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# THERMODYNAMIC AND STRUCTURAL PROPERTIES RELATED TO THE GELATION OF WHEY PROTEINS

BY

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*Suggested short title:* **THERMAL GELATION OF WHEY PROTEINS**

**Dedicated to my husband, Koby, and to my father and mother,**

**Andrew and Paulina.**

**Your love has been my source of encouragement.**

***"Knowledge is of no value until it is shared."***

Joyce Boye, 1990.

## FOREWORD

The thesis is submitted in the form of original papers prepared for journal publications. The first two sections comprise a general introduction and a literature review presenting the theory and previous knowledge on this topic. The next seven sections contain the body of the thesis; each chapter represents a complete manuscript. The last section is a summary of the major conclusions. This format has been approved by the Faculty of Graduate Studies and Research, McGill University, and follows the conditions outlined in the Guidelines for Thesis Preparation, Thesis Specification, section 3 entitled "Traditional and manuscript-based theses" which are as follows:

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In the case of manuscripts co-authored by the candidate and others, **the candidate is required to make an explicit statement in the thesis as to who contributed to such work and to what extent.** Supervisors must attest to the accuracy of such statements at the doctoral oral defense. Since the task of the examiners is made more difficult in these cases, it is in the candidate's interest to make perfectly clear the responsibilities of all authors of the co-authored papers. **Under no circumstances can a co-author of any component of such a thesis serve as an examiner for that thesis.** "

Although all the work reported in this thesis is the responsibility of the candidate, the project was supervised by Dr. Inteaz Alli, Department of Food Science and Agricultural Chemistry, Macdonald Campus of McGill University.

## ABSTRACT

The gelling characteristics of whey proteins is governed by factors which affect the structural properties of the protein. To understand this structure gelling relationship, the following factors were investigated; protein concentration, heating temperature and time, pH, NaCl and sugars. The effect of these factors on the molecular structure and gelation properties of whey protein concentrate (WPC), β–lactoglobulin (β–lg), α–lactalbumin (α–lac) and bovine serum albumin (BSA) were studied using polyacrylamide gel electrophoresis, HPLC, mass spectrometry, differential scanning calorimetry and Fourier transform infrared spectroscopy. The results showed that protein concentration affected textural properties without affecting the molecular structure of the whey proteins while heating temperature, pH and NaCl affected both molecular structure and textural characteristics. NaCl and sugars increased the stability of whey proteins to thermal denaturation but decreased gel formation. β-lg formed an opaque gel at pH 3 and a translucent gel at pH 9; the peak temperature of denaturation was 84°C at pH 3 and 70°C at pH 9. At both acid and alkaline pH, denaturation of β–lg resulted in the formation of intermolecular β–sheet structures associated with aggregation. These β–sheet aggregate structures were also observed when α-lac was heated at pH 3 and 5 but not at pH 7 and 9. At pH 7, heating α–lac resulted in a loss of α–helix, $3_{10}$-helix and β–sheet and an increase in turns. DSC showed two reversible transitions at 39.6°C(A) and 64.8°C (B). At pH 3, transition A was partially reversible (14%) while transition B was completely reversible. At pH 9, transitions A and B were completely irreversible and a translucent gel was formed. Bovine serum albumin (BSA) showed maximum stability to thermal denaturation at pH 5. Denaturation of BSA resulted in the loss of α-helical structure and the formation of non-native β–sheet structure. Heating to 85°C resulted in gel formation at both acid (pH 3) and alkaline pH (pH9).

## RÉSUMÉ

Les caractéristiques de la coagulation des proteines des protéines du petit-lait sont gouvernées par des facteurs qui affactent les propriétées de la protéine. Pour comprendre la relation entre la structure et la coagulation, les facteurs suivant ont été investiguér; la concentration de la protéine, la température de chauffage et le temps, le pH, NaCl, sucrer. L'effet de ces facteur sur la structure moléculaire et les propriétées de coagulation de la protéine du petit-lait rassemblés (WPC), β–lactoglobuline (β–lg), α–lactalbumin (α–lac) et la serum-albumine de bovine (BSA) ont été étudiés en utilisant un électrophorèse de gelation polyacrylamide, HPLC, spectrométrie de masse, calorimétrie d'échographie différentielle, et spectroscopie d'infrarouge transformé de Fourier. Les résultats ont démontrés que la concentration de la protéine affecte les propriétées de la texture sans affecter la strucuture moléculaire de la protéine du petit-lait. Par contre la temperature de chauffage, le pH et le NaCl affectent la structure moléculaire et les caractéristiques de la texture. Le NaCl et les sucrer ont augmentes la stabilité des protéines du petit-lait jusqu'a la dénaturation thermale mais ont diminués la formation de gel. La β–lg a formé un gel opaque à un pH 3 et un gel translucide à un pH 9; la température maximale de la dénaturation était 84°C à un pH 3 et 70°C à un pH 9. À un pH acide et alkalin, la dénaturation de la β–lg a produit la formation de β–sheet intermoléculaire associés avec structures de l'aggrégation. Ces structures d'aggrégat de β–sheet en aussi été observées lorsque la α–lac fut chauffée à un pH de 3 et 5 mais pas à un pH 7 et 9. À un pH 7, le chauffage de la α–lac a produit une perte de α–helix, $3_{10}$-helix et β–sheet et une augmentation par la suite. DSC à démontré deux transitions réversible a 39.6°C (A) et à 64.8°C (B). À un pH 3, la transition A était partiallement réversible (14%) et la transition B était complètement réversible. À un pH 9, les transitions A et B étaient complètement irréversible et un gel translucide s'est formé. La sérum-albumine de bovin (BSA) a démontré une stabilité maximale à la dénaturation thermale à un pH 5. La dénaturation de BSA a produit une perte de la structure α–helical et la formation de structure β–sheet non-native. Le chauffage à 85°C a resulté dans la formation d'un gel à un pH acid (pH 3) et alkalin (pH 9).

# PREFACE

## CLAIM OF ORIGINAL RESEARCH

1) This study represents the first study directed at relating the thermal transitions of whey proteins as determined by differential scanning calorimetry to the specific changes occurring in their secondary structure as monitored by amide band signals from infrared spectroscopy.

2) The exact contributions of β–lactoglobulin, α–lactalbumin, and bovine serum albumin, individually and in combination, to the gelling of whey protein concentrate have been demonstrated under the pH range 3 - 11 and NaCl concentration of 0.4 - 2.0M.

3) This is the first study to demonstrate that glucose and sucrose promote the unfolding of β–lactoglobulin but inhibit aggregate formation by stabilizing the partially denatured protein.

4) Evidence is presented to show that the 1692 $cm^{-1}$ band in the amide I' band profile of β–lactoglobulin is due to a β–sheet structure shielded from surrounding solvent in the interior of the protein.

5) This study has shown that the two endothermic peaks observed in α–lactalbumin represent the denaturation of $Ca^{++}$-free (apo-) and bound (holo-) forms and is the first study to correlate the first endothermic transition of α–lactalbumin to the breakdown of the $3_{10}$-helix structure and increase in β–sheet formation of apo-α–lactalbumin and the second transition to complete unfolding

of holo-α-lactalbumin.

6) This study has shown that sulfhydryl-oxidation may not be essential in the aggregation phase of gel formation but may be necessary in the crosslinking of the aggregates formed; hydrophobic interactions may be the most important in the initial aggregation phase of gel formation.

**Part of this work has been published or submitted for publication as follows:**

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Boye, J.I., Ismail, A., Alli, I., "Effect of Physico-chemical factors on the Secondary Structure of β-lactoglobulin". *J. Dairy Res.* (Accepted, **March 1995**).

Boye, J.I., Alli, I., Ismail, A., " Use of Differential Scanning Calorimetry and Infrared Spectroscopy to study the Thermal and Structural Stability of α-lactalbumin". *Int. J. Peptide Protein Res.* (Submitted, **May 1995**).

Boye, J.I., Alli, I., Ramaswamy, H., Raghavan, V. S. G., "Interactive effects of factors affecting gelation of whey proteins". *J. Food Sci.*(Submitted, **June 1995**).

Boye, J.I., Alli, I., Ismail, A., "Interactions involved in the Gelation of Bovine Serum Albumin". *J. Agric. Food Chem.* (Submitted, **June 1995**).

Boye, J.I., Alli, I., "Thermal interactions of α-lactalbumin, β-lactoglobulin and bovine serum albumin: A differential scanning calorimetric study". *Food Res. Int.* (Submitted, **June 1995**).

Alli, I., Boye, J.I., Gibbs, B.F., Konishi, Y., "Mass spectrometric evaluation of whey proteins". *J. Agric. Food Chem.* (Submitted, **July 1995**).

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## ABBREVIATIONS

| | | |
|---|---|---|
| **α-lac, α-la** | - | α-lactalbumin |
| **β-lg** | - | β-lactoglobulin |
| **BSA** | - | bovine serum albumin |
| **CCRD** | - | Central composite rotatable design |
| **ΔH** | - | change in enthalpy |
| **DSC** | - | differential scanning calorimetry |
| **EDTA** | - | Ethylene diamine tetra acetic acid |
| **EGTA** | - | Ethylene olycol bis-(β-aminoethylether)-N,N'-tetra acetate |
| **FTIR** | - | Fourier transform infrared spectroscopy |
| **i.r, IR** | - | infrared |
| **IPN** | - | Interpenetrating network |
| **LDL** | - | Low density lipoprotein |
| **MW** | - | molecular weight |
| **NEM** | - | N-ethylmaleimide |
| **PAGE** | - | Polyacrylamide gel electrophoresis |
| **RP-HPLC** | - | reversed phase high performance liquid chromatography |
| **RSM** | - | Response surface methodology |
| **SDS** | - | Sodium dodecyl sulfate |
| $T_d$ | - | peak temperature of denaturation |
| $T_w$ | - | width of peak at half-height |
| **WHC** | - | water holding capacity |
| **WPC** | - | whey protein concentrate |

# CHAPTER 1

## INTRODUCTION

### 1.1 General

Whey protein concentrate (WPC) obtained after separation of the caseins from milk (Evans & Gordon, 1980; Ziegler & Foegeding, 1990) contains approximately 10-24% of the total protein content of milk (Kinsella, 1984; Fox, 1989). The major protein constituents of whey are β-lactoglobulin (comprising 50% of the proteins of whey), α-lactalbumin (comprising 21%), immunoglobulins (10%), bovine serum albumin (5%) and residual amounts of proteose-peptones (Evans & Gordon, 1980; Kinsella 1984). These proteins posses excellent functional properties (e.g., gelation, foaming, solubility and emulsification) which make them valuable ingredients in food formulation. However, of the 100 million tons of whey produced from cheese making and 15 million tons from casein production, only half is utilized and consequently well over 250, 000 tons of high quality protein is wasted each year (Pomeranz, 1991).

Whey protein gelation is of particular importance in food formulation since it contributes significantly to the sensory, textural and rheological properties of foods. The mechanism of protein gelation has been described as a two stage process (Ferry,1948; Shimada & Matsushita, 1980) involving initial denaturation of the protein followed by protein-protein interactions which result in the formation of a rigid macroscopic structure, resistant to flow under pressure(Flory, 1974; Kinsella, 1976; Schmidt, 1981; Clark & Lee-Tuffnell, 1986). When heated, whey proteins unfold, reorient themselves and trap solvent as well as other food components within their gel matrix to form viscoelastic gels (Schmidt, 1981; Kinsella & Whitehead, 1989; Ziegler & Foegeding, 1990). Examples of

foods in which whey proteins are used include breads, cakes, starch puddings, whipped toppings and meat products (Kilara, 1994).

Several workers have attempted to relate the functional property of whey proteins to their structure and amino acid composition and sequence (Kinsella, 1976; Damodaran & Kinsella, 1982; Ziegler & Foegeding, 1990; Damodaran, 1994 ). The difficulty in providing a simple mechanistic explanation of functional behavior as related to protein structure arises from the fact that whey proteins are multicomponent systems (Tolstoguzov, 1986) and the functional property of one component may be altered by the presence of the other macromolecules and chemicals in the system. Another difficulty is that native whey is usually modified during processing (Cheftel *et al*, 1985); whey protein concentrate prepared by different techniques (such as electrodialysis, ultrafiltration, reverse osmosis, gel filtration and reagent complexation), are therefore highly variable in their composition and functionality (Schmidt *et al.*, 1984). In addition, the texture and strength of whey protein gels have been demonstrated to be affected by intrinsic factors such as the composition and concentration of the proteins and by extrinsic factors such as heating temperature, pH and ionic strength (Schmidt, 1981; Damodaran, 1989; Ziegler & Foegeding, 1990).

Although various studies have facilitated greater understanding of the gelation of globular proteins, much of the fundamental relationship between the molecular properties of whey proteins and their gelation behavior still remains unknown.

## 1.2 Rationale and Objectives of Study

The nutritional and functional significance of whey proteins in food processing applications and food formulation can not be overemphasized. Knowledge of the gelation mechanism of whey proteins will contribute to their

increased use in food formulation. There is considerable information in the literature which partly explains the relationship between the physical properties and the gelation characteristics of proteins, however the molecular basis for many of these changes is still poorly understood. Besides, very little work has been done on the effects of other food components on the gelation properties of whey proteins and the interactive effects of factors that affect whey protein gelation. The lack of standardized methods for characterization of gel properties has further compounded these problems.

In this study, commercial whey protein concentrate (WPC) was used as a model system to monitor factors affecting gelation and the changes that occur in protein conformation during gelation in an attempt to provide a clearer understanding of the fundamental relationship between the molecular structure, molecular characteristics, physical and physico-chemical properties involved in whey protein gelation.

The objectives of the study therefore, were:

1)To monitor the effects of heating temperature and time, concentration of protein, NaCl and pH on the gelation characteristics of whey protein concentrate, and to relate these effects to changes in molecular structure at the sub-unit level.

2) To establish the quantitative effects of the factors mentioned above on whey protein gelation and evaluate the interactive effects of the factors studied.

3) To monitor the effects of the factors mentioned above on the three major proteins in bovine whey (β-lactoglobulin, α-lactalbumin, bovine serum albumin)and to determine the thermodynamic properties involved in their gelation, and the changes occurring in their secondary structure during gelation.

4) To determine the interactions of β-lactoglobulin, α-lactalbumin and bovine serum albumin during heat treatment and the effects of environmental conditions (pH, NaCl, heating), non-protein components (sugars, Na bicarbonate, Na ascorbate), denaturing agents (cysteine, urea, sodium dodecyl sulfate) and thiol-blocking agents (N-ethylmaleimide) on their gelation.

## CHAPTER 2

## LITERATURE REVIEW

### 2.1 Protein Functionality

In recent years, the increased dependence of food processing industries on the manufacture of fabricated foods has drawn much attention to the functional properties of individual ingredients used in food formulation and in product development. The term "functionality" as applied to food ingredients has been defined as any property aside from nutritional attributes that influences an ingredient's usefulness in foods (Pour-El, 1981). Proteins are of particular significance since they play a major role in determining the sensory, textural as well as the nutritional characteristics of various food products and have the ability to act as a matrix for holding water, lipids, sugars, flavors and other ingredients during gelation (Kinsella, 1976).

Functional properties of a protein are related to the physical, chemical and conformational properties which include size, shape, amino acid composition and sequence, charge and charge distribution. The properties of food proteins which affect functionality include hydrophilicity/hydrophobicity ratio, secondary structure content and their distribution (e.g. alpha-helix, beta-sheet and aperiodic structures), tertiary and quaternary arrangements of the polypeptide segments, inter- and intra- subunit cross links ( e.g. disulfide bonds) and the rigidity/flexibility of the protein in response to external conditions (Damodaran, 1989). Most functional properties affect the textural qualities of a food and play an important role in determining the physical behavior of a food during preparation, processing and storage.

Functional properties of food proteins can be classified into three main groups: (a) hydration properties (dependent on protein-water interactions);

(b) properties related to protein-protein interactions and (c) surface properties. The first group relates to such properties as water absorption and retention, wettability, swelling, adhesion, dispersibility, solubility and viscosity. The second group is associated with precipitation, gelation and rheology. The third group relates primarily to surface tension, emulsification and foaming characteristics. These groups are not totally independent : for example gelation involves not only protein-protein interaction but also protein-water interaction, and viscosity and solubility both depend on protein-water and protein-protein interaction (Cheftel *et al.*, 1985). Kinsella (1982), divided the functional properties of proteins in food applications into the following general classes: organoleptic, kinesthetic, hydration, surface, binding, structural, rheological, enzymatic, blendability, antioxidant; these classes, however, fall under the three broad classifications i.e., protein-water, protein-protein and surface properties.

Apart from physicochemical properties, various other factors influence the functional properties of food proteins. These factors are related to environmental conditions encountered during processing. Processing factors that affect functionality include the protein source and variety, extraction procedures, temperature, drying, impurities and storage (Pour-El, 1981).

## 2.2 Protein Gelation

Gelation plays a major role in the preparation of many foods, including various dairy products, soybean protein gels, vegetable proteins texturized by extrusion or spinning and bread doughs. Proteins are utilized for the formation of viscoelastic gels, for improved water absorption, thickening and particle binding (adhesion) (Cheftel *et al.*, 1985).

### 2.2.1 Mechanism of Heat-Induced Gelation

Gelation is the association or crosslinking of long polymer chains to form a three-dimensional continuous network which traps and immobilizes the liquid within it to form a rigid structure that is resistant to flow under pressure (Glicksman, 1982). The most important food processing operation which contributes to protein gelation involves heat treatment. This review is devoted to thermally induced gelation.

The mechanism by which food proteins form a thermally induced gel matrix consists of the following three sequential events, denaturation, aggregation and crosslinking (Edwards *et al.*, 1987), as follows:

denaturation ——> aggregation ——> crosslinking

Protein *denaturation* is any modification in conformation (secondary, tertiary or quaternary) not accompanied by the rupture of peptide bonds involved in primary structure (Cheftel *et al.*, 1985). This process results in the formation of new short-lived intermediary conformations. *Aggregation* involves the formation of higher molecular weight complexes from the denatured protein (Schmidt, 1981) which then *crosslink* by specific bonding at specific sites on the protein strands or by nonspecific bonding occurring along the protein strands.

Protein denaturation is a prerequisite for the formation of an ordered gel structure. The first steps in heat gelation of an aqueous protein solution are:

a) Reversible dissociation of the quaternary structure into subunits or monomers (reversible dissociation of native polymers may also take place as the first step of denaturation).

b) Irreversible denaturation of secondary and tertiary structures (unfolding generally remains partial)

The final gel state corresponds to aggregates of partly denatured proteins and involves the following mechanism:

$$nP_N \longrightarrow nP_D \longrightarrow [P_D]n \qquad \text{(Eqn 1)}$$

where n is the number of protein molecules, $P_N$ is the native protein and $P_D$ is denatured protein.

### 2.2.2 Crosslinking in Gel Structure

The characteristics of a protein gel are affected by intra- and inter- strand crosslinking which, combined with the fluidity of the immobilized solvent gives gels their characteristic strength, elasticity and flow behavior. Both covalent and non-covalent bonding are involved in the crosslinking of protein gels.

In the absence of denaturation, the native state of proteins is maintained by a delicate balance of chain interaction energies involving electrostatic interactions, hydrogen-bonding, disulfide bonds and hydrophobic interactions (Fig 2.1). Dissociation and/or unfolding of protein molecules generally increases the exposure of reactive groups especially the hydrophobic groups of globular proteins. Protein-protein hydrophobic interactions are, therefore, favored and are usually the main cause of subsequent aggregation (Cheftel *et al.*, 1985) provided that the protein concentration, thermodynamic conditions and other conditions are optimal for the formation of the tertiary matrix (Schmidt, 1981).

Crosslinking of protein aggregates usually involves one or more of the following four mechanisms (Parker & Dalgleish, 1977 a,b):

a) oxidative chemical reactions of proteins resulting from the covalent interaction of their functional groups;

b) crosslinking of proteins by polyfunctional agents (including metal ions) in solution;

c) physicochemical conditions resulting in limited solubility;

d) chemical modification of the proteins leading to limited protein solubility

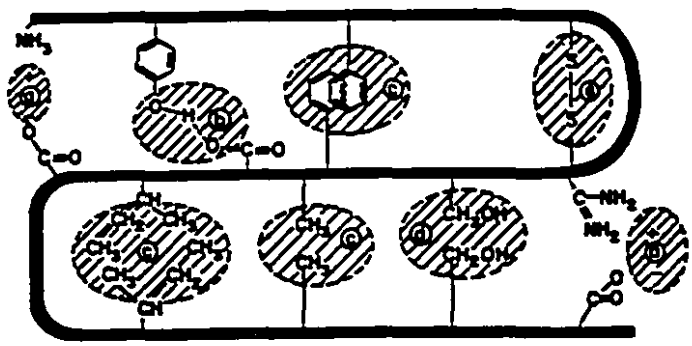

**Fig 2.1:** Bonds which stabilize protein structure (a) electrostatic interactions; (b) hydrogen bonding; (c) hydrophobic interactions; dipole-dipole interaction; (e) disulfide bonding. (Whitaker, 1977).

Insufficient crosslinking or overdependence on crosslinking results in undesirable gel structure (Schmidt, 1981). Both disulfide and hydrogen bonding, as well as ionic interactions are involved in the crosslinking of protein gels (Catsimpoolas & Meyer, 1970; Morrissey *et al.*, 1987).

The ability of a protein to form intermolecular disulfide bonds during the heat treatment is a prerequisite for gelation of the proteins (Huggins *et al.*, 1951, Nakamura *et al.*, 1984; Utsumi & Kinsella, 1985). Heat treatment results in cleavage of existing disulfide bond structure or "activation" of buried sulfhydryl groups through unfolding of the protein. These newly formed or activated sulfhydryl groups can form new intermolecular disulfide bonds which are essential for the formation of a highly ordered gel structure in some protein

systems (Tombs, 1974). Catsimpoolas and Meyer (1970) suggested that disulfides play a major role in the progel to gel transformation in soybean globulins; in more reversible soy protein gelation processes, disulfide bridging may be involved in the formation of the gel structure. The role of disulfide-sulfhydryl interchange reactions in the crosslinking of protein gels is shown in Fig 2.2.

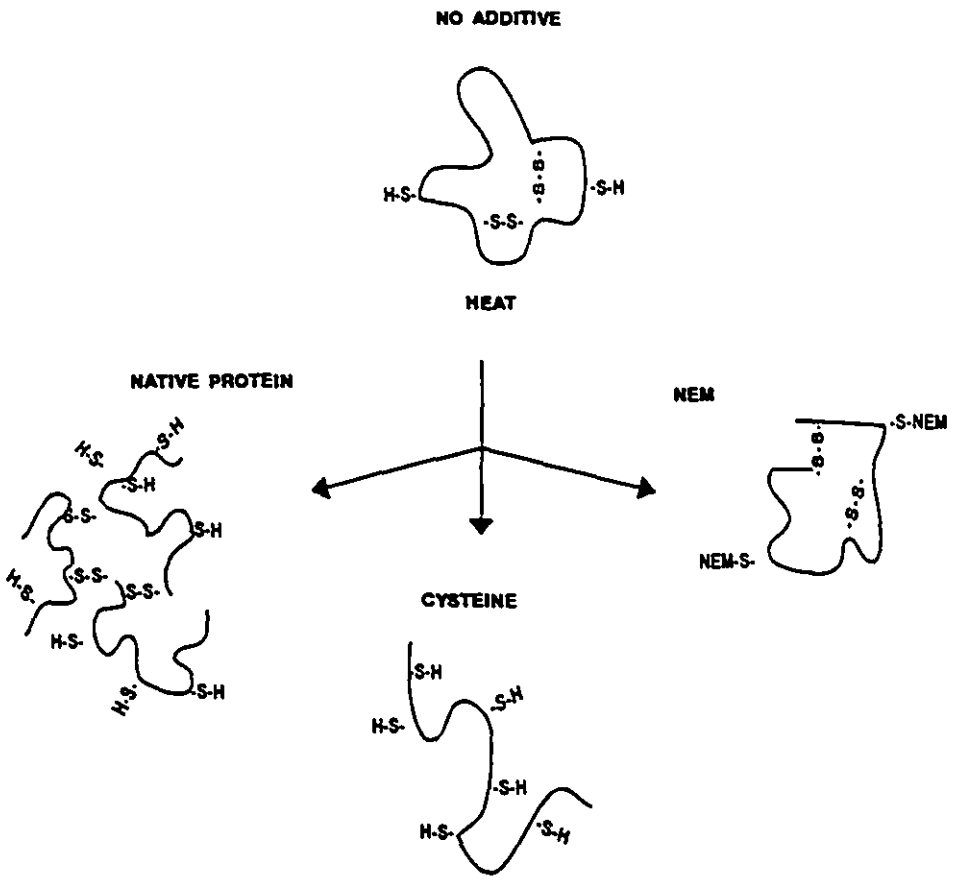


**Fig 2.2: Role of sulfhydryl-disulfide interchange reactions during gelation in the presence and absence of N-ethylmaleimide (NEM), a sulfhydryl blocking agent, and cysteine, a reducing agent. (Wang & Damodaran, 1990).**

Hydrogen bonding plays a major role in increased viscosity which precedes the onset of gelation and in stabilizing the gel structure This type of crosslink allows for an open orientation necessary for water immobilization (Schmidt, 1981) and may be the most important type of crosslinking in reversible and gelatin gels (Stainsby, 1977).

Ionic bonding has been suggested to be of primary importance at the protein-solvent interface and to solvent immobilization (Cheftel *et al.*, 1985). Gel solvation increases with increased salt addition due to decreased protein-protein attractions and increased protein-solvent attractions; at higher ionic strength, gels exhibit more protein-protein attractions as the ions compete with the protein for solvent and results in the formation of a more aggregated gel (Hermansson, 1979; Schmidt, 1981).

Hydrophobic interactions are not true bonding but result from apolar side chains associating to avoid contact with polar solvents. The number of apolar groups, the degree of hydrophobicity, and the location in the chain determine the overall contribution of hydrophobic interactions to protein structure (Stanley & Yada, 1992) and gelation. Nonspecific hydrophobic interactions have been implicated for the dissociative-associative reactions which initiate the gelation process and also for improved strength and stability of the protein gels on cooling (Schmidt, 1981; Stainsby, 1977).

Flory (1941) and Stockmayer (1943, 1944) suggested that gel formation occurs only when the degree of crosslinking reaches a critical value. Prior to this, there is a lag period during which the system becomes increasingly viscous as simple molecular condensation takes place. At the gel point, where the critical cross-linking threshold is reached, viscosity diverges to infinity and a gel is formed. It has been argued that this theory deals with the idea of connectivity only and does not give aggregates a space-filling geometrical significance which

allow them to be embedded in real space (Clark, 1992). An alternative theoretical approach to gelation is the percolation theory. The essence of this approach is to place monomers on a lattice and then randomly introduce a certain proportion of bonds connecting these; as this process proceeds, clusters of monomers (aggregates) develop naturally, and at a critical threshold of bonding, the gel point divergence property is observed in the form of a "molecule" spanning the entire percolation lattice (Clark, 1992).

## 2.3 Characteristics of Gel Structure

A diagrammatic representation of the heat induced changes which occur during thermal gelation of globular proteins is shown in Fig 2.3. There are two basic types of heat-induced gel structures depending on the conditions involved, as follows: 1) thermo-set (or "set") or irreversible and 2) thermoplastic or reversible gels. In reversible gelation, a sol or progel condition (Fig 2.4), is obtained upon heating and is usually accompanied by increased viscosity. This progel "sets" to form a gel upon cooling. The aggregation step in this type gel is usually reversible and thermoplastic gels can usually be remelted to form the progel upon subsequent heating. Excessive heating of the protein sol at temperatures much higher than the denaturation temperature e.g., 125$^{o}$C, however, leads to the formation of a metasol (Fig 2.4), which does not set into a gel when cooled (Catsimpoolas & Meyer, 1970). Examples of reversible gels include soya, conalbumin and gelatin. Irreversible gels on the contrary, do not revert to the progel state on reheating; they may, however, soften or shrink (Schmidt, 1981; Morrissey *et al.*, 1987; Damodaran, 1989). Examples of thermo-set gels are whey protein and hemoglobin gels.

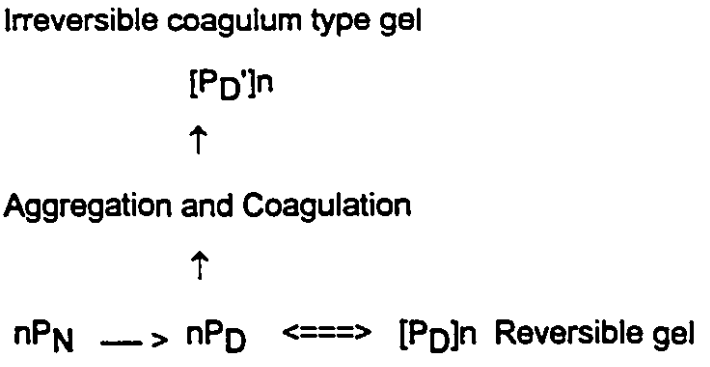


**Fig 2.3:** Heat induced changes during thermal gelation of globular proteins ($P_N$) - native protein; $P_D$ - denatured protein; $(P_D)n$ - translucent type gel; $(P_D')n$ - coagulum type gel; (Damodaran, 1989).

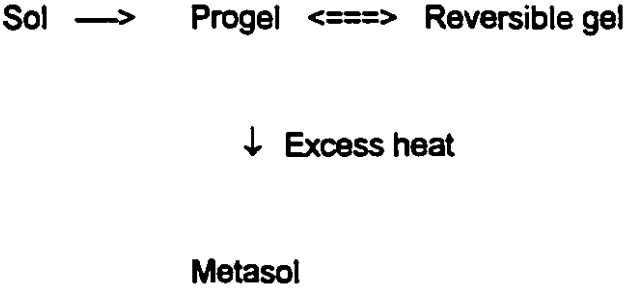


**Fig 2.4:** Reversible gel formation.

Gels can also be classified as translucent (fine-stranded) or opaque (coarse particulate aggregates) (Ziegler & Foegeding, 1990;Hermansson, 1994). Fine-stranded gels (Fig 2.5) are formed by an ordered association of molecules, and small dimensions make these gels transparent (Hermansson, 1994). In conditions favoring denaturation over aggregation (e.g., high net protein charge at low or high pH, very low ionic strength, presence of certain salts, presence of bond-breaking agents, such as urea, guanidine and detergents) heating results in the formation of a translucent gel that is ordered, homogenous, of smooth consistency, highly expanded, highly elastic, transparent and stable toward syneresis and exudation. The slow aggregation step, enables the partially unfolded polypeptides to orient themselves in an ordered manner before aggregation.

When aggregation is favored over denaturation, gels consisting of coarsely aggregated protein particles are formed. The gels are opaque, lacking in elasticity and susceptible to destabilization (syneresis and weeping) (Cheftel *et al.*, 1985). This often occurs near the isoelectric point (Poole & Fry, 1987).

Whether or not a protein forms a coagulum type gel or a translucent type gel is related to the percentage of hydrophobic residues, the net charge of the protein and the ratio of net charge to hydrophobicity (Shimada & Matsushita, 1980; Bigelow, 1967; Damodaran, 1989). According to Shimada & Matsushita (1980), proteins which contain above 31.5 mole percent of hydrophobic residues (Val, Pro, Leu, Ile, Phe, and Trp) form coagulum type gels, whereas those that contain less than 31.5 mole percent of the above apolar residues form translucent type gels. The problem with this generalization however, is that thermal denaturation does not necessarily expose all hydrophobic residues; furthermore, denatured proteins can refold during heat treatment and shield hydrophobic groups.

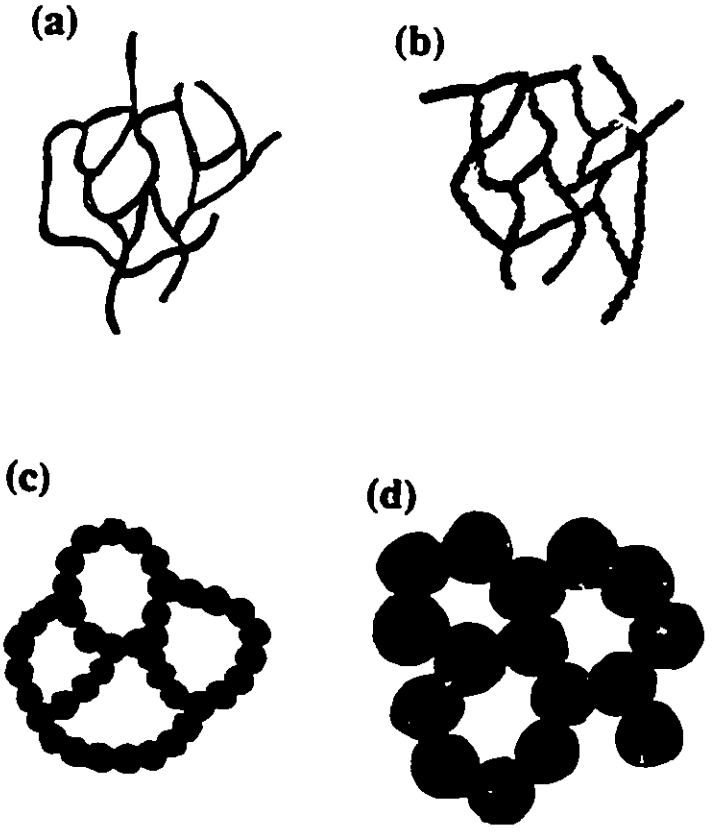


Fig 2.5: Schematic representation of gel networks: a) a fine-stranded gel where the strands are made up of macromolecules aligned into supramolecular assemblies; b) a fine-stranded network of globular proteins; c) and d) particulate gels. (Hermansson, 1994).

In addition, certain proteins such as β-lactoglobulin which have about 32% (42% including Ala, Met, and Tyr) of the apolar amino acid residues form a translucent gel in the presence of NaCl, at as low as 0.05M. Damodaran (1989), therefore, proposed that a better indicator in predicting the texture of a gel is the ratio of net charge to hydrophobicity.

## 2.4 Gelation Models

A gel matrix can be represented as consisting of interconnected cage-like unit structures, with the solvent continuous throughout the matrix. The matrix geometry, flexibility of the polymer and strength of the junctions (chemical nature and extent of protein-protein interactions) determine its rheological characteristics (Foegeding, 1989). A hierarchy of gel structures has been proposed to model this complex molecular network formed by the protein components within the food system. These structures range from simple single component networks used to describe the behavior of individual biopolymers, through binary networks or mixed gels used as the simplest models for multicomponent structures present in actual foods and composite or filled gels used to assess the role of particulates such as fat globules, liquid droplets, fibers, gas bubbles, crystallites or cellular components present in food systems (Brownsey & Morris, 1988).

### 2.4.1 Single Component Gels

Single component polymeric networks are the simplest molecular models for biopolymer gels. This represents a simple end to end polymerization and can only be considered as realistic models for very simple food systems such as flans or table jellies (Brownsey & Morris, 1988). Flory (1974), described gelation as the formation of an infinite network by aggregation of trifunctional and

bifunctional units. This definition adequately describes the single component gel model shown in Fig 2.6. The simple model of a protein polymerizing to form a homogenous network is not valid when there is more than one protein present. Such gels represent the first step towards a description of more complex multicomponent gels.

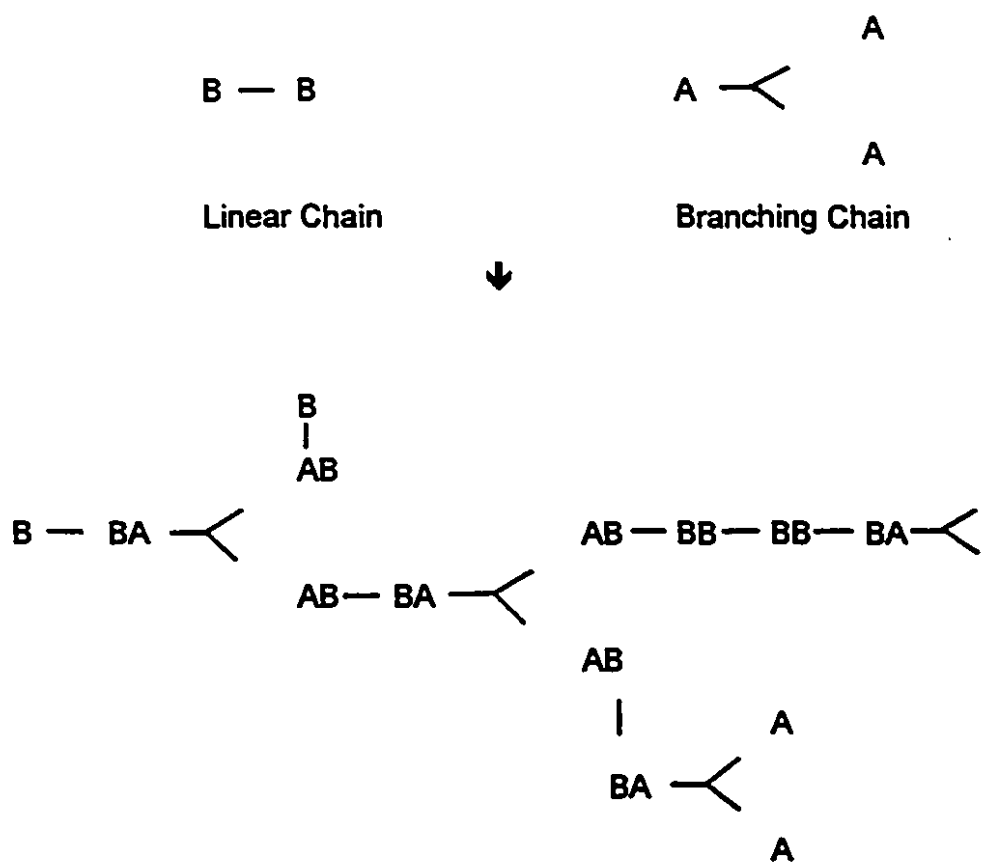


**Fig 2.6: Formation of an infinite gel matrix (Flory, 1974; Foegeding, 1989).**

### 2.4.2 Multicomponent Gels

Multi component gels can be categorized into three separate gels namely filled gels, complex gels and mixed gels (Tolstoguzov & Bravdo, 1983).

**A. Filled Gels**

In filled gels one macromolecule forms the gel matrix while the other molecules act as fillers within the interstitial spaces (Fig 2.7a). The matrix gel may be a single component or multicomponent gel. In this kind of gel the filler molecules (for example non-gelling proteins) can affect certain textural properties and/or water binding.

(a) (b)

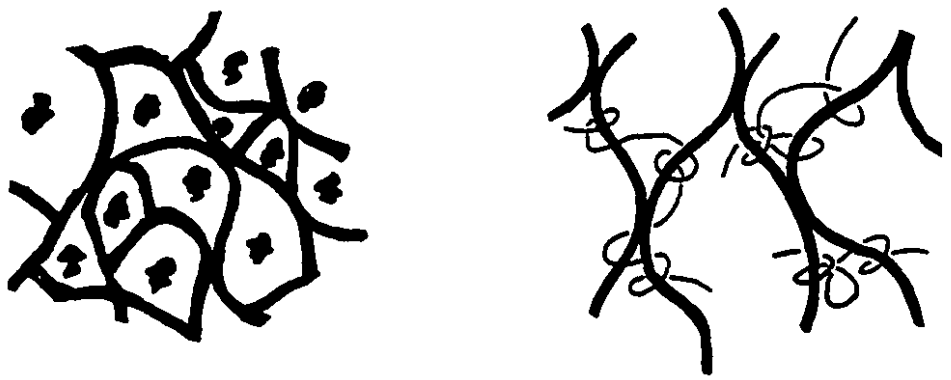

**Fig 2.7:** (a) Schematic representation of a filled gel. (b) Gel network showing a soluble polymer entrapped inside a polymeric network. (Brownsey & Morris, 1988; Foegeding, 1989).

Another kind of gel similar to a filled gel consists of a polymer network entrapping a second soluble polymer (Fig 2.7b). In this type gel only one of the two polymers actively forms part of the polymer network. The presence of the

entrapped soluble polymer may influence gelation of the network polymer, affect its conformational transitions and/or swell the polymeric network.

**B. Two Component Mixed Gels**

Mixed protein gels are those in which the macromolecules form two or more three-dimensional networks with or without interactions amongst the polymers (Brownsey & Morris, 1988; Foegeding, 1989). Three kinds of networks can be categorized under this model: a) coupled networks; b) phase-separated networks; and c) interpenetrating networks.

**I. Coupled Networks**

Coupled networks are formed from two polymers under conditions which favor gelation of each component. If the two polymers are designated A and B, a coupled network is formed by interactions between A and B (Fig 2.8). For example, specific ion interactions occur between kappa-casein and kappa-carrageenan; such interactions result in mixed coupled gel networks responsible for stabilizing milk products (Snoeren, 1976). Coupled networks are attractive commercially because they offer the prospect of developing

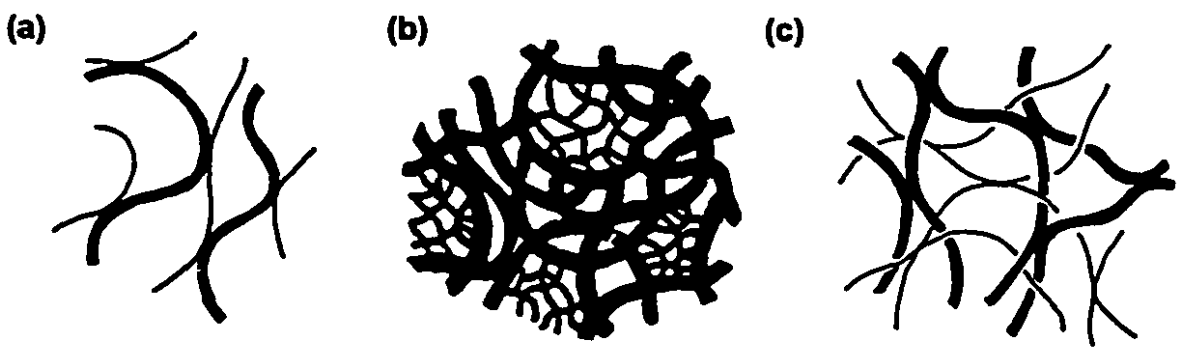


**Fig 2.8:** a) Coupled b) Phase-separated c) Interpenetrating Networks (Brownsey & Morris, 1988).

new mechanical or textural properties to foods. It is sometimes possible to mix two polymers, (which will not gel by themselves alone) under conditions which promote intermolecular (A-B) binding and obtain subsequent gelation of the mixture. This is a useful tool in the fabrication of food products.

**II. Phase-Separated Networks**

Phase-separated networks are models suggested for the gelation of binary polymer mixtures in which A-B interactions are not favored. For a mixed gel formed from two polymers A and B there is a particular ratio (A/B) called the phase inversion point where the system changes from a matrix of Gel A containing inclusions of Gel B to a matrix of Gel B containing inclusions of Gel A. At the phase inversion point the system consists of two continuous interpenetrating networks. Fig 2.8b shows a phase separated network model. This type of network is very useful when dealing with immiscible components (Brownsey & Morris, 1988).

**III. Interpenetrating Networks**

The interpenetrating network (IPN) (Fig 2.8c), is formed when two gelling polymeric species form independent networks with at least one being prepared in the presence of the other. A common type of IPN has been reported for systems in which a preformed network is swollen and a second system is crosslinked in situ; a high degree of miscibility results in an IPN. When the components are less miscible, phase separation occurs and an IPN structure is observed only at the boundaries.

The models described above show that food proteins can form a wide variety of gel structures which exhibit diverse mechanical and microstructural properties. These properties are influenced by the characteristics of the

individual proteins, inter- and intra- crosslinking mechanisms as well as the nature and flexibility of the protein chains formed. However, processing factors (e.g., heating temperature, heating time) and environmental factors (e.g., pH, ionic strength) also affect denaturation and crosslinking during gel formation.

## 2.5 Factors that Affect Protein Gelation

The formation of a self-supporting gel network that is stable against thermal and mechanical motions is dependent on the number of crosslinks per monomer or unit cell of the gel which in turn is dependent on both protein concentration and the number of loci available per molecule (Damodaran, 1989). The hydrodynamic (protein shape and size) and thermodynamic (energy required to expose reactive sites during unfolding) properties of food proteins are two of the most important properties that affect gel formation (Table 2.1). Protein hydrophobicity, net charge and content of sulfhydryl groups also affect gelation (Fligner & Mangino, 1991) and have already been discussed. In addition, factors that influence the conformation of the protein molecule, such as ionic strength and reducing agents, pH, the presence of certain salts as well as other food components in multicomponent food systems, for example lipids, sugars, starches and other non-gelling proteins, interfere with or modify gelation reactions (Kinsella, 1984; Mangino, 1984; Foegeding, 1989; Stanley & Yada, 1992). Changes in heat treatment conditions (heating rate, time and temperature) have also been shown to affect both the macroscopic and microscopic interactions of protein gels (Schmidt, 1981). These interactions often result in new structural matrices of significance in food formulation.

### 2.5.1 Hydrodynamic Properties of Proteins

The hydrodynamic properties of a food ingredient includes its shape, size

and molecular weight (Regenstein & Regenstein, 1984) and are independent of the details of the amino acid composition and distribution. Wang & Damodaran (1990) showed that the hardness or strength of globular protein gels is largely dependent on the weight-average molecular weight and the shape of the protein rather than their amino acid composition and distribution.

**Table 2.1: Properties of Proteins that Affect Gelation.**

| | |
|---|---|
| Hydrodynamic Properties | |
| | - shape of protein |
| | - three-dimensional size of protein |
| | - molecular weight |
| | - chain length |
| Thermodynamic Properties | |
| | - energy state of "native" protein |
| | - temperature of denaturation |
| | - heat capacity |
| Net Protein Charge | |
| Protein Hydrophobicity | |
| Sulfhydryl Groups | |

Studies on the gelation of soy proteins and BSA in the presence of excess cysteine - which prevents formation of inter and intra molecular disulfide bonds during thermal gelation - showed that the square root of gel strength is proportional to the weight-average molecular weight of the polypeptide in the

sample. Further studies also showed that for a globular protein to form a stable gel network the molecular weight of the protein should be greater than 23000 Da; below this critical molecular weight no globular protein would form a gel at any reasonable concentration (Wang & Damodaran, 1990). The gelling properties of a protein can, therefore, be improved by modifying their effective chain length and shape via chemical cross-linking methods.

Kratochvil *et al.* (1961) developed an equation (Eqn 2) to describe the heat induced aggregation step of the globular protein human serum albumin.

$$Mw = \frac{M(1 + 2fVokt)}{(1-f(f-2)Vokt)} \qquad \text{(Eqn 2)}$$

where M is the monomer molecular weight, f(>2) is the number of equivalent binding sites per monomer, Vo is the concentration of monomer initially present, k is the rate constant and t is the time. The weight average molecular weight Mw, was followed as a function of time and the data analyzed using a kinetic model based on the statistical theory of gelation and a second order rate process, describing coupling of crosslinking sites. Equation 2 shows that the rate of aggregation/gelation is proportional to the size of the protein monomers, the concentration, heating time and number of equivalent binding sites per monomer suggesting that any reagent that alters the number of binding sites on the monomer will affect gelation of the protein.

### 2.5.2 Thermodynamic Properties

Proteins assume a configuration that expends the least amount of free energy. This causes the peptide chain to orient its hydrophilic amino acid towards the outside of the molecule and to bury the hydrophobic amino acids in the interior of the molecule with the exclusion of water from this core (Fig 2.9).

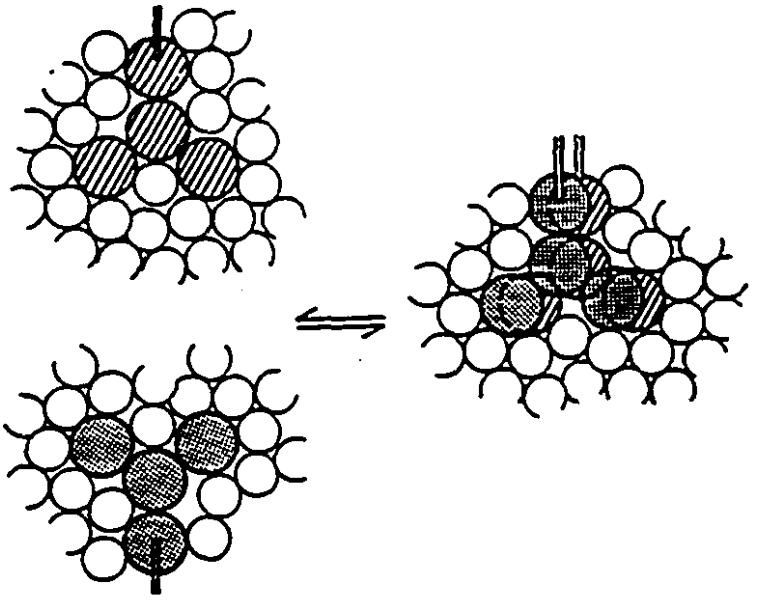

**Fig 2.9:** Schematic representation of interaction of hydrophobic groups in proteins with water. Shaded circles represent hydrophobic side chains of the protein; white circles represent water molecules (Whitaker, 1977).

The conformation adopted by a given polypeptide sequence is that of minimum free energy for the system; that is, of all the minimal energy states, the native protein inhabits the lowest one or "global minimum" (Fig 2.10).

A common observation is that denatured proteins, upon restoration of the "native" environment, can regain precisely and spontaneously the native conformation as depicted in Fig 2.10.C (van Holde, 1977). However, if the random coiled peptide chain undergoes a random search for the global minimum the number of conformations to be explored would require times of the order of $10^{26}$ years (Anfinsen & Scherga, 1975); an alternative suggestion is that the folding and refolding of a protein chain occurs along certain kinetically

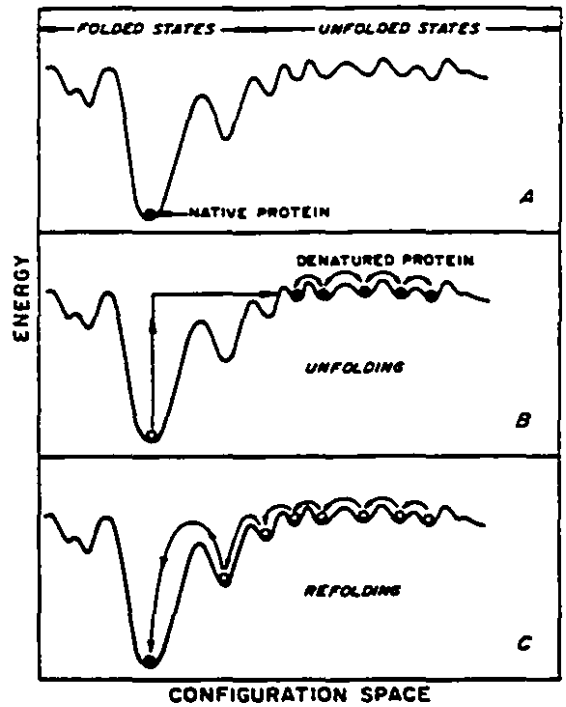


Fig 2.10: Diagrams of the energy of a protein molecule as a function of chain conformation (van Holde, 1977).

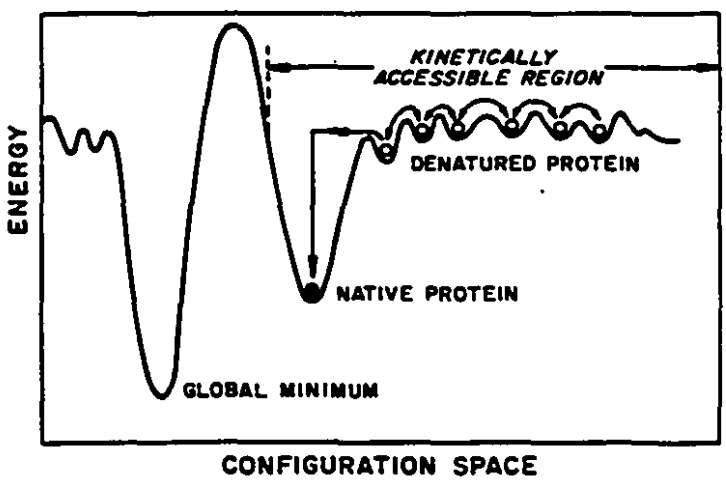


**Figure 2.11**: Kinetic view of "Renaturation" (van Holde, 1977).

accessible pathways that cover only a very small fraction of the potentially possible conformation as shown in Fig 2.11.

The change of state of a substance is generally accompanied by a change in energy level and is manifested by the absorption or liberation of heat. The exclusion of water from contact with hydrophobic residues in the interior of the protein structure causes a large increase in entropy while the close packing of protein interiors leads to low enthalpy. The net stability of the native protein is a result of the lowered enthalpy and the large increase in entropy.

The thermodynamic parameters for changing a protein from a native state (N) to a denatured state (D) can be developed as:

$$N \xrightarrow[k]{\text{heat}} D$$

The equilibrium constant can be written:

$$K = (D)/(N) \qquad \text{(Eqn 4)}$$

The thermodynamic parameters $\delta G^o$, $\delta H^o$, $\delta S^o$ and $\delta Cp^o$ can be obtained from the usual equations:

$$\delta G^o = -RT\ln K \qquad \text{(Eqn 5)}$$

$$dH^o = R[(\ln K)/(1/T)] \qquad \text{(Eqn 6)}$$

$$\delta S^o = (\delta H^o - \delta G^o)/T \qquad \text{(Eqn 7)}$$

$$\delta Cp^o = T(\delta S^o/\delta T)_p \qquad \text{(Eqn 8)}$$

where $\delta G^o$ is the change in standard free energy (difference between the free energy of the native system containing all reactants at equimolar concentration and the free energy of the same system at equilibrium after denaturation), R is the gas constant, T is absolute temperature, $\delta H^o$ is the change in enthalpy at constant pressure, $\delta S^o$ is the change in entropy and $\delta Cp^o$ is the change in heat capacity at constant pressure.

The activation energy (energy required to denature the protein) may be calculated from the Arrhenius law

$$\delta(\ln K)/\delta T = Ea/RT^2 \qquad \text{(Eqn 9)}$$

where K is the rate constant, T is absolute temperature, R is the gas constant and Ea is the activation energy.

From the equations above, it can be observed that $\delta H$ (enthalpy) and $\delta S$ (entropy) are dependent on temperature. Increase in entropy corresponds to the disorder accompanying unfolding and the increase of $\delta Cp^o$ is related to the transfer, during denaturation of aliphatic or aromatic apolar groups to an aqueous environment, and the energy associated with the disruption of water structure (Cheftel *et al.*, 1985). Increase in enthalpy upon denaturation is indicative of a much lower energy level of the native conformation of the protein (Kilara & Sharkasi, 1986).

In the denatured state and at low temperatures, water is oriented into structures of solvation around non-polar groups, whereas at high temperatures a more random and disorganized solvation interaction occurs. The net result of increasing the temperature, therefore, leads to a more positive contribution to the entropy of unfolding caused by solvation effects (Kilara & Sharkasi, 1986).

The susceptibility of proteins to denaturation by heat depends on other factors such as the nature of the protein, protein concentration, water activity, pH, ionic and or reducing agents and heat treatment conditions (Table 2.2). Some of these factors are briefly discussed.

**Table 2.2: External Factors that Affect Protein Gelation**

| |
|---|
| Protein Concentration |
| Heating Temperature |
| Heating Time |
| Heating Rate |
| pH |
| Ionic, Oxidizing and Reducing Agents |
| Other Protein Components |
| Non Protein Components |

### 2.5.3 Protein Concentration

For a given type of protein, a critical concentration (least concentration endpoint LCE) is required for the formation of a gel and the type of gel formed also varies with the protein concentration. Below the critical concentration, a precipitate or soluble aggregate is formed (Ziegler & Foegeding, 1990). Gelatin and polysaccharide solutions, form gels at relatively low concentrations of the gelling material. On the contrary, considerably higher protein concentrations are usually required for the gelation of globular proteins (Schmidt, 1981).

Ferry (1948) showed that the rigidity of gelatin gels is proportional to the square of the concentration; this relationships is true for globular proteins such as serum albumin and soy proteins (Damodaran, 1989). Mori *et al.* (1981) reported that low levels of 11S soy protein favored disaggregation while high levels led to gel formation. Iwabuchi & Yamauchi (1984) and Nakamura *et al.*, (1986) also reported that heated 7S soy proteins dissociate and then recombine into soluble aggregates at high concentrations and ionic strength; reducing these conditions led to the proteins remaining dissociated.

The least concentration end point, however, does not guarantee gel formation. Other factors such as heating conditions, ionic and pH environment must be optimized for gel formation to occur.

