2003) Functional properties of whey, whey components and essential amino acids mechanisms underlying health benefits for active people. *J. Nutrition Biochem*. **14**, 251 - 258.

Halling, P.J. (1981) Protein stabilized foams and emulsions. *CRC Crit. Rev. Food Sci. Nutrition.* **21**, 155 - 203.

Hambraeus, L. (1982) Nutritional aspects of milk proteins. in *Developments in Dairy Chemistry: I, proteins* (Ed: Fox, P.F.), pp. 289 - 213, Applied Science Publishers, London, UK.

Hans, F., Petsko, G.A., and Demetrius, T. (1979) Temperature – dependent X-Ray diffraction as a probe of protein structural dynamics. *Nature*. **280**, 556 - 563.

Haschemeyer, R.H., and Haschemeyer, A.E. (1973) Primary structure of proteins. in *A guide to study by physical and chemical methods,* pp. 54 - 92, John Wiley and Sons ,USA.

Havea, P., Carr, A.J., and Creamer, L.K. (2004) The roles of disulfide and non-covalent bonding in the functional properties of heat-induced whey protein gels. *J. Dairy Research*. **71**, 330-339.

Hermansson, A.M., Olson, D., and Holmberg, B. (1974) Functional properties of foods-Modification studies on Rapeseed protein concentrate. *Lebebsm. Wiss. Technol*. **7**, 176 -181.

Hill, S.E. (1996) Emulsion. in *Methods of testing protein functionality* (Ed. Hall, G.M.), pp. 153 - 182, Blackie Academic & Professional, London.

Hong, Y.H., and Creamer, L.K. (2002) Heat induced aggregation behaviour of bovine β-Lactoglobulin. *Food Sci. Biotechnol*. **11**, 161 - 164.

Hoshi, Y., and Yamauchi, F. (1983) Further studies on the aggregation and insolubilization of soybean 11S globulin with humidity storage. *Agric. Biol. Chem.* **47**, 1473 -1479.

Hoshi, Y., and Yamauchi, F. (1983) Determination of sulphydryl and disulfide contents of soybean 11S globulin and their change by lyophilization. *Agric. Biol. Chem*. **47**, 2435-2439.

Huang, X.L., Catignani, G.L., and Swaisgood, H.E. (1996) Improved emulsifying properties of β-barrel domain peptides obtained by membrane fractionation of a limited tryptic hydrolyzate of β-Lactoglobulin. *J. Agric. Food Chem.* **44**, 3437 - 3443.

Jai, K.K., and Bhatt, R. (1998) Thermal stability of protein in aqueous polyol solutions. Role of surface tension of water in the stabilizing effect of polyols. *J. Phys. Chem* .**102**, 7058 - 7066.

Javier, V., Raul, S.V., Alfonso, C., Justo, P., and Francisco, M. (2000) Partially hydrolyzed rapeseed protein isolates with improved functional properties. *J. Am. Oil. Chem. Soc*. **77**, 447 - 450.

Jencks, W.P. (1969) *Catalysis in chemistry and enzymology*, pp. 351 - 392, Mc Graw Hill, New York, USA.

Jones, D.B., and Gersdorff, C.E.F. (1927) Proteins of sesame seed, (*Sesamum indicum L). J. Biol. Chem*. **75**, 213 - 225.

Johnson, D.W. (1970) Functional properties of oilseed proteins. *J.Am.Oil. Chem. Soc*. **47**, 402 - 407.

Johnston, C. C., Jr., Miller, J. Z., Slemenda, C. W., Reister, T. K., Hui, S., Christian, J. C., and Peacock, M. (1992) Calcium supplementation and increases in bone mineral density in children. *N. Engl. J. Med*. **327**, 82 - 87.

Kato, A., and Nakai, S. (1980) Hydrophobicity determined by a fluorescence probe method and its correlation with surface properties of proteins. *Biochem. Biophys. Acta*. **624**, 13 - 20.

Kato, A., Komatsu, K., Fujimoto, K., and Kobayashi, K. (1985) Relationship between surface functional properties and flexibility of proteins detected by protease susceptibility. *J. Agric. Food Chem*. **33**, 931 - 934.

Khalil, M., Ragab, M., and Hassanien, F.R. (1985) Some functional properties of oil seed proteins. *Nahrung*. **29**, 275 - 282.

Kim, S.H., Carpenter, J.A., Lanier, T.C., and Wicker, L. (1993) Polymerization of beef actomyosin induced by transglutaminase. *J. Food Sci*. **58**, 473 - 474.

