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Study of the Gelation of Whey Protein Isolate by FTIR Spectroscopy and Rheological Measurements

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A thesis submitted to the Faculty of Graduate Studies and Research in Partial fulfillment of the requirements for the Degree of Master of Science

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I WOULD LIKE TO DEDICATE THIS THESIS IN MEMORY OF MY GRAND FATHER, **FOUAD E. GEARA**. WITH LOVE.

MARCH 14TH, 1999

Suggested Short Title

Study of the Gelation of Whey Protein Isolate

Abstract

Variable-temperature Fourier transform infrared spectroscopy can be employed to monitor the denaturation and aggregation of proteins during heat treatment. Information on the changes that occur in protein secondary structure upon heating is provided by detailed examination of the amide I band, as different protein conformations have characteristic amide I frequencies. The objectives of the present study were: (*i*) to study the changes in protein structure that occur during gelation of whey protein isolate (WPI) and (*ii*) to correlate the changes in protein structure observed under different physico-chemical conditions to rheological properties of WPI gels prepared under the same conditions.

The FTIR spectra of D_2O solutions of WPI at different pHs, ranging from 3 to 10, were recorded as the temperature of the solution was increased from room temperature to 95°C in 5°C increments. In all the solutions studied, the formation of two new bands at 1618-1623 cm^{-1} and 1678-1684 cm^{-1} was observed upon heating; these bands are characteristic of aggregate formation and have been previously assigned to antiparallel β-sheet structure. As the pH increased from 3 to 10, the aggregation temperature of WPI decreased from 85 °C to 65 °C.

The rheological properties of WPI gels were studied by employing an Instron Universal Testing Machine. The Instron measurements demonstrated that protein concentration, heating temperature, and heating and cooling time are directly related to gel strength. The changes in gel strength as a function of cooling time (for gels prepared by heating at 75°C for 45 min) were correlated to FTIR spectral data for WPI solutions subjected to the same treatment.

Résumé

La Spectroscopie d'Infrarouge Transformée de Fourier (SITF) à température variable peut être utilisée afin de surveiller la dénaturation et l'agrégation des protéines lors d'un traitement thermique. L'information sur les changements ayant lieu dans la structure secondaire de la protéine, à la suite du traitement thermique, est fournie par un examen détaillé de la bande amide I, puisque différentes conformations de protéines possèdent des fréquences amide I. Les objectifs de cette étude furent: i) d'étudier les changements dans la structure de la protéine ayant lieu lors de la formation de gel de la Protéine du Petit Lait (PPL); ii) de corréler les changements dans la structure de la protéine, observés sous différentes conditions physico-chimiques, versus les propriétés rhéologiques des gels de la PPL, préparés sous ces mêmes conditions.

Les spectres obtenus par SITF des solutions D_2O de la PPL, à différents pHs, allant de 3 à 10, furent inscrits pendant que la température de la solution était augmentée depuis une température ambiante jusqu'à 95°C, 5°C à la fois. Dans toutes les solutions étudiées, la formation de deux nouvelles bandes à 1618-1623 cm^{-1} et 1678-1684 cm^{-1} fut observée lors du traitement thermique; ces bandes sont charactéristiques d'une formation agglomérée et furent auparavent assignées à une structure de β-sheet antiparallele. Lorsque le pH fut augmenté de 3 à 10, la température de l'agrégation de la PPL diminua de 85 °C à 65 °C.

Les proriétés rhéologiques des gels de la PPL furent étudiées en utilisant la machine Instron® Universal Testing Machine. Les mesures de l'Instron® démontrèrent que la concentration de la protéine, la température de réchauffement, et les temps de réchauffement et de refroidissement sont directement reliés à la force du gel. Les changements dans la force du gel, étant fonction du temps de refroidissement (pour les gels préparés par réchauffement à 75°C pendant 45 min) furent corrélés aux données spectrales de la SITF pour les solutions de la PPL qui étaient sujettes au même traitement.

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Chapter 1 Introduction

Whey is the "serum part of milk, that is separated from the curd (the thick fraction) especially in the process of making cheese" (Oxford Dictionary). Whey composition varies depending on milk composition and method of casein removal, but whey typically contains 50% of the solids of the original milk and includes fat, lactose, proteins, minerals, and vitamins. Whey protein concentrates (WPCs) and whey protein isolates (WPIs) contain approximately 70 and 95% protein, respectively. Whey protein has excellent nutritional and functional properties, the term functional property being defined as any physicochemical property which affects the processing and behavior of proteins in food systems, and judged by the quality attributes of the final product (Kinsella, 1976). Whey protein is a blend of different proteins with numerous and various functional properties, and hence may have many potential uses (Fig 1.1). Some of the uses of whey protein products in the food industry are listed in Table 1.1.