### 2.5.4 Heat Treatment Conditions

The final texture of a gel depends not only on its gelation temperature, but also on the heating time and the rate of heating (Table 2.2). Alteration of these conditions affects both the gels macroscopic and microscopic structural attributes (Schmidt, 1981). In general, the heating time required to form a gel, at a given temperature, decreases with increasing protein concentration.

Heating rate affects both the type and texture of the gel formed. Gelation, as explained earlier, is a two stage process involving the initial denaturation of native protein into uncoiled polypeptides, which then associate gradually to form the gel matrix, if attractive forces and thermodynamic conditions are suitable. When the heating conditions are extreme, protein molecules may not have time to align themselves in an ordered fashion. In these circumstances, poorly hydrated aggregates or precipitates which lack the continuous matrix gels are formed (Morrissey *et al.*, 1987).

### 2.5.5 pH

Another important physico-chemical parameter that controls the functional properties of whey proteins is pH (De Wit, 1989). Both temperature of denaturation and gel strength are affected by pH (Kilara & Sharkasi, 1986). Exposure of moderately high pH followed by readjustment to neutral pH has been shown to "activate" the protein molecules thereby improving their gelling ability (Pour-El & Swenson, 1976; Ishino & Okamoto, 1975). This could be related to unfolding of the protein and/or activation of buried sulfhydryl groups.

At the isoelectric pH, the lack of repulsive forces leads to a less expanded, less hydrated and less firm gel (Cheftel *et al.*, 1985). At pH values well removed from the isoelectric points repulsive electrostatic forces are induced by the large net charge of the proteins resulting in the formation of an expanded hydrated firm gel. Prolonged exposure to extremely high pH suppresses aggregation (Schmidt & Illingworth,1978) . At strongly basic pH values, carboxylate-phenolic group and carboxylate-protonated amino ($NH_3^+$) group interactions are inhibited (Catsimpoolas & Meyer, 1970); this interferes with the crosslinking stage of gel formation and affects gel texture.

### 2.5.6 Ionic , Oxidizing and Reducing Agents

Gel formation of globular proteins is affected by ionic strength and the presence of certain salts. Hermansson (1979) showed that the pH dependent aggregation of 0.9% WPC was dependent on the ionic strength in the range 0.05-0.2. Addition of 0.2M NaCl to soy protein gels at pH 7.0, was also shown to enhance protein-protein interactions (Hermansson, 1979; Hermansson & Bucheim, 1981). Hickson *et al.*, (1980) reported that the viscosity index of 8.0% bovine blood plasma protein suspensions increased in the presence of 0.2M $Na^+$ and 0.2M $Ca^{++}$; the increase was significantly higher (1.5-2 times) for $Ca^{++}$

than for $Na^+$. This effect of $Ca^{++}$ was attributed to the formation of bridges between molecules, generating a stronger gel. The rigidity of low density lipoproteins gels has also been shown to increase with the addition of small amounts of salt; maximum gel rigidity was observed at ionic strength of about 0.06 and subsequently decreased with further increase of ionic strength.

The effect of various reagents on the rheological properties of heat induced gels formed from plasma proteins solutions (pH 9.0) were studied by O'riordan *et al.*, (1989). Both propylene glycol (5-20% w/v) and ethanol (5-20% w/v) which enhance hydrogen and electrostatic interactions increased gel compressive strength, whereas mercaptoethanol (25-100 mM) which reduces sulfides and the sulfhydryl-blocking agent, *p*-hydroxymercuribenzoate (25-100 mM) reduced gel strength. High levels of guanidine hydrochloride (>1M) and urea (>2M), which weaken both hydrogen and hydrophobic interactions, decreased gel strength. Itoh *et al.*, (1976), observed that addition of reducing agents (sodium sulfite, thiosulfite or nitrite, 0-20 mM/kg) to Alaska pollack surimi prior to cooking increased gel strength.

### 2.5.7 Other Protein Components

Other proteins in complex food systems (gelling or non-gelling) may, through direct or indirect interactions interfere with or modify gelation reactions. This interference has been observed with whey, casein, soy, meat and other proteins upon heating. Kojima & Nakamura (1985) investigated the influence of BSA, ovalbumin and lysozyme on the heat gelling properties of hen's egg yolk low density lipoprotein (LDL) and observed that ovalbumin or BSA greatly increased the rigidity of LDL gels at pH 6.2 - 7, while lysozyme increased it at alkaline pH 9. In the acidic region, lysozyme suppressed the gel formation of

LDL . Addition of ovalbumin or BSA gave weak gels with a paste-like appearance quite different from that obtained using only LDL.

### 2.5.8 Non Protein Components

The gelation of food protein is affected by the presence of non-protein components such as lipids and sugars (Catsimpoolas & Meyer,1971). Wheatmeal, for example, contains a high percentage of both starch (about 80%) and the protein gluten. In traditional products of wheatmeal (e.g., bread, pasta etc.) the gluten protein makes up the continuous and the starch the discontinuous phase both before and after cooking (Hermansson, 1988) to form the structural matrix of these foods. In some red meat and poultry products, fats are a major constituent. Protein coating of divided fat particles, often referred to as "emulsification", is initially important in attaining an even distribution of fat particles throughout the product batter; when heated, fat is encased in the gel matrix formed by the myofibrillar proteins and thus stabilized against migration within and from the product (Lanier, 1991) and affects gel texture.

## 2.6 Characterization of Protein Gels

### 2.6.1 Direct Structural Measurements

Infrared absorption spectroscopy in the 1400 to 1700 cm-1 spectral region has been used to study the secondary structure of polypeptides in the solid state (Krimm, 1962) and in deuterium oxide solutions (Susi, 1972; Ruegg *et al.*, 1975). Deuterium oxide is usually employed instead of water because of its greater transparency in the region of interest (Susi & Byler, 1983).

The Amide I' band in the region of 1600-1700 cm-1 (Susi & Byler, 1986, 1988), has been found to be the most useful for protein structure studies by infrared spectroscopy. This region involves C=O stretching vibrations of peptide

groups (Krimm, 1962; Susi, 1972). The broad infrared bands of proteins, which result from the vibrations of the peptide groups in this region, are composed of several overlapping bands due to the various protein segments with different secondary structures (Surewicz & Mantsch, 1988). Each type of substructure, such as the α-helix, β-strands and the various kinds of turns (Richardson, 1981), gives rise to different C=O stretching bands, as a result of differences in the orientation of molecular subgroups or changes in interchain and intrachain hydrogen-bonding interactions of the peptide groups. These subbands usually cannot be resolved by conventional spectroscopic techniques because their inherent widths are greater than the instrumental resolution. Resolution enhancement of the band, also known as Fourier self-deconvolution, results in a narrowing of the Amide I' band to reveal the hidden peaks which can then be qualitatively and quantitatively characterized. This technique not only enriches the qualitative interpretation of infrared spectra, but also provides a basis for the quantitative estimation of protein secondary structure. The detailed principles

**Table 2.3: Properties and techniques for characterizing protein gels**

| | |
|---|---|
| Thermal properties | Differential scanning calorimetry |
| Structural Properties | Fourier Transform infrared spectroscopy |
| Rheological properties | Dynamic viscoelastic measurements |
| Gel texture | Gel strength measurements |
| Water holding capacity | Centrifugation |
| Turbidity | Spectrophotometry |

and instrumentation of FTIR have been extensively reviewed (Krimm & Bandekar, 1986; Susi & Byler, 1986;Surewicz & Mantsch, 1988).

Infrared studies of protein to date, have addressed the determination of secondary structure, using the structure sensitive Amide I' band in the infrared spectrum (Byler & Farell, 1989; Casal *et al.*, 1988; Prestrelski *et al.*, 1991a, b). Some workers have examined the side chain vibrations of proteins for additional information on interactions within the protein (Holloway & Mantsch, 1988). In addition, events leading to protein denaturation and reorganization (Ismail *et al.*, 1992) as well as intermolecular reactions not involving conformational changes have also been studied using the amide I' region of the infrared spectrum (Casal *et al.*, 1988).

Infrared spectroscopy can be used to monitor aggregation during protein gelation. The particular changes that occur in the protein secondary structure and the manner in which the networks build from individual molecules during heat treatment affect gel texture (Kinsella & Whitehead, 1989). The formation of non-native ordered structure of hydrogen-bonded β-sheets has been observed when gelling proteins were heated above their transition temperatures (Smith, 1994). Clark *et al.*, (1981) suggested that the formation of a band at 1620 $cm^{-1}$ correlated with protein aggregation and the formation of hydrogen-bonded β-sheet. These authors observed that formation of a second, less intense band at 1680 $cm^{-1}$ together with the band at 1620 $cm^{-1}$ indicated formation of anti-parallel β-sheet.

### 2.6.2 Indirect Structural Measurements

Differential scanning calorimetry (DSC) (Table 2.3) has emerged as the technique of choice for the study of thermal transitions of food proteins (Arntfield

& Murray, 1981; De Wit & Klarenbeek, 1981; Chan *et al.*, 1992). DSC can detect the heat denaturation of a protein in complex protein systems as an endothermic peak in its thermogram (Katsuji *et al.*, 1988). In this technique, the substance of interest and an inert reference are maintained at the same temperature while the temperature of both is gradually raised, usually at a programmed linear rate. Any thermally induced changes occurring in the sample are then recorded as a differential heat flow displayed normally as a peak on a thermogram (Wright, 1982). Integration of the differential heat flow curve, yields a value for the change of enthalpy with respect to time or temperature, and is a direct sequence of the change of enthalpy associated with the heat-induced transitions of state of the sample (Fig 2.12).

The thermally induced process exhibited by most proteins and detectable by DSC is the conformational change (denaturation) resulting from the disruption of the native protein structure. When proteins denature, both inter- and intramolecular bonds are disrupted, often in a cooperative manner; the conformation of the protein changes from a highly ordered to a less ordered state. The DSC enthalpy value obtained is a composite value of both endothermic and exothermic contributions arising from this unfolding. For the transition

Native (N) ←→ Denatured (D) → Aggregate (A)

the N-to-D transition will entail disruption of both hydrophobic interactions (exothermic) and polar interactions (endothermic); overall, a net endotherm is usually observed. For the D-to-A transition, the formation of hydrophobic interactions will be mainly endothermic and the formation of polar interactions, exothermic; overall, a net exotherm normally results. For the complete N-to-A

transition, one may expect a net exo- or endothermic transition; usually, net endotherms are observed (Myers, 1988).

Parameters obtained from the DSC thermogram include the overall enthalpy change (ΔH) (Fig 2.12), which is an estimate of the thermal energy required to denature the protein and is calorimetrically measured from the peak area of the thermogram ; the temperature of denaturation ($T_d$) which is the peak maximum or minimum temperature (at this temperature the change in Gibbs free energy (dG), between the folded and unfolded protein structure, is zero - dG is

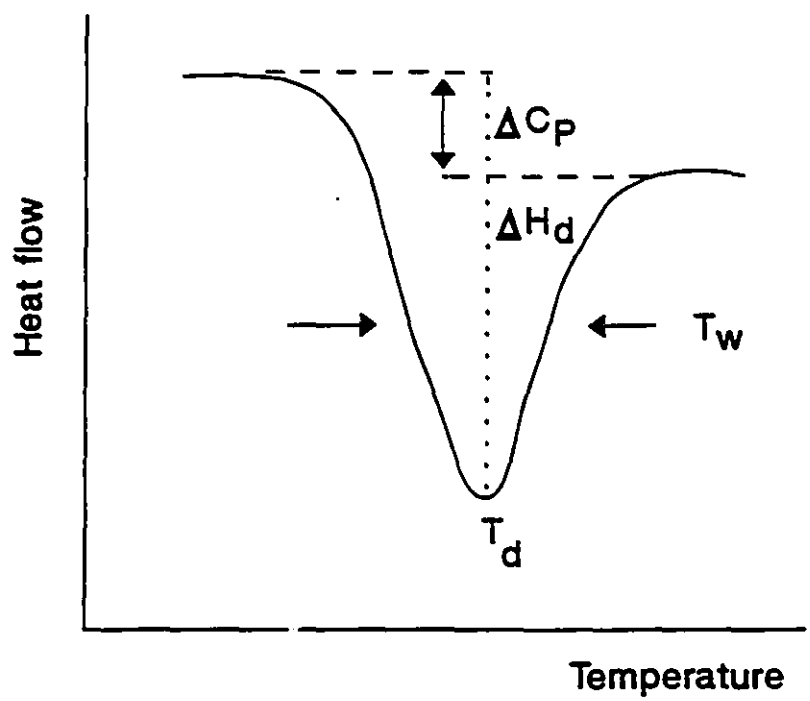


**Fig 2.12:** DSC thermogram showing change in heat capacity (ΔCp), change in enthalpy, ($\Delta H_d$), peak temperature of denaturation ($T_d$) and width of the peak at half-height ($T_w$) (Kato *et al.*, 1990).

the net result of two large counteracting free energy contributions, enthalpy and entropy); width of the peak at half-height ($T_w$), which is essentially an estimate of the cooperativity of the process - a broad peak (high $T_w$ value), is indicative of an increased degree of difficulty for protein unfolding/denaturation. If denaturation occurs within a narrow range of temperature (low $T_w$ value), the transition is considered highly cooperative (Wright, 1982; Harwalkar & Ma, 1989; Myers, 1990; Arntfield *et al.*, 1990).

In addition, DSC can give information on the reversibility of the protein denaturation. The extent of reversibility is easily estimated by rescanning samples after heat denaturation and comparing relative areas of the transitions produced after the first and second heating (Relkin *et al.*, 1993). Kinetic parameters of protein denaturation can be determined by DSC using the same data as in the thermodynamic studies (Myers, 1990). In this case, emphasis is placed on the dynamics of the reaction as opposed to its energetics (Stanley & Yada, 1992).

The basic equation for rate of change of a species (A) with time (t) can be written as:

$$\pm dA/dt = kA^n \qquad \text{(Eqn 10)}$$

$$k = Ze^{-Ea/RT} \qquad \text{(Eqn 11)}$$

where k is the rate constant (time -1), dependent upon temperature, n is the apparent reaction order, and Ea is the activation energy. The temperature dependence of the rate constant can be determined from the Arrhenius equation (Eqn 11).

### 2.6.3 Rheological Properties

Heat treatment of gelling proteins often results in increased water uptake and swelling as proteins unfold. This results in an increase in hydrodynamic volume and increased resistance to flow (Kinsella, 1984). The increase in viscosity also reflects intermolecular interactions resulting from attractions between adjacent molecules with the formation of weak transient networks. Where there is no interaction between particles, flow properties depend only on the volume fraction or concentration of the suspended material and show Newtonian behavior. In concentrated solutions the hydrodynamic domains of the protein molecules come into contact, resulting in interactions between suspended particles. Concentrated protein solutions, therefore, exhibit non-Newtonian behavior and show viscoelastic properties (Rha & Pradipasena, 1986). Several reviews on the rheology of foods have been published (Charm, 1962; Holdsworth, 1971; Rao, 1977; Rha, 1975; Rha, 1978).

Protein gels are generally difficult to evaluate because of their viscoelastic nature i.e., their textural attributes vary with time under deformational forces (Stanley & Yada, 1992). A creep compliance curve which relates changes in the ratio of strain to shear stress of protein gels as a function of time (Aguilera & Stanley, 1990) is shown in Fig 2.13. In general, rheological measurements are made by the application of stress to induce small or large deformations to the test sample. Small deformation tests probe viscoelastic parameters and are commonly derived by dynamic oscillatory testing in the linear viscoelastic region of the test material (dynamic viscoelastic measurements); large deformation tests measure stress, strain and failure properties of a given material (gel strength measurements) (Hermansson, 1994).

### 2.6.4 Dynamic Viscoelastic Measurements

The preferred methods for characterizing viscoelastic foods involve dynamic testing in which sinusoidally oscillating stress or strain is applied to the sample (Stanley & Yada, 1992). Dynamic measurements allow gelation to be monitored since the induced deformations are usually so small that their effect on structure is negligible (Ross-Murphy, 1984). Two independent parameters are obtained from dynamic measurements: the storage modulus (G') describing the amount of energy that is stored elastically in the structure and the loss modulus (G") which is a measure of the energy loss or the viscous response. The phase angle (δ) ,is a measure of how much the stress and strain are out of phase with each other. For a completely elastic material, the phase angle is 0°; for a purely viscous fluid the δ is 90° (Hermansson, 1994).

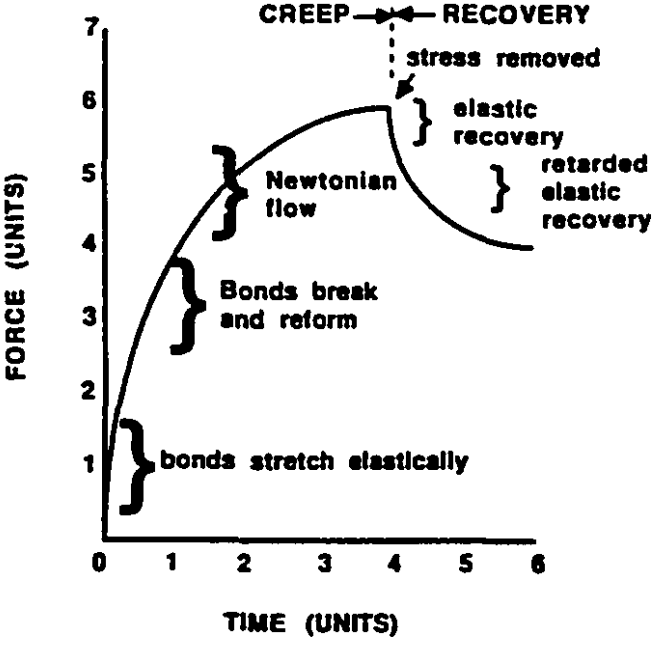


**Figure 2.13:** Typical creep compliance curve (Adapted from Stanley & Yada, 1992).

The ratio G"/G' is called the loss tangent and is equal to the tangent of the phase angle:

$$\tan \delta = G''/G' \qquad \text{Eqn 12}$$

This is proportional to the (energy dissipated)/(energy stored) per cycle (Hamman *et al.*, 1990).

Dynamic viscoelastic measurements have been used to monitor the gel properties of various food proteins including, myosin, β-lactoglobulin, casein and muscle protein (Egelandsdal *et al.*, 1986; van Vliet *et al.*, 1989; Paulsson *et al*, 1990; Stone & Stanley, 1994).

### 2.6.5 Gel Strength Measurements

Gel texture has been described with terms such as soft, elastic, brittle, tough, wobbly and stiff; these perceptions have been found to correlate with different rheological measurements such as shear modulus and break strength (Oakenfull, 1984). Gels have also been characterized by visual observation depending on the ease with which they break when shaken, the absence of flow when tubes containing gels were inverted and immobilization of small air bubbles in the protein solution (Schmidt, 1981; Nonaka *et al.*, 1993).

Large deformation tests are the standard procedure for gel strength measurements. This type of test permits differentiation between regular and irregular particulate networks and between fine-stranded gels below and above the pH range for particulate gels (Stading & Hermansson, 1991). The mechanism of this technique involves the application of a stress to either deform or break a gel (Patel & Fry, 1987) and can be performed both in compression and tension. The total stress during compression is the sum of both tensile and

shear stresses, whereas the shear stresses in tensile testing are negligible (Hermansson, 1994).

Several workers have used the Instron Universal Testing machine in a compressive mode to measure the hardness, cohesiveness and elasticity of protein gels (Matsudomi *et al.*, 1991; Mulvihill *et al.*, 1991; Wang & Damodaran, 1991). With this instrument, the rigidity of gels is measured as the initial slope of a force-deformation curve made by compression of the test sample (Stanley & Yada, 1992). In other studies, gels have been twisted to failure on a torsion apparatus to measure the rheological properties of true shear stress at failure and true shear strain at failure (Diehl *et al.*, 1979; Kuhn & Foegeding, 1991). Characteristics, such as gumminess, chewiness, springiness and adhesiveness have also been measured using the General Foods Texturometer (Bourne, 1978).

### 2.6.6 Water Holding Properties

The ability of a protein to take up water without dissolving is a key functional property in foods such as comminuted meat products, doughs and batters. The term water holding capacity (WHC) has been used to describe the maximum amount of water that a protein gel can hold (Patel & Fry, 1987) and may be theoretically defined as the grams of water held per 100 grams of protein (Kinsella, 1984). The WHC of gelling proteins increases with gel formation. Heating above the gelation temperature, results in increased degree of phase separation and poorer water holding properties for certain proteins (e.g., whey proteins) due to the formation of a coarser structure with larger aggregates and pores (Hermansson, 1988, 1994; Stanley & Yada, 1992). In the case of 11S soy proteins, however, increased heating led to a transition from an

aggregated disordered gel to an ordered structure consisting of strands (Stanley & Yada, 1992).

Common methods for measuring water holding properties of foods are based on the application of an external force such as pressure and centrifugation or on capillary suction of a porous material in contact with the sample (Labuza & Lewicki, 1978). The important factor is to minimize the degree of deformation during the measurement, since large deformations can result in structural changes in the material that alter the water holding properties (Hermansson, 1994). The simplest procedure in WHC measurements is the centrifugation test (Patel & Fry, 1987). In this technique, the protein gel is weighed into a centrifuge tube and centrifuged at low speed. After centrifugation, the supernatant is discarded and the water remaining in the gel is measured by weight (Yasuda *et al.*, 1986; Camou *et al.*, 1989).

### 2.6.7 Turbidity Measurements

Association of denatured molecules during heat treatment produces aggregates that increase the turbidity of protein solutions prior to the formation of the gel matrix (Ziegler & Foegeding, 1990). An increase in the turbidity of protein dispersions during heat treatment has, therefore, been used as an indication of increased aggregate formation (Samejima *et al.*, 1984; Ziegler & Acton, 1984; Sano *et al.*, 1990). If the energy barrier against random aggregation is sufficient, molecules arrange themselves into strands resulting in the formation of a fine stranded network structure which is transparent in nature; an opaque gel is formed otherwise (Kinsella & Whitehead, 1989). The size and density of aggregates as well as the mechanisms by which aggregates align into a three dimensional network structure, is dependent on environmental conditions such as pH, ionic strength and heating conditions (Hermansson,

1988). β-lactoglobulin for example, can form transparent fine stranded gel structures as well as opaque aggregated structures depending on the pH of the solution.

The change in the clarity of protein dispersions during heat treatment can be measured spectrophotometrically (Samejima *et al.*, 1984). Several studies have correlated an increase in absorbance measurements of protein solutions with aggregation (Ziegler & Acton, 1984; Sano *et al.*, 1990). In these studies, the absorbance of protein solutions are measured as the samples are heated either isothermally or as a function of temperature.

## 2.7 Techniques for Analyzing Changes in Molecular Structure of Proteins

In addition to the techniques mentioned above for characterizing gels, electrophoresis and reversed phase high performance liquid chromatography (RP-HPLC) have been used to monitor the changes that occur in the molecular structure of proteins during gel formation. The principles and instrumentation for these techniques have been extensively reviewed (Macrae, 1982; Newton, 1982; Simpson, 1982; Takács & Kerese, 1984).

### 2.7.1 Electrophoresis

In general, electrophoresis involves the separation of charged ions on the basis of their mobility under the influence of an externally applied electric field and can be used to evaluate the changes occurring in food proteins during heat treatment. When proteins aggregate during gelation, they form complex structures with large molecular weights that can not migrate through the separation gel used in electrophoretic analysis. Differences between the electrophoretic patterns of proteins before and after gelation (or aggregation)

have been used to determine changes in the molecular structure of protein during aggregation (Li-Chan, 1983; Kim *et al.*, 1987; Parris *et al.*, 1991).

### 2.7.2 Reversed Phase High Performance Liquid Chromatography (RP-HPLC)

RP-HPLC has been utilized to estimate the changes that occur in food proteins during processing (Pearce, 1983; Morr, 1985). The separation of proteins during RP-HPLC is based on an adsorptive interaction between the hydrophobic side chains of the amino acid residues of proteins and the alkyl function of the stationary phase, as well as ionic and polar interactions between the hydrophilic side chains of the amino acid residues and the free silanols of the stationary phase (Lemieux & Amiot, 1989). The amino acid composition, the polypeptide chain length and the net hydrophobicity of the protein molecules contribute to the retention of the proteins during the separation process (Young & Merion, 1989). Any changes induced in these properties of the protein molecules during gelation can be easily monitored by HPLC. Parris *et al.*, (1991), monitored the extent of protein denaturation by comparing the sum of the normalized absorbance peaks of heated whey proteins eluted from the RP-HPLC column to those of an unheated control sample. In another study (Li-Chan, 1983), integration of peak areas obtained from RP-HPLC and comparison of peak retention times with a calibration plot of log molecular weight versus retention time of known standards allowed for characterization of whey protein concentrate and analysis of heat treatment effects.

CHAPTER 3

# FACTORS AFFECTING MOLECULAR CHARACTERISTICS OF WHEY PROTEIN GELATION

## 3.0 CONNECTING STATEMENT

Processing factors frequently encountered in food formulation which affect gelation include protein concentration, heating temperature and time, pH and NaCl. The effects of these extrinsic factors result from changes in the conformation and physicochemical properties of proteins during processing. The work described in this chapter is a comprehensive evaluation of the effects of these factors on the molecular, structural and gelation properties of whey protein concentrate and addresses the first objective discussed in the "Rationale and Objectives of Study" section of Chapter 1.

**Note:** This chapter is the text of a paper which has been published as follows:

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***Contributions of co-authors***: Alli,I. (thesis supervisor); Ismail,A. (provided expertise in FTIR); Gibbs, B.F. and Konishi, Y. ( provided HPLC instrumentation).

## 3.1 ABSTRACT

The effects of pH, protein concentration, NaCl, heating temperature and time on the gelation of whey protein concentrate (WPC) and the associated changes in the molecular conformation of the individual whey proteins were studied using polyacrylamide gel electrophoresis, high performance liquid chromatography and Fourier transform infrared spectroscopy. Heat denaturation was studied using differential scanning calorimetry. The results obtained showed that varying WPC concentration affected textural properties of gels without any observed differences in the molecular behavior of α-lactalbumin and β-lactoglobulin, while heating temperature, pH and NaCl affected both molecular and textural characteristics. WPC formed firm gels at temperatures above 70°C, at alkaline pH range and in the absence of NaCl. When the whey proteins were heated, α-lactalbumin did not aggregate above pH 7 but denatured readily to form aggregates at acid pH while β-lactoglobulin aggregated at both acid and alkaline pH regions. Denaturation of α-lactalbumin at neutral pH resulted in an increase in viscosity while denaturation of β-lactoglobulin resulted in gel formation. The aggregation of WPC, particularly at alkaline pH, was attributed mainly to β-lactoglobulin and only minimally to α-lactalbumin.

## 3.2 INTRODUCTION

Protein gelation plays a major role in the preparation and acceptability of many foods; these include various dairy, egg, vegetable and other products. Proteins are utilized not only for the formation of solid viscoelastic gels in foods but also for improved water absorption, thickening, particle binding (adhesion), emulsion and foam-stabilizing effects (Cheftel *et al.*, 1985).

In general, heat-induced gelation of proteins is governed by a balance between attractive and repulsive forces (Egelandsdal, 1980). Kojima *et al.*, (1985) attribute the repulsive forces to surface charges and the attractive forces to various functional groups exposed by the thermal unfolding of the protein. The heat-mediated gelation of protein-water systems has been described as a two stage process (Ferry,1948; Shimada & Matushita,1980); the denaturation of the native protein is followed by protein-protein interaction resulting in a three-dimensional protein network which forms the final gel structure.

The texture and strength of protein gels are affected by intrinsic factors such as the composition and concentration of the proteins and by extrinsic factors such as heating temperature, pH, ionic strength and the presence of other food components, for example, lipids, sugars, starches etc., (Schmidt,1981; Damodaran,1989; Foegeding,1989). Heat treatment can result in cleavage of existing disulfide bond structure or "activation" of buried sulfhydryl groups through unfolding of the protein. This results in the formation of new intermolecular disulfide bonds (bridging) which are required for the formation of a highly ordered gel structure in some protein systems (Catsimpoolas & Meyer, 1970). Alteration of heat treatment conditions (temperature and time) affects the gel's macroscopic and microscopic structural attributes (Schmidt, 1981), by changing the rates and mechanisms of denaturation and aggregation. The

macrostructure of protein gels resulting from the crosslinking of several aggregates is concentration dependent, and at any given temperature, a critical concentration exists above which gelation will be observed (Schmidt, 1981; Damodaran, 1989). The pH and salt concentration of protein dispersions have a profound effect on gelation reactions, by influencing the balance of polar and non polar residues. Protein-protein interactions are generally favored under conditions which reduce the net charge on the molecules, i.e., pH values near the isoelectric point. High ionic strength tends to reduce electrostatic repulsion between proteins due to the shielding of ionizable groups by mobile ions (Ziegler & Foegeding, 1990). Any changes in pH and salt concentration will ultimately affect gelation characteristics by interfering with protein solubility, heat stability and protein-protein interactions during gel formation (Bigelow, 1967; Harwalkar & Ma, 1989). When the pH is near the isoelectric point and the ionic strength low, denatured proteins randomly aggregate by hydrophobic interaction. At pH far from the isoelectric point and at low ionic strength, electrostatic-repulsive forces hinder the formation of random aggregates, resulting in the formation of linear polymers (Doi *et al.*, 1989).

In this study the effects of NaCl, pH, protein concentration, heating temperature and time on the gelation characteristics of WPC were investigated. Changes in the thermal stability of WPC as affected by these factors were measured by differential scanning calorimetry. Changes in conformation of the components of WPC were monitored by polyacrylamide gel electrophoresis and high performance liquid chromatography, while changes in the secondary structure of the whey proteins were monitored by Fourier transform infrared spectroscopy. Observed molecular transitions were subsequently related to the gelling ability of the whey proteins.

## 3.3 MATERIALS AND METHODS

### 3.3.1 Materials

A commercial sample of whey protein concentrate (WPC), (75% protein, 11% lactose, 7% fat, 4% moisture, 3% ash) from Calpro Ingredients (Corona, CA) was obtained and stored in airtight containers at room temperature (25°C), until used. Protein standards of α-lactalbumin (bovine, L6010), ß-lactoglobulin (bovine, L2506), bovine serum albumin (BSA)(A7511), immunoglobulins (bovine, 15506) and peptone (bovine, P7750) were purchased from Sigma Chemical Co. (St. Louis, MO). $D_2O$ was from Aldrich (Milwaukee, WI).

### 3.3.2 Preparation of Gels

Aqueous dispersions of WPC of desired concentrations were prepared in test tubes (20mm ID, 150mm length) by agitating until a uniform suspension was obtained. The test tubes were covered with aluminium foil to prevent evaporation during heating and were heated in a water bath; the temperature and times of heating are given below. The effects of the following factors on gelation were investigated:

*Protein Concentration*: WPC dispersions (20-300 mg WPC/g $H_2O$) were made by dispersing the protein in distilled water (pH unadjusted). Samples of each dispersion (20 ml) were heated in test tubes at temperatures ranging from 60 to 90°C for a period of 60 min.

*Temperature*: 20 ml samples of WPC dispersion (150 mg WPC/g $H_2O$), pH unadjusted, were heated at temperatures ranging from 25 to 100°C for 60 min.

*Heating time:* WPC dispersions (150 mg WPC/g $H_2O$) were prepared in distilled water and heated for 15, 30, 45 or 60 min at 75°C.

*pH:* The pH of WPC dispersions (150 mg WPC/g $H_2O$) was adjusted from 2 to

10 with acid (1M HCl) or alkali (1M NaOH). Twenty ml samples of each dispersion were heated at 75°C for 60 min.

*Ionic strength:* WPC dispersions (150 mg WPC/g NaCl solution) were prepared in 0 - 2M NaCl solutions and heated at 75°C for 60 min.

The heated dispersions were cooled to room temperature (25°C) and observed for gelation by inverting the test tubes and shaking the samples as described by Patel and Fry (1987). Firm gels (Table 3.1) were defined as gels that did not flow on vigorous shaking; soft gels flowed on vigorous shaking and viscous dispersions flowed on gentle tilting of the test tubes. Samples of the heated dispersions were lyophilized and stored at 4°C until they were analyzed by electrophoresis (PAGE) and reversed-phase high performance liquid chromatography (RP-HPLC).

### 3.3.3 Differential Scanning Calorimetry

A preliminary experiment was carried out to determine the optimum concentration of WPC required for satisfactory instrument sensitivity. On the basis of the results obtained, all DSC analyses were performed on WPC dispersions containing 300 mg WPC per gram of $H_2O$. The following samples were subjected to DSC analysis: WPC dispersions made in phosphate buffers at pH 3, 5, 7, 9; WPC dispersions made in 0 - 2M NaCl solutions. All phosphate buffers in this study were made using $H_3PO_4$, $KH_2PO_4$ and $Na_2HPO_4$. The DSC (TA3000, METTLER, Mettler Instrument Corporation, Switzerland) was calibrated by use of indium standards. Samples (25ul) to be analyzed were placed in preweighed DSC aluminium pans (Mettler Instrument Corporation, Switzerland), hermetically sealed and weighed. The samples were placed in the DSC cell and scanned from 30°C to 120°C at a heating rate of 5°C/min with an empty pan as

a reference. Each sample was analyzed in duplicate.

### 3.3.4 Fourier Transform Infrared Spectroscopy

Infrared spectra were recorded with a 8210E Nicolet FTIR equipped with a deuterated triglycine sulfate (DTGS) detector. The spectrometer was purged with dry air from a Balston dryer (Balston, Lexington, MA). To study the effect of pH, WPC dispersions (150 mg/g phosphate buffer) were prepared in phosphate buffered $D_2O$ solutions (pH 3 - 9) . For ionic strength studies, the dispersions were prepared in $D_2O$ containing 0 - 2M NaCl. For the effect of concentration, WPC dispersions containing 20 - 300 mg WPC/g $D_2O$ were prepared without any pH or ionic strength adjustments. The samples (7ul) were held in an IR cell with 25μm path and $CaF_2$ windows. The temperature of the sample was regulated by placing the IR cell in a temperature-controlled holder employing an Omega temperature controller (Omega Engineering, Stanford, CT). Temperatures were ramped in 5°C increments (starting from 25 to 100°C) and the IR cell was allowed to equilibrate for 15 min prior to data acquisition at each temperature. A total of 512 scans were added at $4cm^{-1}$ resolution. Deconvolution of the spectra was carried out as described by Kauppinen *et al.*, (1981).

### 3.3.5 Electrophoresis

Polyacrylamide slab gel electrophoresis was performed using a Protean II xi slab cell (Bio-Rad, Richmond, CA) according to the method of Davis (1964). Polyacrylamide resolving gels (7%) were prepared in 3M Tris/HCl buffer (pH 8.9) and stacking gels (3%) in 0.5M Tris/HCl buffer (pH 6.7); electrophoresis was carried for 3h at a constant current of 3 mA/sample using 5mM Tris glycine buffer (pH 8.3). After electrophoresis, the gels were stained with Coomassie

Blue (R) (1% w/v) in trichloroacetic acid (12.5% w/v) for 18h, then destained using trichloroacetic acid (10% w/v). Samples were analyzed in duplicate.

### 3.3.6 Reversed Phase High Performance Liquid Chromatography

The lyophilized samples were subjected to reversed phase high performance liquid chromatography (RP-HPLC) to separate the individual whey proteins. The separation was done on a diphenyl reversed phase column (0.46 x 25 cm length, Vydac 219TP54). A quantity (3 mg) of protein sample was solubilized in trifluoroacetic acid (TFA) solution (1 ml, 0.1%) and injected into a liquid chromatograph equipped with a diode array, UV-visible detector (HP1090, Hewlett Packard). Elution was done at 1 ml/min using the following 2-buffer gradient system: Buffer A - 0.1% TFA in water, Buffer B - 0.1% TFA in 70/30 acetonitrile/water, starting at 10% B and increasing to 90% B in 1 min and holding at 90% B for 10 min. The eluate was monitored at 210 nm. The fractionated proteins were collected and dried in a Speed-vac Concentrator (Savant, N.Y.) under vacuum. Each sample was analyzed in duplicate.

## 3.4 RESULTS AND DISCUSSION

Table 3.1 shows the effects of various factors on the gelling ability of the WPC dispersions. Firm gels were obtained under the following conditions: (i) heating dispersions containing 150 and 300 mg WPC/g $H_2O$, at 75°C for 60 min, (ii) heating dispersions (150 mg WPC/g $H_2O$) for 60 min at 70°C and above, (iii) heating dispersions (150 mg WPC/g $H_2O$) at 75°C for 45 and 60 min (iv) heating dispersions (150 mg WPC/g $H_20$) at pH between 7.0 and 9.0 at 75°C for 60 min, and (v) heating dispersions (150 mg WPC/g $H_2O$) at 75°C for 60 min in

**Table 3.1: Effect of pH, NaCl, protein concentration, heating temperature and time on the gelling ability of WPC.**

| Factors Investigated | | | | |
|---|---|---|---|---|
| Conc[a] (mg WPC/g $H_2O$) | Temp[b] (°C) | Time[c] (min) | pH[d] | NaCl[d] (M) |
| 20 (-) | 25 (-) | 0 (-) | 2.0 (++) | 0.0 (+++) |
| 40 (-) | 50 (-) | 15 (+) | 3.0 (++) | 0.5 (++) |
| 60 (-) | 60 (-) | 30 (++) | 4.0 (cc) | 1.0 (++) |
| 80 (+) | 65 (+) | 45 (+++) | 4.5 (cc) | 1.5 (+) |
| 100(+) | 70 (+++) | 60 (+++) | 5.0 (cc) | 2.0 (-) |
| 120(++) | 75 (+++) | | 6.0 (cc) | |
| 150(+++) | 80 (+++) | | 7.0 (+++) | |
| 300(+++) | 90 (+++) | | 8.0 (+++) | |
| | 100 (+++) | | 9.0 (+++) | |

a) WPC dispersions (20 -300 mg/g $H_2O$), heated at 75°C for 60 min;
b) WPC dispersions (150 mg/g $H_2O$), heated at 25 - 100°C for 60 min;
c) WPC dispersions (150 mg/g $H_2O$), heated at 75°C for 0 - 60 min;
d) WPC dispersions (150 mg/g $H_2O$), heated at 75°C for 60 min;

(-) - no gel formation
(+) - viscous solution
(++) - soft gel
(+++) - firm gel
(cc) - curdlike coagulum

the absence of NaCl. For each of the factors investigated, it is apparent that there was some gradual transition from the state of no gelation to that where a firm gel was obtained. Consequently, the treated WPC dispersions were subjected to analysis of molecular characteristics to determine whether there were transitions in these characteristics that could be related to the observed transitional states between no gel formation and firm gel formation.

### 3.4.1 Temperature/Time Effects

Fig 3.1 shows electrophoregrams of WPC dispersions (150 mg WPC/g $H_2O$, pH 6.8) heated for 60 min over the temperature range 25°C to 100°C. The native (unheated) WPC showed four main bands identified as ß-lactoglobulin A and B, α-lactalbumin and bovine serum albumin (BSA). Traces of immunoglobulins and proteose-peptones, also known to be present in WPC, were identified. Below 65°C, the electrophoretic pattern of the heated whey proteins did not vary much from that of the native protein. However, at 65°C, only traces of the bands for bovine serum albumin and α-lactalbumin could be detected on the electrophoregram (Fig 3.1). Using calorimetry, the denaturation temperature of bovine serum albumin, pH unadjusted, has been found to be 62°C (Myers, 1990) and 65°C for α-lactalbumin (Evans and Gordon, 1980). The loss of intensity of the BSA and α-lactalbumin bands from the electropherograms of WPC dispersions heated at 65°C, can therefore be attributed to their aggregation. The ß-lactoglobulin A and B bands were not affected until 70°C at which temperature only a faint ß-lactoglobulin A band was observed. With WPC heated above 70°C, very little of the proteins were detected in the gels suggesting extensive aggregation of the proteins at these higher temperatures.

Results from the RP-HPLC support the findings obtained by

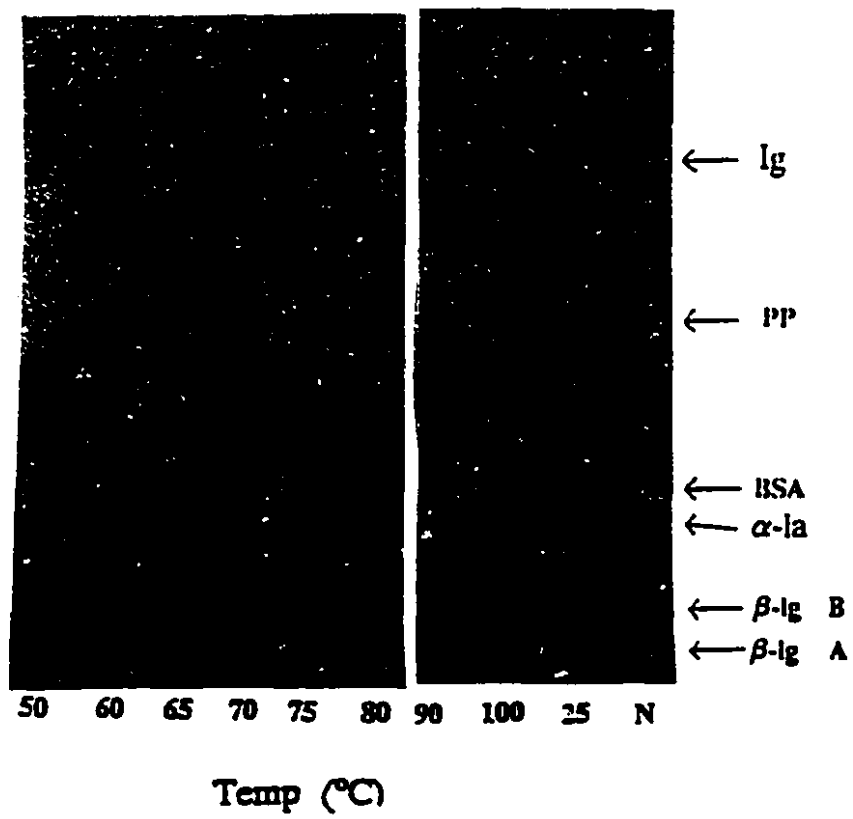



Fig 3.1: Electropherogram of WPC gels (150 mg WPC/g $H_2O$, pH unadjusted) heated for 60 min at various temperatures: α-la - (α-lactalbumin); ß-lg - (ß-lactoglobulin); BSA - (Bovine Serum Albumin); Ig - (Immunoglobulins); PP - (Proteose Peptones); N - (Native WPC).

electrophoresis. Fig 3.2 shows the HPLC chromatogram of WPC dispersions (150 mg WPC/g $H_2O$, pH 6.8) heated for 60 min at 60, 65 and 70°C. The α-lactalbumin was not detected in WPC heated at 65°C while the intensity of the ß-lactoglobulin peak was substantially reduced in WPC heated at 70°C. In relation to gelation behavior, no gelation was observed below 65°C; however an increase in the viscosity of the WPC dispersion was noted at 65°C, the temperature at which α-lactalbumin denatured. Gelation was only observed above 70°C, very close to the ß-lactoglobulin denaturation temperature of 73°C found using the DSC in this study. Evans & Gordon (1980) reported a similar thermal transition midpoint of 72.9°C for ß-lactoglobulin using the DSC. The gelation of WPC when heated above 70°C, at neutral pH, could be attributed predominantly to molecular changes of ß-lactoglobulin and only minimally to changes in α-lactalbumin. It is also possible that BSA and α-lactalbumin aggregate first but due to their low concentration, they were not incorporated into a gel matrix until ß-lactoglobulin denatured.

Fig 3.3 shows the effect of temperature on the FTIR spectra of WPC in the 1700 - 1600 $cm^{-1}$ region. Bands in this region, known as the amide I region, are attributed to the carbonyl (C=O) stretching vibrations of peptide groups (Krimm & Bandekar, 1986; Susi,1972). The six main bands in this region that are characteristic of WPC are shown in Table 3.2. The bands at 1624, 1636 and 1676 $cm^{-1}$ have been assigned to ß-sheet (Susi & Byler, 1988). The band at 1650 $cm^{-1}$ has been assigned to α-helix; its broadness in the infrared spectrum of WPC can be attributed to its overlap with the band due to random coils (Casal *et al.*, 1988; Susi & Byler, 1988). The bands at 1664 and 1692 $cm^{-1}$ have been assigned to turns (Susi & Byler, 1988); however this assignment is still a matter of controversy (Kawai & Fasman, 1978, Krimm & Bandekar, 1986; Casal *et al.*,

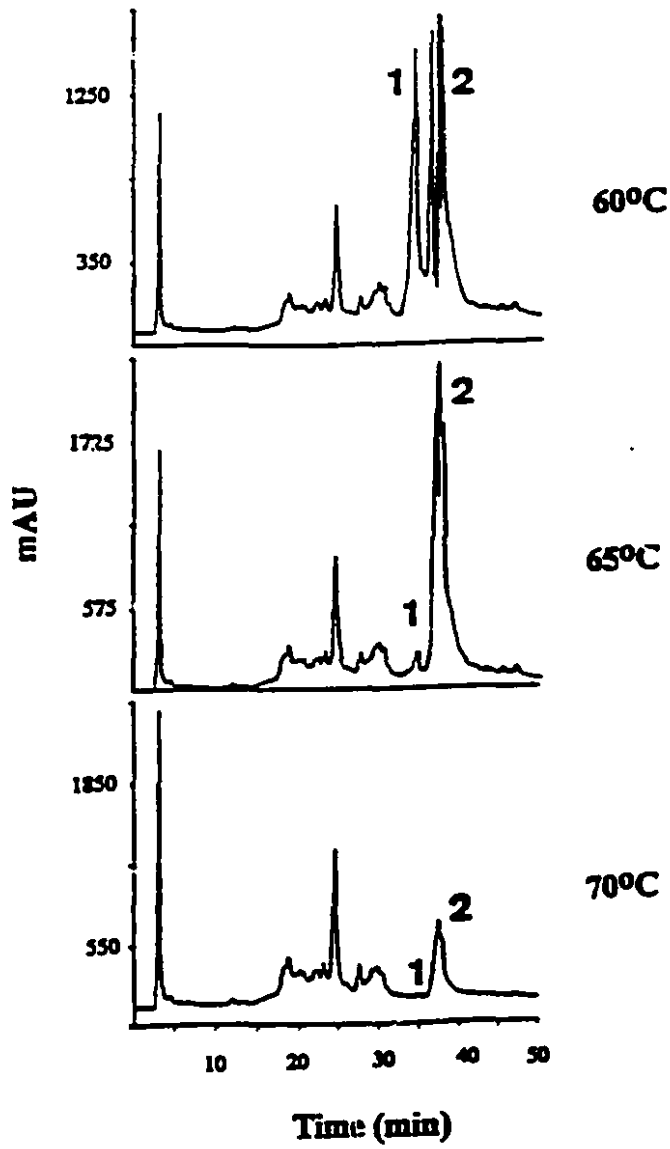



Fig 3.2: HPLC chromatogram of WPC gels (150 mg WPC/g $H_2O$, pH unadjusted), heated for 60 min at 60, 65 and 70°C. 1) α-lactalbumin; 2) ß-lactoglobulin

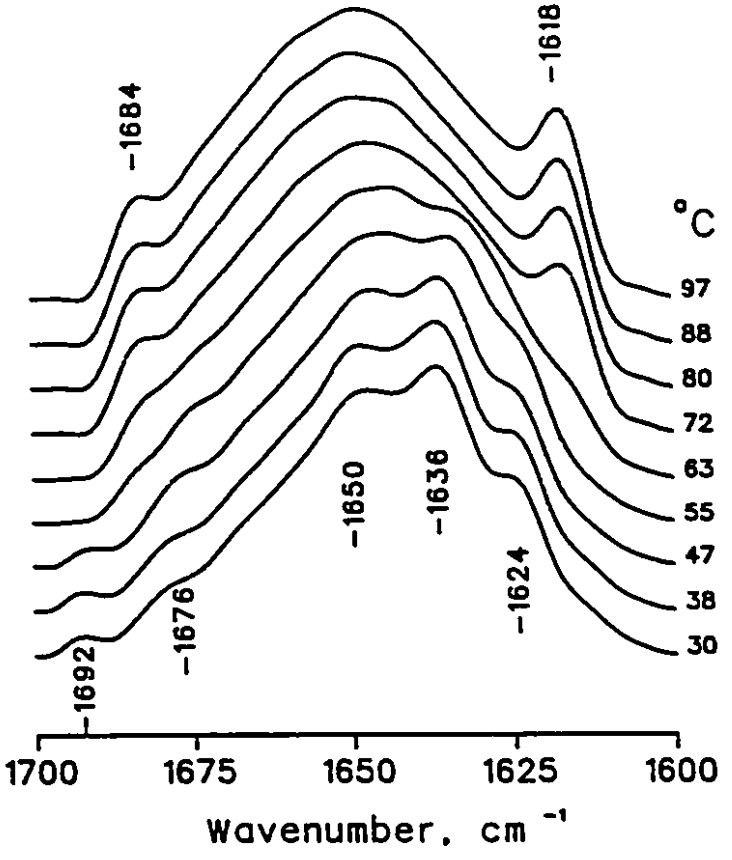



**Fig 3.3:** Stacked plot of the deconvoluted infrared spectra of WPC (150 mg /g $D_2O$, pH unadjusted) at the indicated temperatures.

**Table 3.2: Frequencies and band assignments of α-lactalbumin(α-la), ß - lactoglobulin (ß - lg), bovine serum albumin (BSA), immunoglobulins(Ig), peptones(Pp) and WPC in the Amide I (1600 - 1700 $cm^{-1}$) region.**

| Wavenumber ($cm^{-1}$)[a] | | | | | | |
|---|---|---|---|---|---|---|
| α-la | ß-lg | BSA | Ig | PP | WPC | Band Assignment[e] (Tentative) |
| | 1692 | | | | 1692 | turns[b] |
| | | 1684 | 1689 | | | ß-sheet/turns |
| 1674 | 1676 | 1673 | | | 1676 | ß-sheet/turns |
| 1664[d] | 1663[d] | | 1660 | 1658 | 1664[d] | turns |
| 1651 | 1648[c] | 1654 | | 1649 | 1650[c] | α-helix |
| 1645 | | 1646 | | 1643 | | random coil |
| 1638 | 1634 | 1636 | 1638 | 1634 | 1636 | ß-sheet |
| 1629 | 1623 | | | 1624 | 1624 | ß-sheet |
| | | | 1615 | 1616 | | ß-sheet |

a - Spectra taken at 26°C in $D_2O$
b - Band assignment is still controversial
c - overlap of α-helix and random coil
d - shoulder
e - (Krimm & Bandekar, 1986; Casal et al., 1988; Susi & Byler, 1988)

1988; Byler & Farrell, 1989). The advantage of studying the IR response of WPC alongside those of the two major whey proteins (α-lactalbumin and ß-lactoglobulin) is that any transitions involving these two proteins on heating WPC (Evans & Gordon, 1980) can be accounted for. Table 3.2 shows the contributions of α-lactalbumin and ß-lactoglobulin in the spectra of WPC. All the bands observed in WPC are present in the spectra of both proteins, except for the 1692 $cm^{-1}$ band which was only observed in the spectrum of ß-lactoglobulin.

The spectra in Fig 3.3 show the effect of increasing temperature on the transitions which occur in WPC on heat treatment. Heating WPC dispersions (150 mg WPC/g $D_2O$) to 55°C resulted in the disappearance of the 1692 $cm^{-1}$ band (Fig 3.3) without any major changes observed in the other bands; this can be attributed to an initial unfolding of the protein not accompanied by any major changes in the secondary structures of the whey proteins. This unfolding enhances hydrogen-deuterium exchange (Hvidt & Nielsen, 1966), which results in the disappearance of the 1692 $cm^{-1}$ band. The band at 1636 $cm^{-1}$ attributed to ß-sheet (Susi & Byler,1983) disappeared between 59 and 63°C, suggesting denaturation and the loss of secondary structure (Ismail *et al.*, 1992). The appearance of bands at 1618 and 1684 $cm^{-1}$ (67°C - 72°C) may be the result of formation of an intermolecularly hydrogen-bonded anti-parallel ß-sheet structure (Ismail *et al.*, 1992) associated with aggregation; these two "aggregation" bands observed at 1618 and 1684 $cm^{-1}$ in the WPC spectra (Fig 3.3) are absent in the spectrum of pure α-lactalbumin heated to the same temperature, but are present in that of pure ß-lactoglobulin. This suggests that aggregation of WPC at neutral pH might be due primarily to interactions involving ß-lactoglobulin; if ß-lactoglobulin/α-lactalbumin interactions occur, these were not detected by FTIR analysis.

The DSC thermogram of WPC (300 mg/g phosphate buffer, pH 7), shows an endothermic peak at 76.8°C characteristic of ß-lactoglobulin. A comparison of the DSC peak temperature with the FTIR transition temperatures shows that the transition indicative of denaturation in the FTIR spectra actually occurred between 59 - 63°C (Fig 3.3), which is a much lower temperature than the denaturation temperature of 76.8°C (peak maximum) observed with the DSC. Myers (1988) reported that at the high concentrations required for measurements with most commercial calorimeters, thermal denaturation is usually accompanied by aggregation. The transition from the native to the aggregated state, which is normally observed as a net endothermic reaction, is in fact a sum of the endothermic denaturation and the exothermic aggregation of the protein; the peak temperature observed in the DSC is therefore not representative of the denaturation of WPC but a net transition temperature of denaturation and aggregation; this is supported by the results from the FTIR analysis.

### 3.4.2 WPC Concentration Effects

WPC concentrations below 150 mg/g $H_2O$ did not give firm gels when heated at 75°C for 60 min (Table 3.1). No gelation was observed at any concentration (20-300 mg WPC/g $H_2O$) below 65°C; this could be related to the fact that denaturation is a prerequisite for gelation (Ferry, 1948; Hermansson, 1979; Morrissey *et al.*, 1987). Between 75-100°C, gelation occurred at concentrations as low as 80 mg WPC/g $H_2O$, although the gel formed was very soft compared to that formed at higher protein concentrations.

The FTIR spectra of the WPC showed the aggregation band (1618 $cm^{-1}$) at all concentrations studied (20 - 300 mg WPC/g $D_2O$). This band is considered to result from the presence of an intermolecularly hydrogen-bonded

ß-sheet structure, which represents the irreversible re-association of unfolded peptide segments and subsequent aggregation (Ismail et al., 1992). Although aggregates were formed at all the concentrations studied, no gels were formed at concentrations below 80 mg WPC/g $D_2O$, suggesting that the aggregation step was not dependent on protein concentration, but that, formation of gels from the aggregates was concentration dependent. This is in agreement with findings of other workers (Ferry, 1948; Trautman, 1966) who suggest that, for a given type of protein, a critical concentration is required for the formation of a gel and also that the type of gel formed at a given temperature varies with protein concentration (Table 3.1).

### 3.4.3 Effect of pH

Table 3.1 shows the effect of pH on gelling ability of WPC dispersion (150 mg/g $H_2O$) heated for 60 min at 75°C. At pH values between 4 - 6, an opaque coarse coagulum was formed. This pH range corresponds to the isoelectric region of α-lactalbumin and ß-lactoglobulin. In this pH region the lack of repulsive forces could lead to less expansion, less hydration and therefore the formation of weaker gels (Cheftel *et al.*, 1985). Firm gels were obtained in the alkaline region (pH 7 -10). In this region, the whey proteins can be expected to undergo extensive denaturation and expansion and entrap more water. The gels formed in the acidic region, pH 2 - 3, were less firm as compared to those formed in the alkaline region. This could be related to the observation that higher temperatures were required to denature the proteins at acid pH values as was determined from the DSC analysis. Fig 3.4 shows a plot of the DSC peak temperatures of WPC as a function of pH.