Kim, J.I., Choi, D.Y., and Row, K.H. (2003) Separation of whey proteins by anion–exchange membranes. *Korean. J. Chem.Eng*. **20**, 538 - 541.

Kinsella, J.E. (1976) Functional properties of proteins in foods. A survey. *CRC Crit. Rev. Food Sci. Nutrition*. **9**, 219 - 280.

Kinsella, J.E. (1981) Functional properties of proteins: possible relationships between structure and function in foams. *Food Chem*. **7**, 273 - 288.

Kinsella, J.E. (1982) Relationship between structure and functional properties of food proteins. in *Food proteins*, (Eds. Fox, P.F. and Condon, J.J.), pp. 51, Applied science, New York, USA.

Kinsella, J.E., and Whitehead, D.M. (1989) Proteins in whey: chemical, physical and functional properties. *Adv. Food. Nutrition Res.* **33**, 343 - 438.

Kinsella, J E., Damodaran, S. and German, J.B. (1985) Physicochemical and functional properties of oil seed proteins with emphasis on soy proteins. in *New protein foods*: *seed storage proteins*, (Eds. Altschul, A.M. and Wilcke, H.L.), pp. 108, Academic press, New York, USA.

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

Kitamura, K., and Shibasakhi, K. (1975) Isolation and some physico-chemical properties of the acidic subunits of soybean 11S globulin. *Agric. Biol. Chem*. **39**, 945 - 951.

Kilara, A., and Panyam, D. (2003) Peptides from milk proteins and their properties. *CRC Crit. Rev. Food Sci. Nutrition*. **43**, 607 - 633.

Kolar, C.W., Cho, I.C., and Watrous, W.L. (1979) Vegetable protein application in yogurt, coffee creamers and whip toppings. *J.Am.Oil. Chem. soc.* **56**, 389 - 391.

Kontopidis, G., Holt, C., and Sawyer, L. (2004) β-Lactolgobulin-binding properties, structure and function. *J. Dairy Sci*. **87**, 785 - 796.

Kuntz, I.D., and Kauzmann, W. (1974) Hydration of proteins and polypeptides. *Adv. Protein chem*. **28**, 239 - 345.

Kuraishi, C., Sakamoto, J., Katsutoshi, Y., Yasuyuki, S., Chiho, k., and Takahiko, S. (1997) Production of restructured meat using microbial transglutaminases, without salt or cooking. *J. Food Sci.* **62**, 488 - 490.

Kussendrager, K.D., and Hooijdonk, A.C. (2000) Lactoperoxidase: physicochemical properties, occurrence, mechanism of action and applications. *Brit. J. Nutrition*. **42**, 353 - 375.

Laemmli, U.K. (1970) Cleavage of structural proteins during the assembly of the head of bactoriophage $T_4$. *Nature*. **227,** 680 - 685.

Lakkis, J., and Villota, R. (1992) Comparative performance of chemically and enzymatically modified whey proteins. *Food Chemistry*. **43**, 93-105.

Lakshmi, T.S., Nandi, P.K., and Prakash, V. (1985) Effect of electrolytes on the heat coagulation of α-globulin from *Sesamum indicum*. *Ind. J.Biochem.Biophys.* **22**, 135 - 141.

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

Lee, S.Y., Morr, C.V., and Seo, A. (1990) Comparison of milk-based and soymilk-based yogurt. *J. Food Sci.* **55**, 532 - 536.

Lorenzen, P.C. (2000) Techno-functional properties of transglutaminase-treated milk proteins. *Milchwissenschaft.* **55**, 667 - 670.

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

Ma, C.Y., and Harwalkar, V.R. (1988) Study of thermal denaturation of oat globulin by UV and fluorescence spectroscopy. *J. Agric. Food Chem*. **36,** 155 - 160.

MacRitchie, F. (1992) Physicochemical properties of wheat proteins in relation to functionality. *Adv. Food Nutrition Res*. **36**, 1 - 87.

Mahajan, A., and Dua, S. (1998) Improvement of functional properties of rapeseed (Brassica campestris Var toria) meal by reducing antinutritional factors employing enzymatic modification. *Food Hydrocolloids*. **12**, 349 - 355.

Manderson, G.A., Hardman, M.J., and Creamer, L.K. (1998) Effect of heat treatment on the conformation and aggregation of *β*-lactoglobulin A, B, and C. *J. Agric. Food Chem.* **46**, 5052 – 5061.

Manion, B., and Corredig, M. (2006) Interactions between whey protein isolate and soybean protein fractions at oil-water interface: Effects of heat and concentration of protein in aqueous phase. *J. Food Sci*. **71**, E343 - E349.