Similarly processed whey proteins may have different functional properties. Based on their work with WPI, Melachouris (1984) concluded that WPIs vary in their composition and properties due to differences in: (1) the composition of milk used for making cheese; (2) the composition of whey from companies that manufacture different kinds of cheese; (3) the composition of different lots of whey from the same company; (4) the length and the temperature of whey storage; and (5) the processing conditions. An improved understanding of the variability of the functional properties of whey protein products could provide a basis for predicting the functional properties of a particular WPC or WPI.

Fig 1.1 Cheese whey composition. Concentrations are in g/liter. NPN = products comprised of non-protein nitrogen. (Copied from Food Protein and their Applications, p.226)

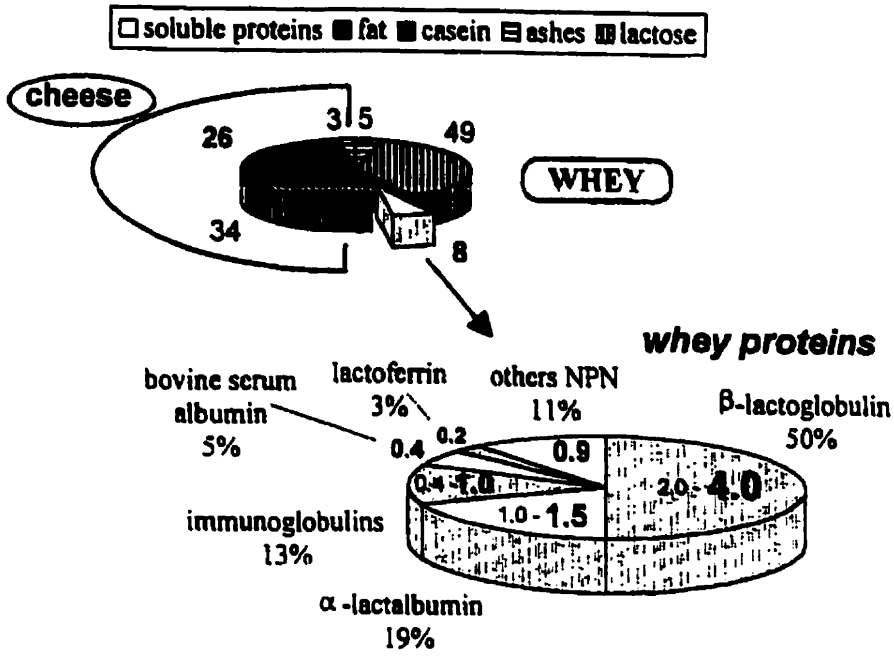


Table 1.1 Whey Protein Uses in Human Food. (Adapted from Food Proteins and Their Applications, p. 227).

Industrial applications	Functional properties Expected	Proteins used
Alcoholic beverages	Cream stabilization	WPC + Caseinates
	Cloudy aspects	WPC or WPI
Bread making	Water holding	WPC or WPC + Caseinates
Breakfast cereals	Emulsion stabilization	Whey
	Gelling properties	
	Browning	
Meat products	Emulsion making	WPC, WPI alone or with caseinate
Nutritional uses	Protein intake	Whey, WPC, or WPI
	Enteral nutrition	WPC hydrolysates

Gelation is a very important functional property of food proteins. It plays a major role in the preparation of many foods, including many dairy products, coagulated egg white, gelatin gels, various heated, comminuted meat or fish products, soybean protein gels, vegetable proteins, and bread dough. Protein gelation is utilized not only for the formation of solid viscoelastic gels, but also for improved water absorption, thickening, particle binding, and foam-stabilizing effects. Protein products derived from whey, such as WPCs and WPIs, have a tendency to form irreversible heat-induced gels that are important for commercial applications in foods.