Fig 3.5 shows the electrophoregrams of WPC (150 mg/g $H_2O$) heated for

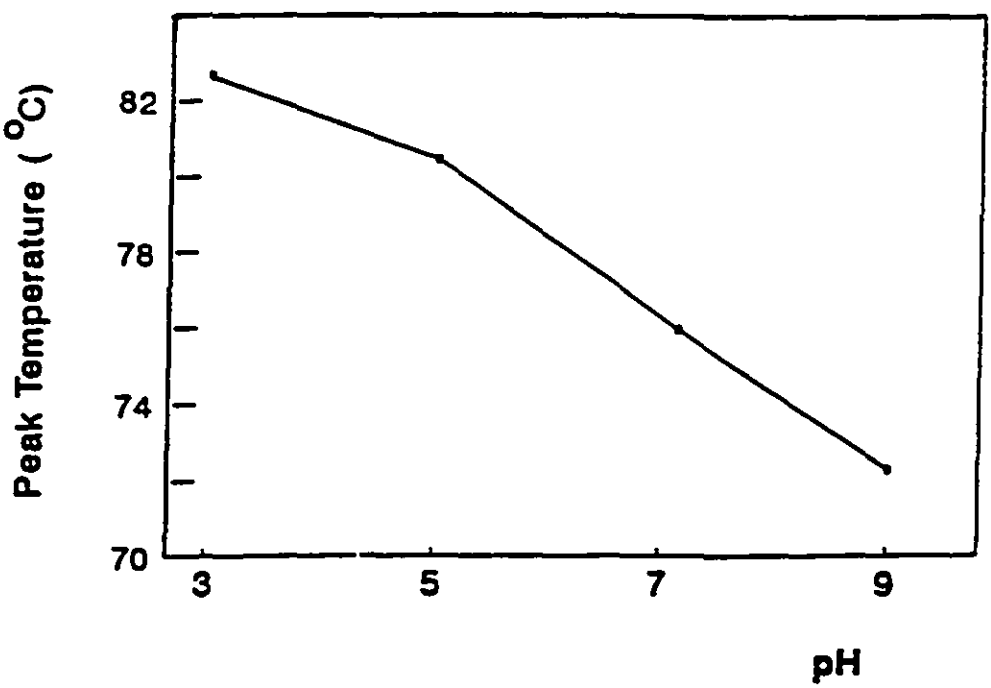



**Fig 3.4:** Effect of pH on DSC characteristics of WPC dispersions (300 mg WPC/g phosphate buffer), heated from 30°C to 120°C at 5°C/min.

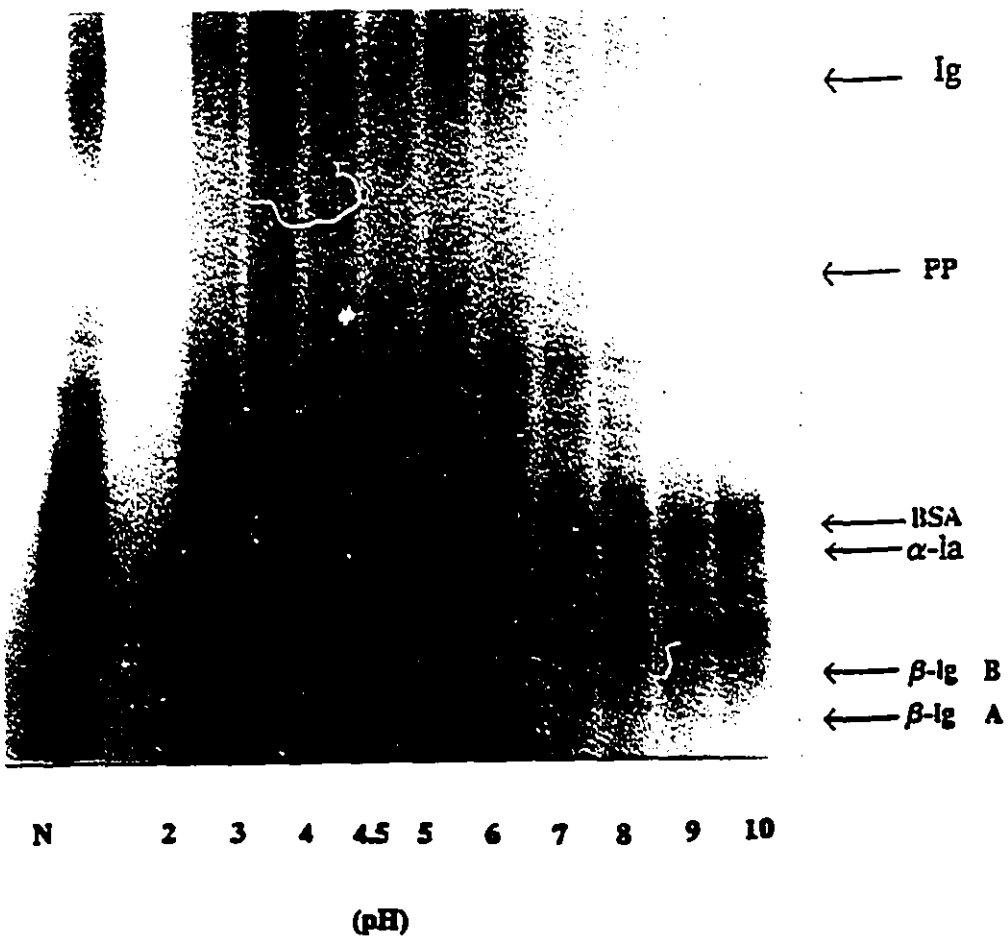



**Fig 3.5:** Electropherogram of WPC gels (150 mg WPC/g $H_2O$) heated (75°C, 60 mins) at pH values indicated.

60 min at 75°C at different pH values. At pH 3, 4 and 4.5, the electrophoretic patterns of the heated WPC dispersions were very similar to that of the native WPC, suggesting that at these pH values the proteins were only minimally affected by heat. At pH 6-10 there was complete disappearance of ß-lactoglobulin bands. At pH 2, the bands due to BSA, α-lactalbumin and ß-lactoglobulin could still be detected, indicating only partial denaturation of these proteins. The two bands with the highest migration (A, B) (Fig 3.5) were identified as ß-lactoglobulin bands. The first ß-lactoglobulin band (A) migrated the same distance between pH 2 and 5; however, between pH 3 and 4.5, the second ß-lactoglobulin band (B) migrated at a slower rate than at pH 2 and 5.

The highest peak temperature (82.7°C) in the DSC analysis (Fig 3.4) was observed at pH 3. The temperature then decreased with increasing pH, with the lowest peak temperature (72.3°C) at pH 9. This finding is supported by work done by Varunsatian *et al.*, (1983), who reported that WPC was denatured extensively by the heating at the alkaline side of the isoelectric region but was very stable when heated at pH values on the acidic side of the isoelectric region. The denaturation temperature-pH pattern for WPC is in line with the one of pure ß-lactoglobulin. The width at half peak height of the DSC thermogram at pH 3 was very small indicating that the transition was very cooperative (occurring over a small temperature range), whilst that at pH 9 was rather broad showing less cooperativity. This broadening of the DSC peak with increasing pH is also found for pure ß-lactoglobulin.

This decrease in cooperativity at pH 9 was also observed in the FTIR spectra (Fig 3.6). The FTIR spectra of WPC heated from 26 to 97°C at pH 3 and 9 are shown in Fig 3.6. The disappearance of the 1692 $cm^{-1}$ band is seen at 47°C at pH 9, but not until 63 - 67°C at pH 3. Major changes were observed in

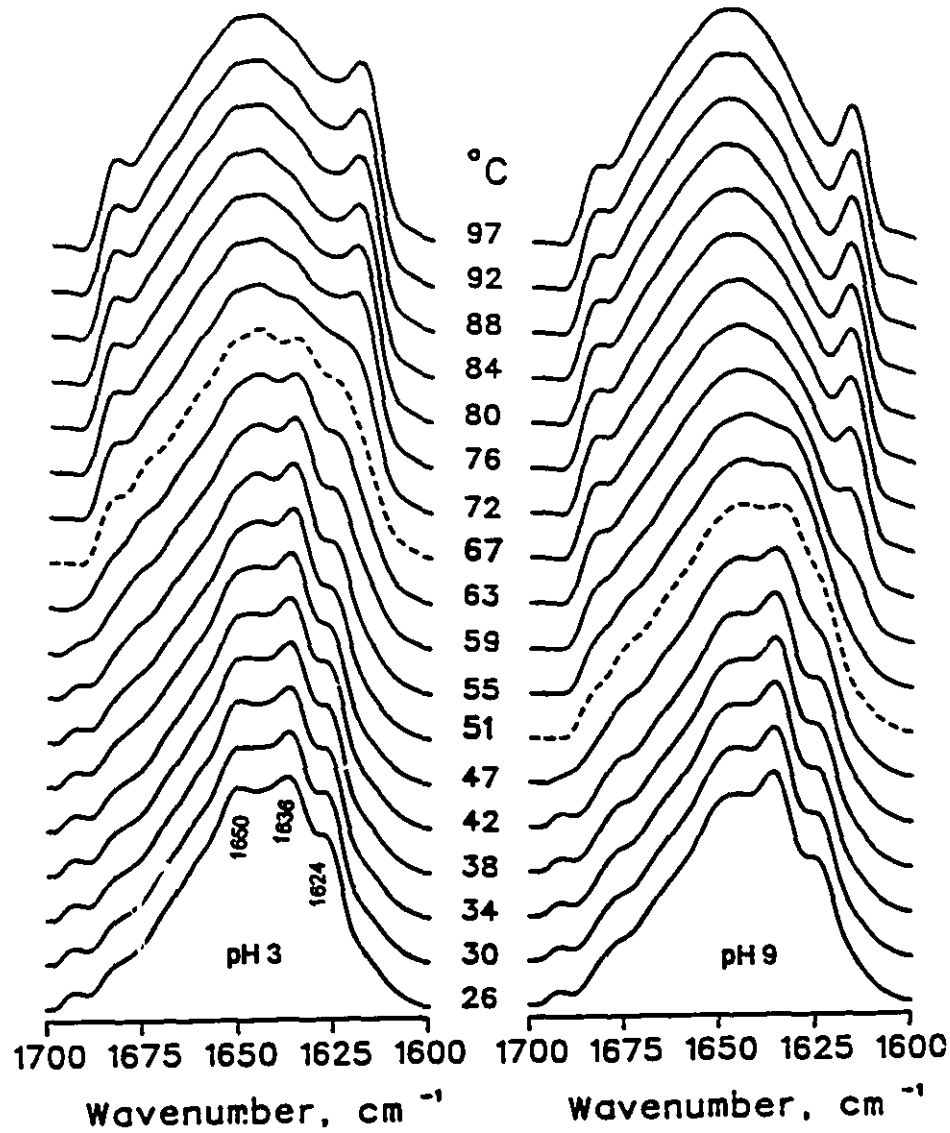



Fig 3.6: Stacked plot of the deconvoluted infrared spectra of WPC (150 mg/g deuterated phosphate buffer) at the indicated temperatures; a) pH 3 b) pH 9. (—— - major changes observed in the bands at 1624, 1636 and 1650 $cm^{-1}$).

the 1624, 1636 and 1650cm$^{-1}$ bands at 51°C for pH 9 and at 67°C for pH 3. These changes represented a loss of secondary structure, suggesting that WPC has higher thermal stability at pH 3 than at pH 9. The aggregation band at 1618 cm$^{-1}$ appeared immediately after denaturation at pH 3, but somewhat later at pH 9. This suggests that the transition from denaturation to aggregation is less cooperative (occurs over a much broader temperature range) at pH 9 than at pH 3. A very broad band (at ca. 1648 cm$^{-1}$) was observed between the two aggregation bands at 1618 and 1684 cm$^{-1}$, at pH 3. This band appeared to become narrow at pH 9, suggesting that during denaturation the proteins unfold into more extensive random coiled structures at pH 9 than at pH 3. Ismail *et al.*, (1992) reported that an increase in band width may be indicative of a more flexible structure and/or an increase in the number of different subconformations. The broadness of this peak at pH 3 could therefore be attributed to the presence of a higher number of subconformations on denaturation, or less extensive denaturation of WPC at this pH. FTIR spectra (Fig 3.7) of ß-lactoglobulin and α-lactalbumin heated to 97°C revealed that ß-lactoglobulin denatured and formed aggregates at both pH 3 and 9, with the denaturation occurring at a much lower temperature at pH 9 (51°C), than at pH 3 (72 - 76°C) (unpublished data). However, α-lactalbumin denatured and formed aggregates only at pH 3 and 5 but not at pH 7 and 9. This suggests that at neutral pH and higher, the aggregation and subsequent gelation of WPC is due primarily to molecular transitions involving only ß-lactoglobulin; if α-lactalbumin transitions are involved in aggregation they were not detected by the FTIR. At pH values below 7, molecular transitions involving both α-lactalbumin and ß-lactoglobulin contribute to the gelation of WPC.

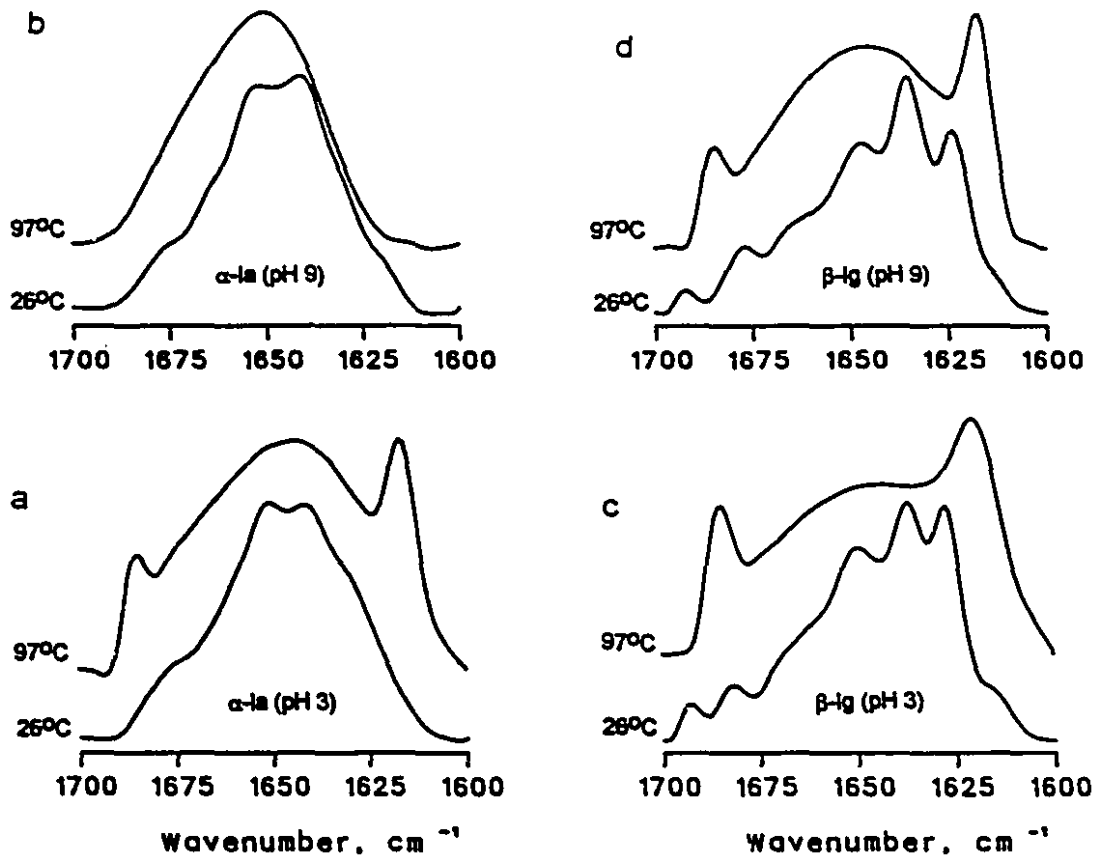



**Fig 3.7:** Plot of the deconvoluted infrared spectra of a) α-lactalbumin, pH 3 (bottom - 26°C; top - 97°C); b) α-lactalbumin, pH 9 (bottom - 26°C; top - 97°C); c) β-lactoglobulin, pH 3 (bottom - 26°C; top - 97°C); d) β-lactoglobulin, pH 9 (bottom - 26°C; top - 97°C).

### 3.4.4 Effect of NaCl

Table 3.1 shows that there was considerable variation in the gelling ability over the NaCl concentration range of 0-2M. Firm gels were obtained in the absence of NaCl; below 1M NaCl concentration, soft gels were obtained; no gelation was observed at NaCl concentrations above 1M.

The effect of NaCl on the DSC characteristics of WPC are shown in Fig 3.8. The denaturation temperature of WPC increased from 76.3°C in the absence of NaCl, to 85.3°C with the addition of 2M NaCl, an increase of about 9°C; this observation is supported by findings of other workers (Itoh *et al.*, 1976; Damodaran & Kinsella; 1982, Harwalkar & Ma, 1989). This explains the fact that higher temperatures were required to denature the WPC in the presence of NaCl, and also explains the absence of gel formation in the presence of 1-2M NaCl; heat treatment of the WPC dispersions was carried out at 75°C, a lower temperature than that required to denature WPC at high salt concentrations.

The spectra of WPC (150 mg/g) in 0.5M and 2.0M NaCl solutions heated from 26 - 97°C are shown in Fig 3.9. At 63°C, the bands at 1624, 1636, 1650$cm^{-1}$ disappeared from the spectra of WPC dispersed in 0.5M NaCl solution, signifying a loss in secondary structure; in 2M NaCl solution, the corresponding loss of secondary structure occurred at 72°C. The "aggregation" band shifted from 1618 $cm^{-1}$ in the 0.5M NaCl solution to 1620 $cm^{-1}$ in the 2M NaCl solution. A shift of this band to higher wavenumbers can be attributed to a strengthening of the C=O bond stretching vibration resulting from a decrease in hydrogen bonding (Krimm & Bandekar, 1986). "Salting-out" effects resulting from the competition between the protein and the salt ions for water molecules may result in protein-protein interactions leading to aggregation followed by precipitation (Cheftel *et al.*, 1985) and this could explain the decrease in

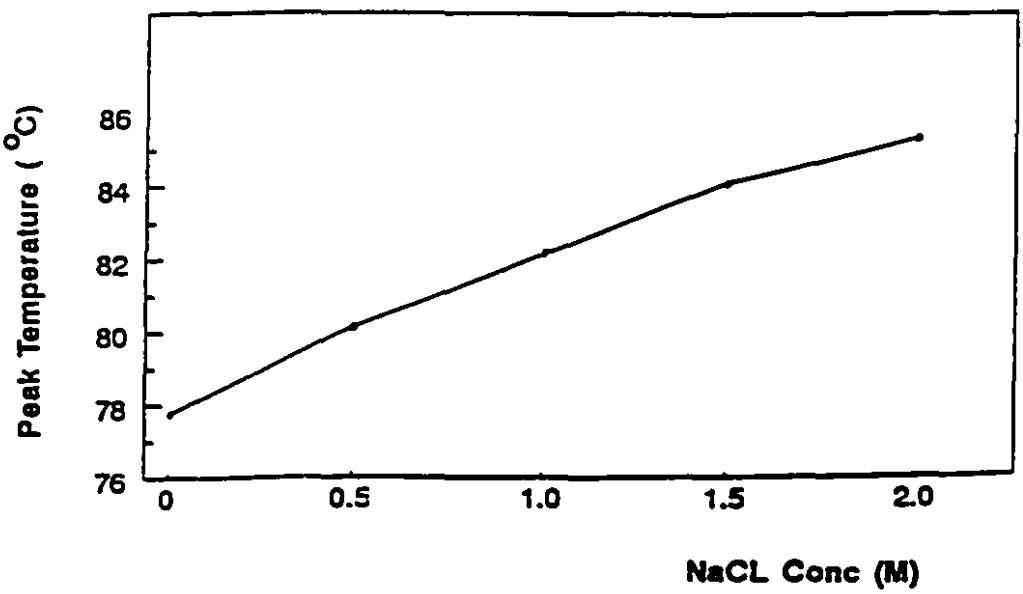



**Fig 3.8:** Effect of NaCl on peak temperature of denaturation of WPC dispersions (300 mg WPC/g NaCl solution), heated from 30°C to 120°C at 5°C/min.

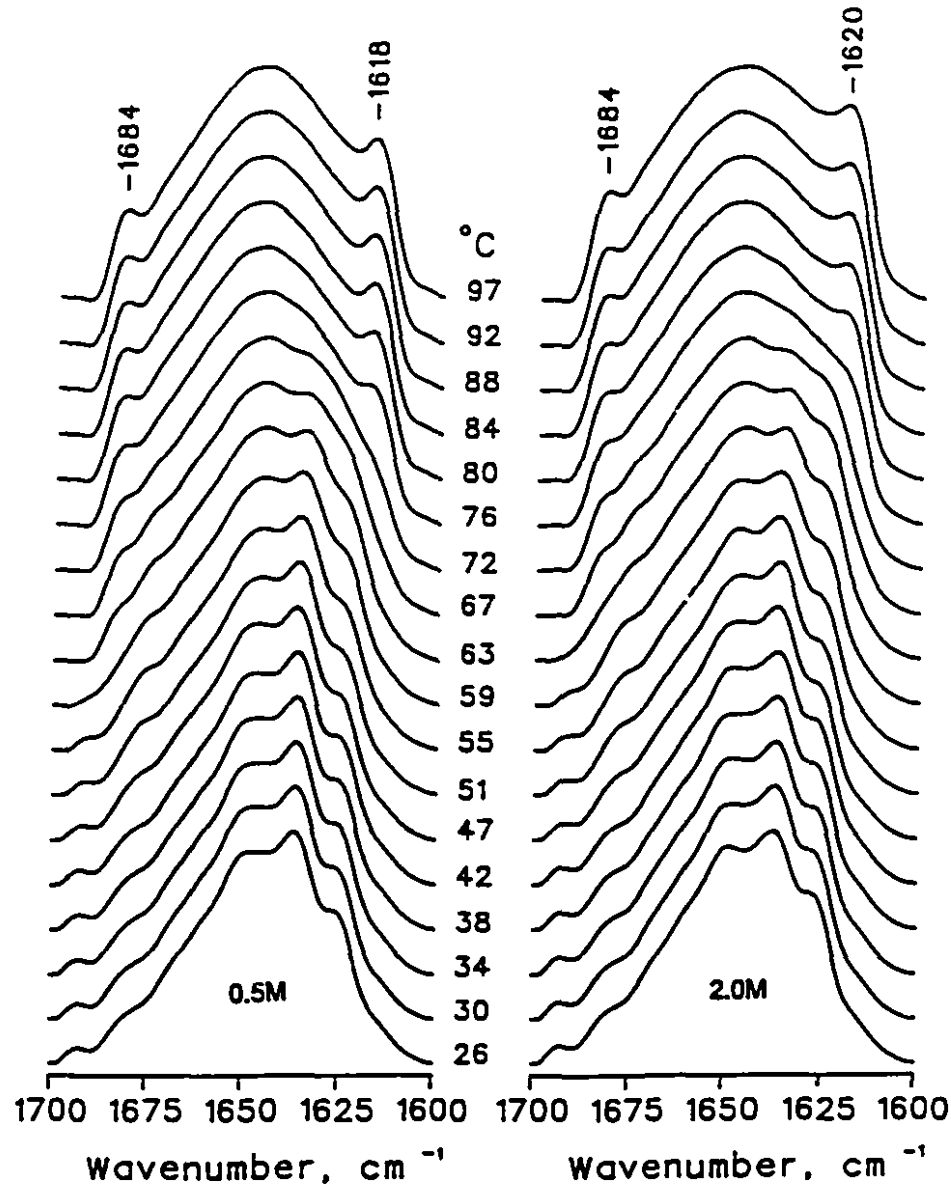



**Fig 3.9:** Stacked plot of deconvoluted infrared spectra of WPC (150 mg/g NaCl solution, in $D_2O$); a) 0.5 M b) 2.0 M NaCl, at the indicated temperatures.

hydrogen bonding observed with increasing NaCl concentration. The spectra of the WPC in the 2M NaCl solution at 97°C also show considerably weaker signals in the region between the 1684 and 1620 $cm^{-1}$ bands than those obtained in the 0.5M NaCl solution; this suggests that the protein unfolds to a lesser extent in the 2M solution than it does in the 0.5M solution.

## 3.5 CONCLUSION

Heating temperature, heating time, pH and NaCl affect the texture of WPC gels by altering the secondary structure of the individual proteins of whey during heat treatment. Denaturation of α-lactalbumin increased the viscosity of WPC dispersions. Denaturation of β-lactoglobulin resulted in gel formation. Lowering the pH of WPC appeared to inhibit unfolding of the protein and increase its stability to thermal denaturation. High pH enhanced unfolding and subsequent aggregation of WPC. Unfolding of β-lactoglobulin resulted in the formation of intermolecularly hydrogen-bonded β-sheet structures at both low and high pH. This was only observed at low pH in the case of α-lactalbumin. The type and texture of WPC gels can be altered by changing the factors that control the behavior of the secondary structures of the individual whey proteins during gel formation.

# CHAPTER 4

# INTERACTIVE EFFECTS OF FACTORS AFFECTING GELATION OF WHEY PROTEINS

## 4.0 CONNECTING STATEMENT

In Chapter 3, the individual effects of pH, protein concentration, NaCl, heating temperature and time on the molecular and structural properties of heated whey protein concentrate (WPC) dispersions were studied. The results obtained showed that these factors markedly affected gel formation and altered both the temperature of denaturation and the secondary structures of the whey proteins during gel formation. The work described in this chapter is an evaluation of the quantitative and interactive effects of the factors studied in Chapter 3 on the textural characteristics and rheological properties of WPC gels and addresses the second objective discussed in the "Rationale and Objectives of Study" section of Chapter 1.

**Note:** This chapter is the text of a manuscript which has been submitted for publication as follows:

Boye, J. I., Alli, I., Ramaswamy, H., Raghavan, V. S. G., "Interactive effects of factors affecting gelation of whey proteins". *J. Food Sci.* (Submitted, **June 1995**).

***Contributions of co-authors***: Alli,I. (thesis supervisor); Ramaswamy, H. (provided instrumentation for rheological studies); Raghavan, V. S. G. (provided instrumentation for gel strength measurements).

## 4.1 ABSTRACT

The individual and interactive effects of pH (3-11), NaCl (0-2M), protein concentration (10-30% w/v) and heating temperature (60-80°C) on the strength, turbidity and water holding capacity of whey protein concentrate (WPC) gels, were investigated by use of a four-variable five-level central composite rotatable design (CCRD) analyzed by response surface methodology (RSM). The effect of sucrose (5-30% w/v) on whey protein gelation was also monitored. Differential scanning calorimetry was used to monitor changes in thermal stability. In the presence of 1.6M NaCl, an increase in the viscosity of the WPC dispersion was observed without any gel formation. Gels formed in the presence of 0.8M NaCl were viscous at pH 7 and more elastic at alkaline pH (pH 11); at acid pH, gel formation was not observed. An elastic gel was formed in the absence of NaCl; in the first phase of gelation, the WPC gels were more viscous and less elastic, as heating progressed, gel elasticity increased and dominated over gel viscosity. Gel elasticity , gel strength and water holding capacity generally increased with increasing protein concentration and heating temperature. Sucrose and NaCl decreased the strength of WPC gels but increased water holding capacity. Increasing NaCl concentration increased the strength and water holding capacity of the WPC gels at low pH (<5) but resulted in weaker gels at high pH (>7). The strongest gels were obtained at pH 9 and 0.4M NaCl concentration and at pH 5 and 1.2M NaCl. In the absence of NaCl, increasing pH resulted in a decrease in denaturation temperature. At any given pH, increasing NaCl concentration resulted in an increase in denaturation temperature.

## 4.2 INTRODUCTION

Whey proteins, obtained as by-products from cheese and casein manufacture, posses excellent gelation properties. In solution, whey proteins are capable of forming viscoelastic gels after sufficient heat denaturation (Schmidt, 1981; Kinsella & Whitehead, 1989; Ziegler & Foegeding, 1990). In this type of heat-induced gelation, there is an initial change in protein conformation from the native state to a progel state (Damodaran, 1994). This results in partial denaturation of the protein molecules which subsequently associate to form the gel matrix, if attractive forces and thermodynamic conditions are suitable (Mulvihill & Kinsella, 1987; Ziegler & Foegeding, 1990).

The functional role of a protein as a food ingredient is a manifestation of complex interactions of various factors. The texture of whey protein gels, for example, is affected by processing conditions such as heating and cooling rates, protein concentration and environmental conditions such as pH, ionic strength and interactions with other food components e.g., sugars (Kinsella *et al.*, 1985; Damodaran, 1989; Smith, 1994). Previous studies (Boye *et al.*, 1995 a, b, c) showed that NaCl, glucose and sucrose increased the thermal stability of whey proteins; it was observed that whey proteins formed firmer gels at temperatures above 70°C and at alkaline pH values. Hines & Foegeding (1991) reported that whey proteins formed translucent gelatin-like gels with low shear stress and high shear strain in the presence of 25-30 mM NaCl and opaque curd-like gels with low shear stress and low shear strain values in 7.5 mM $CaCl_2$. Schmidt *et al.*, (1979) demonstrated that free sulfhydryl groups have a significant effect on properties of whey protein gels; at low concentrations, gel strength increased as free sulfhydryl group increased until a maximal concentration beyond which gel strength decreased with further increase of free sulfhydryls, this depending on pH, ionic strength and calcium content of the system.

There are numerous reports on research directed at the effects of individual factors on whey protein gelation. It is now recognized that a more realistic approach for understanding the functional behavior of proteins in food systems should include multivariate studies in which the combined effects of various factors on a functional property are studied simultaneously (Damodaran, 1994).

The objective of the present study was to investigate the interactive effects of pH, temperature, ionic strength and protein concentration on the gelation of whey protein concentrate (WPC) using response surface methodology (RSM). Cooper *et al.*, (1977) applied RSM to the properties of WPC in a detailed investigation of the effect of whey protein concentration, NaCl, $CaCl_2$ and sucrose concentration on gel formation; the gloss, color, brittleness and firmness of the WPC gels were related to combinations of the factors studied. In another study (Schmidt *et al.*, 1979), the effects of $CaCl_2$ and cysteine on WPC gels were studied using multiple regression and RSM; it was shown that interactions between calcium and concentration of free sulfhydryl groups significantly affected WPC gelation. In this study, the denaturation temperature, strength, water holding capacity and turbidity of WPC gels were monitored. These properties have been classified as important in characterizing protein gels (Kinsella, 1984; Ziegler & Foegeding, 1990).

## 4.3 MATERIALS & METHODS

### 4.3.1 Materials

A commercial sample of whey protein concentrate (WPC) (75% protein, 13% lactose, 3% ash, 8% fat) was obtained from Saputo (St.-Hyacinthe, Quebec) and stored in air tight containers at room temperature.

### 4.3.2 Experimental Design

*Individual Factors Affecting Whey Protein Gelation:* The quantitative effects of pH (3-9) , temperature (65-90°C), heating time (0-120 min) and concentration of NaCl (0-2M), sucrose (5-30% w/v) and protein (10-30% w/v) on the gelation of WPC were investigated (Table 4.1). In a previous study, (Boye *et al.*, 1995a) it was established that these factors individually produced marked effects on whey protein denaturation and gelation.

*Interactive Effects of Factors Affecting Gelation:* Based on the results obtained from the effects of the individual factors above, the interactive effects of pH, temperature and concentration of NaCl and protein on WPC gelation were investigated. The levels at which these factors were studied is given in Table 4.2. A four-variable, five-level central composite rotatable design (CCRD) was used, and analyzed by response surface methodology (RSM) (Box *et al.*, 1978). The coded values of the independent variables and the actual values are given in Tables 4.3 and 4.4. The complete design consisted of 25 experimental points performed in random order. The four responses (Y, dependent variables) measured were: gel strength (Y1), water holding capacity (Y2), turbidity (Y3), denaturation temperature (Y4).

**Table 4.1: Values of individual factors used in the study of WPC gelation**

| Variables | Range |
|---|---|
| Concentration(% w/v) | 10 - 30 |
| Temperature($^{o}$C) | 65 - 90 |
| Heating Time (min) | 15 - 120 |
| pH | 3 - 9 |
| NaCl Conc (M) | 0 - 2.0 |
| Sugar Conc (% w/v) | 0 - 30 |

**Table 4.2: Values of coded levels used in the CCRD for WPC gelation**

| | Levels | | | | |
|---|---|---|---|---|---|
| Variables | -2 | -1 | 0 | 1 | 2 |
| Temperature($^{o}$C) (X1) | 60 | 70 | 80 | 90 | 100 |
| Concentration(% w/v) (X2) | 10 | 15 | 20 | 25 | 30 |
| pH (X3) | 3 | 5 | 7 | 9 | 11 |
| NaCl Conc (M) (X4) | 0.0 | 0.4 | 0.8 | 1.2 | 1.6 |

**Table 4.3: Coded level combinations for a 4 variable CCRD for WPC gelation**

| | Variables** | | | |
|---|---|---|---|---|
| Run #* | X1 | X2 | X3 | X4 |
| 1 | -1 | -1 | -1 | -1 |
| 2 | 1 | -1 | -1 | -1 |
| 3 | -1 | 1 | -1 | -1 |
| 4 | 1 | 1 | -1 | -1 |
| 5 | -1 | -1 | 1 | -1 |
| 6 | 1 | -1 | 1 | -1 |
| 7 | -1 | 1 | 1 | -1 |
| 8 | 1 | 1 | 1 | -1 |
| 9 | -1 | -1 | -1 | 1 |
| 10 | 1 | -1 | -1 | 1 |
| 11 | -1 | 1 | -1 | 1 |
| 12 | 1 | 1 | -1 | 1 |
| 13 | -1 | -1 | 1 | 1 |
| 14 | 1 | -1 | 1 | 1 |
| 15 | -1 | 1 | 1 | 1 |
| 16 | 1 | 1 | 1 | 1 |
| 17 | -2 | 0 | 0 | 0 |
| 18 | 2 | 0 | 0 | 0 |
| 19 | 0 | -2 | 0 | 0 |
| 20 | 0 | 2 | 0 | 0 |
| 21 | 0 | 0 | -2 | 0 |
| 22 | 0 | 0 | 2 | 0 |
| 23 | 0 | 0 | 0 | -2 |
| 24 | 0 | 0 | 0 | 2 |
| 25 | 0 | 0 | 0 | 0 |

* Each run replicated twice for a total of 50 runs.

** X1 = Temp; X2 = Conc; X3 = pH; X4 = NaCl Conc

**Table 4.4: Experimental design for a 4 variable CCRD study of whey protein gelation.**

| RSM # | Temp($^{o}$C) | Conc(%w/v) | pH | NaCl(M) |
|---|---|---|---|---|
| 1 | 70 | 15 | 5 | 0.4 |
| 2 | 90 | 15 | 5 | 0.4 |
| 3 | 70 | 25 | 5 | 0.4 |
| 4 | 90 | 25 | 5 | 0.4 |
| 5 | 70 | 15 | 9 | 0.4 |
| 6 | 90 | 15 | 9 | 0.4 |
| 7 | 70 | 25 | 9 | 0.4 |
| 8 | 90 | 25 | 9 | 0.4 |
| 9 | 70 | 15 | 5 | 1.2 |
| 10 | 90 | 15 | 5 | 1.2 |
| 11 | 70 | 25 | 5 | 1.2 |
| 12 | 90 | 25 | 5 | 1.2 |
| 13 | 70 | 15 | 9 | 1.2 |
| 14 | 90 | 15 | 9 | 1.2 |
| 15 | 70 | 25 | 9 | 1.2 |
| 16 | 90 | 25 | 9 | 1.2 |
| 17 | 60 | 20 | 7 | 0.8 |
| 18 | 100 | 20 | 7 | 0.8 |
| 19 | 80 | 10 | 7 | 0.8 |
| 20 | 80 | 30 | 7 | 0.8 |
| 21 | 80 | 20 | 3 | 0.8 |
| 22 | 80 | 20 | 11 | 0.8 |
| 23 | 80 | 20 | 7 | 0.0 |
| 24 | 80 | 20 | 7 | 1.6 |
| 25 | 80 | 20 | 7 | 0.8 |

### 4.3.3 Gel Formation

Aqueous dispersions of WPC (10 ml) of desired concentrations were prepared in centrifuge tubes (25 mm ID, 100 mm length, for water holding capacity measurements) and in 50 ml beakers (for gel strength measurements). The pH (3-11) of the dispersions were varied by use of phosphate buffers (ionic strength 0.2). NaCl (0.4-2M) and sucrose (0-30% w/v) were added to the suspension and stirred. The test tubes and beakers were covered with aluminum foil to prevent moisture loss and were heated in a water bath for up to 60 min.

### 4.3.4 Gel Strength Measurements

Gels formed under the different conditions (individual and interactive factors) were allowed to equilibrate at 5$^{o}$C for 24h prior to compression testing. Samples (37mm diameter, 10mm long sections) were uniaxially compressed to 50% deformation using an Instron Universal Testing Machine (Series IX). The cross-head speed of the compressing plate was set at 25mm/min. The gel strength (N) was calculated as the force required to break the gels (load at yield); these measurements were replicated 4 times for each sample. Gels were also made in 5 ml plastic hypodermic syringes (Becton-Dickinson) and the force required to extrude the gels was measured as an indication of gel strength.

### 4.3.5 Water Holding Capacity (WHC)

WPC gels formed in the centrifuge tubes were centrifuged at 15,000 g for 10 min. The supernatant was removed and the water retained in the WPC gel was determined gravimetrically. Water holding capacity was expressed as the water retained in the residue after centrifugation per 100 gram of the original protein (Kinsella, 1984; Patel & Fry, 1987).

### 4.3.6 Turbidity

Turbidity of the gels was measured with a LKB Biochrom Ultrospec II spectrophotometer (LKB Biochrom Ltd., Science Park, Cambridge CB4 4FJ, England). Samples of WPC gels containing 1.5 mg protein were suspended in distilled water (1.5 mg/ml) and a uniform suspension was prepared by use of a Corning pyrex tissue grinder (15 ml). The suspensions were placed in a cuvette (1cm light pathlength) and the transmission was measured at 660 nm; all measurements were done in triplicate.

### 4.3.7 Dynamic Viscoelastic Measurements

A Haake viscometer (Haake RV 20 Rotovisco, Rheocontroller RC 20) was used to measure the viscoelastic properties of the WPC gels during heat treatment. Samples were held in a heating cup and were subjected to a sinusoidal strain of amplitude $4^{o}$ with an oscillating frequency of 1 Hz. The storage (G') and loss (G") moduli were generated by a Haake software program. Tan delta, which is a ratio of energy dissipated to energy stored per cycle, was calculated as G"/G' (Clark, 1992). Heating was by circulating temperature-controlled water through a water jacket surrounding the sample. The WPC dispersions were covered with a layer of paraffin to avoid dehydration during heating. All measurements were in duplicate.

### 4.3.8 Differential Scanning Calorimetry (DSC)

WPC dispersions prepared as described in Tables 4.1 and 4.4 (25µl) were weighed in aluminum dishes and heated from $30^{o}C$ to $120^{o}C$ at a programmed heating rate of $5^{o}C$/min using a TA 3000 DSC (METTLER, Mettler Instrument Corporation, Greifensee, Switzerland) calorimeter. An empty sample

pan was used as reference. Indium standards were used for temperature and energy calibrations.

### 4.3.9 Statistical Analysis

The results obtained from the CCRD were analyzed by multiple regression to fit the following second order equation to all dependent Y variables:

$$Y = Bo + \sum_{i=1}^{4} B_i X_i + \sum_{i=1}^{4} B_{ii} X_i^2 + \sum_{i=1}^{4} B_{ij} X_i X_j$$

where Bo, Bi, Bii, Bij are constant regression coefficients of the model, and Xi the independent variables in coded values. A second order model, which includes both the interactive and quadratic effects was chosen to provide a better description of the geometric shape of the response surface (Glacula & Singh, 1984). The regression procedure (PROC GLM) of the Statistical Analysis System, Inc., (SAS) was used to generate regression coefficients, analysis of variance and correlation coefficients for the model. Response surfaces using the SAS/Graph program on a McGill University mainframe computer and a Zeta plotter were produced to demonstrate the main effects of independent variables on the characteristics of WPC gels.

## 4.4 RESULTS AND DISCUSSION

### 4.4.1 Individual Factors Affecting WPC Gelation

Fig 4.1 shows the gel strength, water holding capacity and turbidity measurements of WPC gels made at various protein concentrations (Fig 4.1.i), heating temperatures (Fig 4.1.ii) and heating times (Fig 4.1.iii). Gels formed

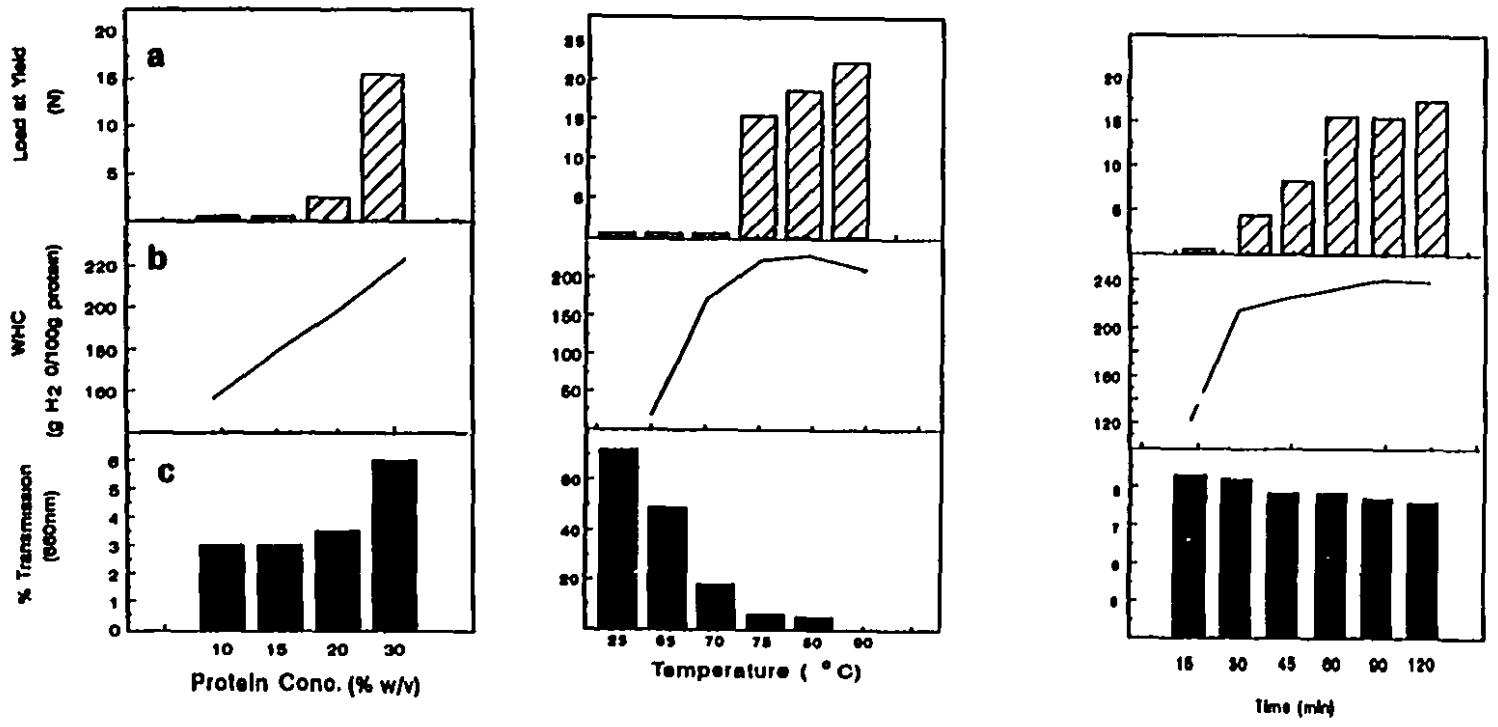



**Fig 4.1:** Effect of (i) protein concentration (ii) temperature and (iii) heating time on the strength, water holding capacity and turbidity of WPC gels.

below 15% (w/v) protein concentration at 75°C and 60 min of heating (Fig 4.1.ia) were very weak (< 1N); as protein concentration increased, gel strength increased from 2.5±.1N at 20% (w/v) protein concentration to 15.5±.1N at 30% (w/v) concentration (Table 4.5).This confirms findings reported in previous studies (Damodaran, 1989; Boye *et al.*, 1995a). One of the most important functional properties of gels is their water holding capacity (Stanley & Yada, 1992). The water holding capacity (WPC) of the 10% (w/v) WPC dispersion heated at 75°C for 60 min, was 156% (Fig 4.1.ib). This increased linearly with increasing protein concentration to 223% at 30% (w/v) protein concentration, suggesting that the water holding capacity of the WPC gels increased as protein concentration and gel strength increased. A number of studies have shown that an increase in the turbidity of protein solutions can be used to demonstrate aggregation (Samejima *et al.*, 1984; Ziegler & Acton, 1984; Sano *et al.*, 1990). A decrease in transmission indicates an increase in turbidity and represents an increase in aggregate and gel formation. The transmission for the 10% (w/v) WPC dispersion heated at 75°C for 60 min was 3.0±0.1% (Fig 4.1.ic); this value increased slightly to 5.87±0.2% for the 30% w/v WPC dispersion, heated under similar conditions. The higher transmission observed for the 30% w/v sample suggests a slight decrease in aggregate formation; the lower transmission value obtained at lower protein concentration indicates an increase in aggregate formation and suggests that in the absence of enough protein molecules required for gel formation, bigger particulates (aggregates) may have been formed which decreased the transmission.

The strength, water holding capacities (WHC) and turbidity measurements of gels from the WPC dispersions (30% w/v) heated at varying temperatures are shown in Fig 4.1.ii. Below 70°C, the WPC gels formed were very soft and gave values below 1N. Heating above 70°C, resulted in an

**Table 4.5: Effect of protein concentration, heating temperature and time on the strength, water holding capacity and turbidity of WPC gels.**

| Treatment | Gel Strength (N) | WHC (%)[1] | Transmission (%) |
|---|---|---|---|
| Protein Concentration (% w/v) | | | |
| 10 | <1 | 156 | 3.00±0.1 |
| 15 | <1 | 178 | 3.00±0.1 |
| 20 | 2.5±.10 | 199 | 3.00±0.1 |
| 30 | 15.5±.10 | 223 | 5.87±0.2 |
| Temperature (°C) | | | |
| 25 | 0 | 0 | 72.0±0.21 |
| 65 | <1 | 15 | 49.2±0.31 |
| 70 | <1 | 169 | 18.4±0.10 |
| 75 | 15.5±.11 | 223 | 5.87±0.21 |
| 80 | 18.7±.20 | 229 | 5.0±0.10 |
| 90 | 22.4±.42 | 210 | - |
| Heating Time (min) | | | |
| 0 | 0 | 0 | 72.0±0.21 |
| 15 | <1 | 122 | 8.5±0.11 |
| 30 | 4.4±.11 | 215 | 8.3±0.10 |
| 45 | 8.3±.90 | 225 | 7.6±0.10 |
| 60 | 15.5±.10 | 223 | - |
| 90 | 15.4±.41 | 240 | 7.3±0.01 |
| 120 | 17.3±.83 | 240 | 7.0±0.20 |

[1] (g $H_2O$/ 100g WPC)

increase in gel strength from 15.5±.1N at 75°C to 22.4±.4N at 90°C which suggests a direct relationship between heating temperature and gel strength. The WHC of the gels heated at 65°C was 15%; this value increased to a maximum of 229% at 80°C and subsequently decreased with further heating (Fig 4.1.iib). The decrease in WHC when WPC was heated above 80°C may be attributed to phase separation associated with the formation of a more coarse structure with larger pores (Stanley & Yada, 1992). The transmission of the unheated WPC dispersion was 72±0.2% (Table 4.5) (Fig 4.1ii.c); this value decreased to 5±0.1% when the sample was heated at 75°C for 60 min. This decrease in transmission is indicative of an increase in the turbidity of the WPC dispersion and suggests an increase in aggregate formation with heating temperature. Above 75°C, no further decrease in transmission was observed which suggests that the aggregation phase was completed. A comparison of the transmission and gel strength results (Figs 4.1.iia, c) suggest that the increase in gel strength observed above 75°C may be due to increased crosslinking of the aggregates formed.

The effect of heating time on the textural properties of gels from the WPC dispersions (30% w/v) heated at 75°C is shown in Fig 4.1.iiia. Gel strength increased from 4.4±.11N after 30 min of heating to 15.5±.1N after 60 min, and to 17.3±.8N after 120 min. The WHC (Fig 4.1.iiib) after 15 min was 122%. After 30 min, the WHC markedly increased to 215% and to 240% after 90 min and then plateaued. This suggests that most of the increase in WHC occurred during the initial stages of gel formation (first 30 min) (Fig 4.1.iiia). The transmission of the samples heated for 15 and 120 min was 8.5±.1% and 7.0±.2% respectively (Fig 4.1.iiic). Comparison of these values with that observed for the unheated sample (72%) (Table 4.5) showed a marked decrease in transmission between 0 and 15 min of heating indicative of an increase in turbidity and suggests that

most of the aggregate formation actually occurred within the first 15 min of heating. Beveridge *et al.*, (1984) concluded from a similar study that aggregates were formed during the initial 3-10 min of heating; these aggregates then associated with continued heating to form a weak viscous network.

The effect of pH on the strength, water holding capacity and turbidity of WPC gels (30% w/v) heated at 75°C for 60 min is shown in Fig 4.2i. Gels formed at pH 9 (Fig 4.2i.a) were stronger (37.6±.36N) than at pH 7 (19.0±.21N). At pH 3 and 5 the gels formed were too soft for deformation measurements; extrusion tests, however, showed that the gels formed at pH 3 were stronger (3.5N) than those formed at pH 5 (1.4N)(Table 4.6). Kinsella & Whitehead (1989) reported that the higher gelation tendency in the region above pH 8 may reflect some disulfide crosslinking and matrix formation via thiol-disulfide interchange. WHC decreased slightly from 186% to 181% when the pH was increased from 3 to 5. Above pH 5 the WHC markedly increased to 284% at pH 7 and to 304% at pH 9. This increase is similar to that observed for the gel strength measurements and suggests a direct relationship between WHC and gel strength. At pH 3, the transmission was 23±.1%. This value decreased to 16±.6% at pH 5 (suggesting an increase in aggregate formation) and then increased to 21±.5%% at pH 7 (suggesting a decrease in aggregate formation) and decreased again to 15±.7% at pH 9 (suggesting an increase in aggregate formation). The increase in aggregate formation observed at pH 5 can be attributed to increased electrostatic attraction at the isoelectric point of the proteins which resulted in precipitation of the proteins (Cheftel *et al.*, 1985). At pH 9, the increase in aggregate formation can be attributed to the formation of a firmer more aggregated gel (Kinsella & Whitehead, 1989).

Fig 4.2.iia shows the effect of NaCl concentration on the textural properties of WPC gels. In the absence of NaCl, the strength of the WPC gels

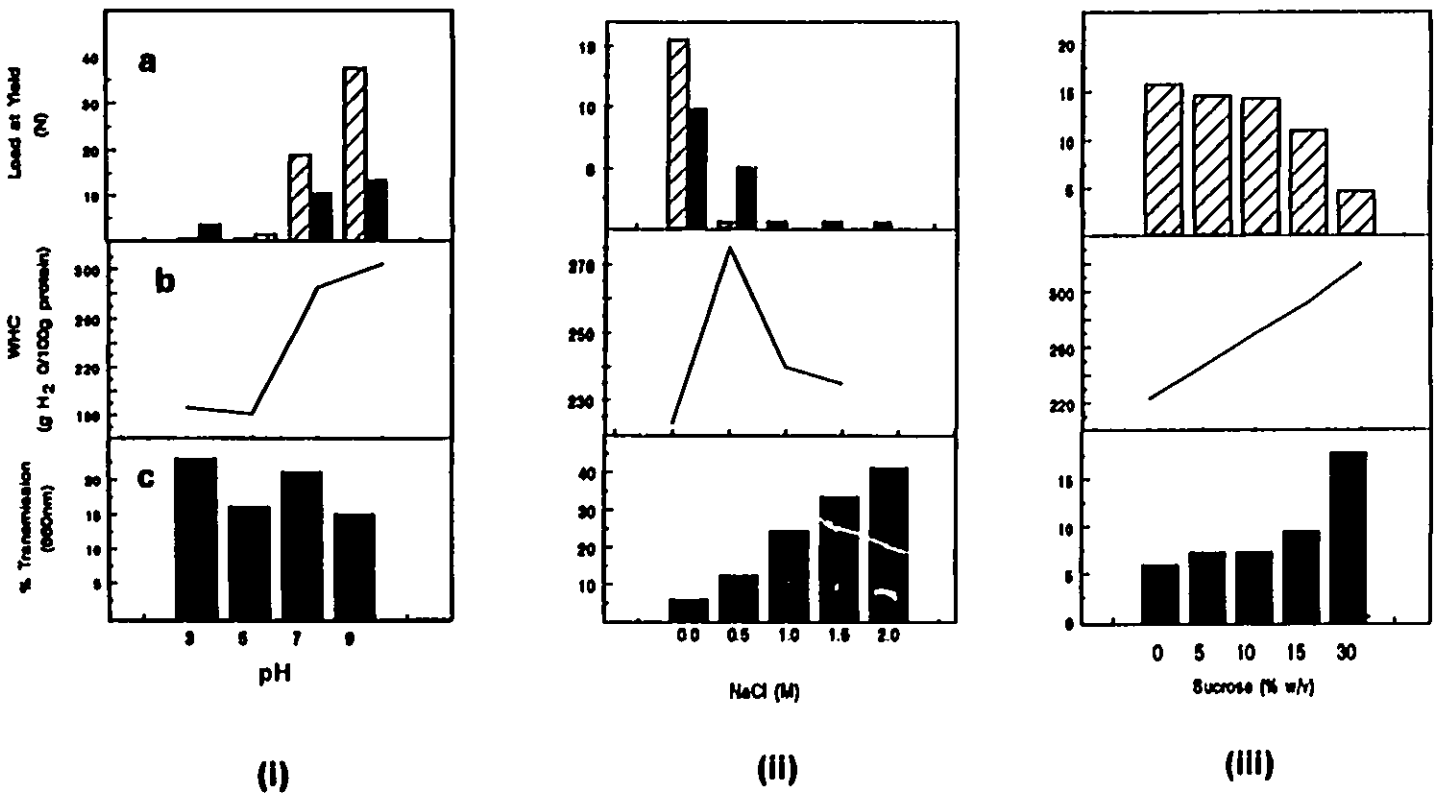



**Fig 4.2:** Effect of (i) pH (ii) NaCl and (iii) sucrose on the strength, water holding capacity and turbidity of WPC gels.

**Table 4.6: Effect of pH, NaCl and sucrose concentration on the strength, water holding capacity and turbidity of WPC gels.**

| Treatment | Gel Strength (N) | | WHC (%)[1] | Transmission(%) |
|---|---|---|---|---|
| | A | B | | |
| pH | | | | |
| 3 | <1 | 3.5 | 186 | 23.0±0.1 |
| 5 | <1 | 1.4 | 181 | 16.0±0.6 |
| 7 | 19.0±.21 | 10.4 | 284 | 21.0±0.5 |
| 9 | 37.6±.36 | 13.5 | 304 | 15.0±0.7 |
| NaCl Concentration | | | | |
| 0.0 | 15.5±.10 | 9.8 | 223 | 6.0±0.2 |
| 0.5 | <1 | 2.1 | 274 | 12.5±0.3 |
| 1.0 | <1 | <1 | 240 | 24.4±0.2 |
| 1.5 | <1 | <1 | 235 | 33.5±0.3 |
| 2.0 | <1 | <1 | - | 41.1±0.1 |
| Sucrose Concentration | | | | |
| 0 | 15.5±.10 | | 223 | 6.0±0.2 |
| 5 | 14.3±.40 | | 246 | 7.2±0.2 |
| 10 | 14.0±.10 | | 270 | 7.3±0.1 |
| 15 | 10.7±.11 | | 2292 | 9.5±0.1 |
| 30 | 4.4±.30 | | 320 | 17.8±0.3 |

[1](g $H_2O$/ 100g WPC)

A - Compression measurements

B - Extrusion measurements

was 15.5±0.1N . With the addition of 0.5-2M NaCl, gel strength markedly decreased (Table 4.6). Extrusion tests showed a notable decrease in gel strength, from 9.8±.5N in the absence of NaCl to 2.1±.7N in the presence of 0.5M NaCl. Gels formed in the presence of greater than 0.5M NaCl, were very soft and gave values below 1N. The effects of NaCl on the water holding capacity and turbidity of the WPC gels are shown in Figs 4.2ii.b and c. In the absence of NaCl the WHC was 223% . This value increased to 274% in the presence of 0.5M NaCl and to 235% in 1.5M NaCl, suggesting that low concentrations of NaCl had a greater effect in increasing the water holding capacity of WPC gels. The transmission of the WPC gels formed in the absence of NaCl was 6±.2% (Fig 4.2ii.c); this increased to 41.1±.1% in the presence of 2M NaCl which is indicative of a decrease in turbidity and suggests a decrease in aggregate formation and may explain the decrease in gel strength observed with increasing NaCl concentration (Fig 4.2iia).