Mannheim, A., and Cheryan, M. (1992) Enzyme modified proteins from corn gluten meal: preparation and functional properties. *J. Am. Oil. Chem. Soc*. **69**, 1163 -1169.

Marshall, K. (2004) Therapeutic applications of whey. *Altern. Med. Rev*. **9**, 136 - 156.

Martinez, K.D., Sanchez, C.C., Ruiz-Henestrosa, V.P., Rodriguez Patino, J.M., and Pilosof, A.M.R. (2007) Effect of limited hydrolysis of soy protein on the interactions with polysaccharides at the air-water interface. *Food Hydrocolloids*. **21**, 813-822.

Matsudomi, N., Oshita, T., and Kobayashi, K. (1994) Synergistic interaction between β-Lactoglobulin and bovine serum albumin in heat induced gelation. *J. Dairy Sci*. **77**, 1487 - 1493.

Matsumora, Y., and Mori, T. (1996) Gelation. in *methods of testing protein functionality* (Ed. Hall, G.M.), pp. 76 - 106, Blackie Academic & professional, London.

Matsura, J.E., and Manning, M.C. (1994) Heat induced gel formation of β-Lactoglobulin. A study on the secondary and tertiary structures as followed by circular dichroic spectroscopy. *J. Agric. Food Chem.* **42**, 1650 - 1656.

Mauron, J. (1990) Influenze of processing on protein quality. *J. Nutrition Sci. Vitaminol. (Tokyo)* **36**, 557 - 569.

McClements, D.J. (2001) Estimation of steric exclusion and differential interaction contributions to protein transfer free energies in aqueous cosolvent solutions. *Food Hydrocolloids*. **15**, 355 - 363.

McKenzie, H.A. (1971) β-Lactolgobulins. in *Milk Proteins: chemistry and molecular Biology* (Ed McKenzie, H.A.), pp. 257 – 330, Academic press, New York.

Mihalyi, E. (1978) in *Applications of proteolytic enzymes to protein structure studies*, CRC Press, Boca Raton.

Miller, G.D., Jarvis, J.K., and McBean, L. D. (2000) in *Handbook of dairy foods and Nutrition, 2nd edition*, CRC Press, Boca Raton.

Mingxia, L., and Srinivasan, D. (1999) Effect of trasnglutaminase-catalyzed polymerization of β-casein on its emulsifying properties. *J. Agric. Food Chem*. **47**, 1514 - 1519.

Mita, T., Ishida, E., and Matsumoto, H. (1978) Physicochemical studies on wheat protein foams II. Relationship between bubble size and stability of foams prepared with gluten and gluten components. *Colloids Inter. Sci.* **64**, 143 -146.

Monaco, H.L., Zanotti, G., Spadon, P., Bolognesi, M., Sawyer, L., and Eliopoulis EE. (1987) Crystal structure of the trigonal form of bovine

β-Lactoglobulin and of its complex with retinal at 2.5 A° resolution. *J. Mol. Biol*. **197**, 695 - 706.

Monahan, E.J., German, J.B., and Kinsella, J.E. (1995) Effect of pH and temperature on protein unfolding and thiol / disulfide interchange reactions. During heat induced gelation of whey proteins. *J. Agric. Food Chem*. **43**, 46 - 52.

Morgan, P.E., Triweck, T.M., Lindner, R.A., Price, W.E., and Carver, J.A. (2005) Casein proteins as molecular chaperonins. *J. Agric. Food. Chem*. **53**, 2670 - 2683.

Mori, T., Utsami, S., and Inabo, H. (1979) Interactions involving disulfide linkages between subunits of soybean seed globulin and between subunits of soybean and sesame seed globulins. *Agric. Biol. Chem.* **41**, 2317 - 2322.

Motoki, M., and Seguro, K. (1998) Transglutaminases and its use for food processing. *Trends Food Sci. Technol*. **9**, 204 - 210.

Mullally, M.M., Meisel, H., and Fitzgerald, R.J. (1996) Synthetic peptides, corresponding to α-Lactalbumin and β-Lactoglobulin sequences with angiotensin- I- Converting enzyme inhibitory activity. *Biochem. Hoppe Seyler*. **377**, 259-260.

Mulvihill, D.M., and Donovan, M. (1987) Whey proteins & their thermal denaturation- a review. *Iri. J. Food Sci . Technol*. **11**, 43 - 75.

Nagaoka, S. (1996) Studies on regulation of cholesterol metabolism induced by dietary food constituents and xenobiotics. *J. Japanese Soc. Nutrition Food Sci.* **49**, 303 - 313.