Fig 4.2.iiia shows the effect of sucrose on the strength, WHC and turbidity of WPC gels. In the absence of sucrose, the strength of the WPC gels was 15.5 ±.1N. This decreased with increasing sucrose concentration to 4.4±.3N in the presence of 30% w/v sucrose, which suggests that sucrose decreased the strength of WPC gels. WHC increased from 223% in the absence of sucrose to 320% in the presence of 30% (w/v) sucrose (Fig 4.2.iiib), which suggests that sucrose enhanced the WHC of WPC gels. The transmission increased from 6±.2% in the absence of sucrose to 17.8±.3% in the presence of 30% (w/v) sucrose (Fig 4.2.iiic), which is indicative of a decrease in the turbidity of the suspension and suggests a decrease in aggregate formation with increasing sucrose concentration; this may explain the decrease in gel strength observed at higher sucrose concentration (Fig 4.2iii.a).

### 4.4.2 Interactive Effects of Factors Affecting WPC Gelation

**Viscoelastic Properties**

The viscoelastic properties measured in this study were the storage (G') and loss moduli (G"). G' is a measure of the energy stored and subsequently released and is related to gel elasticity; G" is a measure of energy dissipated per cycle of deformation and is related to gel viscosity (Hamann *et al.*, 1990). The ratio G"/G' (tan delta) is a measure of the dynamic character of the protein-protein bonds in the gel network; a higher tan delta means that a gel reacts to a stress in a relatively more viscous and less elastic manner and vice versa (Van Vliet *et al.*, 1989). Fig 4.3 shows the effect of protein concentration on the storage, loss moduli and tan delta of WPC dispersions (pH 7, 0.8M NaCl) during heat treatment. No change was observed in the storage modulus of the 10% (w/v) WPC sample heated at 80°C for 30 min. A slight increase was observed in the loss modulus and tan delta, which is indicative of an increase in viscosity without gelation. The storage modulus of the 20% w/v WPC dispersion heated at 80°C, decreased with heating time with a marked decrease observed between 15 and 20 min of heating. The loss modulus however increased, suggesting an increase in the viscous component of the gel network and a decrease in gel elasticity. After 15 min of heating, the tan delta of the 20% (w/v) WPC suspension, markedly increased to a maximum value after 30 min. This increase is indicative of gel network formation and suggests that the gel formed at 20% (w/v) protein concentration was more viscous and less elastic. At 30% (w/v) protein concentration both storage and loss moduli increased with heating time, suggesting an increase in both gel elasticity and viscosity. Tan delta increased to a maximum between 9 and 12 min of heating and then decreased to a minimum after 17 min of heating, which suggests that in the first phase of network formation (0-12 min), the WPC gels were more viscous and less elastic.

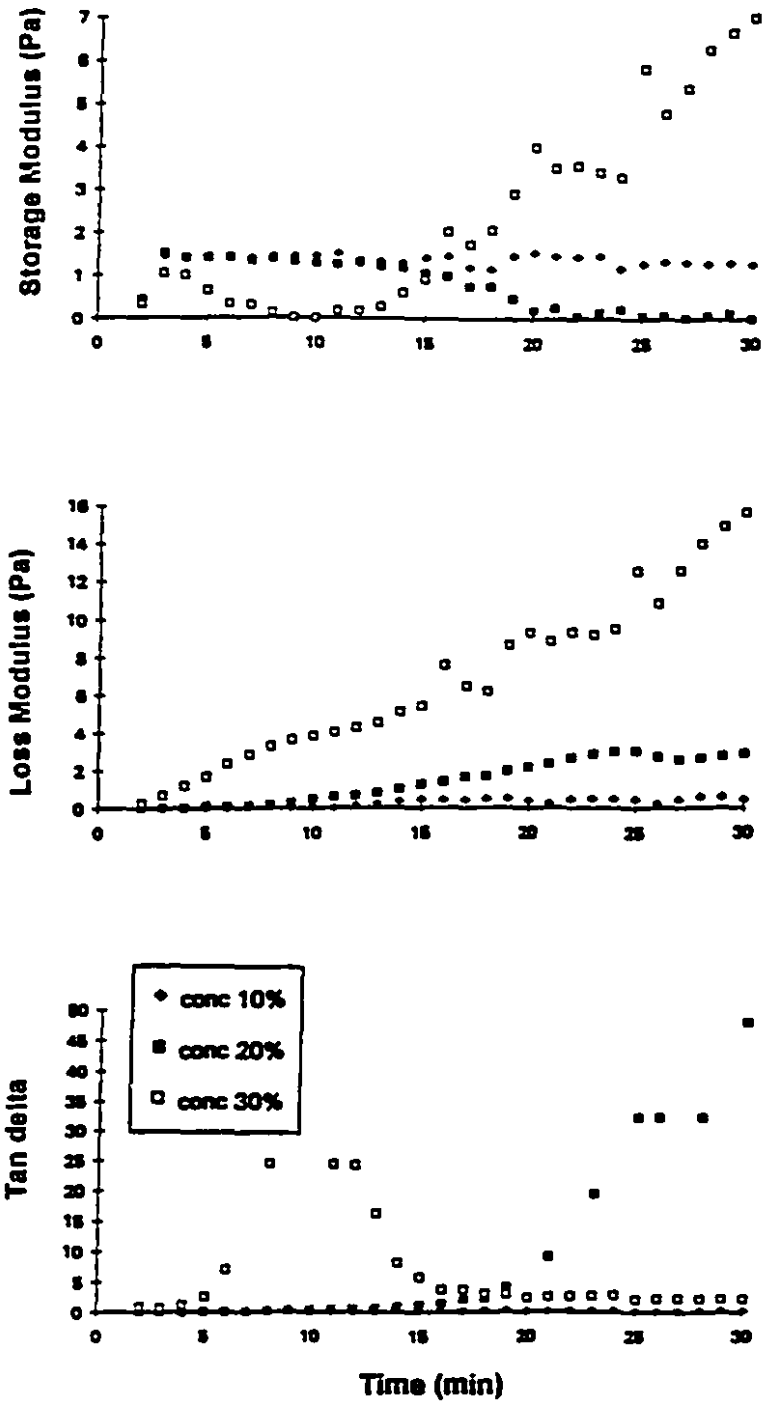



Fig 4.3: Effect of protein concentration on the storage, loss moduli and tan delta of WPC (pH 7, 0.8M NaCl) heated at 80°C, for 30 min.

After 12 min of heating (second phase), gel elasticity increased and dominated over gel viscosity.

Fig 4.4 shows the effect of heating temperature on the viscoelastic properties of WPC (20% w/v, pH 7, 0.8M NaCl) suspensions. No changes were observed in storage modulus, loss modulus or tan delta of the sample heated at 60°C, suggesting that WPC did not form a gel at this temperature. At 80°C, the storage modulus decreased with heating time while the loss modulus increased suggesting a decrease in the elasticity of the gel and an increase in viscosity. The tan delta increased with heating time; after 15 min tan delta values were greater than 1, which is indicative of the formation of a gel network with a high viscous component.

The effect of pH on the storage and loss moduli and tan delta of WPC dispersions (20% w/v, 0.8M NaCl) heated at 80°C for 30 min is shown in Fig 4.5. Very little changes were observed in the storage and loss moduli and tan delta at pH 3 which suggests an absence of gel formation. At pH 7, a decrease in storage modulus was observed with heating, which was accompanied by an increase in loss modulus; this suggests a decrease in gel elasticity and an increase in viscosity. The increase in tan delta (>1) observed after 15 min further suggests the formation of a more viscous and less elastic gel. At pH 11, the storage modulus decreased to a minimum value after 13 min of heating and subsequently increased with further heating. This increase is indicative of an initial decrease followed by an increase in gel elasticity. The loss modulus, consistently increased with heating, suggesting an increase in the viscous component of the gel formed. Tan delta increased to a maximum value between 13 and 14 min of heating and then decreased with further heating. The initial increase in tan delta suggests that during the first 13 min of heating, the viscous

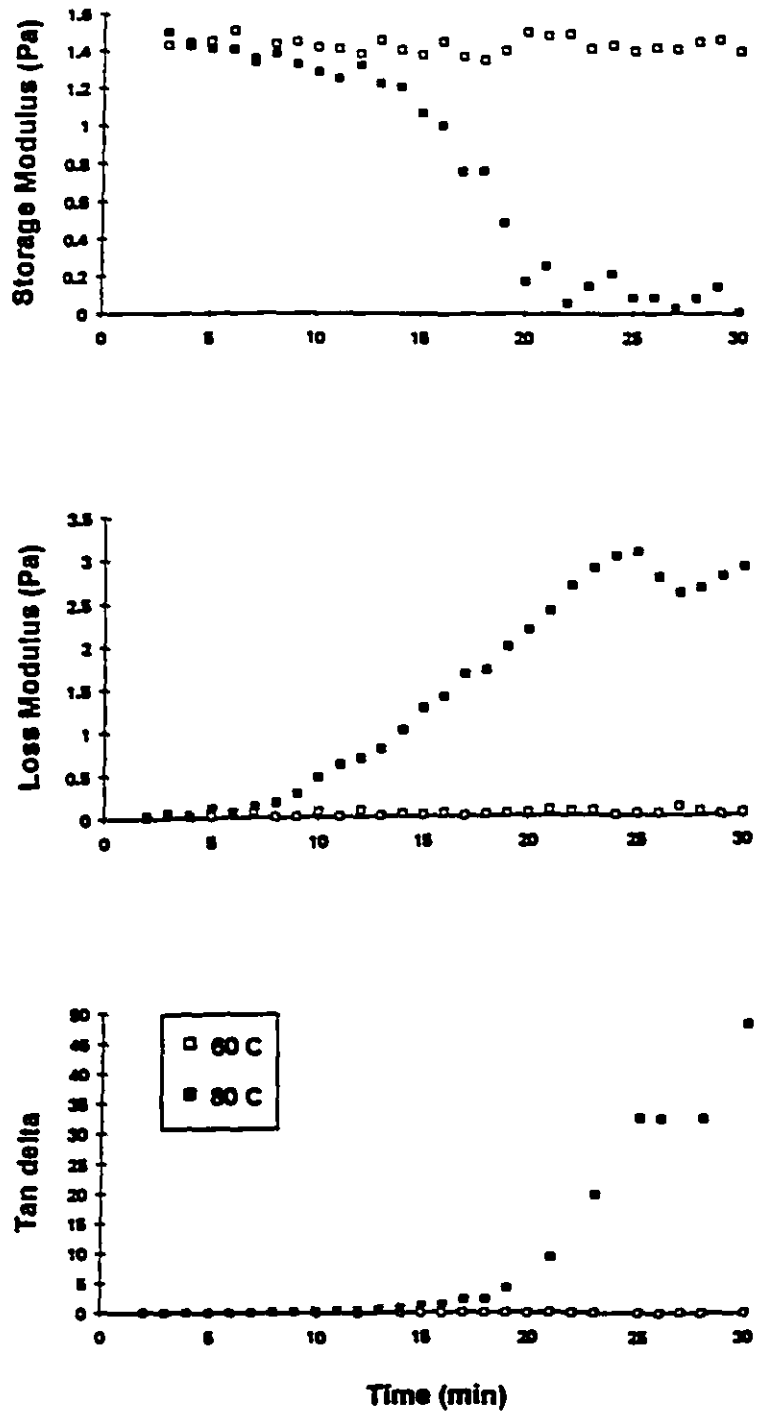



Fig 4.4: Effect of heating temperature on the storage and loss moduli and tan delta of WPC dispersions (20% w/v, pH 7, 0.8M NaCl).

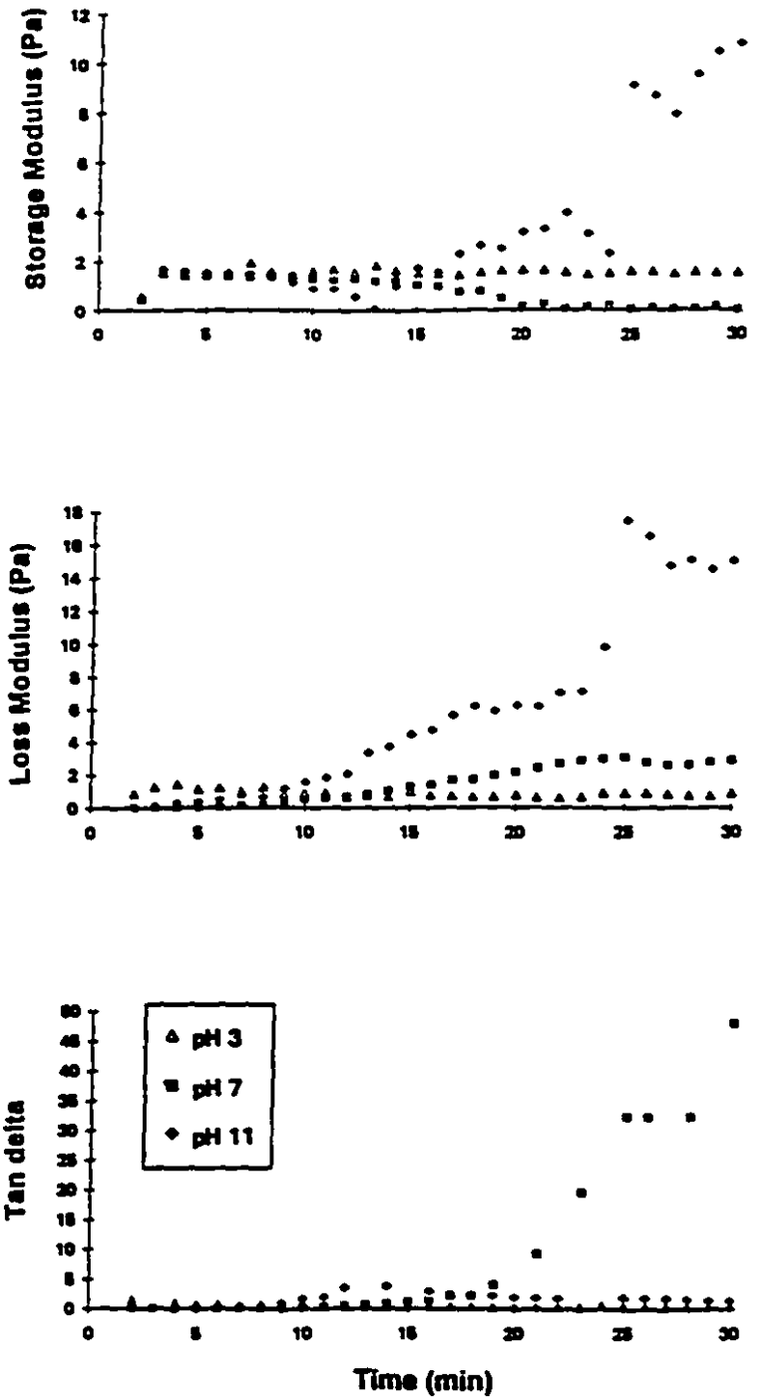



Fig 4.5: Effect of pH on the storage and loss moduli and tan delta of WPC (20% w/v, 0.8M NaCl) heated at 80°C, for 30 min.

component of the WPC gel was greater than the elastic component; after 14 min of heating, a gel with greater elasticity was formed.

The effect of NaCl concentration on the viscoelastic properties of WPC dispersions (20% w/v, pH 7) heated at 80°C for 30 min is shown in Fig 4.6. In the absence of NaCl, a marked increase in storage modulus was observed after 5 min of heating which decreased after 10 min of heating. After 13 min, the storage modulus increased again to a maximum after 20 min of heating and subsequently decreased with further heating. A similar trend was observed in loss modulus. This suggests that as the WPC gels were heated, there was an initial increase in both the viscous and elastic components of the WPC gels. As heating continued, the gel structure weakened, resulting in the loss of both storage and loss moduli. As heating progressed further, the gel increased in viscosity and elasticity; after 20 min, however, both the elastic and viscous components of the gel decreased, which is indicative of a weakening of the gel structure. The tan delta values showed an initial increase within the first 5 min of heating (Fig 4.6), which suggests an increase in the viscous component of the gel. After 5 min, a decrease in tan delta was observed which is indicative of the formation of an elastic gel. In the presence of 0.8M NaCl, storage modulus decreased with heating time while loss modulus increased. Tan delta markedly increased after 15 min of heating suggesting the formation of a more viscous gel. In the presence of 1.6M NaCl, no changes were observed in the storage modulus with heating time. The loss modulus increased slightly with heating time which suggests an increase in the viscosity of the WPC dispersion without gel formation.

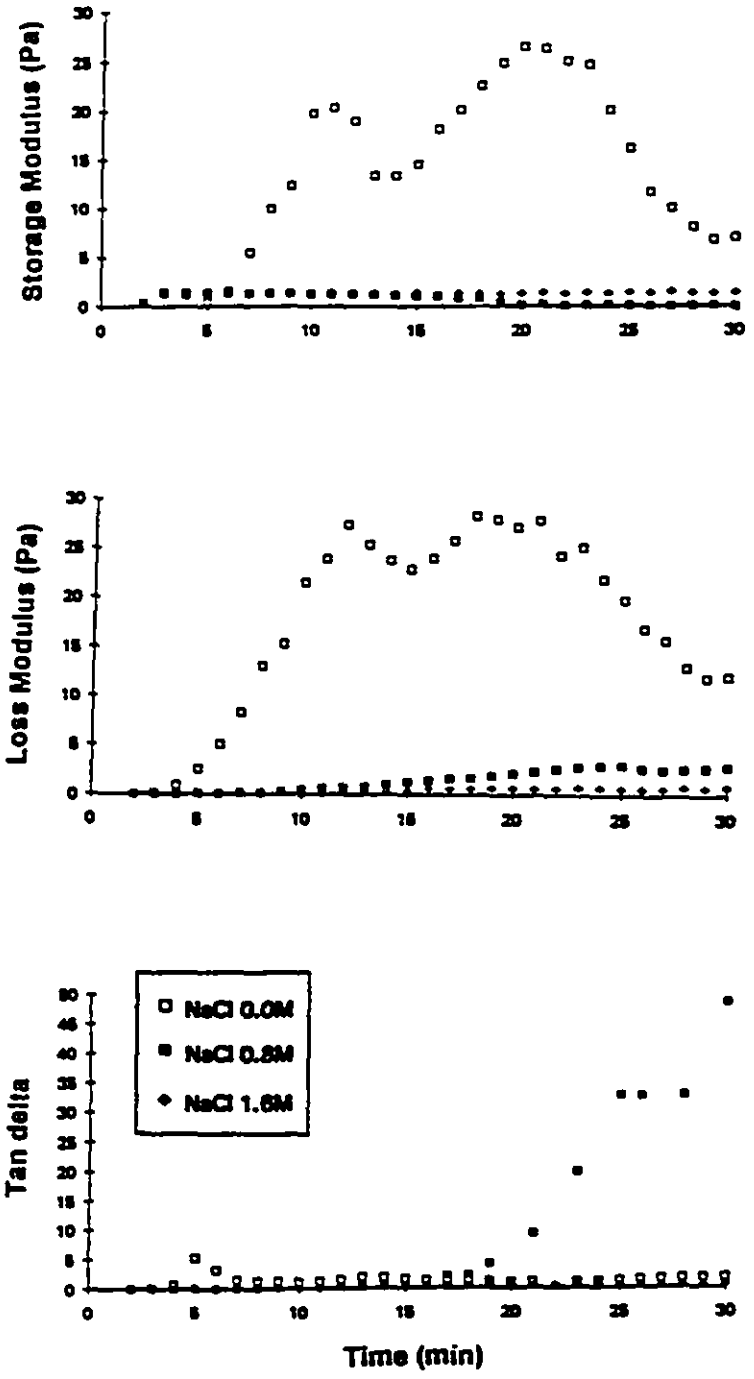



Fig 4.6: Effect of NaCl concentration on the storage and loss moduli and tan delta of WPC dispersions (20% w/v, pH 7) heated at 80°C for 30 min.

**Response Surface**

The coded and uncoded level combinations for the four-variable five-level central composite rotatable design used for this study are shown in Tables 4.3 and 4.4. The gel strength, water holding capacity and turbidity measurements resulting from combinations of conditions corresponding to the uncoded values of protein concentration (X1), pH (X2), NaCl concentration (X3) and heating temperature (X4) are shown in Table 4.7. The linear, quadratic and cross product terms in the second order polynomial were used to generate three dimensional response surface graphs of the responses measured (Diptee *et al.*, 1989) (Figs 4.7 and 4.8).

Fig 4.7a shows the relationship between gel strength and (a) protein concentration and (b) temperature. Statistical analysis showed these factors to be the two significant variables ($p \leq 0.001$) influencing the strength of the WPC gels. Gel strength increased as temperature and protein concentration were increased when pH and NaCl were held constant. At any given temperature, increasing protein concentration increased gel strength and at any given protein concentration, increasing heating temperature also resulted in an increase in gel strength. This confirms findings reported by other workers (Dunkerley & Hayes, 1980; Mulvihill & Kinsella,1987).

Fig 4.7b shows the relationship between gel strength and a) pH and b) NaCl concentration. At low NaCl concentrations (< 0.53M), the strength of the WPC gels increased with increasing pH; the highest gel strength was observed in the absence of NaCl and at pH 11 (Fig 4.7b). At higher NaCl concentrations (>0.5M), gel strength decreased with increasing pH. Protein conformation is altered by the presence of salts which influence electrostatic interactions involving charged species and polar groups, as well as hydrophobic interactions (von Hippel & Schleich, 1969; Damodaran & Kinsella, 1982). At pH values

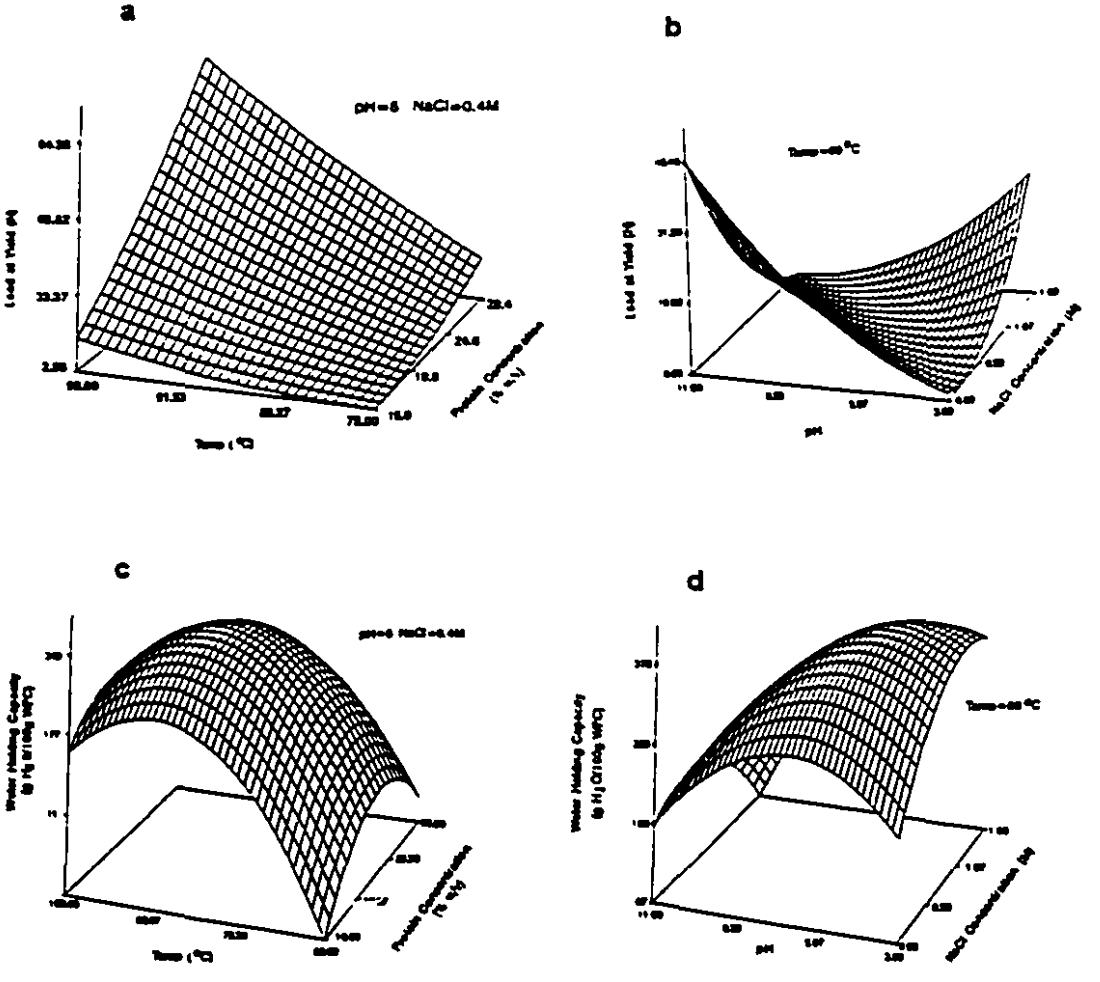



**Fig 4.7:** Relationship between (a) temperature, protein concentration and strength of WPC gels; (b) pH, NaCl concentration and strength of WPC gels; (c) temperature, protein concentration and water holding capacity of WPC gels; (d) pH, NaCl concentration and water holding capacity of WPC gels.

**Table 4.7: Interactive Effects of Factors studied in the CCRD on Water Holding Capacity, Turbidity and Strength of WPC Gels.**

| RSM # | WHC[1] (%) | Turbidity (%) | Gel Strength (N) |
|---|---|---|---|
| 1 | 193 | 49.7 | 0.0 |
| 2 | 322 | 5.3 | 5.4 |
| 3 | 169 | 33.1 | 0.0 |
| 4 | 288 | 5.5 | 32.7 |
| 5 | 1 | 60.8 | 0.0 |
| 6 | 289 | 6.4 | 12.9 |
| 7 | 53 | 49.8 | 0.0 |
| 8 | 56 | 7.4 | 54.6 |
| 9 | 297 | 39.1 | 0.0 |
| 10 | 389 | 5.6 | 9.5 |
| 11 | 256 | 38.1 | 0.0 |
| 12 | 328 | 10.6 | 54.2 |
| 13 | 1 | 59.2 | 0.0 |
| 14 | 314 | 11.2 | 9.6 |
| 15 | 66 | 58.7 | 0.0 |
| 16 | 287 | 9.2 | 30.5 |
| 17 | 1 | 66.7 | 0.0 |
| 18 | 311 | 6.7 | 20.6 |
| 19 | 214 | 35.0 | 2.2 |
| 20 | 233 | 27.3 | 19.6 |
| 21 | 245 | 25.3 | 0.0 |
| 22 | 302 | 23.4 | 16.9 |
| 23 | 375 | 12.2 | 20.2 |
| 24 | 237 | 42.7 | 0.0 |
| 25 | 362 | 30.4 | 5.3 |

[1] (g $H_2O$/100g WPC)

removed from the isoelectric point, intramolecular charge repulsion causes proteins to unfold (Harwalkar & Ma, 1989) which facilitates gel formation and may explain the increase in gel strength observed with increasing pH.

Fig 4.7c shows the effect of temperature and protein concentration on the WHC of WPC gels. Statistical analysis showed these two factors to be significant variables ($p \leq 0.001$) influencing the water holding capacity of the gels while pH and NaCl were not. In addition, the two factor interaction, pH x Temp and pH x pH significantly affected WHC ($p \leq 0.001$). In general, water holding capacity increased with increasing temperature and protein concentration, however, at a given protein concentration there was an optimum temperature above which no further increase in WHC was observed. Shimada & Cheftel (1988), reported in an earlier study that increasing the temperature of gels made from whey protein isolate, from 75-125°C had no effect on water holding capacity. The WHC of WPC (pH 3) heated at 80°C, increased with increasing NaCl concentration (Fig 4.7d); as the pH was increased, the WHC decreased to a minimum value at pH 11 and in the presence of 1.6M NaCl. In the absence of NaCl, the WHC increased to an optimum value when the pH was between 5.67 and 8.33 and then decreased with further increase in pH.

Changes in the turbidity measurements of the WPC gels with temperature are shown in Fig 4.8a. Statistical analysis showed the linear and cross product responses were significant ($p \leq 0.001$) while the quadratic response was not. Temperature and pH gave significant effects on the turbidity of the gels. With increasing temperature, a decrease in transmission (increase in turbidity, Fig 4.8a) was observed, which suggests an increase in aggregate and gel formation. Fig 4.8b shows the relationship between turbidity of WPC gels and a) pH and b) NaCl concentration. At a given pH, increasing NaCl concentration resulted in a decrease in the turbidity of the WPC gels suggesting

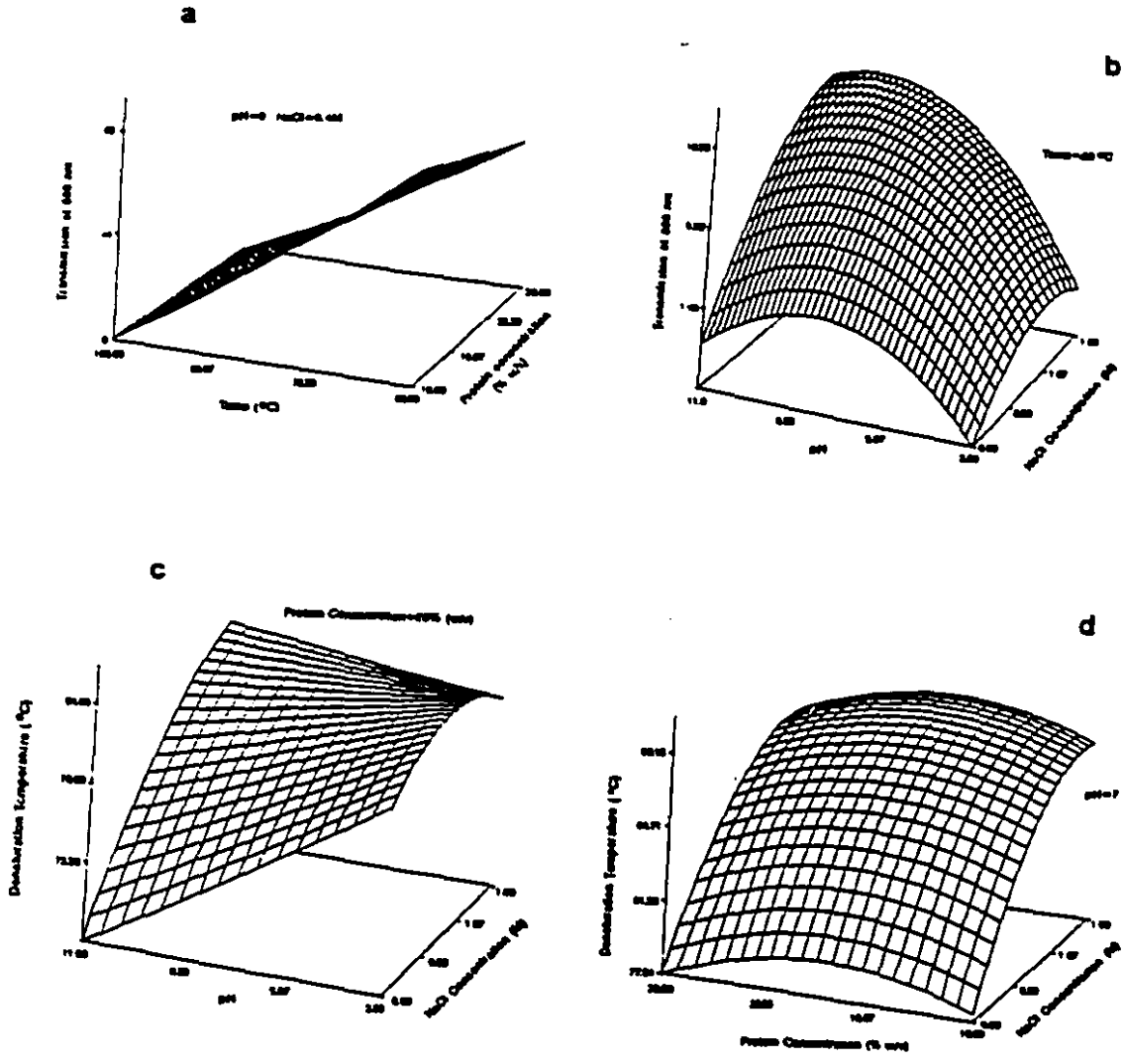



**Fig 4.8: Relationship between (a) temperature, protein concentration and turbidity of WPC gels; (b) pH, NaCl concentration and turbidity of WPC gels; (c) temperature, protein concentration and denaturation temperature of WPC; (d) pH, NaCl concentration and denaturation temperature of WPC gels.**

a decrease in gel formation. At low NaCl concentrations (<0.5M NaCl), higher transmission values (lower turbidity) were observed between pH 5.67 and 8.33, suggesting a decrease in aggregation, or reduced gel formation.

The temperature of denaturation is an important index in the study of protein gelation (Stanley & Yada, 1992). Below this temperature, no gelation occurs at any concentration. The effects of pH and NaCl on the temperature of denaturation of whey proteins is shown in Fig 4.8c. In the absence of NaCl, increasing pH resulted in a decrease in the denaturation temperature with the lowest denaturation temperature of 73.3$^{o}$C observed at pH 11; at a given pH, increasing NaCl concentration resulted in an increase in the denaturation temperature. Between pH 3 and 5, the denaturation temperature increased with increasing NaCl concentration to an optimum level at 1M NaCl. Above this concentration, no further increase in denaturation temperature was observed. Above pH 8, denaturation temperature increased linearly with increasing NaCl concentration with a maximum value at 1.6M NaCl.

## 4.5 CONCLUSION

The results obtained showed a direct relationship between gel formation, gel strength, turbidity and water holding capacity. These properties were affected by protein concentration, heating temperature and time, pH, NaCl and sucrose. At a given pH and protein concentration, gel strength and water holding capacity decreased with increasing NaCl concentration, while the temperature of denaturation increased. Increasing protein concentration resulted in an increase in gel strength. For any given treatment, increasing the heating temperature increased the strength of the WPC gels. At lower temperatures, increasing pH decreased the water holding capacity of WPC gels, but increased it at higher temperatures.

## CHAPTER 5

# EFFECTS OF PHYSICO-CHEMICAL FACTORS ON THE SECONDARY STRUCTURE OF β–LACTOGLOBULIN

## 5.0 CONNECTING STATEMENT

In Chapters 3 and 4, the individual and interactive effects of factors that affect whey protein gelation (e.g., heating temperature, pH) were studied. The results obtained showed that differences in the texture and strength of WPC gels were attributable to the responses of the individual whey proteins to the factors studied. The major proteins in whey protein concentrate are β-lactoglobulin, α-lactalbumin and bovine serum albumin; during gelation, the secondary structures of these proteins are altered. The specific changes induced in the structure of these proteins ultimately determine the textural characteristics of the WPC gel formed. The work described in this chapter addresses the effects of the factors studied in Chapters 3 and 4 on the denaturation, aggregation and gelation of β-lactoglobulin, the primary gelling protein in whey and satisfies objective 3a described in the "Rationale and Objectives of Study" section of Chapter 1.

**Note:** This chapter is the text of a paper which has been accepted for publication as follows:

Boye, J. I., Ismail, A., Alli, I., "Effect of physico-chemical factors on the secondary structure of β-lactoglobulin". *Journal of Dairy Research.* (Accepted, **March 1995**).

***Contributions of co-authors***:Ismail, A. (provided expertise in FTIR); Alli, I. (thesis supervisor).

## 5.1 ABSTRACT

Fourier transform infrared spectroscopy (FTIR) and differential scanning calorimetry (DSC) were used as complementary techniques to study changes in the secondary structure of β–lactoglobulin under various physico-chemical conditions. The effects of pH (3-9), NaCl (0 - 2M), and lactose, glucose and sucrose (100 - 500 g/l) in the temperature range 25 - 100°C on the conformation-sensitive amide I band in the infrared spectrum of β–lactoglobulin in $D_2O$ solution were examined. The 1692 $cm^{-1}$ band in the amide I band profile had not been definitively assigned in previous studies of the infrared spectrum of β–lactoglobulin. The decrease in this band at ambient temperature with time or upon mild heating was attributed to slow H-D-exchange, indicating that it was due to a structure buried deep within the protein. The disappearance of the 1692 $cm^{-1}$ band on heating was accompanied by the appearance of two bands at 1684 $cm^{-1}$ and 1628 $cm^{-1}$, assigned to β–sheets. The 1692 $cm^{-1}$ band was therefore attributed to a β–type structure. β–Lactoglobulin showed maximum thermal stability at pH 3 and was easily denatured at pH 9. On denaturation, the protein unfolded into more extensive random coil structures at pH 9 than at pH 3. After 10 h at pH 9 (25°C), β–lactoglobulin was partially denatured. Heating to 60-80°C generally resulted in the loss of secondary structure. At all pH values studied, two new bands at 1618 and 1684 $cm^{-1}$, characteristic of intermolecular β–sheet structure and associated with aggregation, were observed after the initial denaturation. DSC studies indicated that the thermal stability of β–lactoglobulin was enhanced in the presence of sugars. The FTIR results obtained provide evidence that sugars promoted the unfolding of β–lactoglobulin via multiple transition pathways leading to a transition state resisting aggregation.

## 5.2 INTRODUCTION

β–Lactoglobulin, the major protein component in the whey fraction of milk, is largely responsible for the aggregation and gelation of whey protein. The expression of these functional properties directly results from changes in the structure and conformation of the protein at the molecular level. β–Lactoglobulin has been reported to exist as a monomer below pH 3.5 (Timasheff *et al.*, 1966). Between pH 3.7 and 6.5 the protein reversibly aggregates to form octamers. Over the pH range 8.5 to 9.5 slow time-dependent changes occur leading to irreversible denaturation and aggregation of the protein above pH 9. Under physiological conditions the protein exists as a dimer (Hambling *et al.*, 1992), consisting of antiparallel β–sheets formed by nine β–strands. Circular dichroism and infrared studies suggest an α–helix content of 10-15%, β–structure content of approximately 50%, turns accounting for 20% and the remaining 15% representing amino acid residues in a random non-repetitive arrangement without well-defined structure (Timasheff *et al*, 1966; Susi & Byler, 1986; Casal *et al.*, 1988). Processing conditions such as pH and the concentration of salt, sugar and protein affect the textural characteristics of β–lactoglobulin (DeWit & Klarenbeek, 1981; Harwalkar & Ma, 1988; Arntfield *et al.*, 1990) through their effects on the conformation of the protein during heat treatment. Although the interrelationship between the molecular and functional properties of food proteins is qualitatively understood, quantitative prediction of the functional behaviour of proteins in food systems from a knowledge of their molecular properties has not been achieved (Damodaran, 1994). There is very little information in the literature on the sequence of changes that occur in the secondary structure of β–lactoglobulin during heat treatment, and this has greatly retarded the effective use of the protein in product development.

In this study, Fourier transform infrared (FTIR) spectroscopy and

differential scanning calorimetry (DSC), two techniques that have become widely accepted for studying the structural and thermal behaviour of proteins (Casal *et al.*, 1988; Byler & Farrell, 1989), were employed to monitor the temperature-induced conformational changes in the secondary structure of β–lactoglobulin under various conditions. The use of FTIR spectroscopy for the study of protein secondary structure is based primarily on the examination of C=O stretching bands in the region 1700-1600 $cm^{-1}$ (Susi & Byler, 1988). The positions of these bands are sensitive to differences in the orientation of molecular subgroups or changes in interchain and intrachain hydrogen-bonding interactions of peptide groups (Krimm & Bandekar, 1986). For proteins, each characteristic absorption band is generally a composite, consisting of overlapping components representing α–helical segments, β–sheet sectors, turns and unordered regions. These subbands usually cannot be resolved by conventional spectroscopic techniques because their inherent widths are greater than the instrumental resolution (Susi & Byler, 1986). Resolution enhancement techniques, such as Fourier self-deconvolution, are used to reveal the individual peaks attributable to the various secondary structures. This provides several advantages over conventional dispersive techniques such as higher resolution, sensitivity, signal-to-noise ratio and frequency accuracy (Susi & Byler, 1986). DSC, on the other hand, is used to detect thermally-induced conformational changes that result in unfolding or denaturation of proteins. The disruption of intramolecular hydrogen bonds which results in the initial unfolding of the protein is an endothermic process, while the aggregation and breakup of hydrophobic interactions, on the other hand, are exothermic reactions (Privalov, 1982). The overall enthalpy calculated from DSC measurements thus gives a sum of both the endothermic and exothermic transitions during heat treatment. A detailed study of changes in the secondary structure of β–lactoglobulin was undertaken using FTIR

spectroscopy and the conformational changes observed were correlated with the thermal transitions observed by DSC.

## 5.3 MATERIALS AND METHODS

### 5.3.1 Materials

β–Lactoglobulin (A+B) was obtained from Sigma (St. Louis,MO,63178-9916,USA) and used as received. $D_2O$ was from Aldrich (Milwaukee, WI,53233,USA).

### 5.3.2 Fourier Transform Infrared Spectroscopy

β–Lactoglobulin was dissolved in $D_2O$ at concentrations ranging from 10 to 400 mg/ml to study the effect of concentration on protein secondary structure. To study the effect of pH, the protein (200 mg/ml) was dissolved in deuterated phosphate buffer at a concentration of 0.2M (pH 3 and 5); 0.09M (pH 7) and 0.07M (pH 9). (In this work, pD = pH + 0.4. For the sake of clarity, pH is employed in place of pD). For the effect of ionic strength, NaCl solutions (0.5 - 2.0M) made in $D_2O$ were used in place of phosphate buffer. Infrared spectra were recorded with a 8210E Nicolet FTIR spectrometer equipped with a deuterated triglycine sulphate detector. A total of 512 scans were averaged at 4 $cm^{-1}$ resolution. Wavenumber accuracy was within $\pm$ 0.01$cm^{-1}$. The spectrometer was purged with dry air from a Balston dryer (Balston, Haverhill, MA, 01835-0723, USA). The samples were held in an i.r. cell with a 25-mm pathlength and $CaF_2$ windows. The temperature of the sample was regulated by placing the cell in a holder employing an Omega temperature controller (Omega Engineering, Laval, QC, H7L 5A1, Canada). The temperature was increased in 5°C increments and the cell allowed to equilibrate for 15 min prior to data acquisition. The reported temperatures are accurate to within $\pm$ 0.5°C. The

deconvolution of the infrared spectra was done as described by Kauppinen *et al.*, (1981). The signal to noise ratio was >20,000:1, and the bandwidth used for deconvolution was 13 $cm^{-1}$ with a narrowing factor of 2.4. All FTIR experiments were done in duplicate.

### 5.3.3 Differential Scanning Calorimetry

β–Lactoglobulin solutions (200 mg/ml) were prepared in $D_2O$ containing 0.5 - 2.0M NaCl to study the effect of ionic strength and phosphate-buffered $D_2O$ solutions (at the same pH values described for the FTIR study) for the effect of pH. The DSC (TA3000, Mettler Instrument Corporation, Greifensee, Switzerland)) was calibrated by use of indium standards. Twenty-five μl of each solution were placed in preweighed DSC pans, which were hermetically sealed and weighed accurately. The samples were placed in the DSC cell and scanned from 30°C to 120°C at a programmed heating rate of 5°C/min using an empty sample pan as reference. For satisfactory instrumental sensitivity, a protein concentration of 200 mg/ml was used for this study. All DSC experiments were done in triplicate.

## 5.4 RESULTS

### 5.4.1 Effect of concentration

The deconvoluted FTIR spectrum of deuterated β–lactoglobulin (pH unadjusted) in the 1700 to 1600 $cm^{-1}$ region, at 25°C (Fig 5.1), showed six principal bands for all the concentrations studied. These bands have been previously assigned by other workers (Susi & Byler, 1983; Casal *et al.*, 1988; Byler & Farrell, 1989). Apart from an increase in absorbance with increasing concentration, there appeared to be no differences among the spectra recorded

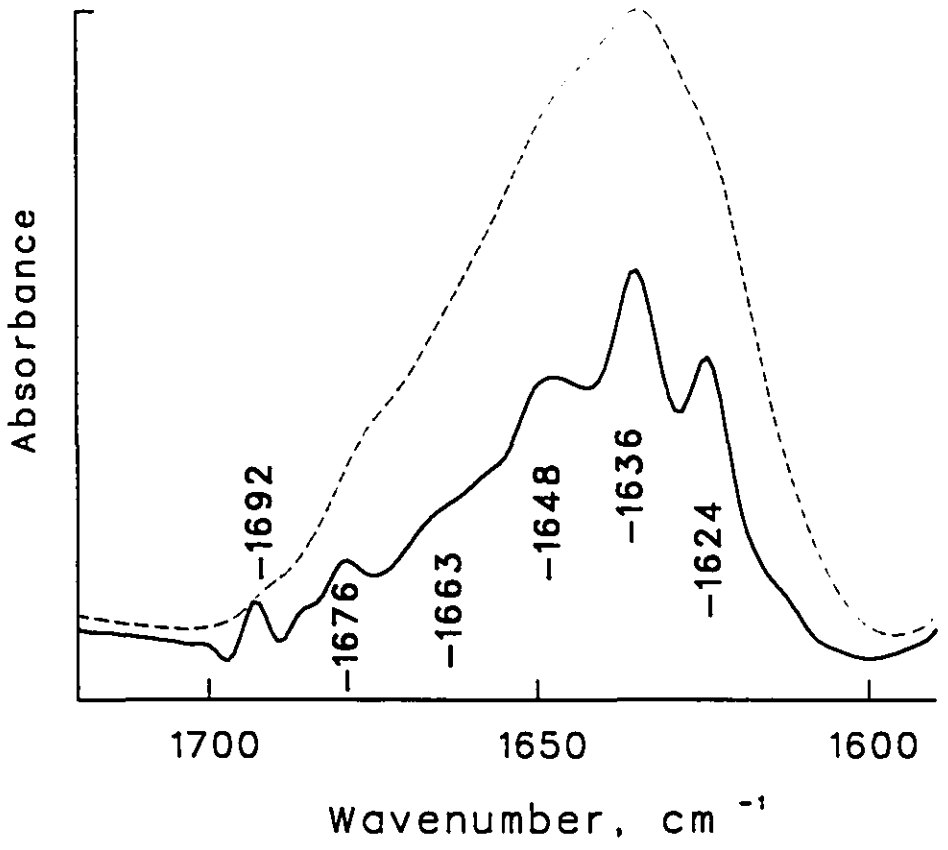



**Fig 5.1: Infrared spectra of ß-lactoglobulin in $D_2O$ (200 mg/ml, pH unadjusted, 26°C) before (----) and after (——) deconvolution. The numbers refer to the wavenumber ($cm^{-1}$) of the six main bands.**

from solutions of different protein concentration in the range of 10 to 400 mg/ml.

### 5.4.2 H-D exchange

Fig 5.2 shows a plot of the integrated intensity of the 1692 $cm^{-1}$ band in the spectrum of β–lactoglobulin in $D_2O$ as a function of time. The 1692 $cm^{-1}$ band gradually decreased in intensity, disappearing completely after approximately 50 h. The amide II' band (1550 $cm^{-1}$), attributed principally to N-H deformation, was observed to decrease at a parallel rate. In order to ascertain whether the disappearance of the 1692-$cm^{-1}$ band was due to H-D exchange or to conformational changes, two solutions of the protein, one in $H_2O$ and one in $D_2O$, were heated for 15 min at 59°C, cooled to room temperature, lyophilized, and redissolved in $D_2O$ and the FTIR spectra recorded. The 1692-$cm^{-1}$ band was absent in the spectrum of the β–lactoglobulin heated in $D_2O$ but present in that of the protein heated in $H_2O$, indicating that the changes in the 1692-$cm^{-1}$ can be attributed to H-D exchange. To confirm this, the heated solution of β–lactoglobulin in $D_2O$ was cooled and the protein was lyophilized, dissolved in $H_2O$, heated for 15 min at 59°C, lyophilized, and redissolved in $D_2O$. The spectrum recorded from this solution showed a band at 1692 $cm^{-1}$.

### 5.4.3 Effect of Elevated Temperatures

The FTIR spectra of β–lactoglobulin in deuterated phosphate buffer, pH 7, at temperatures between 30°C and 95°C (Fig 5.3) showed that the 1692 $cm^{-1}$ band disappeared at 65°C with the appearance of a new band at 1684 $cm^{-1}$ and a shift of the band at 1676 $cm^{-1}$ to 1674 $cm^{-1}$. The intensity of the 1684 $cm^{-1}$ band increased with increasing temperature while the 1674 $cm^{-1}$ band decreased in intensity and broadened between 75°C and 80°C. Major changes in the 1648, 1636 and 1624 $cm^{-1}$ bands were observed above 75°C. Between

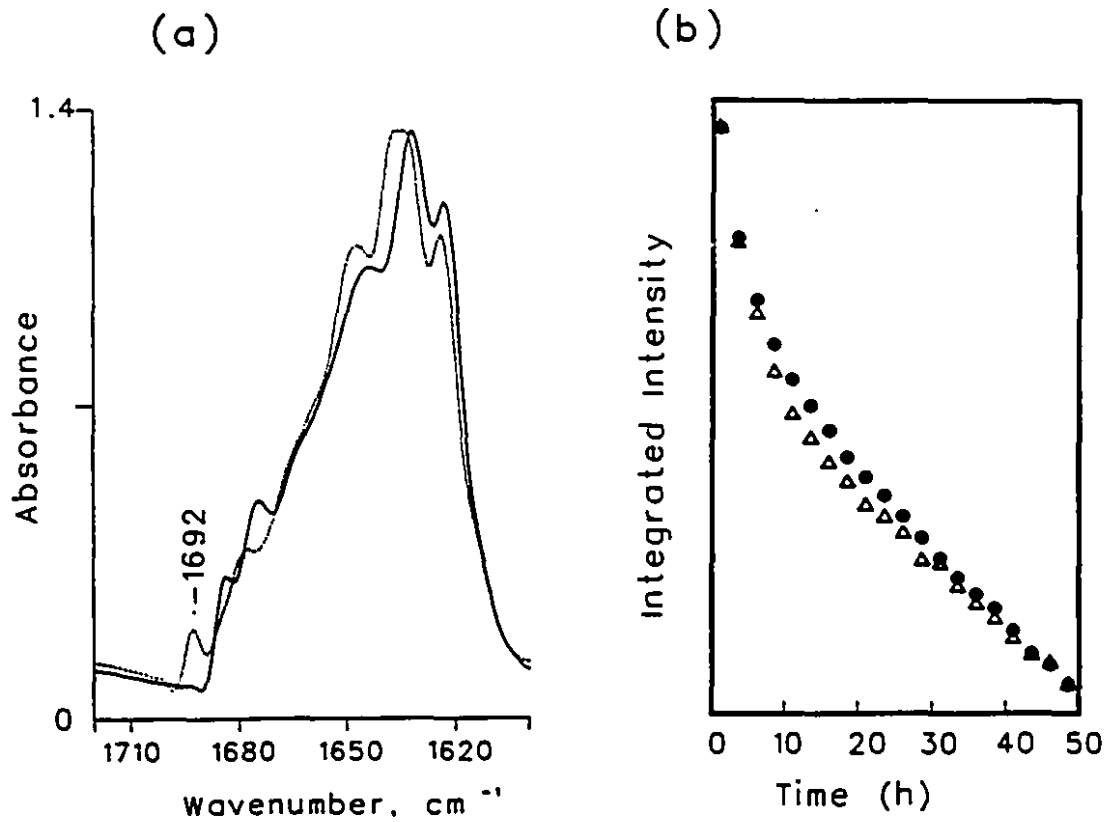



**Fig 5.2:** (a) Deconvoluted infrared spectra of ß-lactoglobulin in $D_2O$ (200 mg/ml, pH unadjusted) heated at 59°C in $H_2O$ (----) and in $D_2O$(——). (b) Integrated intensity of the 1692 $cm^{-1}$ (Δ) and amide II' (●)bands as a function of time at 25°C.

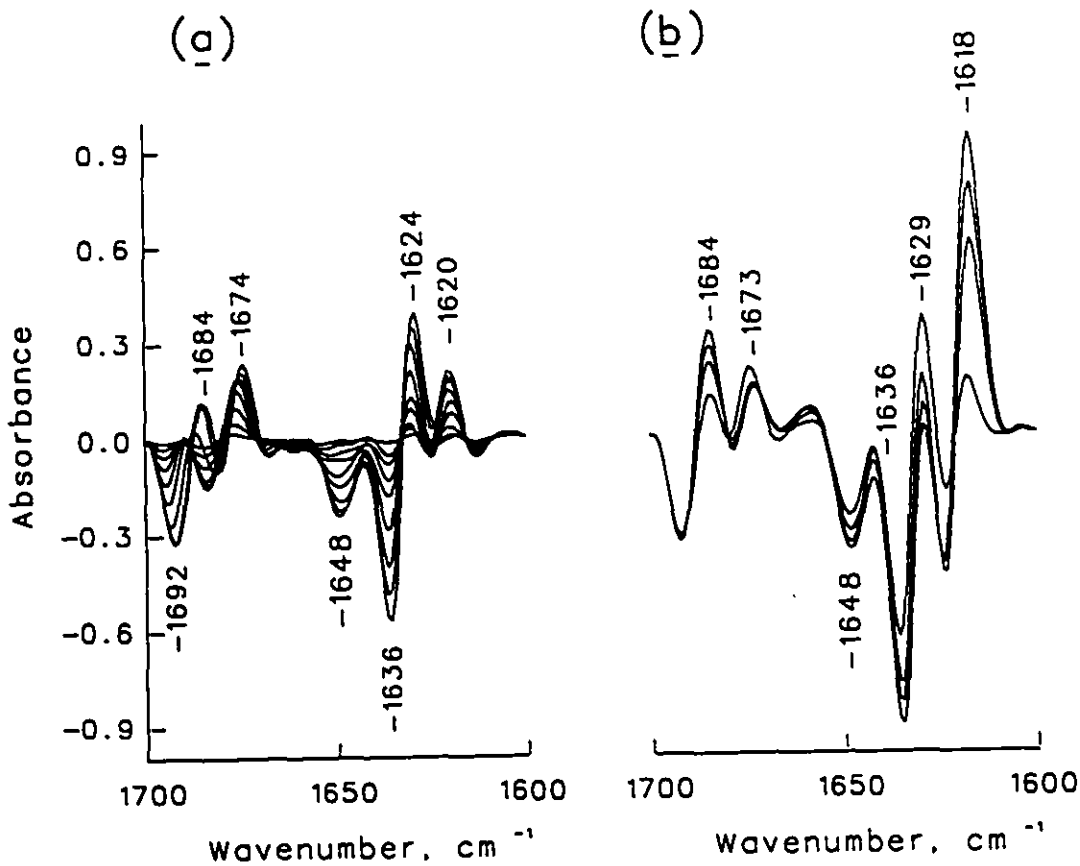



**Fig 5.3:** Superimposed i.r. spectra of ß-lactoglobulin (200 mg/ml) in 0.09M deuterated phosphate buffer(pH 7) as a function of temperature, after subtraction of the spectrum recorded at 30°C. Each line represents spectrum recorded at a given temperature (a) 30 - 75°C; (b) 80 - 95°C (at 5°C increments).

80 and 85°C, the rise of a new band at 1618 $cm^{-1}$ was observed along with a noticeable increase in the intensity of the 1684 $cm^{-1}$ band. On denaturation (Fig 5.3a), the major changes observed in the protein secondary structure were a decrease in the intensity of the 1648 $cm^{-1}$ band which has been attributed to an α–helical structure (Casal *et al.*, 1988; Susi & Byler, 1988); a substantial decrease in the intensity of the 1636 $cm^{-1}$ band attributed to anti-parallel β–sheet structures (Susi & Byler, 1988), an increase in the intensity of the 1629 and 1620 $cm^{-1}$ bands (other β–type structures) (Krimm & Bandekar, 1986), and an increase in the1674 $cm^{-1}$ band attributed to β–sheet structures by some workers (Casal *et al.*, 1988; Susi & Byler, 1988) and turns by others (Krimm & Bandekar, 1986). On aggregation (Fig 5.3b), the major changes observed were an increase in the intensity of the bands at 1684 and 1618 $cm^{-1}$ attributed to intermolecular hydrogen-bonded β–sheet structures (Ismail *et al.*, 1992), a slight decrease in the number of turns (1673 $cm^{-1}$) and further loss of both α–helical and β–sheet structures (1648, 1636 and 1629 $cm^{-1}$ bands). Heat treatment generally resulted in an increase of the half-width of the amide i band with a transition temperature at 75.7°C. The DSC thermogram of β–lactoglobulin (200 mg/ml in 0.09M deuterated phosphate buffer, pH 7) showed a peak temperature at 73°C, very close to the transition temperature obtained in the FTIR experiment.

### 5.4.4 Effect of temperature at different pH values

The FTIR spectra of β–lactoglobulin recorded immediately after dissolving the protein in deuterated phosphate buffer (200 mg/ml) at pH 3, 5, 7 and 9 are shown in Fig 5.4. An increase in the pH from 3 to 9 resulted in an increase in the intensity of the 1636 $cm^{-1}$ band relative to the 1624 $cm^{-1}$ band which can be attributed to an increase in the formation of β–sheet structures. After 10 h at pH

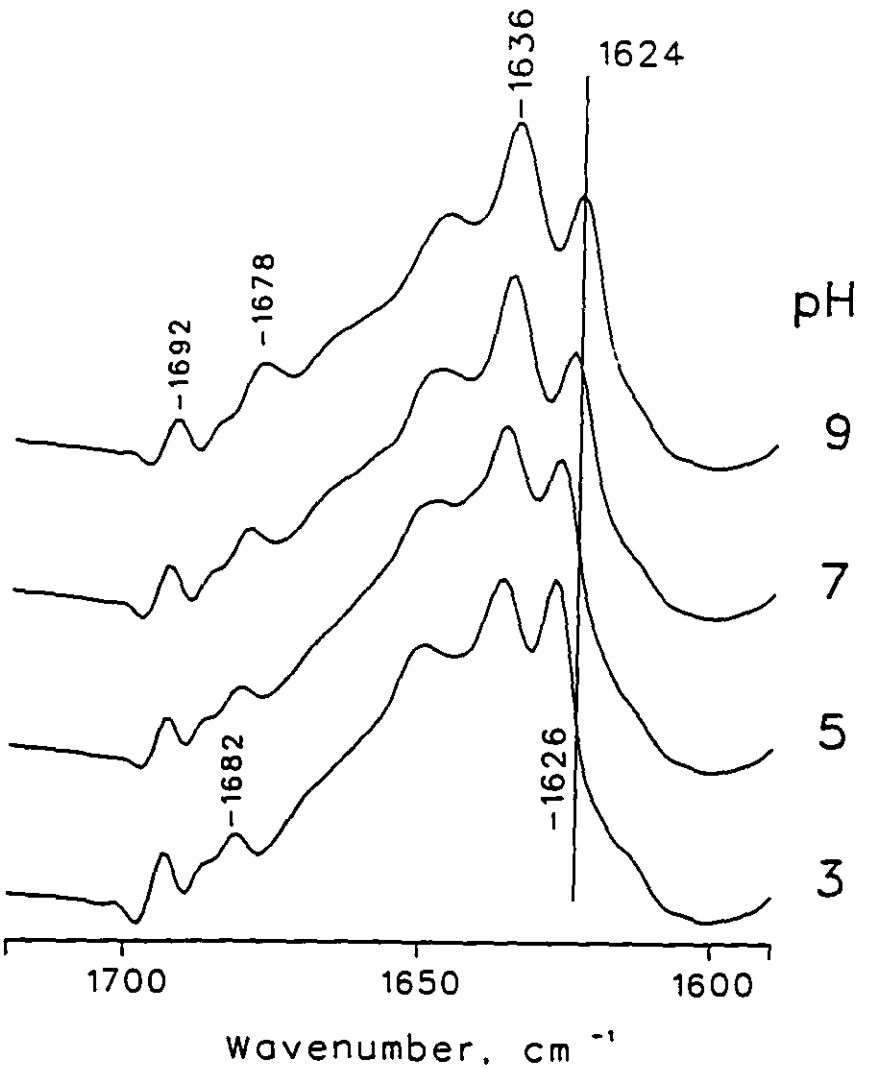



**Fig 5.4: Deconvoluted infrared spectra of ß-lactoglobulin (200 mg/ml, 26°C) after 15 min in deuterated phosphate buffer at the following concentrations 0.2M (pH 3 and 5), 0.09M (pH 7), 0.07M (pH 9).**

3, a decrease in the intensity of the band at 1626 $cm^{-1}$ was noted. This decrease was even more noticeable at pH 5. The changes over time at pH 7 were very minimal. At pH 9, the protein seemed to undergo partial denaturation (split of 1648 $cm^{-1}$ band to 1651 and 1644 $cm^{-1}$) with time, with a shift of the band at 1678 $cm^{-1}$ to 1676 $cm^{-1}$.

Fig 5.5 shows the FTIR spectra of β–lactoglobulin heated in the range of 26 to 97°C at pH 3, 5 and 9. At pH 3, the intensity of the 1636 $cm^{-1}$ band appeared to increase at the expense of the 1626 $cm^{-1}$ band on heating to 67°C. Maximum thermal stability of β–lactoglobulin was observed at this pH. The 1692 $cm^{-1}$ band was observed until 67°C, when it suddenly disappeared from the spectrum, along with the 1626, 1636, 1648, and 1682 $cm^{-1}$ bands. The β–sheet structure apparently "broke down" when the protein denatured at approximately 72°C. At 97°C, however, weak peaks could be observed between the 1618 and 1684 $cm^{-1}$ bands. At pH 5, the protein showed greater susceptibility to heat denaturation. The 1692 $cm^{-1}$ band disappeared above 55°C. A loss of secondary structure, indicated by major changes in the bands between 1682 and 1626 $cm^{-1}$, was observed at 63°C, followed by the formation of the aggregation bands at 1616 and 1684 $cm^{-1}$. The weak bands between the 1684 and 1618 $cm^{-1}$ bands observed at pH 3 (97°C) were not seen at pH 5, indicating a greater degree of unfolding.

β–Lactoglobulin showed most susceptibility to heat denaturation at pH 9. The 1692 $cm^{-1}$ band disappeared from the FTIR spectra above 43°C. Major changes in the bands at 1648, 1635 and 1624 $cm^{-1}$, which are associated with denaturation, were observed above 51°C. Extensive unfolding into random coils resulted in the broadening of the bands at 1635 and 1648 $cm^{-1}$ between 51 and 59°C. The 1618 $cm^{-1}$ band, attributed to aggregation, was not observed until 59°C, indicating that the denaturation was least cooperative at pH 9. Table 5.1

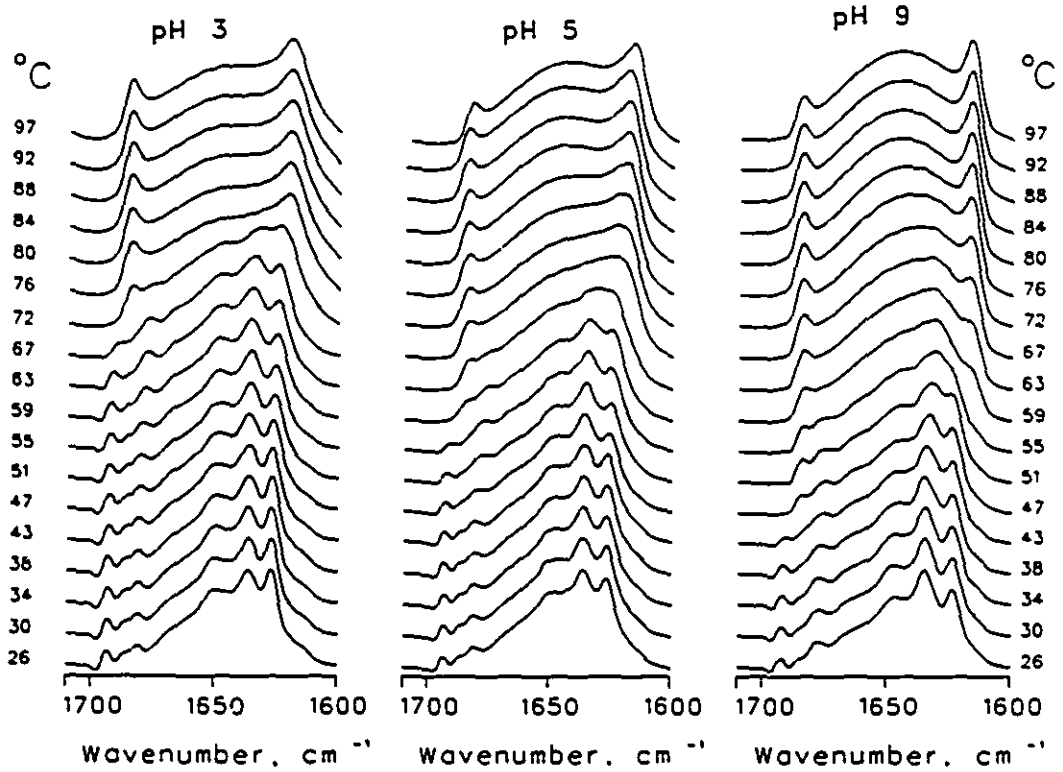



**Fig 5. 5: Stacked plot of deconvoluted infrared spectra of ß-lactoglobulin (200 mg\ml) in deuterated phosphate buffer at the following concentrations 0.2M (pH 3 and 5),0.07M (pH 9) as a function of temperature.**

**Table 5.1** **Transition temperatures on heat treatment of β–lactoglobulin by Fourier Transform Infrared Spectroscopy and Differential Scanning Calorimetry**

| pH* | 1692cm$^{-1}$[a] | Denaturation Temp.(°C) | | Aggregation Temp. (°C) | |
|---|---|---|---|---|---|
| | | FTIR[b] | DSC[c] | FTIR[d] | DSC[e] |
| 3 | 72 | 72 | 70 | 76 | 84 |
| 5 | 59 | 63 | 64 | 72 | 82 |
| 7 | 51 | 55 | 55 | 72 | 73 |
| 9 | 47 | 51 | 56 | 67 | 70 |

a - Temperature at which the 1692 cm$^{-1}$ band disappeared

b - Temperature at which the 1648, 1636 and 1624 cm$^{-1}$ bands disappeared

c - Starting temperature of peak in DSC thermogram

d - Temperature at which 1618 cm$^{-1}$ band distinctly observed

e - Peak temperature in DSC thermogram

* - (ß-lactoglobulin (200 mg/ml) in deuterated phosphate buffer at the following concentrations, 0.2M (pH 3 and 5); 0.09M (pH 7); 0.07M (pH 9).

shows the DSC characteristics of β–lactoglobulin heated from 30°C to 120°C at pH 3, 5, 7 and 9. The starting temperature of the peak of the DSC thermogram was observed at 70°C at pH 3 but decreased to 56°C at pH 9. The FTIR results showed a similar decrease in denaturation temperature with the disappearance of the 1648, 1636 and 1624 $cm^{-1}$ band occuring at 72°C at pH 3 and 51°C at pH 9. The highest peak temperature of the DSC thermogram was observed at 84°C at pH 3 and decreased linearly with increasing pH to 70°C at pH 9.

### 5.4.5 Effect of NaCl

No changes were observed in the secondary structure of β–lactoglobulin dissolved in up to 2M NaCl solution after 20 h at 25°C. Fig 5.6 shows the infrared spectra of β–lactoglobulin heated in 0.5 and 2.0M NaCl solutions. Increasing the concentration of NaCl appeared to increase the thermal stability of β–lactoglobulin. The 1692 $cm^{-1}$ band disappeared above 51°C in 0.5M NaCl solution and above 59°C in the 2M solution. The intensity of the bands at 1648, 1636 and 1628 $cm^{-1}$ was noticeably reduced by 76°C in the 2M NaCl solution but by 67°C in the 0.5M NaCl solution. At 92°C, the aggregation band was observed at 1617 $cm^{-1}$ in the spectrum of the 0.5M NaCl solution and at 1619 $cm^{-1}$ in the spectrum of the 2M NaCl solution. A plot of the width at half-height of the amide I band (not shown) gave a transition point of 69.5°C for the 0.5M NaCl solution and 76.5°C for the 2M NaCl solution, suggesting that higher temperatures were required to disrupt the native protein structure at higher ionic strength. This increase in thermal stability was also observed from the DSC analysis (Fig 5.7). The peak temperature of denaturation increased from 79.5°C in 0.5M NaCl to 85.2°C in 2M NaCl.

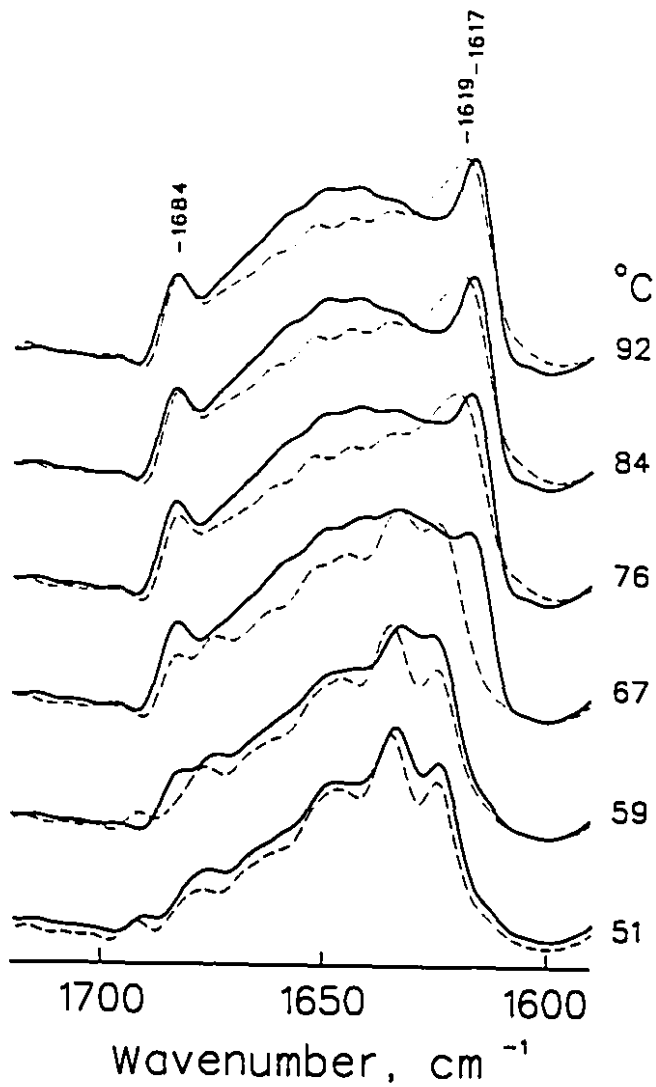



**Fig 5.6: Stacked plot of deconvoluted infrared spectra of ß-lactoglobulin (200 mg/ml, pH unadjusted) as a function of temperature in 2M NaCl (——) and 0.5M NaCl solution (——).**

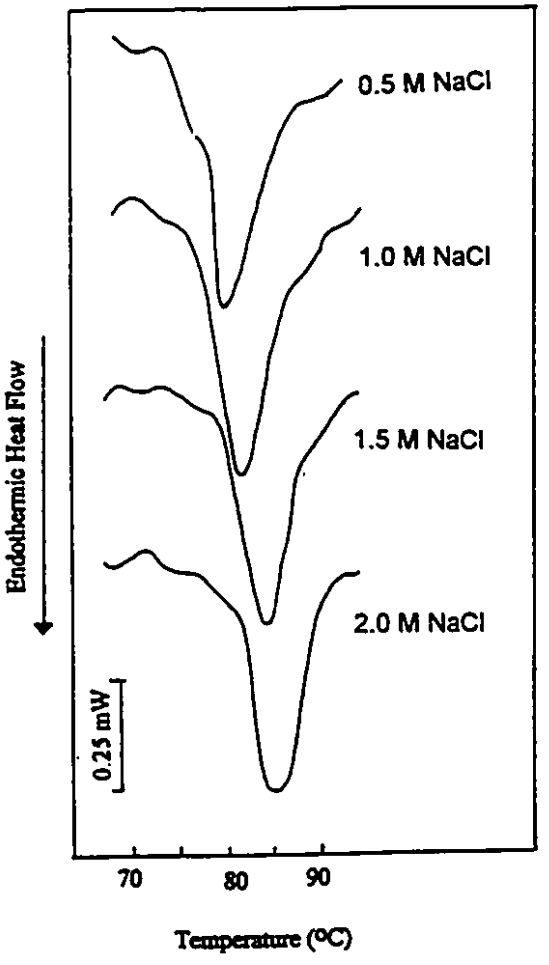



Fig 5.7: Effect of NaCl concentration on DSC characteristics of β-lactoglobulin (200 mg/ml $D_2O$, pH unadjusted). Heating rate was 5°C/min.

### 5.4.6 Effect of Sugars

Fig 5.8 shows the i.r. spectra of β–lactoglobulin heated in 100 and 500 g/l glucose solutions. The amide I band began to broaden at 59°C in the 100 g/l glucose solution and at 63°C in the 500 g/l glucose solution, indicating the loss of native structure. The band associated with aggregation (1618 $cm^{-1}$) was apparent at 67°C in 100 g/l glucose and at 76°C in the 500 g/l glucose. The effect of 500 g/l sucrose was similar to that of glucose except that the characteristic broadening of the amide I band started at 55°C, and substantial aggregation was not observed until 80°C. From the DSC analysis (Fig 5.9), peak denaturation temperatures of 75.5°C and 78.7°C were obtained with the 100 g/l and 500 g/l sucrose solutions, respectively, and 78.3°C and 85.0°C were obtained with the glucose solutions at the same concentrations. A plot of the width of the amide I band at 0.4 height as a function of temperature showed a break in the transition from the native to the aggregated state between 70 and 80°C when β–lactoglobulin was heated in 500 g/l sucrose. No such break was evident in the plot for β–lactoglobulin heated in a 200 g/l lactose. This break in the transition could be due to a stabilization effect of sucrose on the partially denatured protein as was also observed with the 500 g/l glucose. Sucrose, however, seemed to have a greater effect in inhibiting aggregation than glucose. Lactose had the least effect in stabilizing the protein against denaturation and in inhibiting aggregation, and its effect was also not concentration dependent.

## 5.5 DISCUSSION

When $D_2O$ is used as solvent in infrared studies, deuteration of the proteins in solution causes a weakening of the C=O bonds involved in hydrogen/deuterium bonding and a transformation of N-H groups to N-D

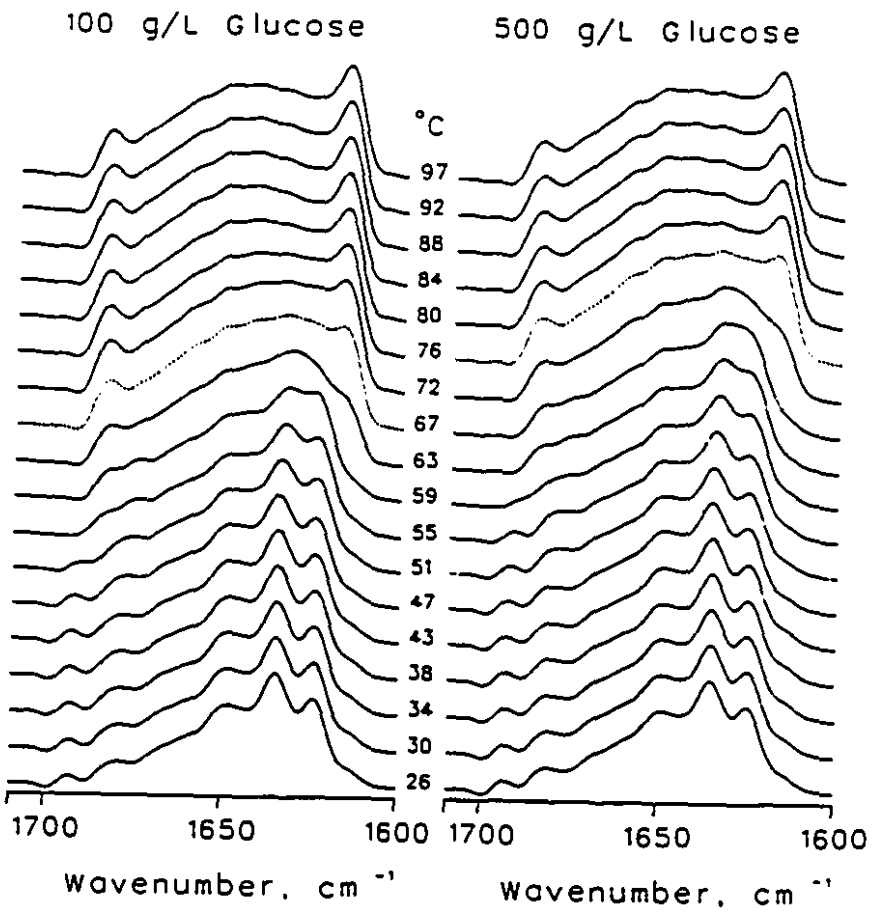



**Fig 5.8: Stacked plot of deconvoluted infrared spectra of ß-lactoglobulin (200 mg/ml, pH unadjusted)as a function of temperature in 100 and 500 g/l glucose solutions. (—— - temperature at which aggregation band was observed).**

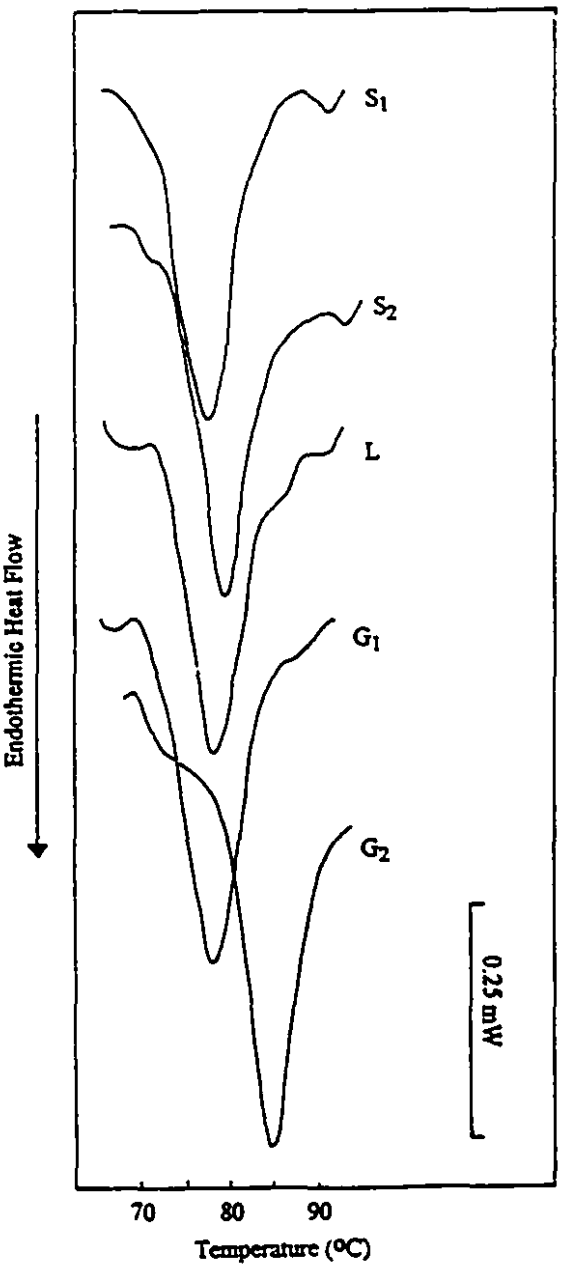



Fig 5.9: Effect of Glucose ($G_1$-100g/l; $G_2$-500g/l), Lactose (L-200g/l), Sucrose ($S_1$- 100g/l; $S_2$-500g/l) on DSC characteristics of ß-lactoglobulin (200 mg/ml $D_2O$, pH unadjusted).

groups. This results in a shift of the amide II band from approximately 1550 $cm^{-1}$ to 1450 $cm^{-1}$ (Krimm & Bandekar, 1986) as well as a shift of some of the amide I bands to lower wavenumbers. The similarity between the rate of disappearance of the 1692 $cm^{-1}$ and the 1550 $cm^{-1}$ bands strongly suggests that its disappearance was due to H-D exchange. The length of time required for it to disappear, coupled with the concomitant appearance of the two new bands at 1684 $cm^{-1}$ and 1629 $cm^{-1}$, suggest that it is due to a β–structure in the interior of the protein, well shielded from the surrounding solvent.

Above pH 7, β–lactoglobulin has been reported to possess increased reactivity which induces a unique phenomenon of intra- or intermolecular thiol-disulfide interchange reactions (De Wit, 1989). This could explain the sharp increase in the β–sheet content (1635/1636 $cm^{-1}$ band) relative to β–strand (1624 $cm^{-1}$ band), at pH 7 and pH 9. McKenzie (1971), found that above pH 8, β–lactoglobulin slowly undergoes time-dependent changes following an initial transition. ORD studies have shown that around pH 11 the α–helix remains intact whilst the β–sheet is converted to a random conformation (Timasheff *et al.*, 1966). In complete contrast, infrared spectroscopy shows that it is the α–helix which unfolds first in the initial stage of alkali denaturation (Casal *et al.*, 1988). The partial denaturation observed at pH 9 is consistent with results reported by McKenzie (1971) and (Casal *et al.*, 1988). The changes observed at alkaline pH could be attributed to changes in the secondary structure of the helical portion of the molecule which result in the partial unfolding of a tight helix and the formation of more unordered structure.

The weak intensity of the aggregation band in the spectrum of the protein in the 2M NaCl solution, in comparison with that of the protein in the 0.5M NaCl solution, suggests that β–lactoglobulin was less aggregated at high salt concentrations than at low salt concentrations. The shift to higher wavenumbers

with increasing NaCl concentration may have been related to a decrease in hydrogen (deuterium) bonding, which may be explained in terms of the unavailability of water ($D_2O$) molecules for protein solvation at higher salt concentrations.

Most of the studies done to date on β–lactoglobulin using the DSC, have reported the peak temperature of the DSC thermogram as the denaturation temperature of the protein (Harwalkar & Ma, 1989; Arntfield *et al.*, 1990). However, a comparison of the DSC results with those obtained from the FTIR spectra showed a similarity between the starting temperatures of the peaks from the DSC and the denaturation temperatures from the FTIR spectra (loss of 1648, 1636, and 1624 $cm^{-1}$ bands). The peak temperatures obtained from the DSC thermogram, on the other hand, were similar to the temperatures at which distinct aggregation bands (1618 and 1684 $cm^{-1}$ bands) could be observed in the FTIR spectra. This suggests that denaturation of β–lactoglobulin actually occurred at an earlier temperature than that signified by the peak DSC temperature, which appears to be more indicative of aggregation than denaturation.

The results obtained from the FTIR generally failed to show a stabilizing effect of sugars on native protein structure. However, the sugars appeared to stabilize partially denatured β–lactoglobulin, inhibiting its aggregation. Lee & Timasheff (1981), suggested that as the level of sugars increased, there is an increase in solvent cohesive force which increases the energy required for cavity formation for the associated structures in the solvent. The energy required for formation of aggregates therefore becomes prohibitive.

The results obtained from this study confirm findings of other workers who used circular dichroism and the related technique of optical rotatory dispersion to study differences in the conformation of β–lactoglobulin at various pH values (Bell & Mckenzie, 1967; Su & Jigersons, 1977; Dufour & Haertlé, 1990). There is

however, very little information, if any, on the actual mechanism by which these treatments induce changes in the secondary structure of β–lactoglobulin during heat treatment. In all the cases studied, our results suggest that the mechanism of heat denaturation of β–lactoglobulin , is an initial unfolding of a β–type structure in the interior of the protein, which results in the formation of more extensive random coil structures, followed by an unfolding of the α–helical structures and formation of intermolecular β–sheet structures, resulting in aggregate formation. The extent of this aggregate formation was found to be dependent on the physico-chemical conditions of the study. This work reveals the potential applicability of FTIR spectroscopy in monitoring changes in the secondary structure of proteins under a variety of conditions. Future work will be directed toward the systematic elucidation of the conformational changes induced in the secondary structure of other whey proteins.

## CHAPTER 6

# USE OF DIFFERENTIAL SCANNING CALORIMETRY AND INFRARED SPECTROSCOPY IN THE STUDY OF THERMAL AND STRUCTURAL STABILITY OF α–LACTALBUMIN.

## 6.0 CONNECTING STATEMENT

The results obtained in Chapter 5 showed the effect of various environmental conditions on the thermal and structural properties of β-lactoglobulin, the major protein in whey. By use of differential scanning calorimetry and Fourier transform infrared spectroscopy, a relationship between the thermal transitions occurring during heat treatment and changes in the secondary structure of β-lactoglobulin was established. The work described in this chapter addresses the response of α-lactalbumin to the factors studied in Chapter 5 and satisfies objective 3b in the "Rationale and Objectives of Study" section in Chapter 1.

**Note:** This chapter is the text of a paper which has been submitted for publication as follows:

Boye, J.I., Alli,I., Ismail,A., " Use of Differential Scanning Calorimetry and Infrared Spectroscopy to study the thermal and structural stability of α-lactalbumin". *Int. J. Pept. Prot. Res.* (Submitted, **May 1995**).

***Contributions of co-authors***: Alli, I. (thesis supervisor); Ismail, A. (provided expertise in FTIR).

## 6.1 ABSTRACT

Structural changes of α–lactalbumin (α–lac) in response to pH, ionic strength, sugars and heat treatment were investigated by use of differential scanning calorimetry (DSC) and Fourier transform infrared (FTIR) spectroscopy. From DSC, two reversible transitions at 39.6°C (A) and 64.8°C (B) were observed when α–lac was heated. At pH 3, transition A was partially reversible (14%) while transition B was completely reversible. Heating α–lac at pH 3 resulted in the formation of aggregate structures with no observed gelation. At pH 9, transitions A and B were completely irreversible with formation of a translucent gel. FTIR showed that at pH 3, denaturation of α–lac resulted in the appearance of two bands at 1616 and 1685 $cm^{-1}$ attributed to intermolecular aggregation. These bands were absent at alkaline pH, in holo-α-lac, in the presence of glucose and sucrose and at NaCl concentrations of less than 1.5M. Apo-α–lac showed a thermal transition at 42.5°C and holo-α–lac at 70.1°C. FTIR showed holo-α–lac to be more reversible than the apo- conformer. The secondary structure of apo-α–lac at 25°C was similar to that of denatured holo-α–lac. The major effects of heat treatment of α–lac were; loss in the bands at 1650, 1639 and 1627$cm^{-1}$ attributed to α–helix, $3_{10}$-helix and β–sheet respectively, and increase in the bands at 1670 and 1661 $cm^{-1}$ attributed to turns.

## 6.2 INTRODUCTION

α–lactalbumin (α–lac) is the second major globular protein in the whey of milks from various mammalian species. It is a low molecular weight (14.2 kDa) compact metalloprotein accounting for 20% of the proteins in bovine whey. α–lac is a modifier protein of the lactose synthase complex in the mammary cell with eight cysteine residues which exist as four intramolecular disulfides. The protein contains 123 amino acid residues with a single bound calcium ion and is capable of binding zinc as well as other metals (Hiraoka *et al.*, 1980; Bernal & Jelen, 1984; Stuart *et al.*, 1986; Kronman, 1989). Crystallographic studies reveal a close structural homology between α–lac and lysozyme (Smith *et al.*, 1987). As in lysozyme, the compact globular structure contains several regions of regular secondary structure including four α–helices, several regions of $3_{10}$-helix, and an antiparallel β–pleated sheet separated by irregular β–turns (Acharya *et al.*, 1989; Brew & Grobler, 1992). The existence of a hydrophobic box structure in bovine α–lac that is very similar to that of hen's egg white lysozyme has been reported (Koga & Berliner, 1985).

α–lac is considered as the most heat-stable whey protein. Removal of the bound $Ca^{++}$ reduces the stability of the native tertiary structure (Hiroaka *et al.*,1980). The conformation around the $Ca^{++}$ bound region consists of a helix-turn-helix motif with residues 76-82 forming the first helix ($3_{10}$-helix) and residues 85-93 forming the second α–helix (Brew & Grobler, 1992). Kuwajima *et al.*, (1986) found that the $Ca^{++}$-bound and -free forms assume essentially the same folded conformation, based on CD and proton NMR studies; they concluded that the stability of the folded state is enhanced by $Ca^{++}$.

In solution, α–lac undergoes intermolecular interactions leading to varying degrees of polymerization on both sides of its zone of insolubility (~ pH 4.8; Shukla, 1973). At acidic pH values, the protein undergoes a rapid reversible

association and slow aggregation (Kronman *et al.*, 1964; Kronman & Andreotti, 1964). Between pH 6 and 8.5 there is very little association and above pH 9.5, there is expansion without aggregation (Kronman *et al.*, 1967).

Denaturation of the protein involves dissociation of $Ca^{++}$ (Mckenzie & White,1991); this occurs regardless of whether the protein is at pH values below 4.0 or above 9.0, is heated above 50°C, exposed to low concentrations of guanidine hydrochloride, or subjected to $Ca^{++}$ removal from the native form. These denaturing conditions result in a transition of the native state to a stable transient state designated as the "A" or the "molten globule" state that is different from the unfolded denatured (U) state (Ptitsyn, 1987; Kuwajima, 1989). The molten globule state has been described as a compact intermediate protein conformation that has a secondary structure content like that of the native state but with a poorly defined tertiary structure (Lala & Kaul, 1992). α-lac has also been shown to refold to its native conformation after exposure to denaturing conditions (Brew & Grobler, 1992); when a chelator (e.g., EGTA) is added to bind endogenous $Ca^{++}$ only 2% or less of active α-lac is regenerated as compared with as much as 90 to 100% in the presence of 100μm $Ca^{++}$. This further suggests a $Ca^{++}$ dependence for refolding.

Results from X-ray scattering experiments have shown that the amino acid residues are densely packed in the interior of the molten globule structure (Timchenko *et al.*, 1986; Damaschun *et al.*, 1986). Hydrogen-deuterium exchange experiments using NMR show that there are specific regions of particular stability in the secondary structure which fluctuate little at room temperature, while other regions are more labile and afford only marginal protection from solvent exchange (Baum *et al.*, 1989).

Inspite of the tremendous advances made in determining the three-dimensional structure and metal-binding properties of α–lac, there is

comparatively less information about its structure and functionality in different environments. Salts for example, alter the native conformation of food proteins and affect their temperature of denaturation (Arntfield *et al.*, 1990). Sugars and polyols stabilize proteins against heat denaturation by enhancing the structuring of water which indirectly strengthens hydrophobic interactions and stabilizes protein conformation (Back *et al.*, 1979). In this study the combination of differential scanning calorimetry (DSC) and Fourier transform infrared spectroscopy (FTIR) were used to investigate the thermal and structural changes in α–lac on heat treatment, in particular its refolding behavior after heat treatment. DSC has been established as a technique for studying thermal denaturation and conformational transition of proteins and is used in the study of various food systems (Arntfield *et al.*, 1990). Infrared spectroscopy constitutes one of the oldest methods for studying the secondary structure of polypeptides and proteins. Several systematic studies of the IR spectra of α–lac in $D_2O$ have been reported (Prestrelski *et al.*, 1991a,b). More recently, the application of resolution enhancement and band narrowing techniques in FTIR spectroscopy has increased its use in protein research. The Amide I' band (1700-1620 $cm^{-1}$) which arises mainly from backbone C=O stretching is sensitive to small variations in molecular geometry and hydrogen bonding. Different patterns of folding of the peptide backbone result in the rise of bands at discrete frequencies (Krimm & Bandekar, 1986; Prestrelski *et al.*, 1991a) within the Amide I' band. These two techniques were used to study the unfolding and refolding behavior of α–lac (both apo- and holo-forms) at various pH values, in the presence of NaCl, sucrose and glucose.

## 6.3 MATERIALS AND METHODS

### 6.3.1 Materials

α–lactalbumin (Product L6010 containing 0.3 moles $Ca^{++}$ per mole of α–lac) was obtained from Sigma Chemical Co. (St.Louis,MO,USA) and used as received. Deuterium oxide (Product 15,188-2, minimum 99.9 atom % D) was purchased from Aldrich (Milwaukee,WI,USA).

### 6.3.2 Sample Preparation

Solutions of α–lac (mixture of apo- and holo-forms) were prepared by dissolving the protein in deuterium oxide to make a 20% (w/v) concentration; holo-α-lac was obtained by adding $CaCl_2$ to induce $Ca^{++}$ binding of the apo-conformers. The final concentration of $CaCl_2$ was 0.5M. The apo-conformer was obtained by dissolving the protein in deuterated $Na_2$-EDTA (ethylene diamine tetra sodium salt, pH 6.8, 0.1M) and EGTA (ethylene glycol bis-(β–aminoethylether)-N,N'-tetra acetate, 0.1M, pH adjusted with NaOH to 6.8) solutions. $Na_2$-EDTA and EGTA were used as chelating agents for $Ca^{++}$ (Relkin *et al.*, 1993; Prestrelski *et al.*, 1991b).

To study the effect of ionic strength and sugars, the protein was dissolved in $D_2O$ containing 0.5 to 1.5M NaCl, 10 and 50% (w/v) glucose and sucrose.

For the pH studies, the protein was dissolved in deuterated phosphate-buffer at pH 3, 5, 7 and 9 (ionic strength-0.2). In this work, pD = pH + 0.4. For the sake of clarity, pH is employed in place of pD.

### 6.3.3 Differential Scanning Calorimetry (DSC)

Twenty-five μl of each solution were placed in preweighed DSC pans, which were hermetically sealed and weighed accurately. The samples were placed in the DSC (TA3000, Mettler Instrument Corporation, Greifensee,

Switzerland) and scanned from 15°C to 100°C at a programmed heating rate of 5°C/min. For each run, a sample pan containing the deuterated buffer used for dissolving α–lac was used as reference. After heating, the samples were allowed to cool to room temperature in the DSC and the heating cycle was repeated under the same experimental conditions. The degree of reversibility was determined from the ratio of the areas under the second and the first endothermal peaks (Relkin *et al.*, 1993). The DSC was calibrated by use of indium standards. All DSC experiments were done in triplicate.

After scanning in the DSC, the pans were opened and visually checked for gelation: a white non-fluid firm texture was classified as an opaque gel; a clear non-fluid firm texture was classified as a translucent gel; a whitish coagulum that exhibited flow properties was classified as an aggregate (Clark & Lee-Tuffnel, 1986; Boye *et al.*, 1995a).

### 6.3.4 Fourier Transform Infrared Spectroscopy (FTIR)

α–lactalbumin (10 - 20% w/v) dissolved in deuterium oxide solutions as described above were subjected to infrared spectroscopy. Infrared spectra were recorded with a 8210E Nicolet FTIR spectrometer equipped with a deuterated triglycine sulfate detector. A total of 512 scans were averaged at 4 $cm^{-1}$ resolution. Wavenumber accuracy was within $\pm$ 0.01$cm^{-1}$. The spectrometer was purged with dry air from a Balston dryer (Balston, Haverhill, MA, 01835-0723, USA). The samples were held in an IR cell with a 25- μm pathlength and $CaF_2$ windows. The temperature of the sample was regulated by placing the cell in a thermostat holder employing an Omega temperature controller (Omega Engineering, Laval, QC, H7L 5A1, Canada). The temperature was increased in 5°C increments and the cell allowed to equilibrate for 3 min prior to data acquisition except in the case of the NaCl concentration and pH studies where

the spectra was recorded at the temperatures indicated. The reported temperatures are accurate to within ± 0.5°C. Deconvolution of the observed spectra were performed using the Nicolet FTIR Soft ware, Omnic 1.2a. The deconvolution of the infrared spectra was done as described by Kauppinen *et al.* (1981). The signal to noise ratio was >20,000:1, and the bandwidth used for deconvolution was 27 $cm^{-1}$ with a narrowing factor of 2.6. All FTIR experiments were done in duplicate.

## 6.4 RESULTS AND DISCUSSION

### 6.4.1 Thermal Stability

Fig 6.1 shows the DSC thermograms of α–lac heated in $D_2O$, $Na_2$–EDTA (0.1M) and $CaCl_2$(0.5M). Two transitions at 39.6±0.2 °C (peak A) and 64.8±0.5 °C (peak B) with enthalpies (calculated as the area under the peaks) of 0.95±0.11 J/g and 0.52±0.01 J/g respectively, were observed with α–lac in $D_2O$ (Fig 6.1i.a). Cooling of the sample, followed by reheating (second heating cycle) (Fig 6.1i.b) resulted in the reappearance of peaks A and B indicating that these transitions were reversible. The enthalpies of transitions A and B in the second heating cycle were 0.58±0.07 J/g and 0.53±0.3 J/g respectively which indicates a percent reversibility of 61% for transition A, and 100% reversibility of transition B. After the fourth heating cycle, the enthalpy of transition A was 0.07±0.1 J/g and 0.55±.04 J/g for transition B (Table 6.1). No transitions were observed in the fifth heating cycle. In $Na_2$–EDTA, only one thermal transition was observed at 42.5±0.4 °C (Peak C, Fig 6.1.ii). Like the transitions at 39.6°C and 64.8°C (peaks A and B) this transition was also reversible. The enthalpy of this transition decreased from 1.5±.001 J/g to 0.74±.003J/g on reheating

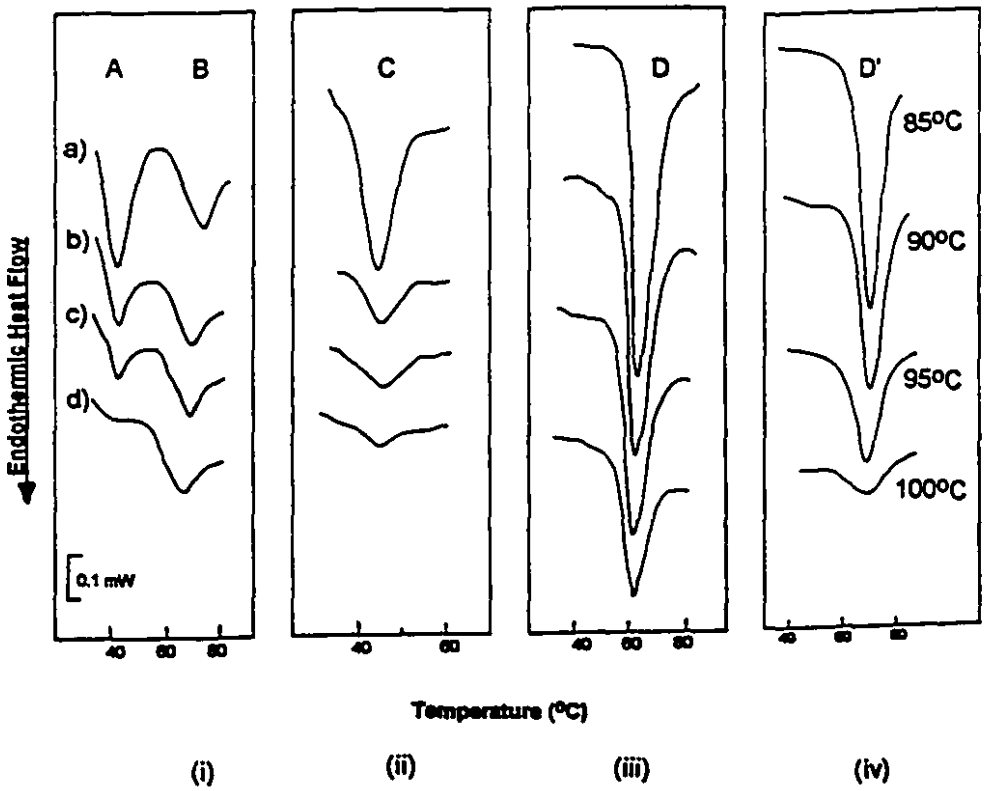



**Fig 6.1:** DSC characteristics of α–lac (20% w/v protein concentration) in (i) $D_2O$ (ii) $Na_2$-EDTA (0.1M) (iii) $CaCl_2$ (0.5M) (iv) $CaCl_2$ (0.5M) preheated at 85°C, 90°C, 95°C, 100°C for 10 min and cooled to room temperature before scanning in the DSC . Heating rate was 5°C/min. (a, b, c, d refer to 1st, 2nd, 3rd and 4th heating cycles respectively).

**Table 6.1: DSC Characteristics of apo-α-lactalbumin and holo-α-lactalbumin**

| Treatment | $T_d$ (A) | H(A) | $T_d$ (B) | H(B) | Reversibitity (%) A | B |
|---|---|---|---|---|---|---|
| $D_2O$ (apo + holo-α-lac) | | | | | | |
| 1st heating cycle | 39.6±.2 | .95±.11 | 64.8±0.5 | .52±.01 | | |
| 2nd heating cycle | 38.7±.4 | .58±.07 | 65.6±1.1 | .53±.03 | 61 | 100 |
| 3rd heating cycle | 39.2±.5 | .45±.02 | 65.1±1.0 | .58±.04 | 47 | 100 |
| 4th heating cycle | 37.4±2 | .07±.1 0 | 63.2±0.1 | .55±.04 | 7 | 100 |
| $Na_2$-EDTA (apo-α-lac) | | | | | | |
| 1st heating cycle | 42.5±.4 | 1.50±.00 | | | | |
| 2nd heating cycle | 43.2±.0 | 0.74±.00 | | | 49 | |
| 3rd heating cycle | 44.0±.5 | 0.52±.05 | | | 35 | |
| 4th heating cycle | 43.3±.2 | 0.38±.04 | | | 25 | |
| $CaCl_2$ (holo-α-lac) | | | | | | |
| 1st heating cycle | | | 70.1±.1 | 2.7±.30 | | |
| 2nd heating cycle | | | 70.5±.1 | 2.2±.02 | | 81 |
| 3rd heating cycle | | | 70.3±.1 | 1.8±.07 | | 66 |
| 4th heating cycle | | | 70.7±.2 | 1.2±.10 | | 44 |

A, B - First and second transitions observed in the DSC thermogram of α-lac

$T_d$ - Peak temperature of transitions A and B

H - Enthalpy of transitions A and B calculated as area underneath the peaks

% Reversibility - (Enthalpy calculated from each heating cycle divided by enthalpy of first heating cycle x 100)

(second cycle) and subsequently 0.38±.04 J/g after the fourth heating cycle; the percent reversibility was 49% after the second heating cycle and 25% after the fourth cycle (Table 6.1). In $CaCl_2$ (0.5M) one thermal transition was observed at 70.1±.1°C (peak D) with an enthalpy of 2.7±.3 J/g. Cooling followed by reheating, indicated reversibility of this transition whose enthalpy decreased to 2.2±.02J/g after the second heating cycle; (81% reversibility); after the fourth heating cycle, the enthalpy value was 1.2±.1 J/g (56% reversibility). The presence of one thermal transition in α-lac treated with $Na_2$-EDTA (peak C) and $CaCl_2$ (peak D) suggests that the first and second transitions in the thermogram of α-lac (peaks A and B) were due to the thermal transitions of $Ca^{++}$ free (apo-) and $Ca^{++}$ bound (holo-) α-lac respectively; the higher transition temperatures of peaks C and D in comparison to A and B might reflect a stabilizing effect of the $Ca^{++}$ and $Na^{+}$ ions (Relkin *et al.*, 1993).

The transitions observed in the DSC analysis demonstrate the reversible denaturation of α–lac in $CaCl_2$ when heat was applied at a temperature gradient of 5°C/min. To confirm that this reversibility is independent of the method of heating, α–lac in $CaCl_2$ (0.5M) was preheated at 85-100°C for 10 min then subjected to the DSC analysis. The results (Fig 6.1.iv) show the transition at 70.4°C (peak D) which confirms the thermal reversibility of α–lac regardless of the mode of heating. The enthalpy decreased from 2.15±.003 J/g (preheating at 85°C) to 0.33±.02 J/g (preheating at 100°C) which indicates 80% and 12% reversibility respectively (Table 6.2). No transitions were observed when α-lac was preheated at 100°C for 30 min suggesting that the protein was irreversibly denatured. The heated solutions were observed for gelation in order to determine if a relationship existed between thermal reversibility of α–lac and its gelling properties. The α–lac solution in $CaCl_2$ heated at 85°C, formed an opaque curd which is indicative of aggregation with no observed gelation. α–lac

**Table 6.2: DSC Characteristics of α-lactalbumin in $CaCl_2$ , NaCl, sucrose and glucose**

| Treatment | $T_d$ (A) | $T_d$ (B) | H(B) | % Reversibitity | |
|---|---|---|---|---|---|
| | | | | A | B |
| $CaCl_2$ (0.5M) | | | | | |
| Preheated at 85°C | | 70.4±.2 | 2.15±.00 | | 80 |
| Preheated at 90°C | | 71.0±.1 | 1.66±.00 | | 61 |
| Preheated at 95°C | | 71.7±.1 | 0.96±.01 | | 45 |
| Preheated at 100°C | | 72.1±.2 | 0.33±.02 | | 12 |
| NaCl (M) | | | | | |
| 0.0 | 39.6±.2 | 64.8±.5 | | 61 | 100 |
| 0.5 | 48.3±.2 | 69.9±.1 | | 63 | 89 |
| 1.0 | 52.6±.1 | 72.2±.3 | | 63 | 82 |
| 1.5 | 53.2±.1 | 73.1±.2 | | 48 | 76 |
| Sucrose (% w/v) | | | | | |
| 10 | 39.6±.2 | 65.5±.1 | | 42 | 78 |
| 50 | 44.6±.1 | 69.7±.3 | | 32 | 100 |
| Glucose (% w/v) | | | | | |
| 10 | 40.0±.1 | 67.4±.2 | | 70 | 94 |
| 50 | 43.7±.2 | 68.1±.1 | | 70 | 100 |

See foctnotes Table 6.1

in $CaCl_2$ when heated at 95°C gave a soft opaque gel and at 100°C gave a firm opaque gel both of which are indicative of aggregation with gelation. In the absence of $CaCl_2$, α–lac did not gel on heating. A soft translucent gel was formed only after the fifth heating cycle and also on heating in the oven at 100°C for 30 min. In the presence of $CaCl_2$ and $Na_2$-EDTA, an opaque gel was formed after the fourth heating cycle. This suggests that the ability of α-lac to renature after heat treatment is directly related to the presence of $Ca^{++}$ and the extent to which it aggregates or gels on heat treatment. When no aggregation or gelation occurs, the percent reversibility increases.

In this study, transitions affecting position and intensities of the Amide I' band (1700-1600 cm-1) and the Amide II band (1550 cm-1) were considered. The Amide I' band involves transitions relating to C=O stretching vibrations while the Amide II involves -CONH vibrations of the peptide backbone and can be used to study rates of hydrogen-deuterium exchange and unfolding of the protein (Clark *et al.*, 1981). Fig 6.2 shows the FTIR spectra of α–lac (20% w/v) in $D_2O$ heated from 25 to 90°C, cooled to 25°C and subjected to four heating/cooling cycles. Spectra were collected both during the heating and the cooling cycles. The spectrum at 25°C of the first heating cycle gave four main bands at 1676, 1662, 1652, 1639 $cm^{-1}$ and a shoulder at 1630 $cm^{-1}$. The assignment of these bands (Table 6.3) are as follows: 1676 $cm^{-1}$ attributed to β–sheet (Casal *et al.*, 1988; Susi & Byler, 1988) and turns (Krimm & Bandekar, 1986); 1662 and 1652 $cm^{-1}$ bands attributed to turns and α–helices respectively (Susi & Byler, 1988); 1639 $cm^{-1}$ previously assigned to antiparallel β–sheet structure and 1630 $cm^{-1}$ attributed to extended antiparallel β–sheet (Susi *et al.*, 1985). Holloway & Mantsch (1989) have recently attributed bands at or near 1639 $cm^{-1}$ to the $3_{10}$-helices of α–lac. The resolution enhancement factor (K) used in this study did not permit us to observe the 1645 $cm^{-1}$ band which was

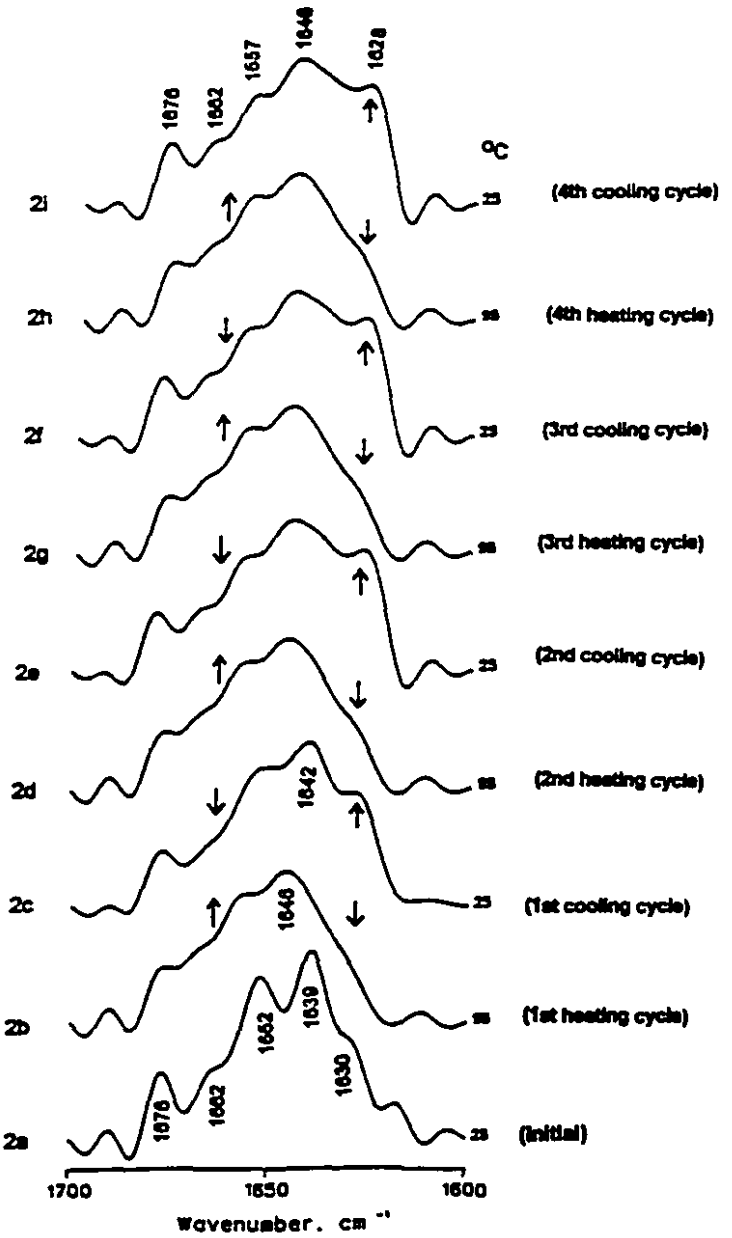



Fig 6. 2: Deconvoluted infrared spectra of α–lac in $D_2O$ (10% w/v, pH 6.8) heated and cooled (four heating/cooling cycles) at the temperatures indicated. The numbers refer to the wavenumbers ($cm^{-1}$) of the main bands observed.

**Table 6.3: Peak positions of the Amide I' regions of α-lactalbumin.**

| Peak Position (cm-1) | Assignment | Peak Position (cm-1) | Assignment |
|---|---|---|---|
| ~ 1613 | side chain | 1644±3 | unordered |
| 1618±3 | β-sheet | 1654±3 | α–helix |
| 1624±4 | β-sheet | 1663±4 | turns |
| 1632±2 | β-sheet | 1669±2 | turns |
| 1638±2 | β-sheet | 1675±4 | β-sheet |
| ~ 1639 | $3_{10}$-helix | ~ 1684 | β–sheet |

References for band assignments given in text.

reported in α–lac by Prestrelski *et al.*, (1991a).

Heating (α–lac in $D_2O$, pH 6.8) to 95°C (first cycle, Fig. 2b) resulted in a loss of the 1630 and 1639 $cm^{-1}$ bands, a split of the 1652 $cm^{-1}$ band accompanied by the appearance of two new bands at 1657 (α-helix) and 1646 $cm^{-1}$ (random coil) and an increase in the band at 1662 $cm^{-1}$ (turns, Table 6.3). Cooling of this heated solution (first cooling cycle, Fig 6.2c), gave a band at 1628 $cm^{-1}$ (β-sheet) accompanied by a shift of the band at 1646 to 1642 $cm^{-1}$ and a decrease in the intensity of the 1662 and 1657 $cm^{-1}$ bands suggesting that on cooling, turns and α-helical structures were transformed to β-sheet. Reheating (2nd heating cycle), gave a spectra (Fig 6.2d) similar to that obtained from the first heating cycle (Fig 6.2b) except for a marked increase in the intensity of the 1676 $cm^{-1}$ band on recooling while the spectra from the second cooling cycle (Fig 6.2e) was similar to that from the first cooling cycle (Fig 6.2c). The third heating cycle (Fig 6.2f), showed a decrease in the intensity of the bands at 1628 and 1676 cm-1 and an increase in the intensity of the bands at 1662 and 1657 cm-1. Cooling (third cycle) (Fig 6.2g) again showed a decrease in the intensity of the 1662 and 1657 cm-1 bands and an increase in the intensity of the bands at 1676 and 1628 cm-1. The transitions in the fourth heating and cooling cycles (Fig 6.2h, i) were similar to that in the third cycle. The data suggests that α-lac has a high degree of thermal stability and that the unfolding observed on heating is reversible (Relkin *et al.*, 1993). The anti-parallel β–sheet at 1628 $cm^{-1}$ is highly mobile and is transformed to turns and unordered structure at high temperatures. Upon cooling these turns re-align to form extended β–sheet with anti-parallel orientation. This supports the results obtained from the DSC which showed reversibility of both transitions (A and B) on reheating.