Nath, R., and Giri, K.V. (1957a) Physicochemical investigations on indigenous seed proteins.1. Studies on the solubilization of nitrogenous constituents of sesamum indicum and characterization of its proteins by electrophoresis. *J. Sci. Ind. Res*. **10**, 5 - 8.

Nath, R., and Giri, K.V. (1957b) Physico-chemical investigation on indigenous seed proteins.2. Fractionation, isolation and electrophoretic characterization of sesame globulin. *J. Sci. Ind. Res.* ***10C***, 51 - 58.

Nemethy, G., Steinberg, I.Z., and Scheraga, H.A. (1963) Influence of water structure and hydrophobic interactions in the strength of side chain hydrogen bonds in proteins. *Biopolymers*. **1**, 43 – 69.

Nio, N., Motoki, M., and Takinami, K. (1986) Gelation mechanism of protein solution by transglutaminase. *Agric. Biol. Chem*. **50**, 851 - 855.

Nishiya, k., and Horwita, D.A. (1982) Contrasting effects of lactoferrin on human lymphocyte and monocyte natural killer activity and antibody dependent cell mediated cytotoxicity. *J. Immunol.* **129**, 2519 – 2523.

Ostojic, S., Pavlovic, M., Zivic, M., Filipovic, Z., Gorjanovic, S., Hranisavljevic, S., and Dojcinovic, M. (2005) Processing of whey- from dairy industry waste. *Environ. Chem. Lett*. **3**, 29-32.

Pace, C.N., Grimsley, G.R., Thomson, J.A., and Barnett, B.J. (1988) Conformational stability and activity of ribonuclease, with zero, one and two intact disulfide bonds. *J. Biol. Chem*. **263**, 11820 - 11825.

Panyam, D., and Kilara, A. (1996) Enhancing the functionality of food proteins by enzymatic modification. *Trends in Food Sci. Technol*. **7**, 120 -125.

Patricia, Z., and Penna, M.S. (2005) Trehalose and glycerol stabilize and renature yeast inorganic pyrophosphatase inactivated by very high temperature. *Arch. Biochem. Biophys*. **444**, 52 - 60.

Pearce, K.N., and Kinsella, J.E. (1978) Emulsifying properties of proteins: Evaluation of a turbidimetric technique. *J. Agric. Food Chem*. **26**, 716 - 723.

Pedro, L., Jesus, C., Jose, L.I., and Arturo, M. (1993) Influenze of polyhydroxylic cosolvents on papain thermostability. *Enz. Microb. Technol*. **15**, 868 – 873.

Peng, I.C., Dayton, W.R., Quass, D.W., and Allen, C.E. (1982) Studies on the subunits involved in the interaction of soybean 11S protein and myosin. *J. Food Sci*. **47**, 1984 - 1990.

Peng, I.C., Dayton, W.R., Quass, D.W., and Allen, C.E. (1982) Investigations of soybean 11S protein and myosin interaction by solubility, turbidity and titration studies. *J. Food Sci*. **47**, 1976 -1983.

Peng, I.C., and Nielsen, S.S. (1986) Protein-protein interactions between soybean beta-conglycinin ($B_1$-$B_1$) + Myosin. *J. Food Sci.* **51**, 588 - 590.

Pofahl, T.R. (1967) Effect of heat on sulfhydryl and disulfide groups of milk proteins as measured by spectrophotometric method. *J. Dairy Sci.* **51**, 1345 -1348.

Poole, S., West, S.I., and Walters, C.L. (1984) Protein-Protein interactions: their importance in the foaming of heterogeneous protein systems. *J. Sci. Food Agric.* **35**, 701 - 711.

Prakash, V., and Nandi, P.K. (1978) Isolation and characterization of α- globulin of sesame seeds (*Sesamum indicum L*). *J. Agric. Food Chem*. **26**, 320 - 323.

Prakash, V. (1982) Partial specific volumes and interactions with solvent components of α-globulin from S*esamum indicum* L. in urea and guanidium hydrochloride. *Bioscience*. **4**, 347 - 359.

Prakash, V., and Rao, M.S.N. (1986) Physicochemical properties of oil seed proteins. *CRC Crit.Rev. Biochem.* **29**, 265 - 363.

Prakash, V. (1985) Hydrodynamic properties of alpha globulin from *Sesamum indicum L. Bioscience*. **4**, 331 - 345.

PriceCarter, M., Hull, M.S., and Goldenberg, D.P. (1998) Roles of individual disulfide bonds in the stability and folding of ω -Conotoxin. *Biochemistry*. **37**, 9851 - 9861.