Prestrelski *et al.*, (1991a), reported that removal of bound $Ca^{++}$ from α–

lac resulted in the transition from a well-defined coordination complex to an essentially orderless structure. To determine the contributions of apo-α-lac ($Ca^{++}$-free) and holo-α-lac ($Ca^{++}$-bound) to the transitions observed in the FTIR and DSC, the FTIR spectra of α-lac in EGTA (pH adjusted with NaOH to 6.8) and $CaCl_2$ were recorded. Fig 6.3 shows the spectrum of α-lac in EGTA; two bands at 1677 and 1649 and a shoulder at 1637 $cm^{-1}$ were observed at 25°C. Heating to 45°C, resulted in a marked decrease in the 1637 $cm^{-1}$ shoulder (attributed to $3_{10}$-helix) accompanied by a substantial increase in the bands at 1685 and 1627 $cm^{-1}$ (attributed to β-sheets, Table 6.3). These bands increased in intensity with increasing temperature to a maximum between 50-55°C then decreased (Fig 6.3). At 65°C a new band at 1616 $cm^{-1}$ was observed along with a concomitant increase in the band at 1685 $cm^{-1}$; these bands which continued to increase in intensity with temperature have been associated with intermolecular hydrogen-bonded antiparallel β-sheet structures resulting from re-association of unfolded peptide segments which lead to formation of gel and aggregate structures (Clark *et al.*, 1981). Fig 6.3b shows the spectrum of the second heating cycle. The spectrum at 25°C of the second heating cycle was similar to that at 85°C of the first heating cycle (Fig 6.3a) suggesting that the thermal transition is irreversible. An increase in the intensity of the band at 1627$cm^{-1}$ was observed between 40 and 45°C; this band decreased in intensity at 50°C with an associated increase in the intensity of the aggregation band at 1615 $cm^{-1}$. The band at 1615 $cm^{-1}$ continued to increase with further increase in temperature; this indicates that although the transitions from the first heating cycle were essentially irreversible, the transitions (denaturation) were not complete and continued during the second heating cycle. Relkin *et al.*, (1993) report that the aggregation observed in apo-α-lac may be due to increased electrostatic interactions between the positive charges of the ethylamino groups

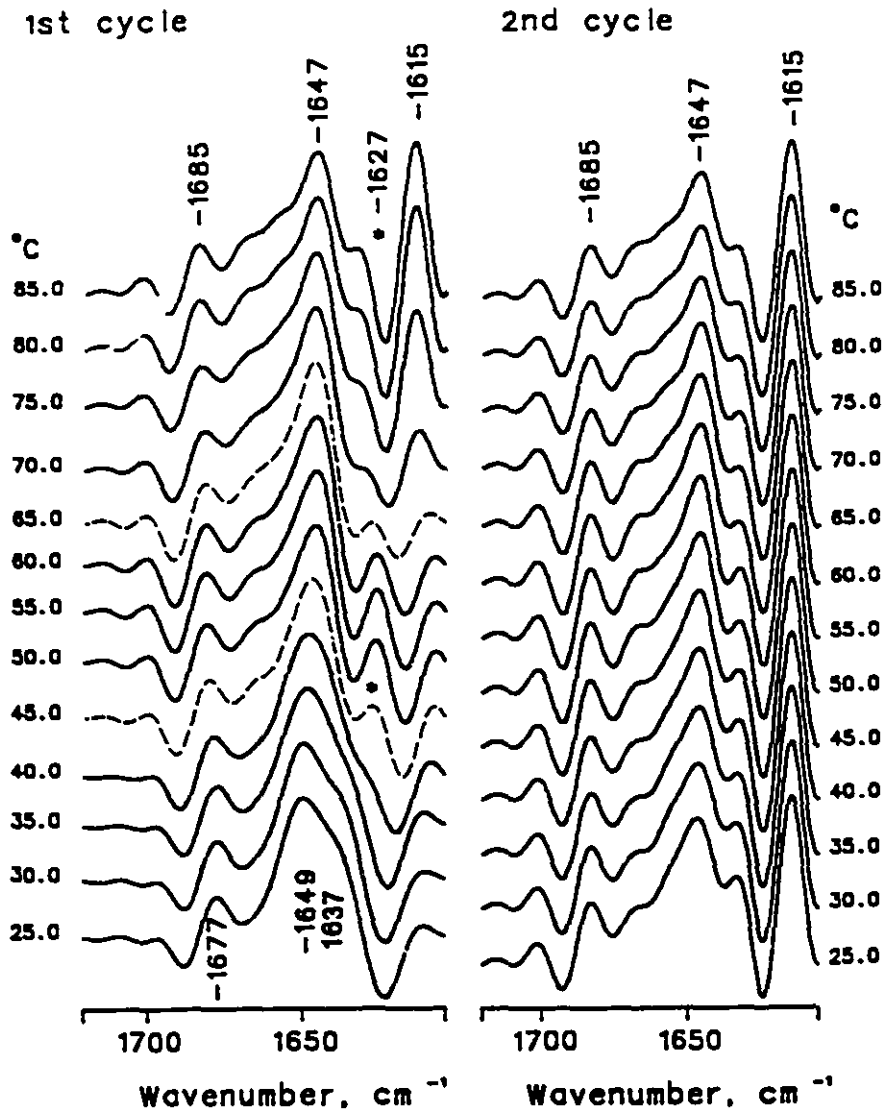



**Fig 6.3:** Stacked plot of deconvoluted infrared spectra of α–lac (20% w/v) heated through two cycles in EGTA (pH 6.8) as a function of temperature (a) first heating cycle (b) second heating cycle.

and the negative charges of α–lac. It may also be that increased exposure of hydrophobic groups on heating enhanced hydrophobic interactions and aided the formation of aggregate structures.

The spectrum in $CaCl_2$ (Fig 6.4a) shows four main bands at 1613, 1640, 1652 and 1676 $cm^{-1}$; the new band at 1613 $cm^{-1}$ has been attributed to side chain absorbances (Table 6.3; Chirgadze *et al.*, 1975). Heating to 50°C gave a band at 1665 cm-1 (attributed to turns) which indicates unfolding of the protein. The band at 1640 $cm^{-1}$, attributed to $3_{10}$-helix, decreased consistently with increasing temperature, suggesting an unfolding of the helical structure probably in the $Ca^{++}$ binding elbow. The α–helix band (1652 $cm^{-1}$) however, was affected only slightly with increasing temperature. Above 50°C, the 1640 $cm^{-1}$ band increased in intensity along with the bands at 1674 $cm^{-1}$ and 1613 $cm^{-1}$. These bands again decreased in intensity above 75°C, suggesting a rearrangement of the protein secondary structure (Casal *et al.*, 1988). The increase in the band at 1649 cm-1, can be attributed to an increase in random coil structures. Fig 6.4b shows the spectra of α–lac in $CaCl_2$ during the second heating cycle. The transitions were similar to that observed during the first heating cycle. The spectrum recorded at 25°C of the second heating cycle was different from that at 95°C of the first heating cycle but similar to that at 25°C of the first heating cycle (Fig 6.4a) suggesting that the thermal transitions are reversible. The results indicate that apo-α–lac at 25°C (Fig 6.3a) has a secondary structure conformation similar to that of denatured holo-α–lac at 90°C (Fig 6.4a); this secondary structure might represent that of the molten globule state reviewed by Kronman (1989).

The above observations have considered transitions in the Amide I' band. In addition to the structural data obtained in this region, information on transitions associated with protein gelation can be obtained by examination of

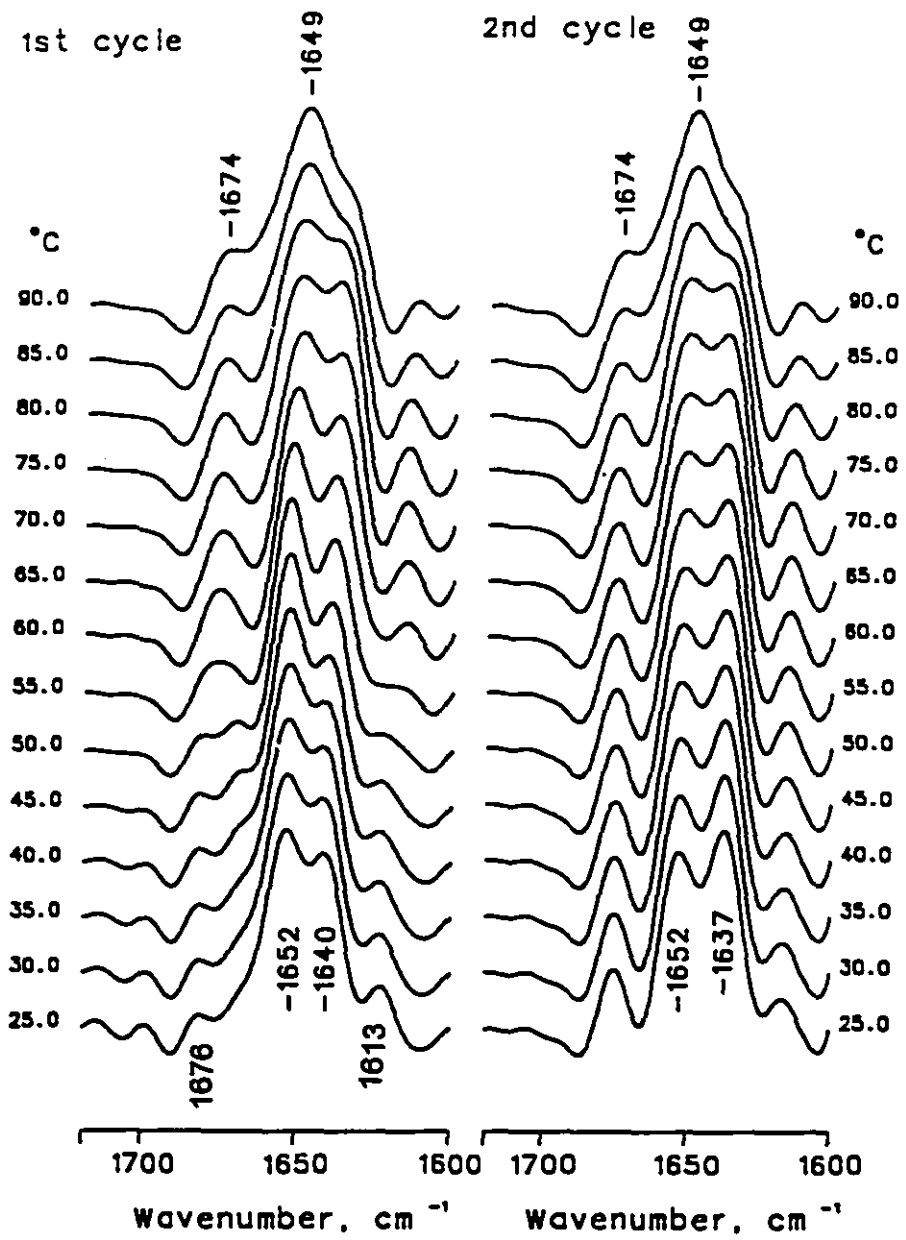



Fig 6.4:Stacked plot of deconvoluted infrared spectra of α–lac (20% w/v) heated through two cycles in $CaCl_2$ (0.5M) as a function of temperature (a) first heating cycle (b) second heating cycle.

the Amide II regions of the spectrum near 1550 $cm^{-1}$ (Clark *et al.*, 1981). To study the rate of hydrogen-deuterium exchange in the presence of $CaCl_2$, the integrated intensity of the band in the 1525-1555 $cm^{-1}$ region (Amide II) of the four heating cycles of α-lac in $CaCl_2$ was plotted (Fig 6.5a). An intense band was observed at 25°C; heating (first heating cycle) resulted in a decrease in the intensity of this band to a minimum value at 75°C; no further decrease in intensity was observed in the second, third and fourth heating cycles. The presence of this band in the first heating cycle indicates that hydrogen-deuterium exchange was incomplete and suggests that the protein structure was compact and inaccessible to solvent (Clark *et al.*, 1981). Heating resulted in unfolding of the protein and enhanced hydrogen-deuterium exchange, which was completed during the first heating cycle (Prestrelski *et al.*, 1991b). The transitions observed in the spectrum, especially in the Amide I' region, after the first heating cycle can therefore be attributed to conformational changes in the protein structure and not to hydrogen-deuterium exchange. In EGTA (spectra not shown) no bands were observed in this region in all four heating cycles suggesting that hydrogen-deuterium exchange was immediate; this indicates that in the presence of EGTA the protein had a more open structure which was more accessible to solvent.

The difference spectra (spectra taken at 25°C subtracted from spectra at increasing temperatures by 5°C increments to 90°C,Fig 6.5b) shows clearly the spectral changes associated with changes in conformation resulting from heating α-lac in $CaCl_2$. The major transitions observed are an increase in the 1665 $cm^{-1}$ band attributed to reverse turns (Prestrelski *et al.*, 1991b) and a decrease in the 1637 $cm^{-1}$ band (attributed to $3_{10}$-helix) which suggests that on heating, the $3_{10}$-helix unfolded into reverse turns. Similar conformational changes were observed in the third and fourth heating cycles suggesting that the changes observed in the secondary structure on heating were reversible.

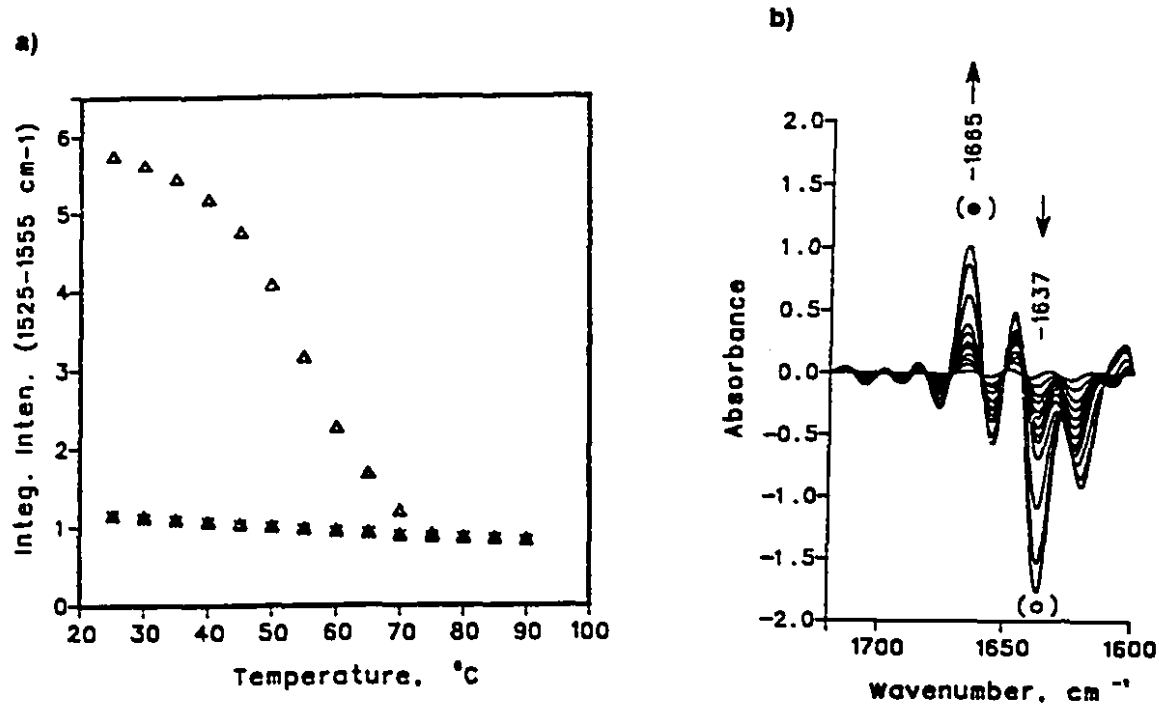



Fig 6.5: (a) Integrated intensity of the 1525-1555 $cm^{-1}$ region of the spectra of α–lac heated in the presence of $CaCl_2$ (0.5M) as a function of temperature. ( Δ ) 1st heating cycle,( ▲ ) 2nd heating cycle,( * ) 3rd heating cycle,(● ) 4th heating cycle. (b)Superimposed infrared spectra of α–lac (20% w/v) in the presence of $CaCl_2$ (0.5M) as a function of temperature. Each line represents spectrum recorded at temperatures between 25 and 90°C at 5°C increments after subtraction of the spectrum recorded at 25°C.

### 6.4.2 Protein-Salt Interactions

The thermograms (Fig 6.6) of α–lac heated in 0.5M NaCl from 15°C to 100°C revealed two transitions at 48.3±.2°C (peak A) and 69.9±.1°C (peak B); this represents a shift from 39.6°C for peak A and from 64.8°C for peak B in the absence of NaCl. In 1.5M NaCl, peak A shifted further to 53.2±.1°C and peak B to 73.1±.2°C which suggests that NaCl has a stabilizing effect on the thermal denaturation of both apo- (peak A) and holo-α–lac (peak B). Table 6.2 shows the effect of NaCl concentration on the reversibility of α–lac. The percent reversibility in 0.5M NaCl was 63% (transition A) and 89% (transition B). In 1.5M NaCl the percent reversibility decreased to 48% (transition A) and 76% (transition B) suggesting that the protein was more irreversibly denatured at higher concentrations of NaCl.

Fig 6.7 shows the FTIR spectra of α–lac heated in the presence of 0.5M NaCl solution from 26 to 97°C. Above 43°C there was a marked decrease in the intensity of the 1676 and 1640 $cm^{-1}$ bands in the spectra of α–lac dissolved in 0.5M NaCl, suggesting a loss of both β–sheet and $3_{10}$–helical structure. Above 47°C, an increase in intensity of the bands at 1672 and 1662 $cm^{-1}$ was observed, which further suggests an increase in turns and unordered structures; this could be due to partial unfolding as the hydrogen bonds in the secondary structures were broken (Ismail *et al.*, 1992). The band at 1640 $cm^{-1}$ increased in intensity above 72°C which suggests a reformation of ordered structure; either $3_{10}$-helix or β–sheet. In the spectra of α–lac in 1.5M NaCl (figure not shown), two bands at 1684 and 1618 $cm^{-1}$ associated with the formation of gel and aggregate structures (Clark *et al.*, 1981) were observed above 55°C. The 1684 and 1618 $cm^{-1}$ bands were absent in the spectrum of α-lac in NaCl concentrations lower than 1.5M, which suggests that α–lac only formed aggregates at high NaCl concentrations. When the solution of α–lac in 0.5M

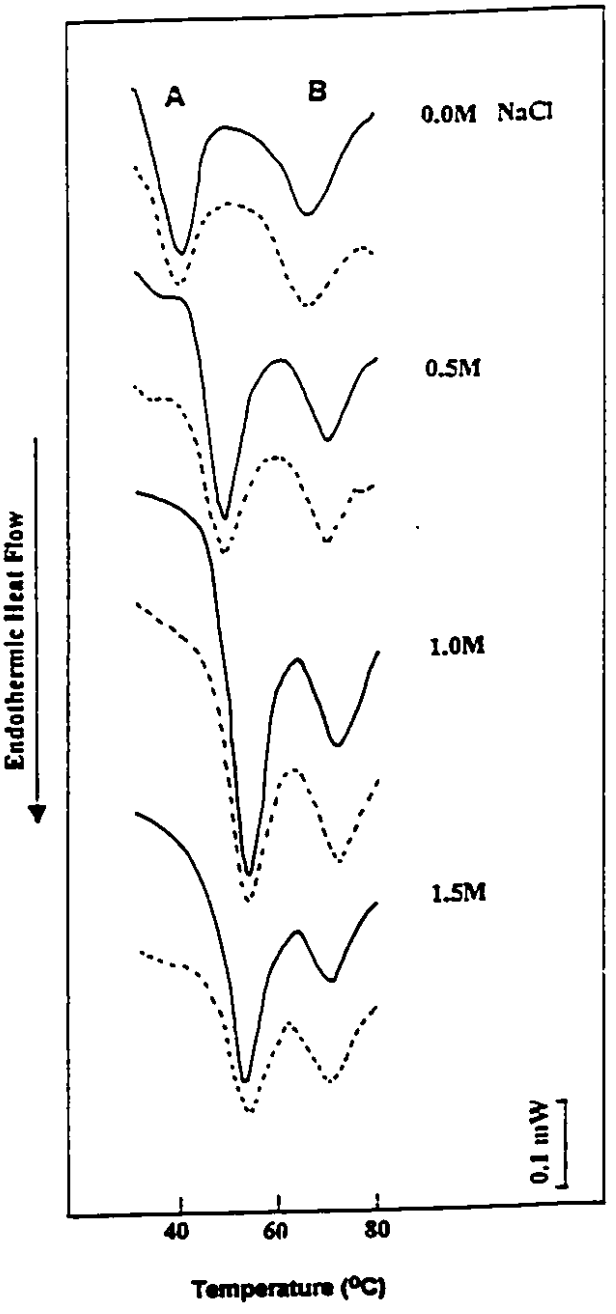



Fig 6.6: Effect of NaCl concentration on the DSC thermogram of α–lac (20% w/v). 1st heating cycle (——), 2nd heating cycle (- - -).

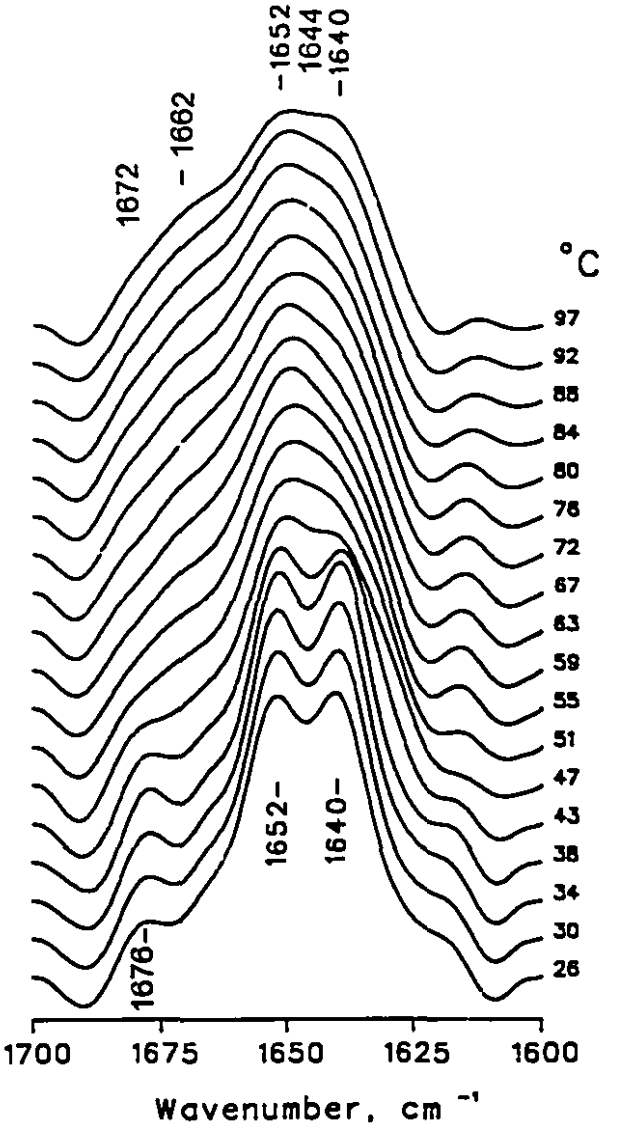



Fig 6.7: Stacked plot of deconvoluted infrared spectra of α-lac (10% w/v, pH unadjusted) as a function of temperature in 0.5M NaCl.

NaCl was cooled from 97°C to 20°C (spectra not shown), the bands at 1672, 1652 and 1640 $cm^{-1}$ decreased in intensity while the β-sheet bands at 1682 and 1624 $cm^{-1}$ (Table 6.3) associated with β–sheet (Susi & Byler, 1988) increased in intensity. This suggests that the denaturation of α–lac in the presence of 0.5M NaCl was reversible; however when cooled the protein could have assumed a conformation different from that of the native protein. The presence of both the apo- and holo- forms of α–lac in the sample used confounds further discussion of the changes observed.

### 6.4.3 Protein-sugar interactions

The thermograms of α–lac heated with sucrose and glucose (10 and 50% solutions w/v in $D_2O$) are shown in Fig 6.8. Two reversible transitions at 40.0±.1°C (peak A) and 67.4±.2°C (peak B) were observed in the 10% (w/v) sucrose solution (Fig 6.8a). These transitions represent a shift from 39.6°C (peak A) and 64.8°C (peak B) in the absence of sucrose (Table 6.2). In the presence of 50% (w/v) sucrose, the transitions shifted to 43.7±.2°C (peak A) and 68.1±.1°C (peak B), which suggests that sucrose had a stabilizing effect on the denaturation temperature of both holo- and apo-α-lac at high concentrations. When the samples were reheated, transition A was 70% reversible in both solutions, while transition B was 94% reversible in the 10% w/v sucrose solution and 100% reversible in the 50% solution. The thermograms of α–lac in 10 and 50% (w/v) glucose solution and in the absence of glucose are shown in Fig 6.8b. Two transitions at 39.6±.2°C (peak A) and 65.5±.1°C (peak B) were observed in the 10% (w/v) glucose solution. These transitions shifted to 44.6±.1°C (peak A) and 69.7±.3°C (peak B) in the presence of 50% (w/v) glucose which indicates that glucose had a greater stabilizing effect on the denaturation of α-lac than sucrose. When the samples were reheated, transition A was 42% reversible

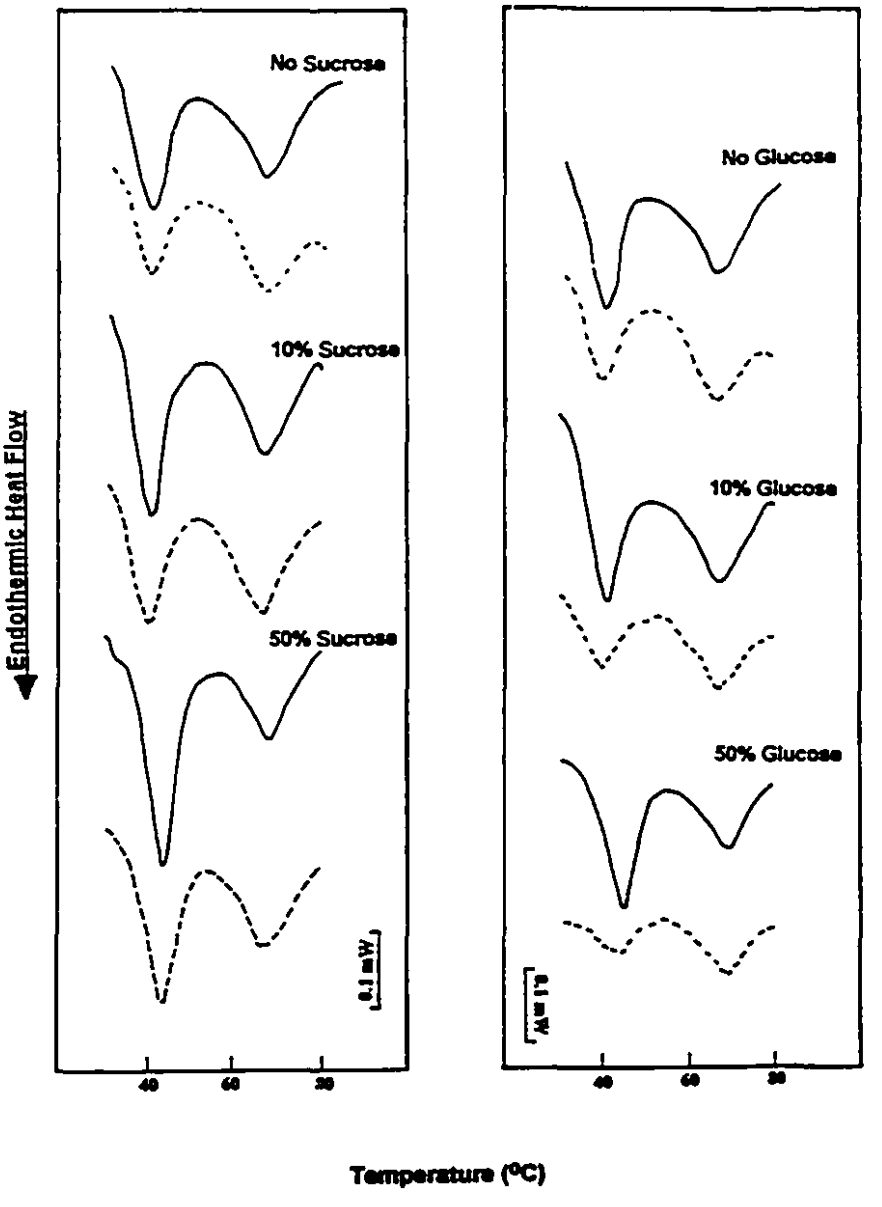



Fig 6.8: DSC characteristics of α–lac (20% w/v protein concentration, pH unadjusted) in the absence and presence of (a) sucrose and (b) glucose (10 and 50 % w/v) in $D_2O$. Heating rate was 5°C/min. 1st heating cycle (——— ), 2nd heating cycle (——).

while transition B was 78% reversible in the 10% (w/v) glucose solution. In the 50% (w/v) solution, transition A was 32% reversible while transition B was 100% reversible. The FTIR spectra (not shown) of α–lac heated in 50% (w/v) glucose and sucrose (heating and cooling cycles) were similar to those observed for α-lac heated in 0.5M NaCl (Fig 6.7a); this indicates that in the presence of these sugars there was no aggregation (absence of 1684 and 1618 $cm^{-1}$ bands) and denaturation on heating was reversible.

### 6.4.4 Effect of pH

Fig 6.9 shows the thermograms of α–lac heated in deuterated phosphate buffer at different pH values. Two thermal transitions were observed at all the pH values studied. At pH 3, the first transition (peak A) occurred at 39.0±.1°C and the second transition (peak B) at 67.5±.1°C (Table 6.4) which indicates an increase in thermal stability in the apo-form but a decrease in the stability of the holo-form. With increasing pH, transition A shifted to 44.4±.1°C at pH 9 while transition B shifted to 65.3±.1°C. At all the pH values studied, the width of the peak at half height ($T_w$) was smaller for transition A than transition B (Table 6.4). With increasing pH, however, the width of the peak $T_w$ for transition B decreased. The $T_w$ for transition B, however, decreased from 13.3°C at pH 3 to 6°C at pH 9. The width of the peak at half-height ($T_w$) is a measure of the sharpness of an endothermic peak and it gives an indication of the cooperative nature of the transition from native to denatured state (Wright *et al.*, 1977); if denaturation occurs within a narrow range of temperature, the transition is considered highly cooperative. This suggests that the transitions occurring in peak A were more cooperative than in peak B; with increasing pH, the cooperativity of transition B increased. When the samples were reheated, transition A was 14% reversible and transition B was 100% reversible at pH 3. At

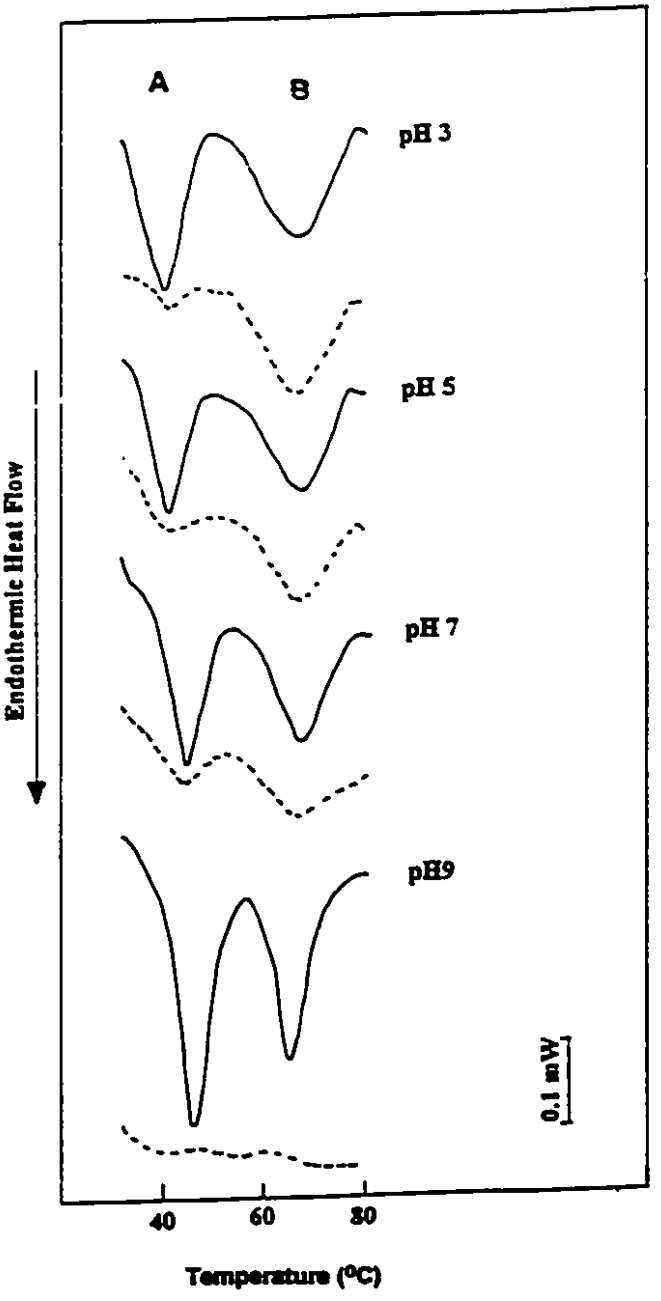



**Fig 6.9**: Effect of pH on the DSC characteristics of α–lactalbumin (200mg/ml deuterated phosphate buffer, ionic strength - 0.2). The numbers refer to the wavenumber ($cm^{-1}$) of the main bands observed. 1st heating cycle (——— ), 2nd heating cycle (——).

**Table 6.4: Effect of pH on the DSC Characteristics of α–lactalbumin.**

| Treatment | $T_d$ (A) | $T_w$(A) | H(A) | $T_d$ (B) | $T_w$(B) | H(B) | % Reversibitity | |
|---|---|---|---|---|---|---|---|---|
| pH | | | | | | | A | B |
| 3 | 39.0±.1 | 7.8 | 0.64±.01 | 67.5±.1 | 13.3 | .40±.02 | 14 | 100 |
| 5 | 39.3±.2 | 7.8 | 0.75±.03 | 67.1±.2 | 11.4 | .42±.02 | 38 | 87 |
| 7 | 43.2±.1 | 7.5 | 1.00±.01 | 66.0±.1 | 9.4 | .56±.01 | 38 | 38 |
| 9 | 44.4±.1 | 7.3 | 1.14±.02 | 65.3±.1 | 6.0 | .57±.01 | - | - |

See footnotes for Table 6.1

$T_w$ - Width of the peaks observed in the DSC at half-height

pH 5 and 7, transition A was 38% reversible, while transition B was 87% reversible at pH 5 and 38% reversible at pH 7. At pH 9 both transitions were completely irreversible. Our data (Table 6.4) show a progressive decrease in enthalpy for both transitions A and B with decreasing pH. At pH 9, the enthalpy was 1.14 and 0.57 J/g for transitions A and B respectively. These values decreased to 0.64 and 0.40 J/g at pH 3. Enthalpy values obtained from the DSC have been correlated with the content of ordered secondary structure of a protein (Koshiyama *et al.*, 1981) and can be used to monitor the proportion of protein in an isolate that is not denatured during processing (Arntfield & Murray, 1981). The decrease in enthalpy with decreasing pH observed in this study may be attributed to denaturation of α–lac at acidic pH, since a partially unfolded protein would require less heat energy to denature completely than a native protein (Ma & Harwalkar, 1988). The decrease in enthalpy may also be due to protein aggregation, which is considered an exothermic reaction (Jackson & Brandts, 1970).

Fig 6.10 shows the FTIR spectra of α–lac at 26°C, heated to 97°C then cooled to 26°C at pH 3, 5, 7 and 9. The spectra at 26°C showed a band at 1627 $cm^{-1}$ at pH 3 which was absent at pH 9; this band assigned to antiparallel β–sheet structure (Table 6.3) suggests an increase in extended β–sheet formation. At 97°C, the spectra showed two bands at 1684 and 1616 $cm^{-1}$ at pH 3 and 5 but not at pH 7 and 9; these bands, attributed to intermolecular β–sheet formation resulting from aggregation of the protein, suggest that α–lac formed aggregates at pH 3 and 5 but not at pH 7 and 9. Lala and Kaul (1992) have reported the exposure of two tryptophan residues which increase the surface hydrophobicity of α–lac in the molten globule state (acid pH). This increased hydrophobicity might be responsible for the intermolecular interactions that result in the irreversible aggregation at acid pH values (1684 and 1618 cm-1

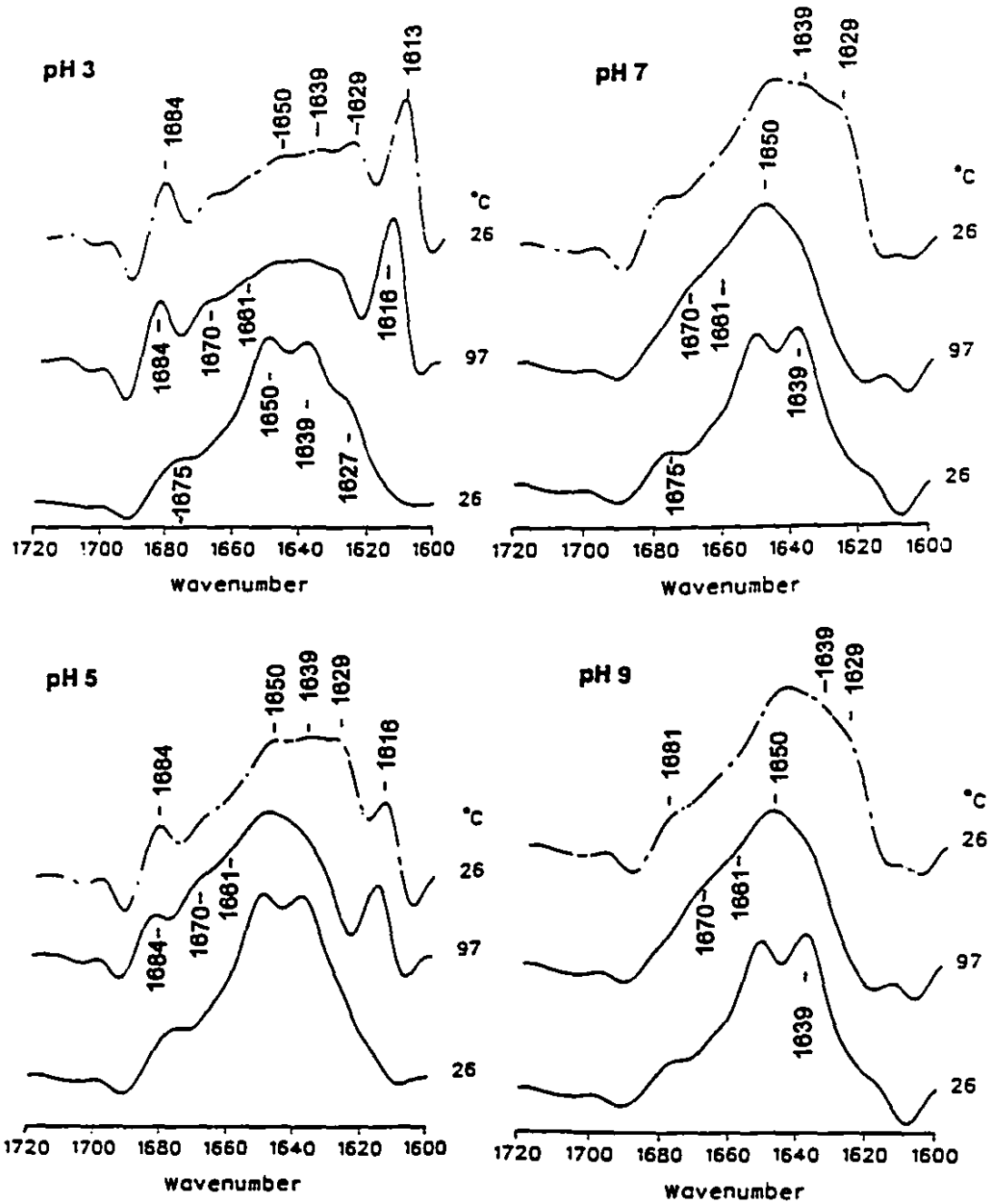



**Fig 6.10:** Stacked plot of deconvoluted infrared spectra of α–lactalbumin (100mg/ml deuterated phosphate buffer, ionic strength - 0.2) at 26°C (———), 97°C (———) and cooled to 26°C ( — · — ).

bands).

At both acid and alkaline pH values, cooling the heated samples resulted in a loss of the 1670 and 1661 $cm^{-1}$ bands (turns) and the appearance of a band at 1629 $cm^{-1}$ band (β–sheet). At pH 7 and 9 there was a notable increase in the 1639 $cm^{-1}$ band on cooling. At pH 5, this band increased in intensity together with the band at 1684 $cm^{-1}$. At pH 3, the band at 1616 $cm^{-1}$ shifted to 1613 $cm^{-1}$ on cooling which suggests an increase in hydrogen bonding and a more compact folding of the aggregates on cooling.

Visual observation of the α–lac gels (Clark & Lee-Tuffnell, 1986) showed that at pH 3 and 5, α–lac aggregated without forming a firm gel. At pH 7 no gel or aggregate formation was observed. At pH 9, a firm translucent gel was formed which did not revert to a solution state on cooling. Hydrophobic interactions normally result in the formation of opaque coagulum-type gels (Damodaran, 1994). The formation of a translucent gel at pH 9, coupled with the absence of a peak in the DSC thermogram on reheating of α–lac at pH 9, suggest that the protein may have undergone irreversible intermolecular disulfide bond formation when heated, in contrast to the hydrophobic interactions that resulted in aggregate formation at pH 3. The absence of the 1684 and 1616$cm^{-1}$ bands in the IR spectra at pH 9, associated with hydrogen-bonded aggregate (gel) structure further suggests that the translucent gel formed at pH 9, does not result from hydrogen-bonding.

## 6.5 CONCLUSION

The two endothermic peaks observed in α–lac, previously associated with the denaturation of the $Ca^{++}$ free (apo-) and bound (holo-) forms by Relkin *et al.*, (1993) were confirmed in this study. Comparison of the DSC and FTIR data,

shows that the first of two transitions from the DSC is related to the breakdown of the $3_{10}$-helix conformation and an increase in β–sheet structure in apo-α–lac as determined by FTIR. The second transition observed from the DSC occurred within the temperature range of 65-70°C which was similar to the temperature associated with complete unfolding of holo-α-lac as determined by FTIR; this unfolding resulted in the loss of $3_{10}$-helix structure and an increase in the formation of turns. The spectra of heated apo-α-lac showed the presence of the two bands at 1615 and 1685 $cm^{-1}$ which indicates that denaturation of apo-α–lac resulted in the formation of β–sheet structures (aggregation), in the case of holo-α–lac denaturation resulted in unfolding into turns (increase in 1665 $cm^{-1}$ band). The ability of holo-α–lac to renature on cooling can be attributed to the absence of aggregation (absence of 1685 and 1616 $cm^{-1}$ bands) on heat treatment.

The present work demonstrated that α-lac is a thermally labile protein whose secondary structure is easily altered by heating but has the ability to refold to a conformation similar to that of its native state in the presence of $CaCl_2$.

## CHAPTER 7

# INTERACTIONS INVOLVED IN THE GELATION OF BOVINE SERUM ALBUMIN.

## 7.0 CONNECTING STATEMENT

The results obtained in Chapters 5 and 6 showed the effect of heating temperature, concentration of NaCl, sugars and pH on the thermal and structural properties of β-lactoglobulin and α-lactalbumin. It was observed that β-lactoglobulin formed intermolecular β-sheet structures associated with aggregation under all the conditions studied while α-lactalbumin formed aggregates only at acidic pH values and in the presence of high concentrations of NaCl. The work described in this chapter addresses the effect of the factors studied in Chapters 5 and 6, as well as effects of several denaturing agents on the thermal and structural properties of bovine serum albumin, the second gelling protein in whey and satisfies objective 3c described in the "Rationale and Objectives of Study" section in Chapter 1.

**Note:** This chapter is the text of a manuscript which has been submitted for publication as follows:

Boye, J. I., Alli, I., Ismail, A., "Interactions involved in the gelation of bovine serum albumin" J. Agric.*Food Chem.* (Submitted, **June 1995**).

***Contributions of co-authors***: Alli, I. (thesis supervisor); Ismail, A. (provided expertise in FTIR).

## 7.1 ABSTRACT

The thermal and structural property of bovine serum albumin (BSA) were studied at different pH values, NaCl, lactose, sucrose and glucose concentrations and in the presence of cysteine, urea, NEM and SDS. Maximum thermal stability was observed at pH 5. Glucose had a greater stabilizing effect on the thermal denaturation of BSA than sucrose. Denaturation of BSA resulted in the loss of the 1654 cm-1 band attributed to α-helical structure and the rise of two bands at 1616 and 1684 cm-1 attributed to the formation of ordered non-native β-sheet structure associated with aggregation. SDS markedly increased the thermal stability of BSA and prevented aggregate formation. The greatest unfolding on heat treatment was observed in the presence of cysteine and the least in SDS.

## 7.2 INTRODUCTION

Bovine serum albumin (BSA) accounts for approximately 10% of the proteins of whey (Fox, 1989); although it is a relatively minor protein component, it is considered as an important gelling protein of whey (Hines and Foegeding, 1993). The other important gelling protein β-lactoglobulin, is the major protein component. Together, these two proteins contribute to the whey protein gelling characteristics (Zeigler & Foegeding, 1990) which can range from viscous fluids, soft, smooth pastes or curds to stiff, rubbery gels which vary in hardness, cohesiveness, stickiness, color and mouthfeel (Hillier & Cheeseman, 1979; Schmidt *et al.*, 1979; Hillier *et al.*, 1980; Kornhost & Mangino, 1985).

BSA is a large globular protein (66,000 Da) consisting of 580 amino acid residues with 17 intrachain disulfide bonds and one free thiol group at residue 34. (Kinsella & Whitehead, 1989). The protein consists of approximately 54% α–helix and 40% β–structure (β–sheet + β–turns) (Kinsella *et al.*, 1989), and contains three domains specific for metal-ion binding, lipid binding and nucleotide binding (Peters & Reed, 1977). McLachlan and Walker (1978) have reported an α–helix content of over 75% for BSA.

The mechanism by which gels are formed when proteins such as BSA are heated has been established (Ferry, 1948; Edwards *et al.*, 1987). This thermally induced gelation is a two-stage sequential process; the first phase involves heat-induced conformational changes in the protein with unfolding of some polypeptide segments followed by a subsequent phase of protein-protein interactions resulting in a progressive build up of a network structure (Bernal & Jelen, 1985). Protein gels can therefore be represented as consisting of intermolecular cage-like unit structures, with the solvent continuous throughout the matrix (Foegeding, 1989). The formation of this matrix is dependent on a balance between attractive and repulsive forces (Schmidt, 1981) which has been

shown to vary with the physical and chemical properties of the protein.

During the first phase of gelation, there is a transition from the native state to progel state; this is associated with dissociation and denaturation of the protein (Damodaran, 1989). During this process functional groups engaged in intramolecular hydrogen bonding and electrostatic interactions in the native state become available for intermolecular interactions (Damodaran, 1994). Exposure of hydrophobic groups results in hydrophobic interactions which are necessary in the aggregation and crosslinking of gel networks (Clark, 1992; Damodaran,1994). Buried sulfhydryl groups can initiate disulfide-sulfhydryl interchange reactions which contribute to crosslinking (Nakamura *et al.*, 1984; Mori *et al.*, 1986; Shimada & Cheftel, 1989).

The particular changes that occur in the secondary structure of proteins and the mechanism by which networks build from individual molecules affect gel texture (Kinsella & Whitehead, 1989). The β–sheet content of native BSA for example is relatively low in comparison to other proteins; when heated, a decrease in α–helix content is observed, with a concomitant increase in β–sheet prior to gelation (Clark *et al.*, 1981 ; Byler & Purcell, 1989); this led to the proposition that β–sheet hydrogen bonding may be important for aggregate formation during the gelation of BSA. Non-native, ordered structures of hydrogen-bonded β–sheets have been observed when BSA was heated above its transition temperatures by infrared spectroscopy (Smith, 1994). Clark *et al.*, (1981), correlated the formation of bands at 1620 $cm^{-1}$ and 1680 $cm^{-1}$ with the formation of anti-parallel hydrogen-bonded β–sheet and protein aggregation.

Proteins must be heated above their denaturation temperature to form gels (Damodaran, 1989). In addition, factors such as pH, ionic strength, protein concentration and the nature and concentration of other solids (for e.g., sugars) affect gel formation and texture (Yasuda *et al.*, 1986; Zeigler & Foegeding,

1990). The secondary structure of denatured proteins are altered by these environmental conditions which affects the temperature of denaturation (Privalov, 1979), an important parameter in protein gelation. Previous work monitored the effects of these environmental factors on the thermal properties and secondary structure of whey protein concentrate, α-lactalbumin and β-lactoglobulin (Boye *et al.*, 1995 a, b, c).

In this work, the effects of pH, NaCl concentration, sucrose, glucose and lactose on the secondary structure and denaturation temperature of BSA were studied. The effects of sodium dodecyl sulfate (SDS), cysteine, urea and N-ethylmaleimide (NEM) which are known to interfere with hydrophobic, disulfide, hydrogen bonding and sulfhydryl oxidation respectively, were determined in order to establish the involvement of these interactions in BSA gelation.

## 7.3 MATERIALS AND METHODS

### 7.3.1 Materials

Bovine Albumin (BSA) (Product A-2153) was obtained from Sigma Chemical Co. (St.Louis,MO,USA) and used as received. Deuterium oxide (Product 15,188-2, minimum 99.9 atom % D) was purchased Aldrich (Milwaukee,WI,USA).

### 7.3.2 Sample Preparation

Deuterium oxide solutions of BSA (20% w/v) were prepared by dissolving the protein in deuterated phosphate-buffer at pH 3 and 9 (ionic strength 0.2). In this work, pD = pH + 0.4. For the sake of clarity, pH is employed in place of pD.

To study the effect of ionic strength and sugars, the protein was dissolved in $D_2O$ solutions containing 0.5 to 2M NaCl, 10 and 50% (w/v) sucrose, 50%

(w/v) glucose and 20% (w/v) lactose.

For the effect of denaturing agents, 50mM solutions of cysteine, urea, SDS (sodium dodecyl sulfate) and NEM (N-ethylmaleimide) made in $D_2O$ and in $H_2O$ were used as solvents for dissolving the protein; 50mM and 2M urea solutions in $D_2O$ were used to study the effect of urea concentrations.

### 7.3.3 Differential Scanning Calorimetry (DSC)

The method for DSC analysis has been described previously (Boye *et al.*, 1995 a,b,c) for α–lactalbumin. Twenty-five µl of each solution were placed in preweighed DSC pans, which were hermetically sealed and weighed accurately. The samples were placed in the DSC (TA3000, Mettler Instrument Corporation, Greifensee, Switzerland) and scanned from 15°C to 100°C at a programmed heating rate of 5°C/min. For each run, a sample pan containing the deuterated buffer used for dissolving BSA was used as reference. After heating, the samples were allowed to cool to room temperature in the DSC and the heating cycle was repeated under the same experimental conditions to check for reversibility. The DSC was calibrated by use of indium standards. All DSC experiments were done in triplicate.

After scanning, the DSC pans were opened and the BSA solutions were visually checked for gelation (Clark & Lee-Tuffnel, 1986). A white non-flowable firm texture was classified as an opaque gel. A clear non-flowable firm texture was classified as a translucent gel.

### 7.3.4 Fourier Transform Infrared Spectroscopy

The method for FTIR analysis has been described previously (Boye *et al.*, 1995 a,b,c). BSA (20% w/v) dissolved in deuterium oxide solutions in the presence of the reagents described above were subjected to infrared

spectroscopy. Infrared spectra were recorded with a 8210E Nicolet FTIR spectrometer equipped with a deuterated triglycine sulfate detector. A total of 512 scans were averaged at 4 $cm^{-1}$ resolution. Wavenumber accuracy was within $\pm$ 0.01$cm^{-1}$. The spectrometer was purged with dry air from a Balston dryer (Balston, Haverhill, MA, 01835-0723, USA). The samples were held in an IR cell with a 25- μm pathlength and $CaF_2$ windows. The temperature of the sample was regulated by placing the cell in a holder employing an Omega temperature controller (Omega Engineering, Laval, QC, H7L 5A1, Canada). The temperature was increased in 5°C increments and the cell allowed to equilibrate for 15 min prior to data acquisition. The reported temperatures are accurate to within $\pm$ 0.5°C. Deconvolution of the observed spectra were performed using the Nicolet FTIR Soft ware, Omnic 1.2a. The deconvolution of the infrared spectra was done as described by Kauppinen *et al.*, (1981). The signal to noise ratio was >20,000:1, and the bandwidth used for deconvolution was 27 $cm^{-1}$ with a narrowing factor of 2.6. All FTIR experiments were done in duplicate.

## 7.4 RESULTS AND DISCUSSION

### 7.4.1 Effect of pH

The effect of pH on the DSC characteristics of BSA is shown in Fig 7.1. At pH 3, the peak temperature of denaturation ($T_d$) was observed at 62.7$\pm$0.3°C. The $T_d$ increased to a maximum of 65$\pm$0.2°C at pH 5 and then decreased to 63.4$\pm$0.1 at pH 7 and 60.8$\pm$0.1°C at pH 9 (Table 7.1). This data suggests that BSA has maximum thermal stability at pH 5 and is more thermally stable at acid pH than at alkaline pH. The highest enthalpies (calculated as the peak area under the peak) were 1.75$\pm$0.02 J/g at pH 7 and 1.45$\pm$0.05 J/g at pH 5 . At pH 3 and 9, the enthalpies were 1.28$\pm$0.04 J/g and 1.37$\pm$0.04 J/g respectively. This

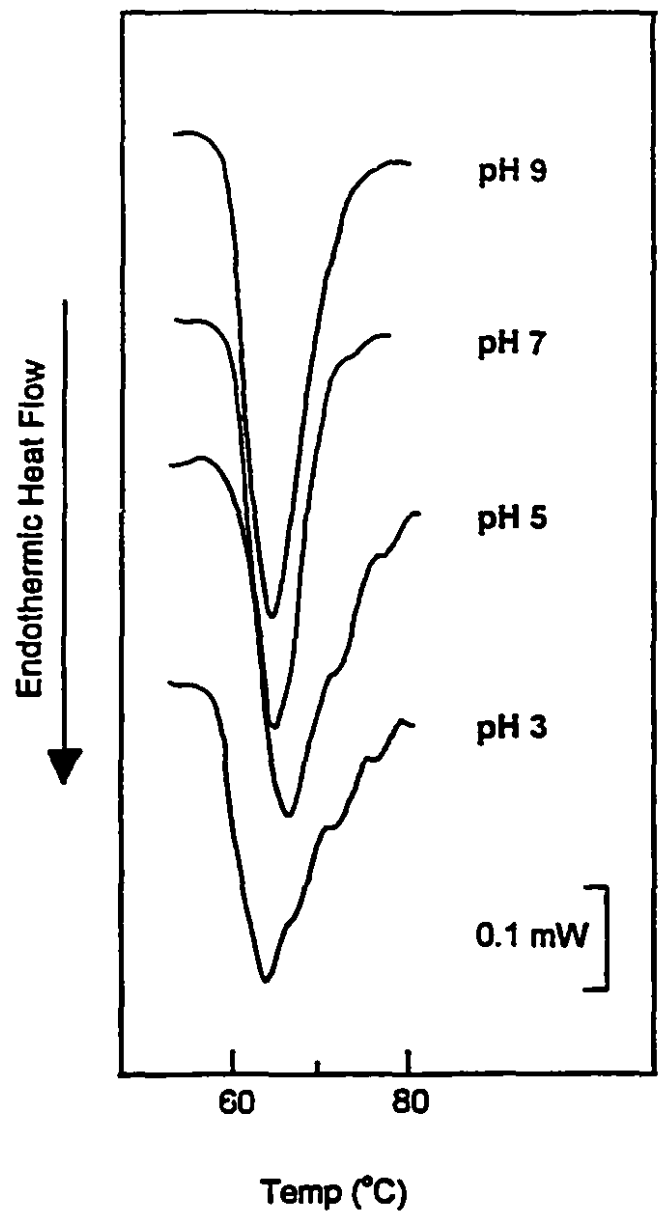



Fig 7.1: Effect of pH on the DSC characteristics of BSA (20% w/v in deuterated phosphate buffer, ionic strength - 0.2). Heating rate was 5°C/min.