Pace, C.N., and Scholtz, J.M**. (**1997**)** Measuring the conformational stability of a protein. in *Protein structure: A practical approach* (ed. T.E. Creighton), pp. 299–321, IRL Press, Oxford, UK.

Qi, X.L., Holt, C., Mc Nulty, D., Clarke, D.T., Brownlow, S., and Jones, G.R. (1997) Effect of temperature on the secondary structure of β-Lactolgobulin at pH 6.7 as determined by CD and IR Spectroscopy: A test of molten globule hypothesis. *Biochem. J*. **324**, 341 - 346.

Quinn, M.R., and Beuchat, L.R. (1975) Functional property changes resulting from fungal fermentation of peanut flour. *J.Food Sci*. **40**, 475-479.

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

Radha, C., Kumar, P.R., and Prakash, V. (2008) Enzymatic modification as a tool to improve the functional properties of heat processed soy flour. *J. Sci. Food. Agric.* **88**, 336-343.

Rahma, E.H., and Rao, M.S.N. (1983a) Effects of acetylation and succinylation of cottonseed flour on its functional properties. *J. Agric. Food Chem*. **31**, 352-355.

Rahma, E.H., and Rao, M.S.N. (1983b) Effects of limited proteolysis on the functional properties of cottonseed flour. *J. Agric. Food Chem*. **31**, 356 - 358.

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

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

Rashid, F., Sharma, S., Baig, M.A., and Bano, B. (2006) Effects of polyols and salts on the acid induced state of human placental cystatin. *Biochemistry (Moscow)*. **71**, 619 - 626.

Rotini, E.A., and Rickey, Y.Y. (1995) Some functional properties of a cowpea (*Vigna unguiculata*) Globulin Isolate treated with transglutaminase. *Biosci. Biotech. Biochem*. **59**, 2298 - 2299.

Renner E. (1983) Milk protein. in *Milk and Dairy Products in Human Nutrition*, Pp. 90 -153, Volkswirtschaftlicher Verlag. Munchen, Germany.

Roesch, R.R., Juneja, M., Monagle, C., and Corredig, M. (2004) Aggregation of soy/milk mixes during acidification. *Food Res. Int.* **37**, 209 - 215.

Roesch, R.R., and Corredig, M. (2005) Heat induced soy-whey proteins interactions: Formation of soluble and insoluble protein complexes. *J. Agric. Food Chem*. **53**, 3476 - 3482.

Roozen, J.P., and Pilnik, W. (1973) Ultra filtration controlled enzymic degradation of soy protein. *Process Biochem*. **8**, 24 - 25.

Sabin, L., Thomas, H., and Klostermeyer, H. (2000) Relationship between the crosslinking of caseins by transglutaminase and the gel strength of yoghurt. *Eur. Food Res. Technol*. **210**, 305 - 309.

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

Sava, N., Van der Plancken, I., Claeys, W., and Hendrickx, M. (2005) The kinetics of heat induced structural changes of β-Lactolgobulin. *J. Dairy Sci*. **88**, 1646 – 1653.

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

Schenz, T.W., and Morr, C.V. (1996) Viscosity. in *Methods of testing protein functionality* (Ed. Hall, G.M.), pp. 61-74, Blackie Academic & professional, London, UK.

Schorsch, C., Carrie, H., and Norton, I.T. (2000) Crosslinking casein micelles by a microbial Transglutaminase. Influenze of crosslinks in acid induced gelation. *Int. Dairy J*. **10**, 529 - 539.

Singh, H. (1991) Modification of food proteins by covalent crosslinking. *Trends Food Sci. Technol.* **2**, 196 - 200.

Singh, R.K., and Balange, A.K. (2005) Characteristics of pink perch (*Nemipterus Japonicus*) surimi at frozen temperature. *J. Food Processing and Preservation*. **29**, 75-83.

Singh, H., and Creamer, L.K. (1993) *In vitro* digestibility of whey protein/$_K$ - Casein complexes isolated from heated concentrated milk. *J.Food Sci*. **58**, 299-302.

Slattery, H., and Fitzgerald, R.J. (1998) Functional properties and bitterness of sodium caseinate hydrolysates prepared with a *Bacillus proteinase*. *J. Food Sci*. **63**, 418-422.

Soares, L.H.B., Albuquerque, P.M., Francine, A., and Marco, A.Z.A. (2004) Physicochemical properties of three food proteins treated with tranglutaminase. *Ciencia Rural.* **34**, 1219 -1223.

Soeda, T. (2003) Effects of microbial transglutaminase for gelation of soy protein isolate during cold storage. *Food Sci. Technol. Res.* **9**, 165 -169.

Sosulski, F.W. (1962) The centrifuge method of determining flour absorption in hard red spring wheats. *Cereal Chem*. **39**, 344 - 350.

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

Stark, Y.Y., Bez, J., Wada, Y., and Wasche, A. (2004) Functional properties, lipozigenase activity and health aspects of *Lupinus albus* protein isolates. *J. Agric. Food Chem*. **52**, 7681-7689.

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

Swaisgood, H.E. (1996) Characteristics of milk. *Food Chem*. **3**, 841 - 818.

Takagi, S., Okamoto, N., Akashi, M., and Yasamatsu, K. (1979) Heat denaturation of soybean proteins. Part 2. Hydrophobic bonding and SS bonding in heat denaturation of 11S of soybean proteins. *Nippon Shokuhin Kogyo Gakkaishi (J. Japanese Soc. Food Sci. Technol).* **26**, 139 – 144.

Tatiana, V.B., Yvan, C., Vinh, T., and Thomas, H. (1998) Role of free Cys 121 in stabilization of bovine β-Lactoglobulin B. *Protein Engineering*. **11**, 1065 - 1073.

Tanimoto, S.Y., and Kinsella, J.E. (1988) Enzymatic modification of proteins: Effects of transglutaminase cross-linking of some physical properties of *β*-lactoglobulin. *J. Agric. Food Chem*. **38**, 281 – 285.

Taravati, A., Shokrzadeh, M., Ebadi, A.G., Valipour, P., Tabar Molla Hassan, A., and Farrokhi, F. (2007) Various effects of sugar and polyols on the protein structure and function: Role as osmolyte on protein stability . *World Applied Sci. Technol*. **4**, 353-362.

Thanh, V.H., and Shibasaki, K. (1976) Major proteins of soybean proteins. A straightforward fractionation and their characterization. *J. Agric. Food Chem*. **24**, 1117 - 1211.

Thomas, C., Daniel, M., Raj, M., and Said, B. (2004) Spectroscopic characterization of heat induced non-native β-Lactolgobulin monomers. *Protein Sci*. **13**, 1 - 7.

Thornton, J.M. (1981) Disulfide bridges in globular proteins. *J. Mol. Biol.* **151**, 261 - 287.

Timasheff, S.N., and Arakawa, T. (1990) Stabilization of protein structure by solvents. in *Protein structure: A practical approach (*Ed. Creighton, T.E.), pp. 331- 345, Oxford, New York, USA.

Timasheff, S.N. (1994) Control of protein stability and reactions by weakly interacting cosolvents: the simplicity of the complicated. *Adv.Protein Chem*. **54,** 355 - 431.

Timasheff, S.N. (2002) Protein–solvent preferential interactions, protein hydration, and the modulation of biochemical reactions by solvent components. *Proc. Natl. Acad. Sci., USA*. ***99***, 9721 - 9726.

Townsend, A.A., and Nakai, S. (1983) Relationships between hydrophobicity and foaming characteristics of food proteins. *J. Food Sci*. **48**, 588 - 594.

Tseng, T.F., Chen, M.T., and Liu, D.C. (2002) Purification of trasnglutaminase & its effects on myosin heavy chain & actin of spent hen. *Meat Sci*. **20**, 267-270.

Tyagi, R., and Gupta, M.N. (1998) Chemical modification and chemical cross linking for protein/ enzyme stabilization. *Biochemistry (Moscow)*. **63**, 334 - 344.

Truong, V.D., Clare, D.A., Catignani, G.L., and Swaisgood, H.E. (2004) Crosslinking and rheological changes of whey proteins treated with microbial transglutaminase. *J. Agric. Food Chem*. **52**, 1170 -1176.

Van der Ven, C., Gruppen, H., de Bont, D.B.A., and Voragen, A.G.J. (2002) Correlations between biochemical characteristics and foam forming and stabilizing ability of whey and casein hydrolyzates. *J. Agric. Food Chem*. **50**, 2938 - 2946.

Vetri, V., and Mihtello, V. (2005) Thermal induced conformational changes involved in the aggregation pathways of β-Lactoglobulin. *Biophys. Chem*. **113**, 83 - 91.

Vojdani, F. (1996) Solubility. in *Methods of testing protein functionality* (Ed. Hall, G.M.), pp. 11 - 55, Blackie Academic & Professional, London, UK.

Walzem, R.L., Dillard, C.J., and German, J.B. (2002) Whey components: Millenia of evolution create functionalities for mammalian nutrition. What we know and what we may be overlooking. *CRC Crit. Rev. Food Sci. Nutri*. **42**, 353 – 375.

Weinberg, E.D. (1996) The role of iron in cancer. *Eur. J. Cancer Prev*. 5, 19-36.