**Table 7.1: DSC Characteristics of BSA - Effect of pH and NaCl.**

| Treatment | | $T_d$ (°C) | ΔH (J/g) | $T_w$ |
|---|---|---|---|---|
| pH | | | | |
| | 3 | 62.7±0.3 | 1.28±0.4 | 11.0 |
| | 5 | 65.0±0.2 | 1.45±.05 | 8.1 |
| | 7 | 63.4±0.1 | 1.75±.02 | 5.7 |
| | 9 | 60.8±0.1 | 1.37±0.04 | 5.7 |
| NaCl | | | | |
| | 0 | 63.1±0.2 | 1.43±0.3 | 8.0 |
| | 0.5 | 70.7±0.1 | 2.08±.04 | 5.0 |
| | 1.0 | 72.2±0.3 | 2.05±.05 | 5.0 |
| | 1.5 | 72.8±0.2 | 1.85±.01 | 5.0 |
| | 2.0 | 72.9±0.3 | 1.77±.04 | 5.0 |

$T_d$ - Peak temperature of denaturation

$T_w$- Peak width at half-height

ΔH- Change in enthalpy

suggests that the protein was more denatured at pH 3 and 9 prior to heat treatment; since partially denatured proteins require less energy for further denaturation (Ma & Harwalkar, 1988). The thermograms showed a broadening of the peaks with decreasing pH. The peak width at half height ($T_w$) increased from 5.7°C at pH 9 to 8.1°C at pH 5 and then to 11°C at pH 3. The $T_w$ which has been used as an indication of the cooperativity (ease) of denaturation (Myers, 1990), suggests that the unfolding and denaturation of BSA at acid pH was less cooperative than at alkaline pH. Two shoulders could be observed above the peak temperature in the BSA thermogram at pH 5 (Fig 7.1). At pH 3, three shoulders could be observed. These shoulders suggest that there are residual structures that continue to unfold (denature) as the heating temperature was increased and may reflect unfolding of the multiple domains in the tertiary structure of the protein. The transition temperature of each domain and the order in which domains unfold within a protein have been reported to change with environmental conditions such as pH (Potekin & Privalov, 1982; Bertazzon & Tsong, 1990).

Fig 7.2 shows the FTIR spectra of BSA (20% w/v) in $D_2O$ (pH 7) and in deuterated phosphate buffer at pH 3, heated from 30 to 95°C. The spectra showed an intense peak at 1654 $cm^{-1}$ (attributed to α–helical structure) and shoulders at 1670 $cm^{-1}$ (attributed to turns), 1640 and 1630 $cm^{-1}$ (attributed to β–sheet) (Susi & Byler, 1988). At both pH values, heat treatment resulted in the rise of two new bands at 1684 and 1616 $cm^{-1}$ and a decrease in the bands at 1654 and 1640 $cm^{-1}$. The 1618 and 1684 $cm^{-1}$ bands have been attributed to the formation of intermolecular hydrogen-bonded β–sheet structures resulting in aggregate or gel formation (Clark *et al.*, 1981). Similar transitions were observed at pH 9 (not shown).

To monitor the changes occurring in the 1645 $cm^{-1}$ band which have

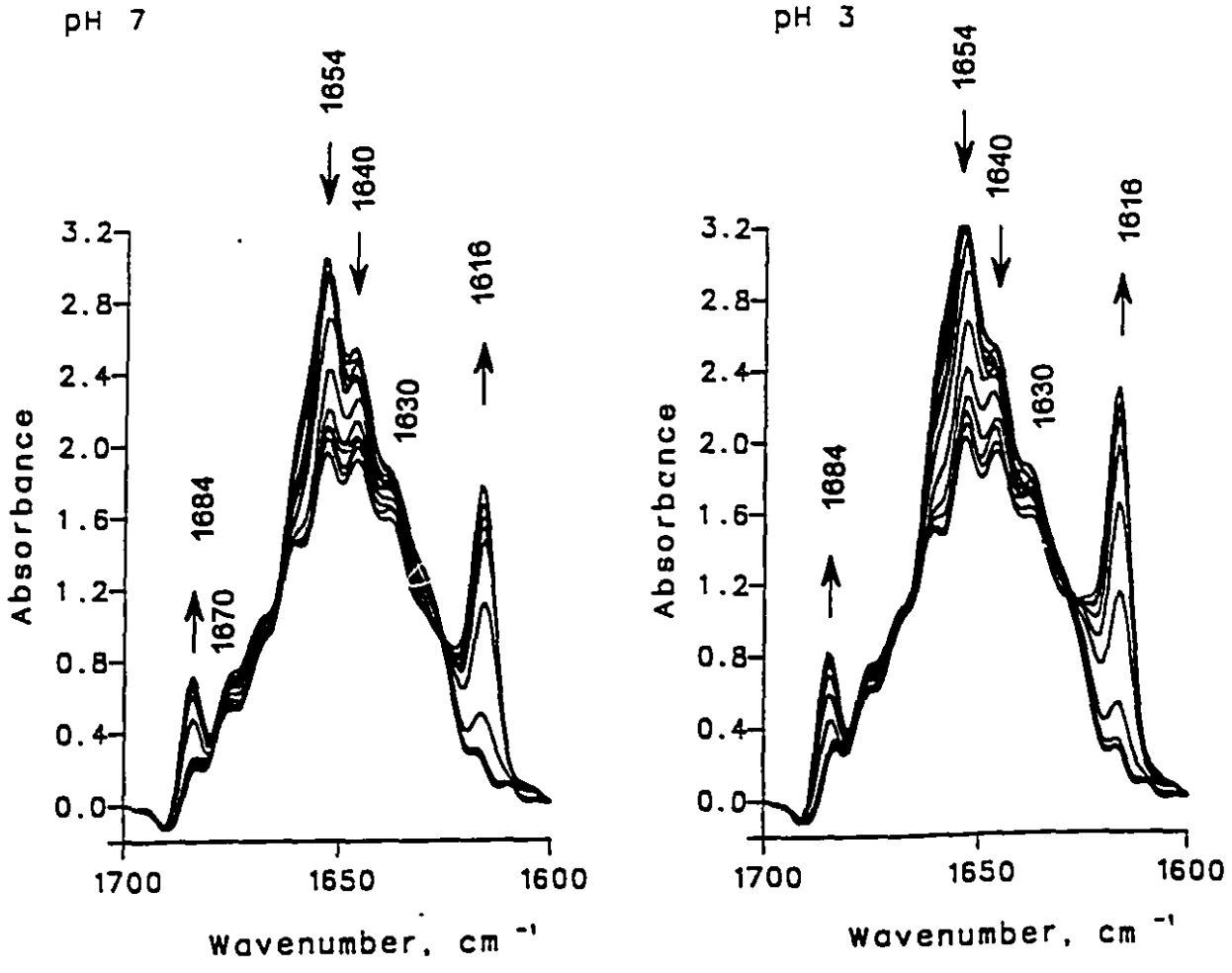



**Fig 7.2:** (a) Deconvoluted infrared spectra of BSA (20% w/v in $D_2O$, pH 7). The numbers refer to the wavenumber ($cm^{-1}$) of the main bands observed. (b) Superimposed deconvoluted infrared spectra of BSA (20% w/v in deuterated phosphate buffer, pH 3, ionic strength - 0.2). Each overlapping spectrum was recorded at temperatures between 30-95°C at 5°C increments. Arrows indicate change in intensity of bands on heating.

been attributed to random coil structures (Susi & Byler, 1988), the integrated intensity of the 1644-1646 $cm^{-1}$ region was plotted as a function of temperature at pH 3 and 7 (Fig 7.3a). The plot shows an increase in random coil formation with increasing temperature from 30°C to a maximum value at 62°C; this temperature was similar to the $T_d$ observed from the DSC analysis of BSA (Fig 7.1) at pH 3 (62.7°C) and pH 7 (63.4°C). This suggests that when heated, BSA unfolded into random coil structures. As the heating progressed above 62°C however, the intensity of the 1646 $cm^{-1}$ band decreased to a minimum at 95°C suggesting a decrease in random coil formation. A plot of the integrated intensity of the 1614-1618 $cm^{-1}$ band (attributed to intermolecular hydrogen-bonded β-sheet aggregation) as a function of temperature (Fig 7.3b) showed a marked increase in intensity above 62°C which coincided with the temperature at which the intensity of the 1644-1646 cm-1 region decreased; this suggests that after the initial unfolding, the protein lost most of its random structure and assumed a more compact and ordered β-sheet structure. Similar findings were reported by Clark *et al.*, (1981) and Byler & Purcell, (1989).

Visual evaluation (Schmidt, 1981; Clark & Lee-Tuffnel, 1986; Patel & Fry, 1987) of the gels formed at each pH value, showed that at pH 3 the gel was firm and opaque while the gel formed at pH 9 was translucent. According to Kinsella and Whitehead (1989), clear gels reflect the formation of uniform networks of fine filaments; greater linear aggregation and frequent crosslinking. As network density becomes less regular, gels increase in turbidity. Our results suggest that the aggregation at pH 3 was more random and unordered than at pH 9.

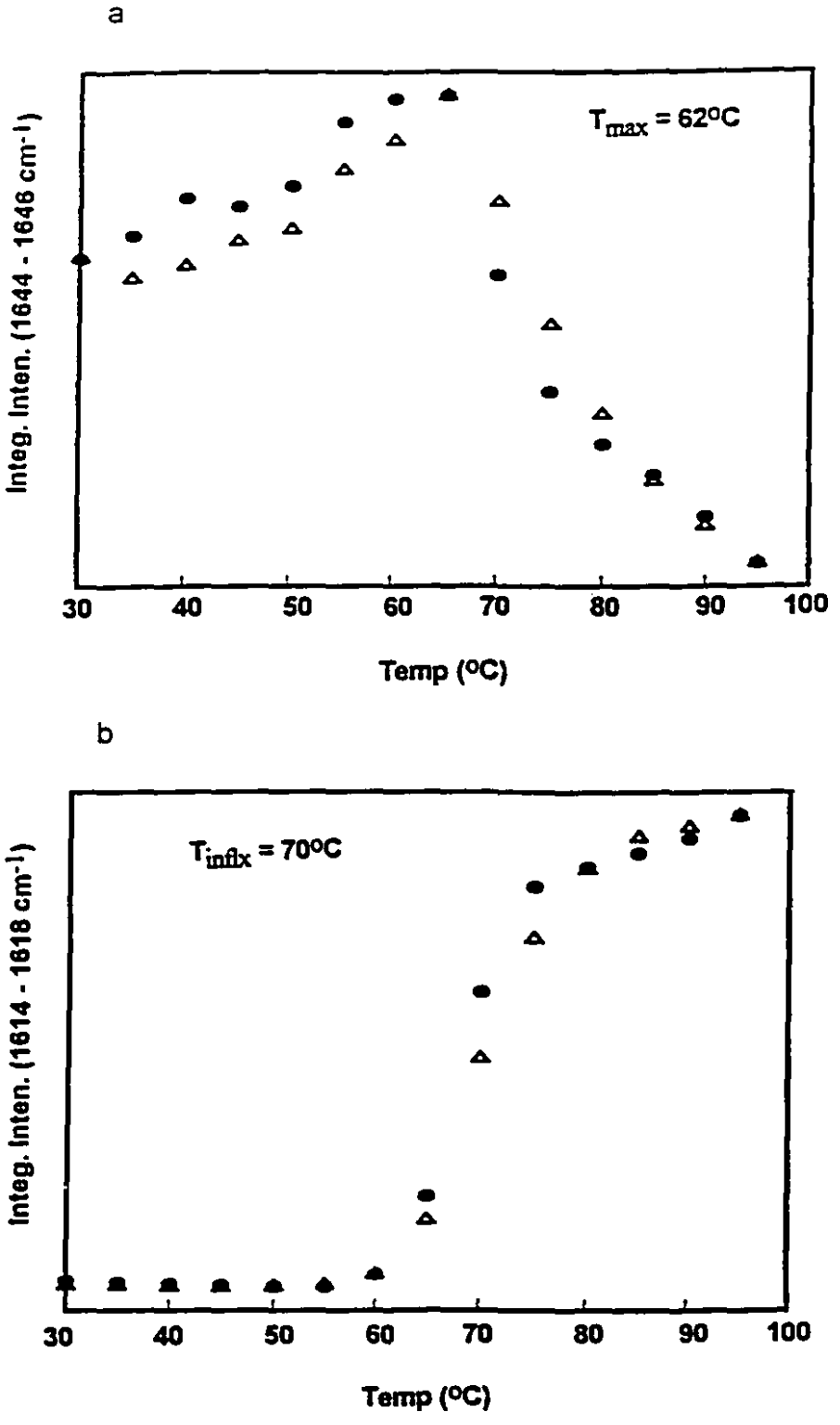



Fig 7.3: Integrated intensity of the (a) 1644-1646 cm$^{-1}$ and (b) 1614-1618 cm$^{-1}$ regions in the amide I' band of BSA (20% w/v); pH 3 (Δ ) and pH 7 (●) as a function of temperature.

### 7.4.2 Protein-salt Interactions

Fig 7.4 shows the thermogram of BSA in various concentrations of NaCl. The $T_d$ increased from 63.1±0.2 J/g (no NaCl) to 70.7±0.1 J/g in 0.5M NaCl (Table 7.1); at 2M NaCl the $T_d$ increased to 72.9±0.3 J/g suggesting a stabilizing influence of NaCl on the thermal denaturation of BSA. Similar findings were reported for β–lactoglobulin and α–lactalbumin in an earlier study (Boye *et al.*, 1995b, 1995c). The enthalpy of denaturation of BSA decreased from 2.08±0.04 J/g in 0.5M NaCl to 1.77±0.04 J/g in 2M NaCl. Our results suggest that at the higher NaCl concentration there was increased aggregation on heat treatment since aggregation of proteins is an exothermic reaction that has been shown to lower enthalpy values (Jackson & Brandts, 1970; Privalov & Khechinashvilli, 1974).

The peak width at half-height ($T_w$) in the absence of NaCl was 8°C. In the presence of 0.5M NaCl, $T_w$ decreased to 5°C then remained constant with increasing NaCl concentration; this suggests that although NaCl increased the stability of BSA to thermal denaturation, the transitions that occur during unfolding are more cooperative in the presence of NaCl.

The FTIR spectra of BSA heated in the presence of 0.5 and 2M NaCl showed the bands at 1616 and 1684 $cm^{-1}$ attributed to aggregate formation (not shown). These bands were observed at 80°C in 0.5M NaCl and at 85°C in 2M NaCl which further confirms that at the higher NaCl concentration, higher temperatures were required to denature and aggregate BSA. These findings were similar to that observed for β–lactoglobulin (Boye *et al.*, 1995b) but not for α–lactalbumin (Boye *et al.*, 1995c) which showed the 1616 and 1684 cm-1 bands only in the presence of NaCl concentrations above 1M.

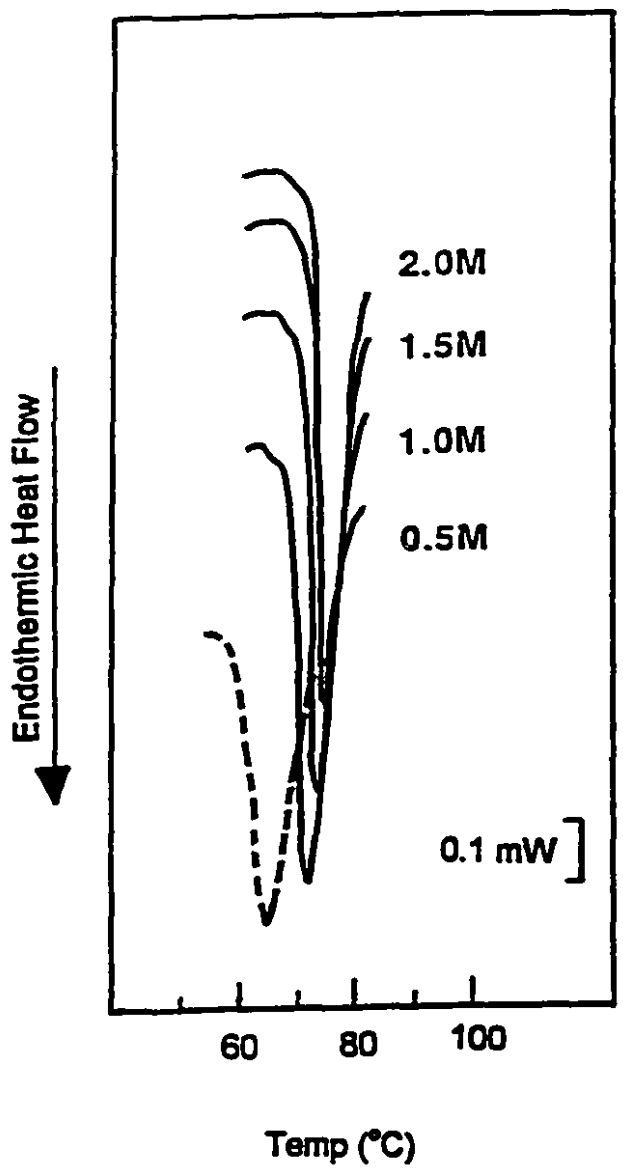



**Fig 7.4**: Effect of NaCl on the DSC characteristics of BSA (20% w/v). (—— - No NaCl added).

### 7.4.3 Protein-sugar Interactions

The effect of sugars on the $T_d$ of BSA is shown in Fig 7.5. In the presence of 10 and 50% (w/v) sucrose, the $T_d$ was observed at 64.6±0.2°C and 67.4±0.1°C respectively (Table 7.2). In 50% (w/v) glucose, the $T_d$ increased to 68.3±0.3°C which suggests that glucose had a greater stabilizing effect on the denaturation of BSA than sucrose. In the presence of 20% (w/v) lactose, the $T_d$ was at 65.5±0.1°C. Very little changes were observed in the width at half peak height of the DSC thermograms in the presence of the three sugars studied, which may suggest that the sugars did not interfere with the cooperativity of unfolding. The stabilizing influence of glucose and sucrose on the thermal denaturation of BSA was also observed for β–lactoglobulin, apo- and holo-α–lactalbumin (Boye *et al.*, 1995b,c); in each instance glucose had a greater stabilizing influence than sucrose.

### 7.4.4 Effect of denaturing agents

Fig 7.6 shows the thermograms of BSA in the presence of cysteine, urea, NEM and SDS. In the presence of cysteine and urea (50mM) the $T_d$ of BSA was 60.1±0.1°C and 61.0±0.2°C respectively. In the presence of NEM the $T_d$ was 63±0.1°C; this is similar to the $T_d$ (63.1°C) of BSA in $D_2O$, (Table 7.2, Fig 7.4), suggesting that NEM had little effect on the $T_d$ of BSA. In the presence of SDS, the $T_d$ increased by 20.8°C to 83.9±0.3°C. SDS has been shown to both increase and decrease the thermal stability of a number of proteins (Hegg *et al.*, 1978; Hegg, 1980; Harwalkar & Ma, 1987). The higher temperature of denaturation observed in this study suggests that SDS increases the thermal stability of BSA. It has been suggested (Hegg & Löfqvist, 1974; Markus *et al.*,1964) that SDS can form a bridge between a positively charged group in one loop of a polypeptide chain and a hydrophobic region in another; this could

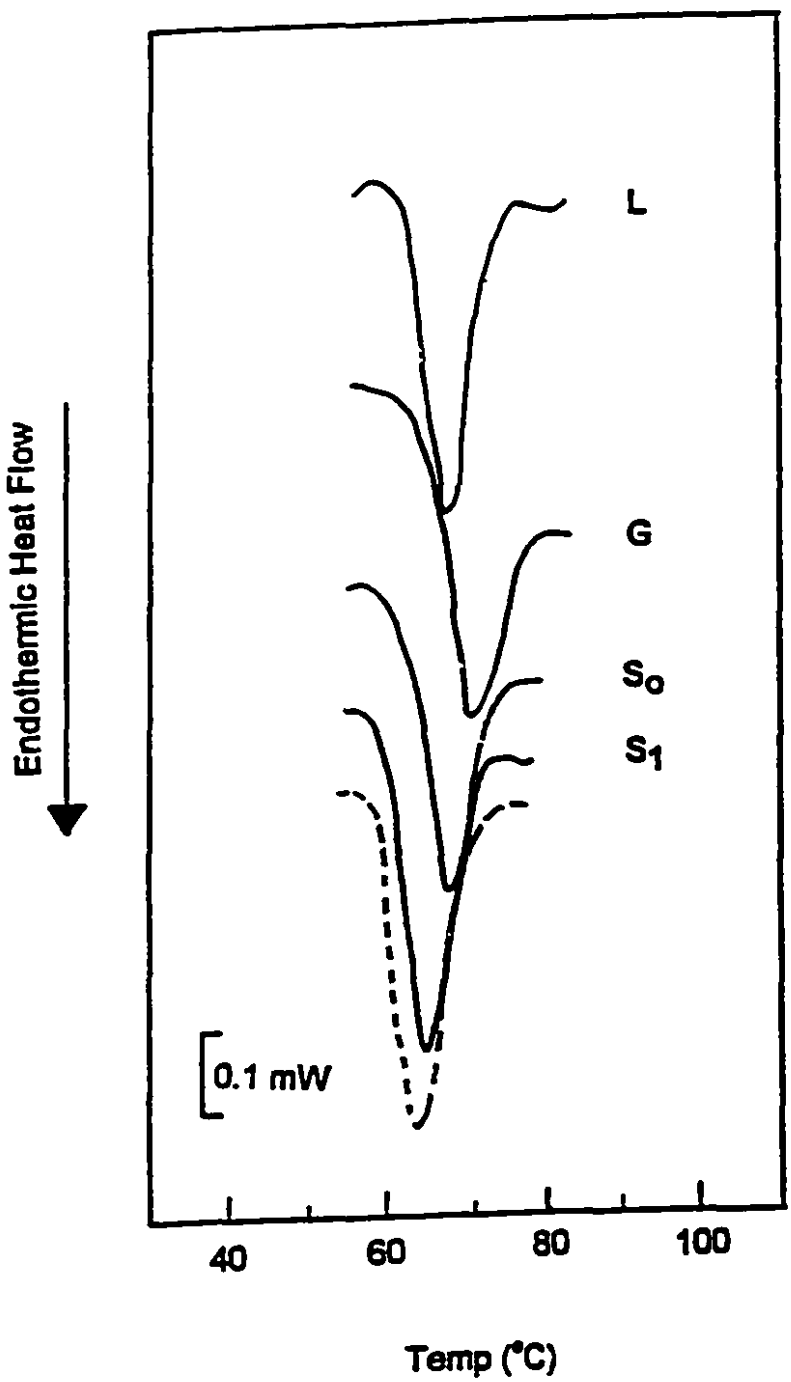



**Fig 7.5:** Effect of 20% (w/v) lactose (L), 50% (w/v) glucose (G), 10% ($S_o$) and 50%($S_1$) (w/v) sucrose on the DSC characteristics of BSA (20% w/v). (—— - No sugar added).

**Table 7.2: DSC Characteristics of BSA - Effect of sugars and denaturing agents.**

| Treatment | | $T_d$ (°C) | ΔH (J/g) |
|---|---|---|---|
| No sugar | 0 | 63.1±0.2 | 1.43±.03 |
| Sucrose | 10% (w/v) | 64.6±0.2 | 1.56±.01 |
| | 50% | 67.4±0.1 | 1.51±.07 |
| Glucose | 10% | 64.8±0.1 | 1.51±.03 |
| | 50% | 68.3±0.3 | 1.34±.04 |
| Lactose | 20% | 65.5±0.1 | 1.44±.02 |
| Cysteine | | 60.1±.01 | 2.00±0.1 |
| Urea | 50mM | 61.0±0.2 | 1.90±.06 |
| | 2M | 56.5±0.3 | 1.15±0.02 |
| NEM | | 63.0±0.1 | 2.50±.04 |
| SDS | | 83.9±0.3 | 2.8±0.02 |

See footnotes Table 7.1

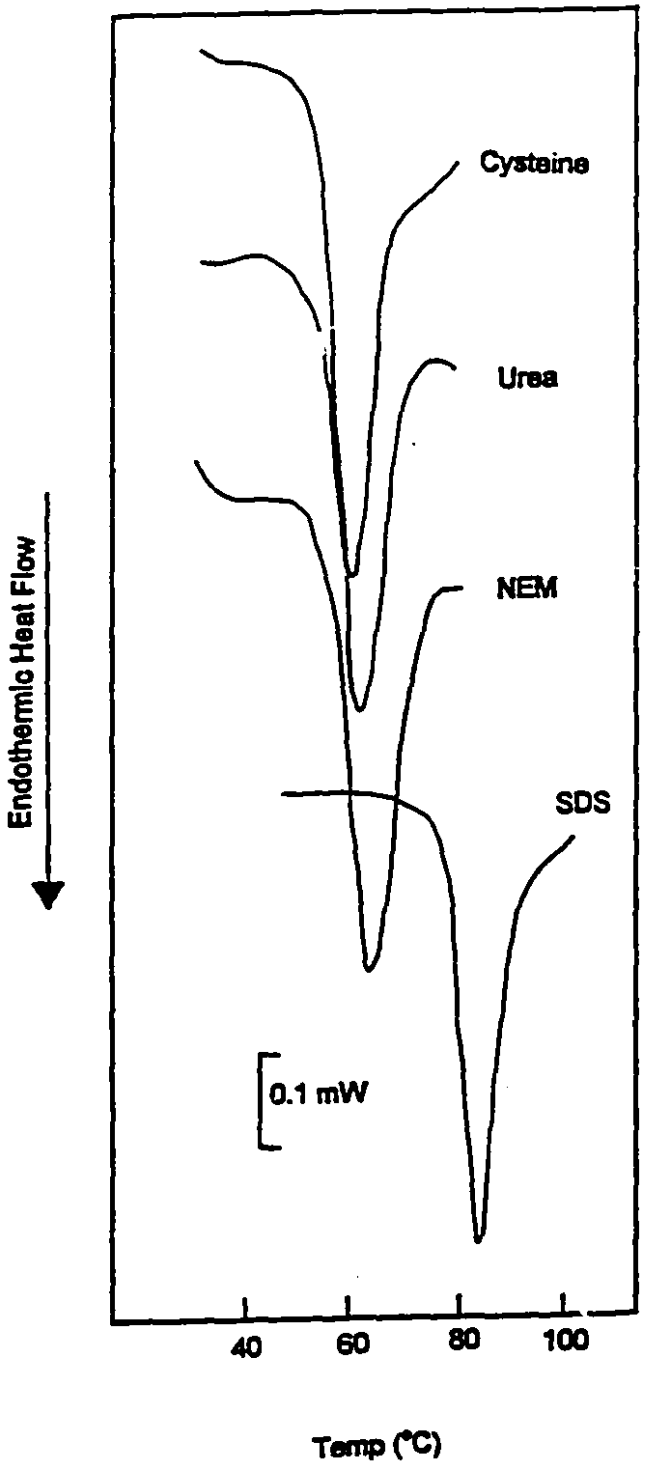



Fig 7.6: Effect of cysteine (50mM), urea (50mM), N-ethylmaleimide (NEM) (50mM) and sodium dodecyl sulfate (SDS ) (50mM) on the DSC characteristics of BSA 20% w/v). (Solutions of denaturing agents were made in $H_2O$).

explain its stabilizing effect on thermal denaturation. The thermogram (not shown) of BSA in 2M urea, showed a $T_d$ at 56.5±0.3°C; this represents a decrease from 61.0°C in the presence of 50mM urea. The lower $T_d$ value observed suggests that BSA was more denatured (unfolded) in 2M urea prior to heat treatment. The highest enthalpy of 2.8±0.02 J/g was obtained in the presence of SDS and the lowest (2.0±0.1 J/g) was in the presence of cysteine and urea. In 2M urea, the enthalpy decreased to 1.15±0.02 J/g. The higher enthalpy value observed in the presence of SDS, reflects weaker hydrophobic interactions in the protein prior to thermal denaturation. Aggregation as well as disruption of hydrophobic interactions are both exothermic reactions which lower the observed enthalpy (Myers, 1990). In the absence of aggregation, a high enthalpy value is an indication of a reduction in the break-up of hydrophobic bonds.

The peak width at half-height ($T_w$) in the presence of 50mM SDS was 4.8°C and in the presence of NEM was 9°C; in urea and cysteine, the $T_w$ values were 8.4°C and 7.4°C respectively. These results suggest that although SDS stabilized the protein against thermal denaturation, the process of unfolding was extremely cooperative in its presence.

The FTIR spectra of BSA heated in the presence of cysteine, NEM, urea and SDS (50mM) is shown Fig 7.7. The spectra recorded at 25°C, shows a shift in the band observed at 1630 cm-1 in the absence of any dissociating agent (Fig 7.2) to 1631 $cm^{-1}$ in the presence of cysteine and 1634 $cm^{-1}$ in urea, NEM and SDS (Fig 7.7). This shift to higher wavenumbers represents a decrease in hydrogen bonding in the presence of the dissociating agents (Krimm & Bandekar, 1986). The band at 1640 $cm^{-1}$ observed in the absence of any dissociating agents (Fig 7.2) and also in the presence of cysteine, NEM and urea (Fig 7.7), was not observed in the SDS spectra. The absence of this band

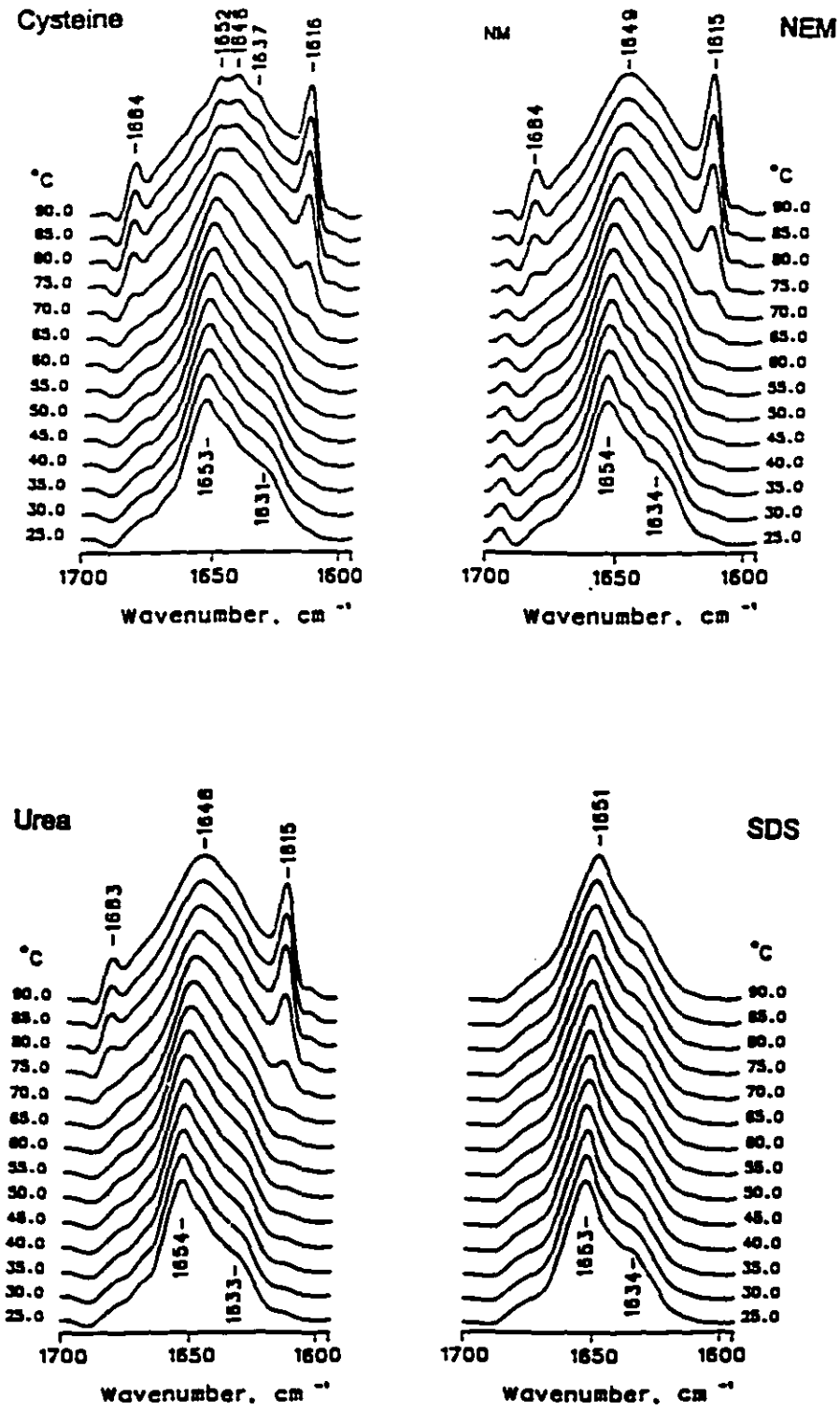



Fig 7.7: Stacked plot of deconvoluted infrared spectra of BSA (20% w/v) in 50mM deuterated cysteine, NEM, urea and SDS solutions at the specified temperatures.

which has been attributed to β–sheet structure (Susi & Byler, 1988) suggests a decrease in β–sheet content in the presence of SDS; this suggests that SDS may have disrupted the bonds responsible for β–sheet formation. Above 65°C, two bands at 1616 and 1684 $cm^{-1}$ (attributed to intermolecular β-sheet aggregation) were observed in the presence of cysteine, NEM and urea, but not in the presence of SDS; this suggests that there was no aggregation on heating BSA to 90°C in the presence of SDS (Clark *et al.*, 1981).

The width of the Amide I' band (1600-1700 $cm^{-1}$ region) has been used as an indication of protein unfolding (Ismail *et al.*,1992). Broadening or blurring of the band which results in an increase of the width of the bands is indicative of the loss or collapse of secondary structural integrity, possibly due to partial unfolding as the hydrogen bonds defining the protein structure are broken (Ismail *et al.*, 1992). To study the extent of BSA unfolding in the presence of the four reagents studied, the width at half-height of the 1600-1700 $cm^{-1}$ region was plotted as a function of temperature (Fig 7.8a). Below 60°C, there was little broadening in the presence of all four denaturing agents. Above 60°C, a marked broadening of the band was observed in cysteine followed by urea and NEM. This increase in width was not observed with SDS. Our results suggest that the greatest unfolding of BSA on heating was in the presence of cysteine and the least unfolding was in the presence of SDS.

The rate of hydrogen-deuterium exchange which also gives an indication of the extent of protein unfolding (Hvidt & Nielsen, 1966) can be studied in the Amide II' region of the infrared spectrum (1549-1542 $cm^{-1}$). To observe the effect of the denaturing agents on the rate of H-D exchange, the integrated intensity of the 1549 to 1542 $cm^{-1}$ region was plotted as a function of temperature (Fig 7.8b). In the presence of urea, cysteine, and NEM, the intensity of the band decreased to a minimum at 75°C which suggests a completion of

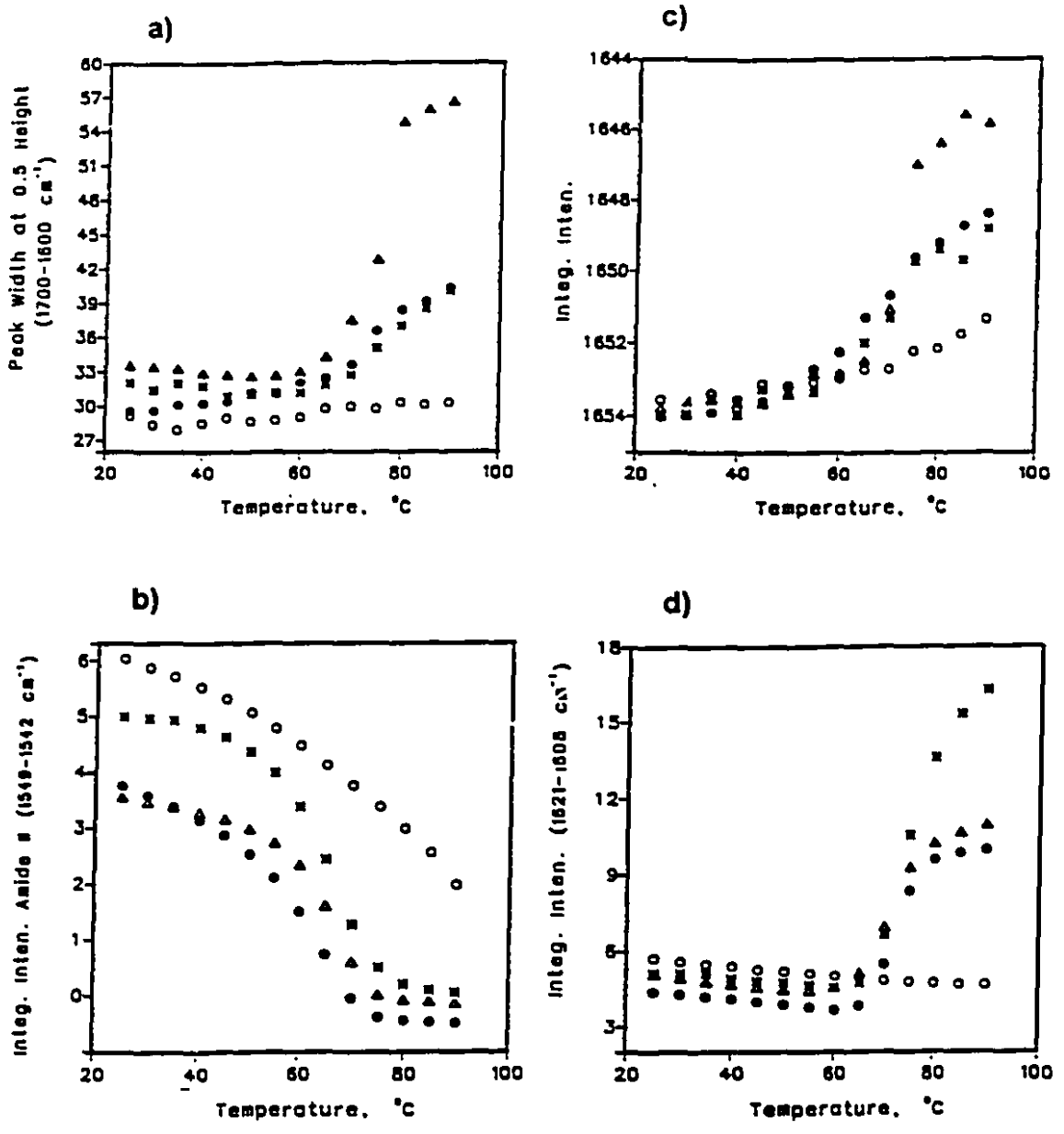



Fig 7.8: (a) Plot of the width at half height of the 1700-1600 $cm^{-1}$ region (Amide I') of BSA in the presence of Cysteine (Δ), urea (●), SDS (O ) and NEM (✱ ). (b) Integrated intensity (Integ. Inten.) of the 1549-1542 $cm^{-1}$ region (Amide II') (c) 1654 - 1644 $cm^{-1}$ bands (d) 1621 to 1608 $cm^{-1}$ region, as a function of temperature.

hydrogen-deuterium exchange. In SDS, however, the decrease in the intensity of the band was slow and a minimum level was not reached. The fastest rate of exchange was in the presence of urea followed by cysteine and NEM. The fast rate of hydrogen-deuterium exchange in the presence of urea might result from the break up of hydrogen bonds which exposed the hydrogen ions and made them more accessible to solvent. In the presence of cysteine, disruption of intramolecular disulfide bonds may have opened up the protein structure for deuterium exchange. NEM, however, did not contribute to the unfolding of the protein below the temperature of denaturation, probably because the native conformation of the protein was not disrupted. In the presence of SDS (which induces a net negative charge on proteins) excessive repulsive forces would have been expected to result in an unfolding of the protein and thus enhance hydrogen-deuterium exchange. The slow rate of exchange observed in SDS may therefore suggest a shielding effect of the dodecyl ions on the hydrogen ions of the protein.

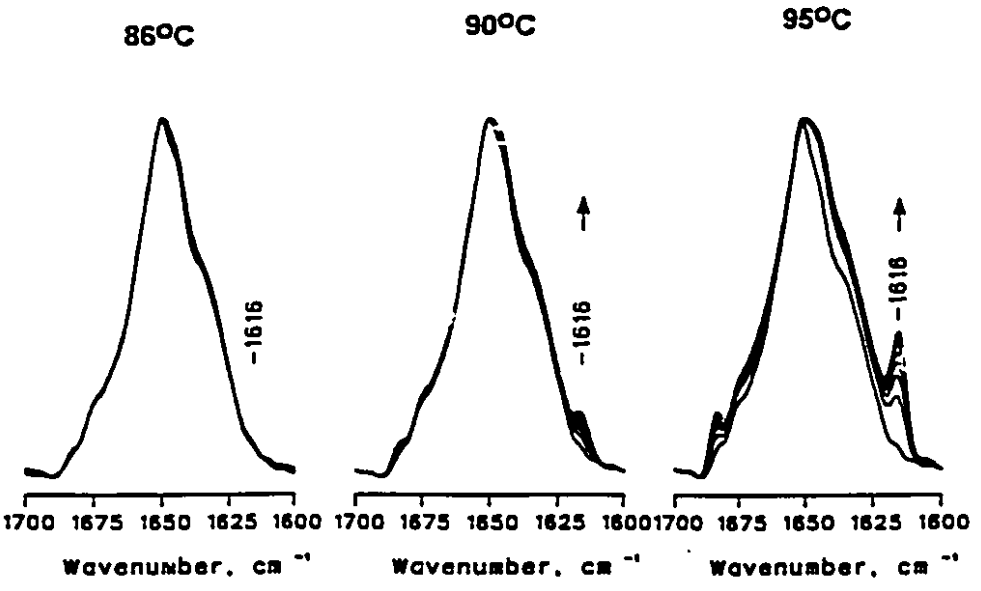
A plot of the integrated intensities of the 1654-1644 $cm^{-1}$ bands showing the shift in these bands as BSA was heated is shown in Fig 7.8c. On heat treatment, the band observed at 1654 $cm^{-1}$ (25°C), shifted to 1648, 1651 and 1652 $cm^{-1}$ in the presence of NEM and urea, SDS and cysteine respectively. In the presence of cysteine, a marked increase in the intensity of the 1646 $cm^{-1}$ band was observed (Fig 7.7 and 7.8c). The shift of the 1654 $cm^{-1}$ band to lower wavenumbers may have resulted from an increase in the intensity of the band at 1646 $cm^{-1}$ attributed to random coil structures, which suggests increased unfolding of the protein on heating. The greatest increase in the intensity of the 1646 $cm^{-1}$ band was observed in the presence of cysteine suggesting that it had greater effect in unfolding BSA.

A plot of the integrated intensity of the 1621-1608 $cm^{-1}$ region (which

represents the area under the 1616 $cm^{-1}$ band attributed to aggregation) is shown in Fig 7.8d. No increase in the intensity was observed below 65°C in the presence of the four denaturing agents. Above 65°C, a marked increase in the intensity of the band was observed in the presence of NEM followed by cysteine then urea. In the presence of SDS, no increase in the intensity of the band was observed at any temperature. This suggests that BSA did not aggregate when heated to 90°C in the presence of SDS, but aggregated in the presence of cysteine, urea and NEM; the aggregation in NEM was more intense than in cysteine and urea.

BSA was heated in the presence of the four denaturing agents at 85°C and 95°C for 10 min to check for gel formation. At 85°C, a translucent gel was formed in the presence of both 50mM and 2M urea. In the presence of cysteine, an opaque gel was formed. The gel formed in NEM was opaque and easily breakable. Matsudomi *et al.*, (1991) reported that in the presence of NEM, there was a marked decrease in the hardness of BSA gels; the gels formed were transparent, fragile and less elastic than control gels. No gel was formed in the presence of SDS on heating at 85°C, however, at 95°C a translucent gel was formed. The absence of the 1616 and 1684 $cm^{-1}$ bands (attributed to aggregate formation) in the spectra of BSA heated in 50mM SDS (Fig 7.7), corroborated the earlier finding which showed no gel formation on heating BSA in the presence of SDS at 85°C for 10 min, since aggregation has been shown to be an integral step in protein gelation (Edwards *et al.*, 1987).

The FTIR spectra of BSA in SDS was recorded as a function of time at 86, 90 and 95°C (Fig 7.9a) to check for aggregation. The spectra (at 86°C) showed no bands at 1684 and 1616 $cm^{-1}$ after 2.5 h of heating indicating an absence of aggregate formation. At 90°C, there was a slight increase in the 1684 $cm^{-1}$ band with time which suggested that the protein had started to aggregate. At 95°C a



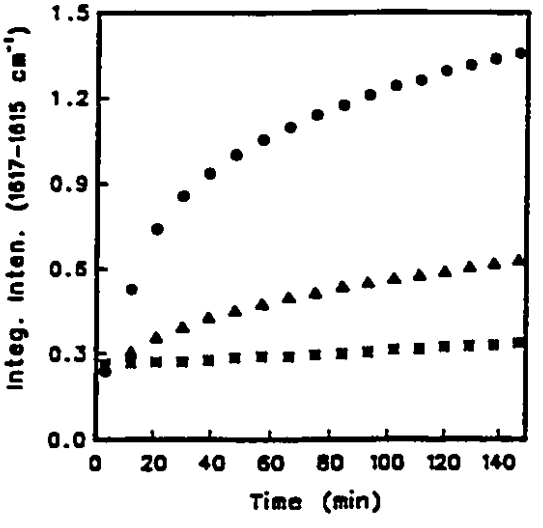




Fig 7.9: (a) Superimposed deconvoluted spectra of BSA in 50mM SDS heated at 86°C, 90°C and 95°C for 2.5 h. (b) Plot of the integrated intensity of the 1617 - 1615 $cm^{-1}$ region of the infrared spectrum of BSA heated at 86°C (✱ ), 90°C (Δ ) and 95°C (● ) as a function of time.

marked increase in the intensity of the 1684 $cm^{-1}$ was observed after 20 min (Fig 7.9b) which increased with time suggesting increased formation of aggregate structures.

No peaks were observed in the DSC thermogram on rescanning the heated BSA sample in the presence of all the reagents studied which suggests that the denaturation was compietely irreversible.

## 7.5 CONCLUSION

In the presence of NaCl, sucrose, glucose, cysteine, urea, NEM and at pH values between 3 and 9, denaturation of BSA resulted in a marked reduction of α–helical structure and an increase in ordered non-native β–sheet structure and random coil structures. Clark *et al.*, (1981) also reported the formation of ordered structure during gelation of BSA.

Based on the enhancement of the 1616 $cm^{-1}$ and 1684 $cm^{-1}$ bands (aggregation bands) in the presence of NEM and cysteine, it can be proposed that sulfhydryl-oxidation (disulfide bond formation) may not be essential in the aggregation step during the formation of BSA gels but may be necessary in the formation of intermolecular crosslinks between the aggregates formed as has been suggested by other workers (Hillier *et al.*, 1980; Xiong & Kinsella, 1990; Matsudomi *et al.*, 1991). Hydrophobic interactions may be the most important in the initial aggregation step since no aggregation bands were observed in the presence of SDS.

## CHAPTER 8

# THERMAL INTERACTIONS OF α–LACTALBUMIN, β–LACTOGLOBULIN AND BOVINE SERUM ALBUMIN: A DIFFERENTIAL SCANNING CALORIMETRIC STUDY.

## 8.0 CONNECTING STATEMENT

In Chapters 5, 6 and 7 the responses of β-lactoglobulin, α-lactalbumin and bovine serum albumin, to various environmental conditions during heat treatment were studied. The results obtained showed for example, that β-lactoglobulin and bovine serum albumin formed translucent gels by themselves at alkaline pH values and opaque gels at acid pH. α-lactalbumin on the other hand formed aggregate structures without gelation at acid pH values and did not gel at neutral pH's, but formed a translucent gel at alkaline pH values. These proteins coexist in whey protein concentrate, and are likely to interact with each other during processing. This chapter addresses the interactions of these proteins during heat treatment and satisfies the fourth objective described in the "Rationale and Objectives of Study" section in Chapter 1.

**Note:** This chapter is the text of a manuscript which has been submitted for publication as follows:

Boye, J.I., Alli,I., "Thermal interactions of α-lactalbumin, β-lactoglobulin and bovine serum albumin: A differential scanning calorimetric study". *Food Res. Int.* (Submitted, **June 1995**).

## 8.1 ABSTRACT

The interactions of α–lactalbumin (α–lac), β–lactoglobulin (β–lg) and bovine serum albumin (BSA) on heat treatment were studied in the presence of lactose, glucose, galactose, fructose, sucrose and at various pH values. The effects of N-ethylmaleimide, cysteine, urea and sodium dodecyl sulfate were also investigated. α–lac and β–lg did not gel on heating at 65°C, but the protein mixture (ratio 1:1) gelled on heating at 65°C for 30 min. The temperature of denaturation of β–lg decreased from 72°C to 69°C in the presence of α–lac. The protein mixture formed an opaque gel on heating at acid pH values but formed a translucent gel at alkaline pH. Glucose conferred thermal stability to β–lg but not apo-α–lac. In the presence of all five sugars a translucent gel was formed. α–lac showed only one irreversible transition at 36°C in the presence of Na bicarbonate and two partially reversible transitions at 45 and 68°C in the presence of Na ascorbate. α–lac showed one broad peak at approximately 55°C in the presence of cysteine and SDS, and two transitions at 34 and 64°C in the presence of NEM and urea. β–lg showed one irreversible transition in the presence of all four denaturing agents. A translucent gel was obtained on heating the α–lac/β–lg mixture in the presence of cysteine, urea and SDS. The results indicated that disulfide-sulfhydryl interchange reactions are primarily responsible for the gelation of α–lac/β–lg mixtures.

## 8.2 INTRODUCTION

Whey protein preparations in the form of concentrates and isolates are important ingredients in many food products, primarily because of their ability to form heat-induced gels (Mulvihill & Kinsella, 1988). The ability of proteins to unfold, aggregate and gel depends upon the amino acid composition (net charge), molecular weight, net hydrophobicity, protein concentration, heating and cooling rates, and a critical balance between attractive and repulsive forces (Shimada & Matsushita, 1980; Schmidt, 1981; Foegeding *et al.*, 1986) as well as interactions with solvent and other proteins in solution. In previous work (Boye *et al.*, 1995a, 1995b, 1995c, 1995d) we reported the effect of various environmental conditions (pH, NaCl, sucrose, lactose, heating temperature) on the denaturation temperature and gel characteristics of the three major proteins in whey, namely, β-lactoglobulin, α-lactalbumin and bovine serum albumin.

β–lactoglobulin (β–lg) is the major component of whey, constituting about 50% of the proteins in WPC. Native β–lg possesses two disulfide bonds and a free thiol group which is normally inaccessible to solvent but displays increasing reactivity at pH values above 6.5 (Morr, 1975; Haque & Kinsella, 1987). Upon heating, the free thiol groups may be oxidized to form intermolecular disulfide bonds or participate in thiol-disulfide interchange reaction (Kinsella, 1982). α–lactalbumin (α–lac) is the second major protein of whey representing approximately 20% of the proteins of bovine whey. α–lac is a calcium-binding protein containing four intramolecular disulfides and no free sulfhydryls (Fox, 1989). Three aspartyl residues participate at the $Ca^{++}$-binding site of α–lac (Stuart *et al.*, 1986). Above pH 5, the carboxyl groups of aspartic and glutamic acid are ionized and serve as effective binding sites for metal ions when held in

appropriate proximity. In the interior of the molecule they may also participate in salt bridges and are capable of serving as hydrogen donors and acceptors in hydrogen bond formation (Kinsella *et al.*,1989). Bovine serum albumin is the longest single polypeptide chain of all the whey proteins, representing about 10% of total whey protein (Fox, 1989). Heat-induced gelation of BSA is initiated by intermolecular thiol-disulfide interchange reactions similar to what happens with β–lg (De Wit, 1989).

There is a relative abundance of information in the literature on factors affecting gel formation and properties of whey protein gels (Mulvihill & Kinsella, 1988; Fox, 1989; Boye *et al.*, 1995a, 1995b, 1995c, 1995d). By contrast, there is relatively limited information on the kinetics of gel formation and gelling properties of the individual whey proteins (Kinsella & Whitehead, 1989) and mixed-gel systems with β–lactoglobulin in combination with other proteins found in whey (e.g., α–lac) (Hines & Foegeding, 1993). α–lac for example, unfolds on heat treatment at temperatures as low as 62°C but on cooling the molecule reverts to its native configuration. The Cys/2 residues which occur as four disulfide bridges, are mainly responsible for this reversible conformational change. This reversibility is lost if the native disulfide bonds are broken, for example by heat-induced thiol-disulfide interactions in the presence of β–lg. Paulsson *et al.*, (1986) found on heating protein solutions at 1°C/min up to 90°C, at pH 6.6, that β–lg formed a gel at 5% (w/v), but α–lac was incapable of gelation at protein concentrations up to 20% (w/v). Hines and Foegeding (1993), however, reported that α–lac and BSA interacted with β–lg in forming mixed protein gels; the β–lg and BSA mixtures formed gels with greater storage modulus than β–lg alone. Elfagm and Wheelock (1978) used size exclusion

chromatography to show that free α–lac disappeared to a greater extent when heated with β–lg than when heated alone, at temperatures above 77°C and in the pH range 6.4-7.2. Legowo *et al.*, (1993), also showed that α–lac can positively influence the heat-induced gelation of mixed proteins (α–lac/β–lg) in the absence of glutathione but the mechanisms by which α–lac, β–lg and BSA interact are still to be investigated.

This report describes the application of differential scanning calorimetry (DSC) to study the effect of denaturing agents such as N-ethylmaleimide, cysteine, urea and sodium dodecyl sulfate, pH, sugars, sodium salts and heat on the heat-induced gelation of mixtures of α–lac, β–lg and BSA.

## 8.3 MATERIALS AND METHODS

### 8.3.1 Materials

WPC samples (75% protein, 13% lactose, 3% ash, 8% fat) from Saputo (St.-Hyacinthe, Quebec) was obtained and stored in air tight containers at room temperature. α–Lactalbumin (Product L6010), β–lactoglobulin A+B (Product L2506) and BSA (Product A–2153) were obtained from Sigma Chemical Co. (St.Louis, MO,USA) and used as received.

### 8.3.2 Sample Preparation

*Interactions of α-lac and β-lg:* Solutions of α–lactalbumin (20% w/v), β–lactoglobulin (20% w/v) and mixtures of α–lac and β–lg [(ratio 1:1), 40% (w/v) final protein concentration of mixture], and whey protein concentrate (20% w/v) were prepared by dissolving the protein in $H_2O$ to obtain the desired mixture. The samples were agitated gently and aliquots (1 ml) were placed in Eppendorf

conical micro centrifuge tubes (1.5 ml capacity) and heated in a convection oven (Fisher Isotemp Oven, Junior Model) at 55, 65 and 75°C for 30 min, cooled to room temperature and visually checked for gel formation (Patel & Fry, 1987).

*Interactions of α-lac, β-lg and BSA*: To study the interactive effects of mixtures of α–lac, β–lg and BSA on their individual denaturation temperatures, solutions containing the three proteins in the ratio 1:1:1 were prepared in phosphate buffers at pH 3 and 9 and scanned in the DSC. The final protein concentration for the mixtures was 30% (w/v). Mixtures of α–lac/BSA and β–lg/BSA in the ratio 1:1 (final protein concentration of mixture was 20% (w/v)) were also studied. Mixtures of the three proteins in the ratio 4:9:1, similar to the ratio in which these proteins are normally present in whey, were also studied. The final concentration for the protein mixture containing the 4:9:1 ratio was 20% (w/v). 100μl of each solution was heated as described above at 85°C for 15 min, cooled, and scanned in the DSC.

*Effect of pH:* To study the effect of pH on the α–lac/β–lg mixtures in the ratio 1:1, the proteins were dispersed in phosphate-buffers at pH 3, 5, 7, 9 and 11 (ionic strength-0.2) .

*Effect of sugars:* To study the effect of sugars, the α–lac/β–lg mixtures were prepared in 20% (w/v) solutions of sucrose, lactose, fructose, glucose and galactose made in $H_2O$.

*Effect of sodium salts:* To study the effect of sodium salts, α–lac (20% w/v), β–lg (20% w/v) and the α–lac/β–lg mixture (40% w/v) were dispersed in 0.5N sodium ascorbate and sodium bicarbonate solutions.

*Effect of denaturing agents:* To study the effect of denaturing agents, α–lac (20% w/v), β–lg (20% w/v) and the α–lac/β–lg mixture (40% w/v) were

dispersed in 50mM solutions of N-ethylmaleimide, cysteine, urea and SDS. The rationale for using these denaturing agents has been discussed in a previous paper (Boye *et al.*, 1995d). The solutions were allowed to stand for 15 min before scanning in the DSC.