Weir, G.S.D. (1986) Protein hydrolyzates as flavourings. in *Developments in food protein* (Ed. Hudson., B.J.F.), pp. 175 – 217, Elsevier, London, UK.

Whittaker, M.M., and Whittaker, J.W. (1988) The active site of galactose oxidase. *J. Biol. Chem*. **263**, 6074 - 6080.

Wolf, W.J., and Tamura, T. (1969) Heat denaturation of soybean 11S protein. *Cereal Chem.* **46**, 331 – 344.

Wong, P.Y.Y., and Kilts, D.D. (2003) A comparison of the buttermilk solids functional properties to non fat dried milk, soy protein isolate, dried egg white and egg yolk powders. *J. Dairy Sci*. **86**, 746-754.

Wouters, J.T.M., Geurts, T.J., and Walstra, P. (2006) Milk components. in *Dairy Science and Technology*, pp. 17 – 108, CRC press, Taylor & Francis. Boca Raton, London.

Wright, H.T. (1991) Non enzymatic deamidation of asparagenyl and glutaminyl residues in proteins. *Crit. Rev. Biochem and Mol. Biol.* **26**, 1-52.

Wu, J.P., and Ding, X.L. (2002) Characterization of inhibitory and stability of soy protein-derived Angiotensin-I-converting Enzyme inhibitory peptides. *Food Res. Int*. **35**, 367 – 375.

Yamamoto, k., Fukazawa, T., and Yasui, T. (1973) The interactions between the muscle protein and the soybean protein. *Hokkaido Daigaku Nogakuba Hobun Kiyo.* **9**, 116.

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

Yang, T., and Olsen, K.W. (1988) Effects of crosslinking on the thermal stability of haemoglobin II. The stabilization of met-,Cyanomet-, carbonmonoxy Hb A&B with bis (3,5-dibromosalicyl) fumarate. *Arch. Biochem. Biophys.* **261**, 283 - 290.

Zayas, J.F. (1997) Solubility of proteins. in *Functionality of proteins in food*, pp 6-67, Springer - Verlag, Berlin.

Zhu, Y., Rinzema, A., Tramper, J., and Bol, J. (1995) Microbial transglutaminases- a review of its production and application in food processing. *Appl. Microbiol. Biotechnol*. **44**, 277 – 282.

**Table 1: Composition of bovine milk**

| Protein fraction | Proportion of milk protein (%) |
|---|---|
| Caseins (total)- | 80.3 |
| $\alpha_s$ - Casein | 43.5 |
| $\kappa$ - Casein | 10.7 |
| $\beta$ - Casein | 24.1 |
| $\gamma$ - Casein | 2.0 |
| Whey proteins- | 19.7 |
| $\beta$-Lactoglobulin | 9.6 |
| $\alpha$-Lactalbumin | 3.7 |
| Serum albumin | 0.9 |
| Immunoglobulins | 2.2 |
| Proteose-peptones | 3.3 |

Kim *et al*., (2003)

**Table 2: Physicochemical characteristics of whey proteins**

| Protein components | Molecular weight (Da) | Approx. Content in skim milk (%) | Isoelectric pH | Cystine groups |
|---|---|---|---|---|
| **β-Lg** | 18600 | 7 -12 | 5.3 | 2 |
| **α-Lac** | 14200 | 2 - 5 | 4.8 | 4 |
| **BSA** | 66000 | 0.7 - 1.3 | 5.1 | 17 |
| **Immunoglobulins** | 15-96x$10^4$ | 1.9 - 3.3 | 5.5-6.8 | ~32 |
| **Proteose-peptones** | | | | |
| **β-CN-5P (1-105)** | 11500 | 2 - 6 | 3.7 | nil |
| **β-CN-5P (1-107)** | 13000 | | 4.5 | nil |
| **β-CN-4P (1-28)** | 4100 | | 3.0 | nil |
| **Lysozyme** | 18000 | 0.13 - 0.32 | 9.5 | 3 |
| **Lactoferrin** | 76500 | 0.02 - 0.35 | 8.0 | 19 |

Source: Kinsella and Whitehead (1989)

**Table 3: Proximate analysis of whey**

| Composition | Gram % |
|---|---|
| Moisture | 3.1 ± 0.1 |
| Protein | 57 ± 1 |
| Fat | 1.5 ± 0.1 |
| Ash | 5.4 ± 0.2 |
| Carbohydrates (glucose) | 2 ± 0.3 |