### 8.3.3 Differential Scanning Calorimetry

Twenty-five µl of each solution were placed in preweighed DSC pans, which were hermetically sealed and weighed accurately. The samples were placed in the DSC (TA3000, Mettler Instrument Corporation, Greifensee, Switzerland) and scanned from 15°C to 100°C at a programmed heating rate of 5°C/min. For each run, a sample pan containing the buffer used for dissolving the proteins was used as reference. After heating, the samples were allowed to cool to room temperature in the DSC and the heating cycle was repeated under the same experimantal conditions. The degree of reversibility was determined from the ratio of the areas under the second and the first endothermal peaks (Relkin *et al.*, 1993). The DSC was calibrated by use of indium standards. All DSC experiments were done in duplicate.

The DSC measurements were made on protein dispersions in $H_2O$. In previous papers (Boye *et al.*, 1995 b,c) DSC measurements of α-lac and β-lg were made in $D_2O$; the $T_d$ observed in $D_2O$ are generally higher by 2-4°C.

After scanning the DSC pans were opened and the solutions were evaluated visually for gelation (Clark & Lee-Tuffnell, 1986; Boye *et al.*, 1995c, d; Nonaka *et al.*, 1993). A white non-flowable firm texture was classified as an opaque gel. A clear non-flowable firm texture was classified as a translucent gel (Boye *et al.*, 1995a, d).

## 8.4 RESULTS AND DISCUSSION

Fig 8.1 shows the thermograms of α–lac, β–lg and a mixture of α–lac/β–lg. The thermogram of α–lac in $H_2O$ shows two transitions with peak temperatures ($T_d$) at 35.0°C (peak A) and 64.3°C (peak B) (Table 8.1) corresponding to the denaturation of apo- ($Ca^{++}$ free) and holo-($Ca^{++}$ bound) α–lac respectively (Relkin *et al.*, 1993; Boye *et al.*, 1995c). β–lg showed one thermal transition at 71.9°C (peak C). The α–lac/β–lg mixture showed two thermal transitions at 37.5°C (corresponding to the denaturation of apo-α–lac) and 69.1°C (corresponding to the denaturation of β–lg) with a shoulder at approximately 63°C (corresponding to the denaturation of holo-α–lac). This represents an increase in the $T_d$ of apo-α–lac by 2.5°C. The $T_d$ of β–lg decreased from 71.9°C to 69.1°C in the presence of α–lac which suggests that β–lg is slightly less stable when heated in the presence of α–lac.

Fig 8.2 shows the thermograms of α–lac, β–lg and the α–lac/β–lg mixture (preheated at 55, 65 and 75°C for 30 min prior to scanning in the DSC). The thermogram of the mixture preheated at 55°C showed only one transition at 69.1°C (attributable to the combined denaturation of both holo-α–lac and β–lg) . This suggests that apo-α–lac (peak A) had irreversibly denatured in the presence of β–lg. The mixtures, preheated at 65°C and 75°C for 30 min resulted in the disappearance of all the peaks, suggesting that the proteins were completely and irreversibly denatured. When β–lg alone was preheated at 55°C for 30 min, the thermogram showed one transition at 72°C with a ΔH of 1.8J/g similar to that of the unheated protein (Table 8.1), which suggests that the protein was not denatured; at 65°C, the ΔH decreased to 0.6J/g suggesting partial denaturation of the protein by the heat treatment. No transitions were

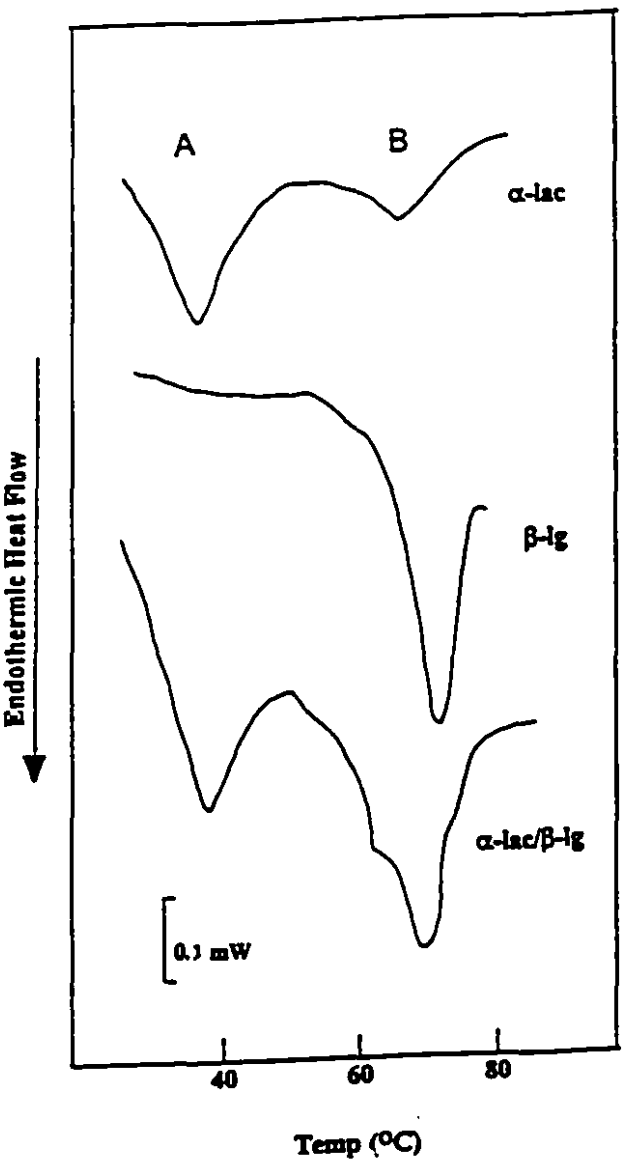



Fig 8.1: Thermograms of α–lac (20% w/v), β–lg (20% w/v) and α–lac/β–lg mixture, ratio 1:1. Heating rate was 5°C/min.

**Table 8.1: Peak temperature of denaturation and gel characteristics of preheated α–lac, β–lg and α–lac/β–lg mixture.**

| Treatment (°C) | Peak Temperature (°C) | | | ΔH (J/g) | Gel Type |
|---|---|---|---|---|---|
| | 1 | 2 | 3 | | |
| α–lactalbumin | | | | | |
| 25 | 35.0 | 64.3 | | | No gel |
| 55 | 34.7 | 61.7 | | | No gel |
| 65 | 35.0 | 62.7 | | | No gel |
| 75 | 35.0 | 62.0 | | | No gel |
| β–lactoglobulin | | | | | |
| 25 | | | 71.9 | 1.83 | No gel |
| 55 | | | 72.0 | 1.83 | No gel |
| 65 | | | 72.0 | 0.60 | No gel |
| 75 | | | - | - | Translucent |
| α–lactalbumin/β–lactoglobulin mixture (1:1) | | | | | |
| 25 | 37.5 | ~63* | 69.1 | | No gel |
| 55 | - | - | 69.1 | | No gel |
| 65 | - | - | - | | Translucent |
| 75 | - | - | - | | Translucent |

* - shoulder

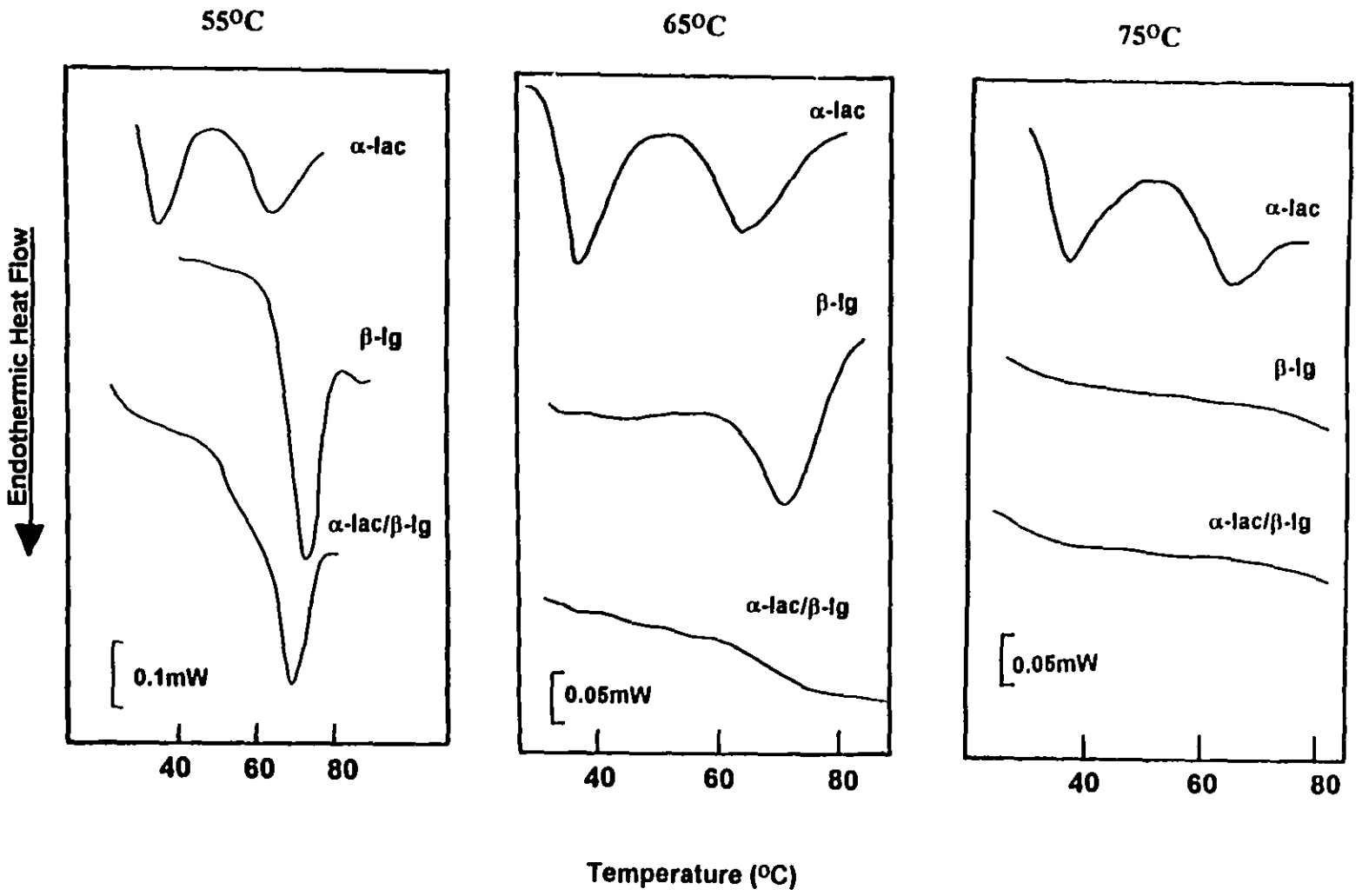



**Fig 8.2:** Thermogram of mixture of α-lac/β-lg (ratio 1:1) heated at temperatures indicated for 30 min and cooled to room temperature prior to scanning in the DSC.

observed in the thermogram when β–lg was preheated at 75°C indicating that the protein was completely denatured. When α–lac was preheated at 55, 65 and 75°C, the thermograms showed two transitions at 35°C and 62°C, suggesting that the protein was not irreversibly denatured by the preheating.

No gelation was observed with the individual proteins preheated at 55 and 65°C (Table 8.1); at 75°C, β–lg formed a translucent gel, α–lac did not form a gel and the α–lac/β–lg mixture formed a translucent gel at both 65°C and 75°C. The results suggest that although α–lac and β–lg individually did not form gels when heated at 65°C, a mixture of the two proteins formed a gel at this temperature. This may result from hydrophobic and disulfide-sulfhydryl interactions between holo-α–lac and β–lg. The high concentration of α–lac (20% w/v) and β–lg (20% w/v) used may also have positively influenced gel formation by increasing the number of loci available per molecule for crosslinking (Damodaran, 1989).

Fig 8.3 shows the thermogram of the α–lac/β–lg mixture at different pH values. Increasing the pH from 3 to 7 resulted in an increase in the $T_d$ of the first transition from 36.0°C at pH 3 to 38.9°C at pH 7 (Table 8.2). At pH 7, 9 and 11, the $T_d$ of the first transition (denaturation of apo-α-lac) remained constant at 39°C. The $T_d$ of the second transition decreased from 79.8°C at pH 3 to 69.1°C at pH 7 and dropped to 64.9°C at pH 11. At pH 7, a shoulder (representing holo-α-lac) was observed at approximately 60°C which was absent at pH 9 and 11. With decreasing pH, the shoulder developed into a peak with a ΔH of 0.4 J/g. The ΔH of the first transition in the α–lac/β–lg mixture at pH 3 was 1.3 J/g; at pH 11, the ΔH increased to 2.3 J/g. The peak area of the second transition increased with pH. The ΔH was not calculated since the peak was considered to

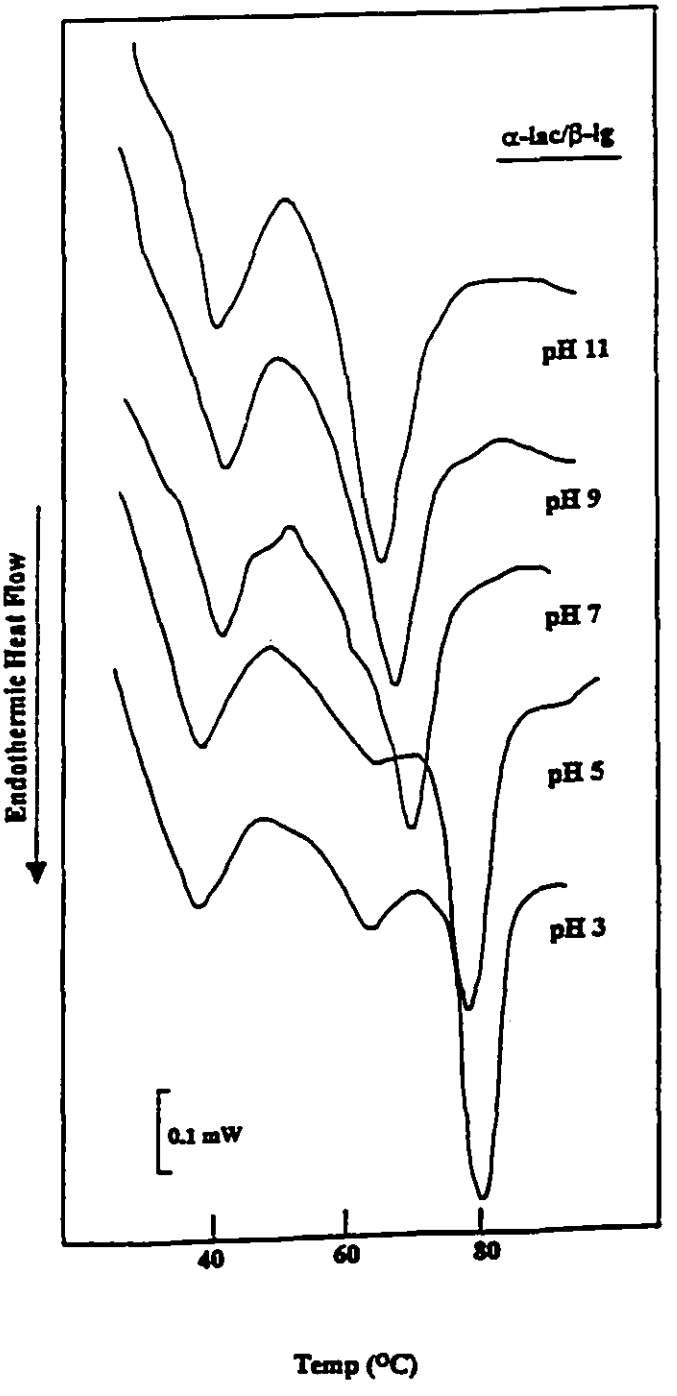



Fig 8.3: Effect of pH on thermal characteristics of α–lac/β–lg mixture (ratio 1:1).

**Table 8.2: Peak temperature of denaturation and gel characteristics of α–lactalbumin and β–lactoglobulin.**

| Treatment | Peak Temperature (°C) | | | $T_w$ | Enthalpy | Gel Type |
|---|---|---|---|---|---|---|
| | 1 | 2 | 3 | | | |
| α–lactalbumin | | | | | | |
| $H_2O$ | 35.0 | 64.3 | | | | No gel |
| Na ascorbate | 45.2 | 68.1 | | | | Translucent |
| Na bicarbonate | 36.0 | | | | | No gel |
| N-ethylmaleimide | 33.9 | 63.8 | | | | No gel |
| Cysteine | | 52.9 | | | | Translucent |
| Urea | 34.5 | 63.1 | | | | No gel |
| SDS | | 55.0 | | | | No gel |
| β–lactoglobulin | | | | | | |
| $H_2O$ | | | 71.9 | 6.2 | 1.83 | Translucent |
| Na ascorbate | | | 78.0 | 6.8 | 1.81 | Translucent |
| Na bicarbonate | | | 66.1 | 5.2 | 1.15 | Translucent |
| N-ethylmaleimide | | | 63.7 | 12.5 | 1.17 | Aggregate* |
| Cysteine | | | 68.2 | 8.5 | 1.97 | Translucent |
| Urea | | | 71.0 | 7.0 | 1.96 | Translucent |
| SDS | | | 75.0 | 13.7 | 0.74 | Translucent |

* - translucent aggregates

$T_w$ - Width of the DSC peak at half-height

represent the sum of the enthalpies of denaturation of both holo-α-lac and β-lg. β-lg has been shown to be more thermally stable at low pH (Harwalkar & Ma,1989; Boye *et al.*,1995b, c); this could explain the high $T_d$ of the second transition at pH 3 in the α-lac/β-lg mixture and suggests that in the presence of α-lac, the thermal stability of β-lg was not affected at low pH values.

When heated, the α-lac/β-lg mixture gave an opaque gel at pH 3 and 5. At pH 7 and 11, a transparent gel was formed (Table 8.3). α-lac heated alone in acid solution results in the exposure of hydrophobic groups and the formation of aggregate structures without gelation (Shukla, 1973). In the presence of β-lg, intermolecular disulfide-sulfhydryl interactions between α-lac and β-lg could have contributed to the formation of an opaque gel.

The thermogram of the mixture of α-lac/BSA (1:1), β-lg/BSA (1:1), α-lac/β-lg/BSA (1:1:1) and WPC at pH 3 and 9 are shown in Fig 8.4. Two thermal transitions were observed in the α-lac/BSA mixture at pH 3 (Fig 8.4a.i). The first transition corresponding to the denaturation of apo-α-lac was observed at 32.2°C (Table 8.3); the second transition, which may represent the denaturation of both holo-α-lac and BSA, was observed at 58.7°C. This represents a decrease in the $T_d$ of both apo- (35.4°C) and holo-α-lac (64.4°C) heated alone at this pH and suggests that BSA decreased the $T_d$ of both apo- and holo-α-lac at acid pH values. At pH 9 (Fig 8.4b.i), the peak of the first transition shifted to 39.0°C while the second transition decreased to 58.2°C. α-lac heated alone at pH 9 gave two transitions at 39.6 and 63.4°C (Table 8.3) which suggests that BSA decreased the $T_d$ of holo-α-lac but not apo-α-lac at alkaline pH. The mixture of β-lg/BSA gave two thermal transitions at 61.9°C (corresponding to the denaturation of BSA) and 82.4°C (corresponding to the denaturation of β-lg)

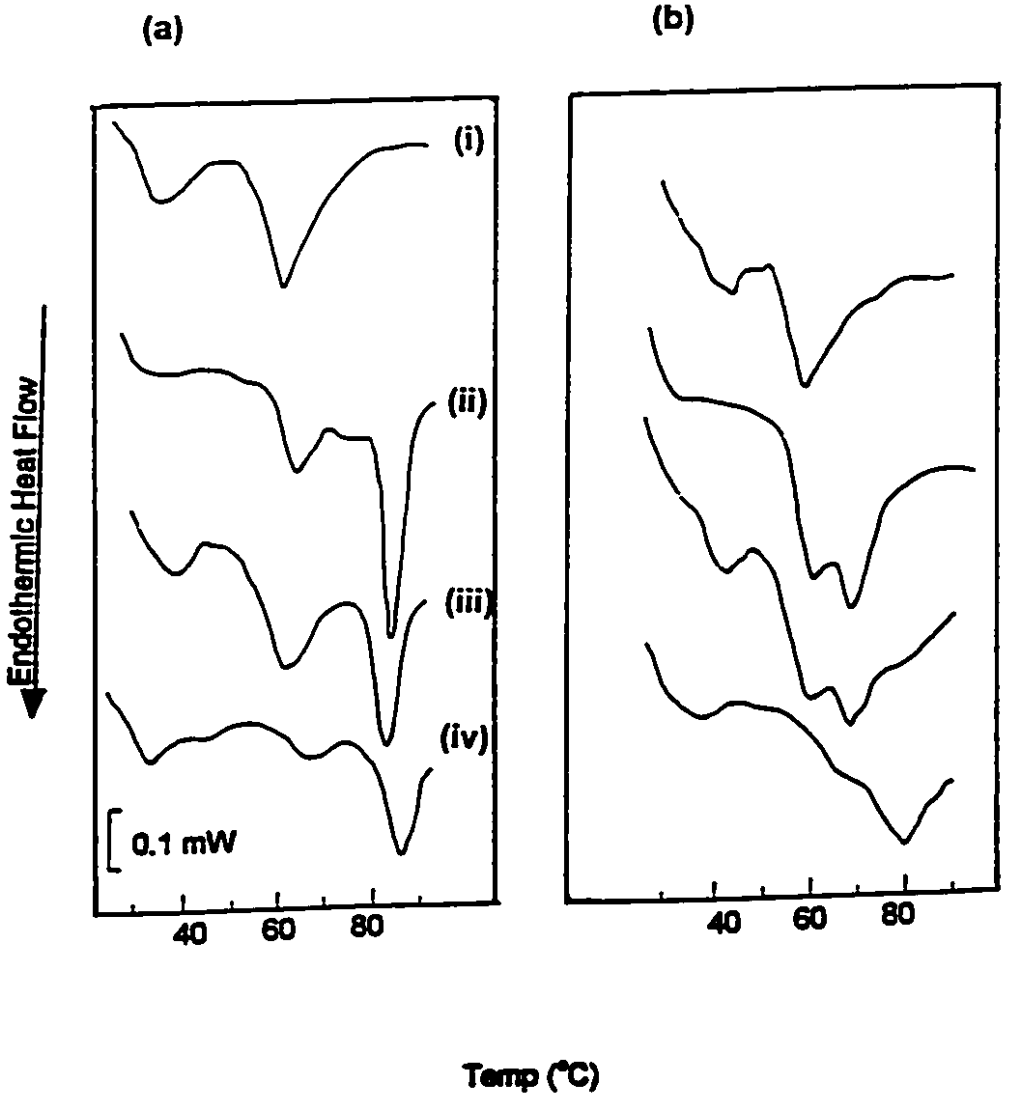



Fig 8.4: DSC characteristics of mixtures of (i) α–lac/BSA (1:1) (ii) β–lg/BSA (1:1) (iii) α–lac/β–lg/BSA (1:1:1) (iv) WPC at (a) pH 3 and (b) pH 9.

**Table 8.3: Peak temperature of denaturation and gel characteristics of α-lac/β-lg mixture (ratio 1:1).**

| Treatment | Peak Temperature (°C) | | | Gel Type |
|---|---|---|---|---|
| | 1 | 2 | 3 | |
| $H_2O$ | 37.5 | ~63* | 69.1 | translucent |
| pH 3 | 36.0 | ~60* | 79.8 | opaque |
| pH 5 | 37.6 | ~60* | 78.1 | opaque |
| pH 7 | 38.9 | ~60* | 69.1 | translucent |
| pH 9 | 38.9 | | 67.0 | translucent |
| pH 11 | 39.0 | | 64.9 | translucent |
| sucrose | 39.2 | ~60* | 71.7 | translucent |
| lactose | 38.6 | ~60* | 73.4 | translucent |
| fructose | 38.9 | ~60* | 73.4 | translucent |
| glucose | 39.0 | ~60* | 76.8 | translucent |
| galactose | 39.2 | ~60* | 77.0 | translucent |
| Na ascorbate | 43.2 | | 75.6 | translucent |
| Na bicarbonate | 36.9 | | 63.0 | translucent |
| N-ethylmaleimide | 32.1 | | 62.2 | aggregate§ |
| Cysteine | | | 67.2 | translucent |
| Urea | 36.3 | | 68.4 | translucent |
| SDS | | | 77.9 | translucent |

* - shoulder; § - translucent aggregate

(Fig 8.4a.ii). At pH 9, the first transition shifted to 59.2°C and the second was observed at 68.5°C. When β–lg was heated alone (thermogram not shown), the $T_d$ was observed at 82.2°C at pH 3 and 67.0°C at pH 9, which suggests that BSA had little effect on the $T_d$ of β–lg at pH 3, but increased it slightly at pH 9. The mixture of α–lac/β–lg/BSA showed three separate transitions at 36.4°C, 61.9°C and 82.3°C at pH 3 (Fig 8.4a.iii) which suggests that BSA had less of an effect in decreasing the $T_d$ of apo-α–lac in the presence of β–lg. At pH 9 (Fig 8.4b.iii), two transitions were observed at 39.4°C and 69.3°C and a shoulder at 60°C which indicates an increase in the $T_d$ of β–lg in the presence of α–lac and β–lg. No ΔH values are reported due to difficulties in adequately defining peak areas in the presence of two transitions.

Fig 8.5 shows the DSC thermograms of α–lac/BSA (4:1), β–lg/BSA (9:1) and α–lac/β–lg/BSA (4:9:1) at pH 3 and 9. These ratios represent the proportions in which these proteins exist in whey. The α–lac/BSA thermogram gave two transitions at 34.7°C and 62.7°C at pH 3 (Fig 8.5a.i) and 40.1°C and 64.2°C at pH 9 (Fig 8.5b.i). This represents a shift from 32.2 and 58.7°C at pH 3 and 39.0 and 58.2°C at pH 9 in the mixture with a 1:1 ratio (Table 8.3), suggesting that higher concentrations of BSA decreased the denaturation temperature of α–lac. The β–lg/BSA mixture gave one major transition at 81.9°C at pH 3 (Fig 8.5a.ii) and 67.6°C at pH 9 (Fig 8.5b.ii). These temperatures are similar to that observed from the mixture with a 1:1 ratio, suggesting that higher concentrations of BSA had little effect on the denaturation temperature of β–lg. In the α–lac/β–lg/BSA mixture, two transitions were observed at 63.2°C and 81.8°C at pH 3. The first transition represents the denaturation of holo-α–lac and BSA, while the second transition represents the denaturation of β–lg. The

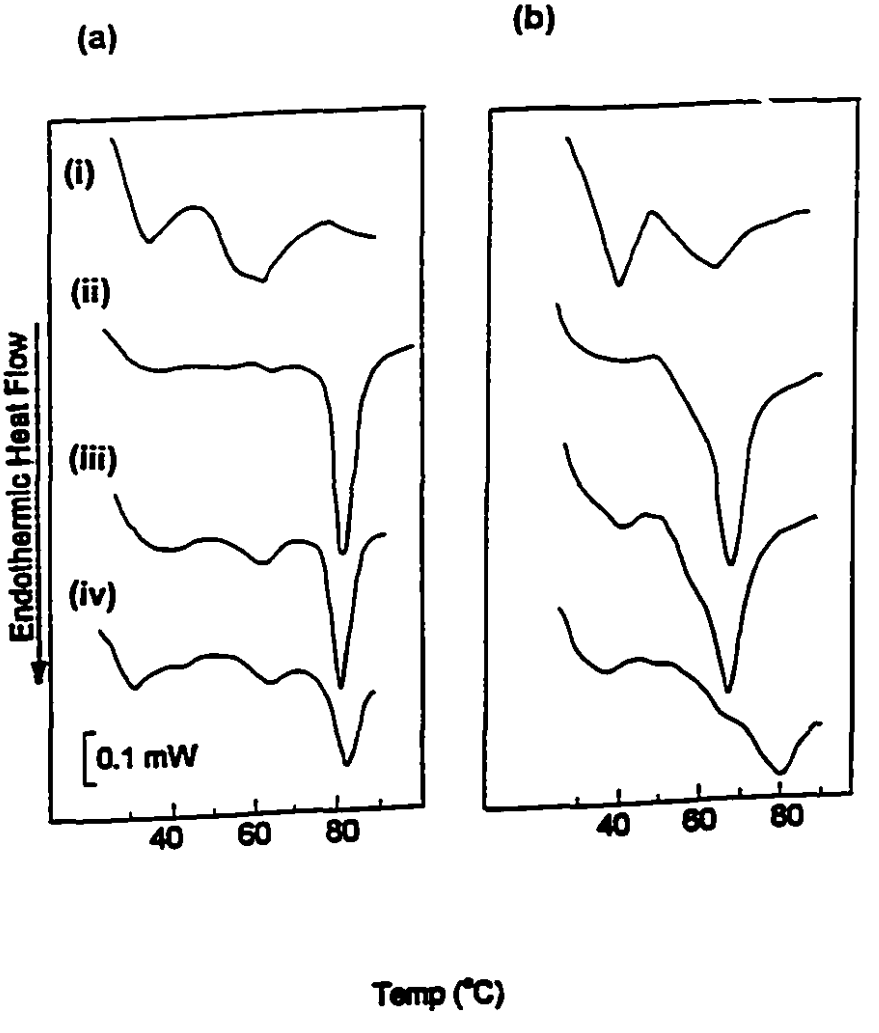



**Fig 8.5:** DSC characteristics of mixtures of (i) α–lac/BSA (4:1) (ii) β–lg/BSA (9:1) (iii) α–lac/β–lg/BSA (4:9:1) (iv) WPC at (a) pH 3 and (b) pH 9.

transition corresponding to the denaturation of apo-α-lac could not be discerned. The peak temperatures observed represent a slight increase from the denaturation temperature of holo-α-lac in the mixture with 1:1:1 ratio and a subtle decrease in the denaturation temperature of β-lg, which confirms that high concentrations of BSA decreased the denaturation temperature of holo-α-lac. At pH 9, one broad thermal transition was observed at 67.7°C. This temperature was close to that observed for β-lg (67.°C) which suggests that both α-lac and BSA had little effect on the Td of β-lg. WPC showed a minor transition at 32.2°C at pH 3 and a major transition at 85.1°C with a shoulder at ~67°C (Figs 8.4, 8.5a.iv). At pH 9 one thermal transition was observed at 79.3°C. These transition temperatures, with the exception of the first transition observed at pH 3 were higher than those observed in the α-lac/β-lg/BSA mixtures which suggests that other components present in whey affect the denaturation temperature of WPC. When the heated mixtures were checked for gel formation, an opaque gel was observed with the mixtures heated at pH 3 and a translucent gel was obtained with the mixtures heated at pH 9.

Figure 8.6 shows the thermograms of the α-lac/β-lg mixture heated in the presence of sucrose, lactose, fructose, glucose and galactose. The effect of sugars on the individual proteins have previously been reported (Boye *et al.*, 1995 b,c). The $T_d$ for the first transition was between 38.6 and 39.2°C in the presence of all five sugars. This represents a slight increase from 37.5°C in the absence of sugar (Table 8.2), suggesting that the effect of sugars on the denaturation temperature of α-lac was minimal. The $T_d$ of the second transition (corresponding to the denaturation of β-lactoglobulin), was 71.7 and 73.4°C for sucrose and lactose respectively, and 76.8 and 77.0°C for glucose and

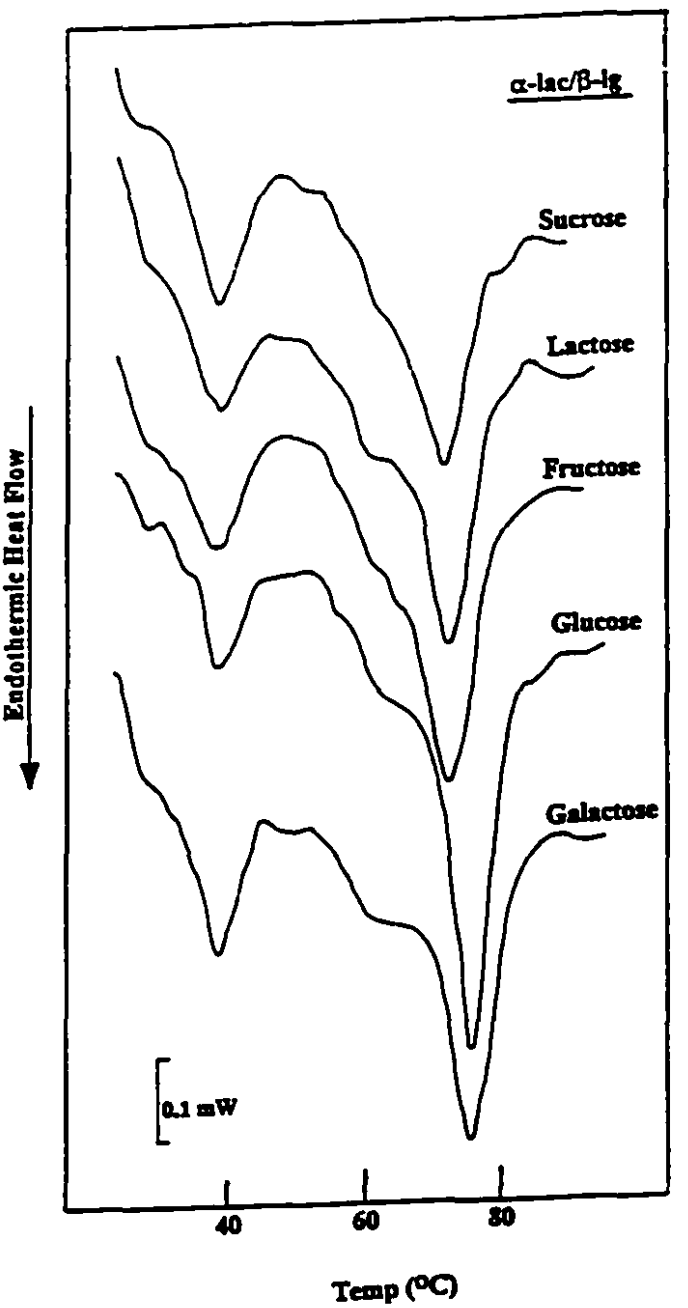



Fig 8.6: Effect of sugars on thermal characteristics of α–lac/β–lg mixture (ratio 1:1).

galactose. This represents an increase from 69.1°C observed in the absence of sugar, and suggests that the sugars conferred greater stability on the thermal denaturation of β-lg than α-lac. Glucose and galactose had the greatest stabilizing effect on the thermal denaturation of β-lg.

Fig 8.7 shows the thermograms of Na ascorbate and Na bicarbonate on the denaturation temperature of α–lac, β–lg and the mixture of α–lac/β–lg. The thermogram of β–lg in Na ascorbate showed a transition at 78.0°C (Table 8.4). This represents a shift from 71.9°C in the absence of Na ascorbate and suggests an increase in the stability of β-lg to thermal denaturation. In the presence of Na bicarbonate this transition shifted to 66.1°C, which suggests that β-lg was less stable to thermal denaturation in the presence of Na bicarbonate. A single transition was observed at 36.0°C in the thermogram of α–lac in Na bicarbonate; reheating (second heating cycle) (Fig 8.7) resulted in the disappearance of the peak, suggesting that the denaturation of α–lac in the presence of Na bicarbonate was irreversible. In the presence of Na ascorbate, two transitions were observed at 45.2°C and 68.1°C. This represents a shift from 35°C and 64.3°C observed in the absence of Na ascorbate and is indicative of a marked increase in the thermal stability of both apo- and holo-α-lac. When the sample was reheated (second heating cycle) there was a marked decrease in ΔH of the first transition from 2.09 J/g (first heating cycle) to 0.57 J/g (second heating cycle), and from 0.49 J/g to 0.43 J/g for the second transition, which suggests that the first transition was only partially reversible while the second transition was almost completely reversible. The α–lac/β–lg mixture showed two thermal transitions at 43.2 and 75.6°C in the presence of Na ascorbate and 36.9 and 63.0°C in Na bicarbonate (Table 8.2), which again shows that Na ascorbate

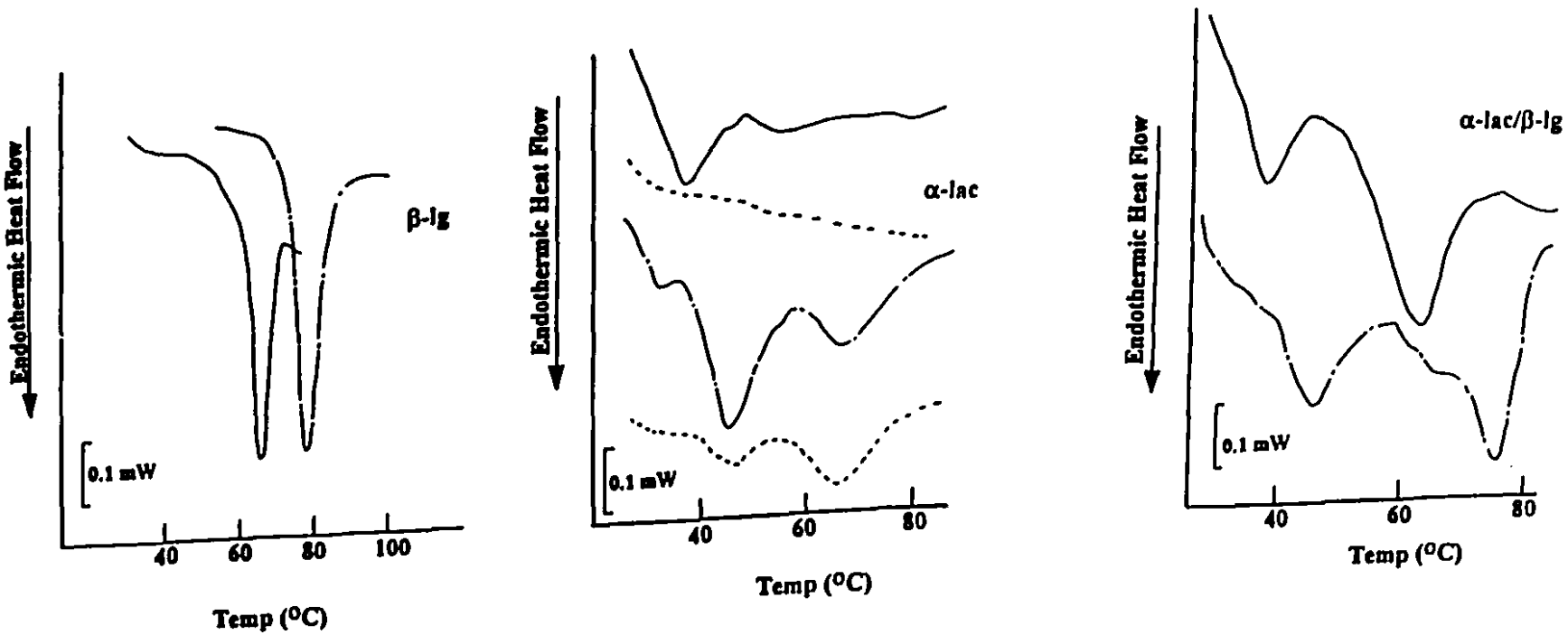



**Fig 8.7:** Effect of sodium ascorbate ( — - — ) and sodium bicarbonate ( —— ) on the thermal characteristics of α–lac, β–lg and α–lac/β–lg mixture. (- - - - Thermograms of second heating cycle).

**Table 8.4: Peak temperature of denaturation and gel characteristics of mixtures of α–lac, β–lg and BSA.**

| Proteins | Treatment | Ratio | Peak Temperature (°C) | | | Gel Type |
|---|---|---|---|---|---|---|
| | | | 1 | 2 | 3 | |
| α–lac | pH 3 | | 35.4 | 64.4 | | aggregate |
| | pH 9 | | 39.6 | 63.4 | | translucent |
| β–lg | pH 3 | | | | 82.2 | opaque |
| | pH 9 | | | | 67.0 | translucent |
| BSA | pH 3 | | | 60.3 | 71.8* | opaque |
| | pH 9 | | | 59.8 | | translucent |
| α–lac/BSA | pH 3 | 1:1 | 32.2 | 58.7 | | opaque |
| | | 4:1 | 34.7 | 62.7 | | opaque |
| | pH 9 | 1:1 | 39.0 | 58.2 | | translucent |
| | | 4:1 | 40.1 | 64.2 | | translucent |
| β–lg/BSA | pH 3 | 1:1 | | 61.9 | 82.4 | opaque |
| | | 9:1 | | | 81.9 | opaque |
| | pH 9 | 1:1 | | 59.2 | 68.5 | translucent |
| | | 9:1 | | | 67.6 | translucent |
| α–lac/β–lg/BSA | pH 3 | 1:1:1 | 36.4 | 61.9 | 82.3 | opaque |
| | | 4:9:1 | | 63.2 | 81.8 | opaque |
| | pH 9 | 1:1:1 | 39.4 | 60.0* | 69.3 | translucent |
| | | 4:9:1 | | | 67.7 | translucent |
| WPC | pH 3 | | 32.2 | 67.0* | 85.1 | opaque |
| | pH 9 | | | | 79.3 | opaque |

* - shoulder

had a stabilizing effect on the thermal denaturation of α-lac and β-lg in the protein mixture. Relkin *et al.*, (1993), showed that in the presence of EDTA (ethylene diamine tetra acetic acid) α–lac shows only one peak at 43.8°C. The second peak corresponding to the $Ca^{++}$- bound protein, disappears completely with the addition of Na–EDTA which chelates the bound $Ca^{++}$. The disappearance of the second peak in the thermogram of α–lac in Na bicarbonate could be attributed to a similar conversion of α–lac from the holo-form to the apo- form in the presence of the $HCO_3^-$ ion.

Fig 8.8 shows the thermogram of α–lac in the presence of N-ethylmaleimide (NEM), cysteine, urea and SDS. Two transitions at 33.9°C and 63.8°C were observed with α–lac dispersed in 50mM NEM in the first heating cycle (Table 8.4), which suggests that the effect of NEM on the thermal denaturation of α-lac was minimal. When the sample was reheated (second heating cycle), a marked decrease in the ΔH of the first transition was observed with no change in the ΔH of the second transition. In the presence of cysteine, the thermogram showed a single broad peak at 52.9°C with a shoulder at approximately 70°C, which is indicative of a marked change in the conformation of the protein. When the sample was reheated (second heating cycle), no transition was observed suggesting that the denaturation in the presence of cysteine was irreversible. In the presence of urea, the first transition was observed at 34.5°C and the second at 63.1°C, which shows that the effect of urea on the denaturation of α-lac was also minimal; when reheated, the two bands were observed suggesting that both transitions were reversible. In the presence of SDS, a single reversible transition was observed at 55°C; the absence of the first transition at 35°C suggests that the conformation of α-lac

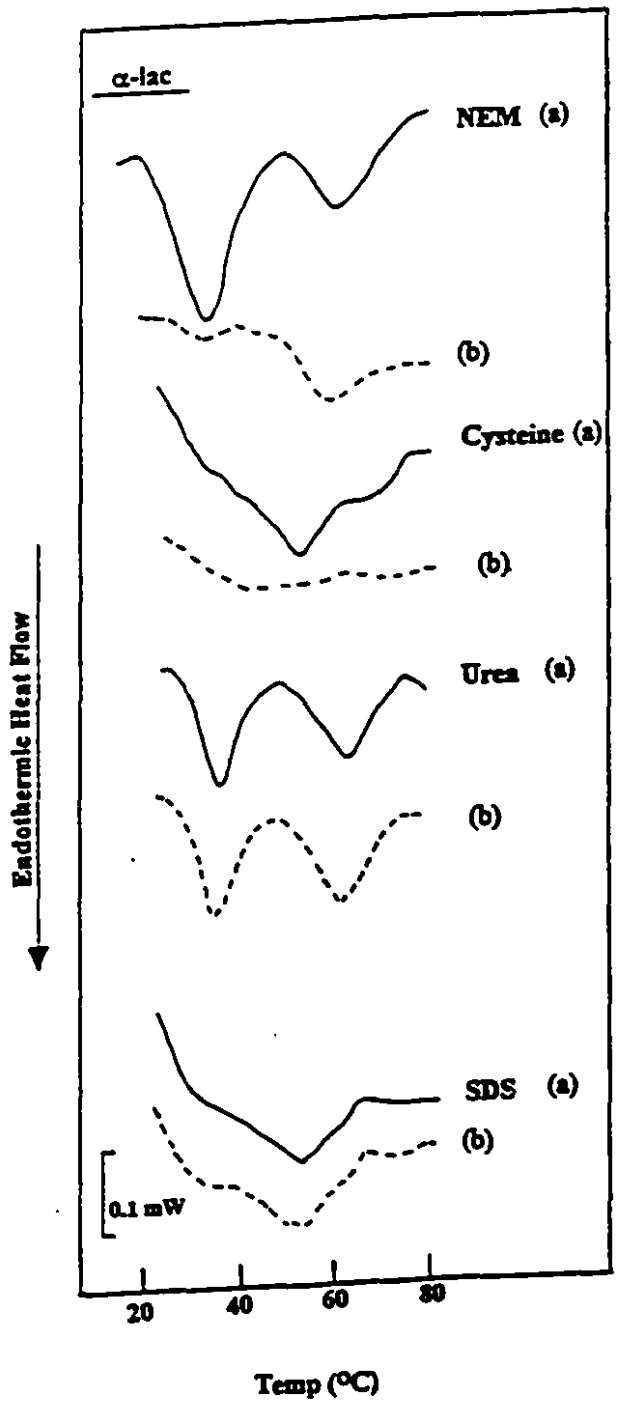



Fig 8.8: Effect of denaturing agents on the thermal characteristics of α–lac. (a) first heating cycle ( —) (b) second heating cycle (- - - -).

was altered in the presence of SDS.

Fig 8.9 shows the thermograms of β–lg heated in the presence of NEM, cysteine, urea and SDS; a single transition was observed with the four denaturing agents. The lowest transition temperature (63.7°C) was observed in the presence of NEM and the highest (75.0°C) in SDS (Table 8.4). The highest ΔH values of 1.97 and 1.96 J/g were observed in the presence of cysteine and urea respectively (Table 8.4). The ΔH values were lower for NEM (1.17 J/g) and SDS (0.74 J/g), suggesting that β–lg was partially denatured in the presence of NEM and SDS. The width at half-peak height was least in the presence of urea (7°C) and cysteine (8.5°C)and greatest in the presence of NEM (12.5°C) and SDS (13.7°C); these values which give an indication of the cooperativity of unfolding (Myers, 1990), suggest that denaturation of β–lg was most co-operative in the presence of urea and cysteine.

Fig 8.10 shows the thermograms of the α–lac/β–lg mixture heated in the presence of NEM, cysteine, urea and SDS. Two transitions were observed at 32.1 and 62.2°C in the presence of NEM and at 36.3 and 68.4°C in the presence of urea (Table 8.2). This represents a shift from 37.5°C and 69.1°C in the absence of these denaturing agents, and suggests that both NEM and urea decreased the stability of the proteins to thermal denaturation, with NEM having a greater destabilizing effect. In the presence of cysteine and SDS, a single transition at 67.2°C (cysteine) and 77.9°C (SDS) were observed, which is indicative of marked changes in the native conformation of the proteins prior to heat treatment.

No gels were formed when α-lac was heated in the presence of urea and SDS and NEM. In the presence of cysteine α–lac formed a translucent gel

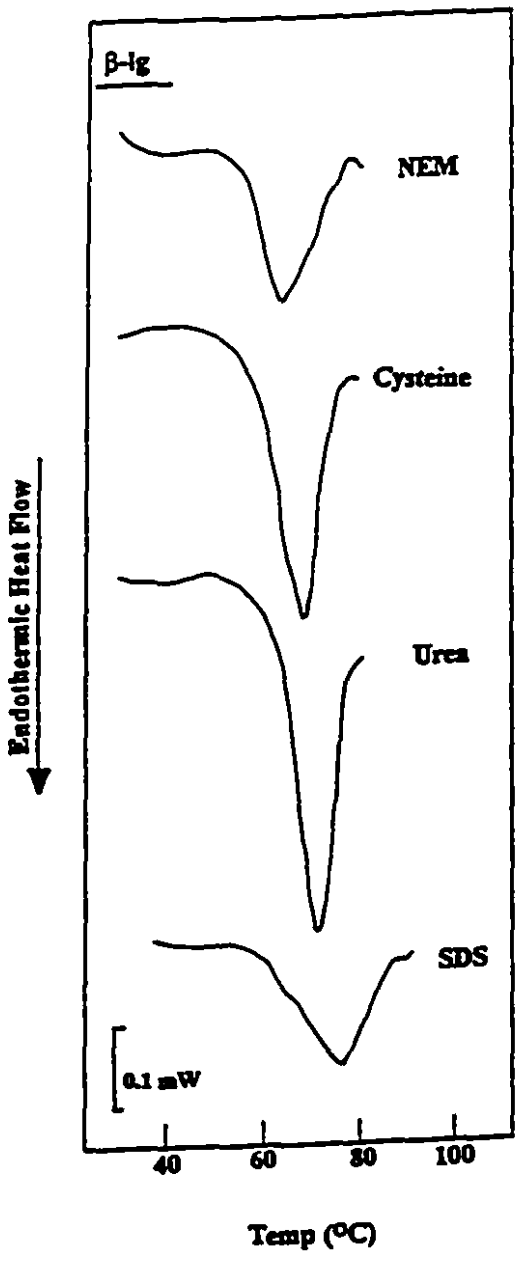



Fig 8.9: Effect of denaturing agents on the thermal characteristics of β–lg.

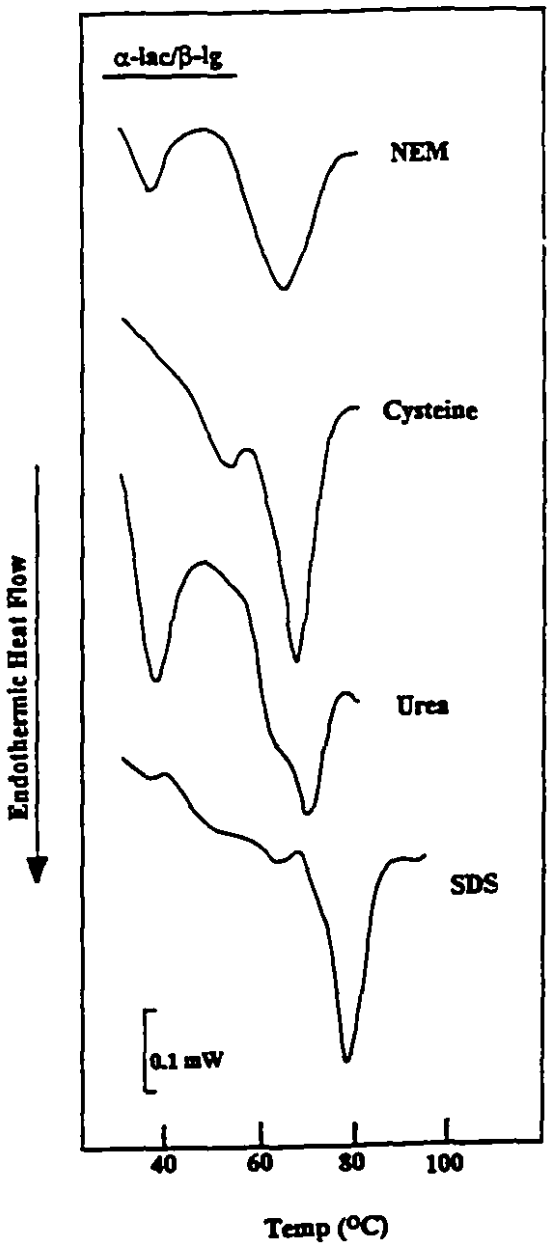



Fig 8.10: Effect of denaturing agents on thermal characteristics of α–lac/β–lg mixture (ratio 1:1).

(Table 8.3), suggesting that disulfide-sulfhydryl interchange reactions were vital in gel formation. In the presence of cysteine, enough S-S bonds may have been broken to initiate disulfide-sulfhydryl interchange reactions. β-lg, and the α-lac/β-lg mixture formed translucent gels when heated in the presence of urea, SDS and cysteine. In NEM translucent gels that were easily breakable were formed; similar gel structures were reported for BSA heated in the presence of NEM (Matsudomi *et al.*, 1991; Boye *et al.*, 1995d), suggesting that NEM interfered with crosslinking during gelation and prevented the formation of firm elastic gels.

## 8.5 CONCLUSION

The above results have shown that the gelation of whey protein concentrate results not only from the individual gelling characteristics of β-lactoglobulin, α-lactalbumin and bovine serum albumin, but also from the interactions of these proteins during heat treatment. The results further support the suggestion that intermolecular sulfhydryl groups are essential for the gelation of α–lac/β–lg mixtures (Elfagm & Wheelock, 1978). Blocking the thiol groups (by using NEM) resulted in the formation of gels that were easily breakable although extensive aggregation was observed. Beveridge *et al.*, (1984) reported that structural aggregates are formed very early during gel formation, prior to development of the self-supporting macroscopic gels; however, actual gelation occurs through intermolecular disulfide bonds formed by sulfhydryl-disulphide interchange reactions or sulfhydryl oxidation and through intermolecular hydrophobic interactions (Fukushima,1980; Shimada & Matsushita,1980,1981).

## GENERAL CONCLUSIONS

Gelation of whey proteins occurs when a change in native protein structure facilitates protein-protein interactions which result in aggregation, this depending on the solvent environment. This change in native structure occurs after sufficient denaturation of the protein. In this study, physicochemical factors which influence the denaturation and gelation of whey protein concentrate, β-lactoglobulin, α–lactalbumin and bovine serum albumin were investigated.

The results obtained showed that heating temperature, heating time, pH and NaCl affect the temperature of denaturation as well as the characteristics of whey protein concentrate gels by altering the secondary structure of the individual whey proteins during heat treatment. Increasing heating temperature and time enhanced gel formation; increasing NaCl and sugar concentration decreased gel formation. At pH values close to the isoelectric point (pH 4-6), whey protein concentrate formed an opaque coarse coagulum; at alkaline pH values, firm gels were obtained .

Analysis of the effect of pH, NaCl and sugars on the denaturation temperature and secondary structure of the individual whey proteins showed that β–lg had maximum thermal stability at pH 3 and was easily denatured at pH 9. Unfolding of β–lg resulted in the formation of intermolecularly hydrogen-bonded β–sheet aggregation at both acid and alkaline pH. This was only observed at acid pH in the case of α–lac. Denaturation of β–lg resulted in gel formation at both acid and pH values

Two conformers of α–lac, $Ca^{++}$-free (apo) and $Ca^{++}$-bound (holo), were identified. Holo-α–lac was more thermally stable than apo-α–lac with a temperature of denaturation of 70.1°C. At pH 3, denaturation of apo-α–lac was partially reversible but completely reversible for holo-α–lac; this resulting in aggregate formation without gelation. At pH 9, both apo-α–lac and holo-α–lac were irreversibly denatured; this resulting in the formation of a translucent gel.

No gel or aggregate formation was observed at pH 7. Denaturation of apo-α–lac resulted in the formation of β–sheet structures associated with aggregation; denaturation of holo-α–lac resulted in reversible unfolding into turns without aggregation.

Bovine serum albumin, the second gelling protein in whey, showed maximum thermal stability at pH 5 and formed an opaque gel at pH 3 and a translucent gel at pH 9. Sodium dodecyl sulfate markedly increased the thermal stability of BSA and prevented aggregate formation. Aggregation was observed when BSA was heated in the presence of N-ethylmaleimide, a thiol-blocking agent, and in cysteine which breaks disulfide bonds. The results showed that sulfhydryl-oxidation (disulfide bond formation) may nct be essential in the aggregation step of gel formation; hydrophobic interactions may be the most important in initial aggregation.

Intermolecular interactions between the three major proteins in whey during gel formation were also investigated. The results showed that although α–lac and β–lg did not gel by themselves at 65°C, a mixture of the proteins gelled. The temperature of denaturation of β–lg was observed to decrease in the presence of α–lac, while high concentrations of BSA decreased the denaturation temperature of α–lac. The data obtained further suggested that disulfide-sulfhydryl interchange reactions are primarily responsible for the gelation of α–lac, β–lg, and BSA mixtures.

The results obtained from this study will allow for increased utilization of whey proteins for novel applications in food gels. The composition of whey protein concentrates and isolates can be altered to increase the protein composition of any one of the whey proteins for the achieving of a desired gel texture under given processing conditions. Alternatively, individual whey proteins can be used in place of whey protein concentrate to enhance specific gel characteristics.

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