Table 4: Amino acid composition of whey

| Amino acid | gram (%) |
|---|---|
| **Aspartic Acid*** | 11.48 |
| **Glutamic acid**** | 21.05 |
| **Tryptophan***** | 2.00 |
| **Serine** | 3.07 |
| **Glycine** | 2.64 |
| **Histidine** | 2.34 |
| **Arginine** | 3.23 |
| **Threonine** | 4.89 |
| **Alanine** | 2.20 |
| **Proline** | 6.56 |
| **Tyrosine** | 1.30 |
| **Valine** | 7.09 |
| **Methionine** | 0.02 |
| **Cystine** | 0.46 |
| **Isoleucine** | 6.33 |
| **Leucine** | 11.44 |
| **Phenylalanine** | 4.69 |
| **Lysine** | 8.61 |

* Aspartic acid & Asparagine
** Glutamic acid & Glutamine
*** Tryptophan estimated by NBS method

Table 5: Functional properties of whey protein concentrate

| Functional properties | Whey protein concentrate | Standard protein (soy protein concentrate) |
|---|---|---|
| (1) Fat absorption capacity | 2.7 ± 0.25 g/g | 2.6 ± 0.1 g/g* |
| (2) Water absorption capacity | Completely soluble in water | 2.7 ± 0.2 g/g** |
| (3) Apparent viscosity | | |
| 10% | 11.5 ± 0.2 cps | Not available |
| 20% | 14.5 ± 0.2 cps | |
| 30% | 18.4 ± 0.5 cps | |
| 4) Gelling capacity | 12% | 8% * |

*Boyarioglu, 2006.
**Wong and Kilts, 2003.

**Table 6: Heat set gelation of various concentrations of whey**

| Whey protein concentration (%) | Peak value (g/sec) ± SD | Adhesiveness (g/sec) ± SD |
|---|---|---|
| 14 | 839 ± 121 | -109.0 ± 4 |
| 16 | 838 ± 46 | - 40.5 ± 2 |
| 18 | 1646 ± 23 | - 29.0 ± 1 |
| 20 | 1753 ± 102 | -25.0 ± 1 |

**Table 7: Amino acid composition of isolated β-Lg**

| Aminoacids | Literature * gram % | Isolated β-Lg gram % | Commercially obtained β-Lg gram % |
|---|---|---|---|
| Asp (D) +Asn(N) | 11.20 | 9.70 | 9.96 |
| Glu (E) +Gln(Q) | 19.20 | 19.60 | 19.15 |
| Ser (S) | 4.50 | 3.50 | 3.00 |
| Gly (G) | 1.40 | 1.20 | 1.10 |
| His (H) | 1.70 | 1.50 | 1.10 |
| Arg (R) | 2.80 | 2.80 | 2.55 |
| Thr (T) | 5.40 | 4.70 | 4.20 |
| Ala (A) | 7.00 | 5.90 | 6.04 |
| Pro (P) | 4.60 | 4.30 | 4.16 |
| Met (M) | 3.10 | 1.92 | 2.50 |
| Ile (I) | 6.70 | 4.80 | 4.06 |
| Leu (L) | 15.30 | 13.80 | 12.57 |
| Cys (C) | 2.50 | 0.38 | 0.50 |
| Phe (F) | 3.50 | 3.36 | 2.70 |
| Lys (K) | 11.70 | 9.03 | 8.80 |
| Trp (W)* | 2.06 | 1.87 | 2.02 |
| Tyr (Y) | 3.80 | 2.19 | 3.03 |
| Val (V) | 5.90 | 4.00 | 4.34 |

*Tryptophan estimated by NBS method.
** Renner (1983).

**Table 8: Foaming capacity of whey protein hydrolysed by fungal protease**

| Time of hydrolysis (min) | Foam volume (ml) |
|---|---|
| 0 | 11 ± 1 |
| 30 | 11 ± 1 |
| 60 | 13 ± 1 |
| 120 | 13 ± 1 |
| 150 | 14 ± 1 |

**Table 9: Emulsifying activity of whey protein hydrolysed by fungal protease**

| Time of hydrolysis (min) | Emulsifying activity expressed as absorbance at 500 nm |
|---|---|
| Control | 0.93 ± 0.02 |
| 30 | 1.02 ± 0.02 |
| 60 | 0.92 ± 0.02 |
| 120 | 0.75 ± 0.02 |
| 150 | 0.64 ± 0.02 |

**Table 10: Foaming capacity of whey protein hydrolysed by papain**

| Time of hydrolysis (min) | Foam volume (ml) |
|---|---|
| 0 | 11 ± 1 |
| 30 | 10 ± 1 |
| 60 | 7 ± 1 |
| 120 | 9 ± 1 |
| 150 | 9 ± 1 |