Thermally induced gelation involves conformational changes in the protein with unfolding of some polypeptide segments followed by a subsequent phase of protein-protein interactions resulting in a progressive buildup of a network structure (Bernal and Jelen, 1985). Protein gels can therefore be represented as consisting of intermolecular cage-like unit structures, with the solvent distributed throughout the matrix (Foegeding, 1989). The formation of this matrix is dependent on a balance between attractive and repulsive forces (Schmidt, 1981), which have been shown to vary with the physical and chemical properties of the protein. At the initial time of gelation, transition from the native state to a progel state occurs, and 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 to become available for intermolecular interactions (Damodaran, 1994). Exposure to hydrophobic groups results in hydrophobic interactions which are necessary in the aggregation and cross-linking of gel networks (Clark, 1992; Damodaran, 1994). Buried

sulfhydryl groups can initiate disulfide-sulfhydryl interchange reactions, which contribute to crosslinking (Shimada and Cheftel, 1989; Mori *et al.*, 1986; Nakamura *et al.*, 1984).

The gelation behavior of whey proteins has been studied by rheological techniques, which are used to measure the viscoelastic properties of gels, and by differential scanning calorimetry, which is used to measure the enthalpy changes associated with protein denaturation and aggregation. Work by Boye et al. (1995a) demonstrated that Fourier transform infrared (FTIR) spectroscopy can be employed to gain a better insight into the changes in protein structure that take place during gelation of whey proteins, and Rejaei et al. (1996) utilized this technique to study gels obtained by thermally induced gelation of the whey protein bovine serum albumin. In the work described in this thesis, a detailed examination of four commercial WPIs by FTIR spectroscopy has been undertaken in order to investigate the extent of variability in their gelation behavior. The possibility of utilizing FTIR spectroscopy to predict the rheological properties of gels prepared by heat treatment of WPIs has also been investigated.

Chapter 2 Literature Review

2.1 Introduction

In this section, the literature relevant to the study of the gelation of whey protein isolate presented in this thesis will be reviewed. Following a brief description of the composition of whey protein, the processes employed in the production of whey protein isolates will be considered. The phenomenon of gelation will then be described, followed by a brief description of the techniques employed in the measurements of rheological properties.

2.2 Whey Proteins

The whey fraction of milk contains four major whey proteins: β-lactoglobulin (β-lg); α-lactalbumin (α-lac); bovine serum albumin (BSA); and immunoglobulins. The whey fraction also contains a number of other proteins, collectively termed the proteose peptone fraction, which includes post-translational proteolysis products of caseins and several minor whey proteins. Table 2.1 illustrates the distribution of the major proteins in whey, and Table 2.2 lists the physicochemical characteristics of the major whey proteins.

Table 2.1 Distribution of Proteins in Whey (Adapted from Mulvihill and Kinsella, 1987)

Protein	Amount g/L milk
β-Lactoglobulin	2.0-4.0
α-Lactalbumin	1.0-1.5
Proteose Peptone	0.6-1.8
Immunoglobulins	0.6-1.0
Serum Albumin	0.1-0.4

β-Lactoglobulin contains 162 amino acids with one thiol group and two disulfide bonds (Table 2.2). The secondary and tertiary structures show a high degree of organization with a great proportion of β-sheet (approximately 50%) and α-helix (10-15%) and 15-20% of β-turns and the remaining 15% represents random (Timasheff *et al.* 1966; Susi and Byler, 1986; Papiz *et al.* 1986 and Casal *et al.* 1988).

α-Lactalbumin is a small molecule with a bound Ca^{2+} and four disulfide bridges that maintain it in a compact ellipsoidal structure. This small molecule consists of 123 amino acids with a molecular mass of 14,174 (Swaisgood, 1982).

Table 2.2 Physiochemical Characteristics of major whey proteins. (Adapted from Food Proteins and their Applications p.228).

Characteristics	β-Lactoglobulin	α-Lactalbumin	BSA	Immunoglobulin
Molar mass (g/mol)	18,362	14,174	69	150,000-1,000,000
Disulfide bond/mol	2	4	17	~4
Amino acid residue/mol	162	123	582	>1000
pI (Isoelectric point)	5.2	4.5-4.8	4.7-4.9	5.5-8.3

The other two important whey proteins are bovine serum albumin (BSA) and immunoglobulin (Ig). BSA is a large globular protein that has a molecular mass of 66,000 Da (Lin *et al.* 1976). The molecule consists of 580 amino acids with one free thiol group and 17 disulfide bonds (Kinsella and Whitehead, 1989). It is believed to be an important

gelling protein of whey (Hines and Foegeding, 1993). It has a considerable highly organized secondary structure: approximately 43% β-structures containing β-sheet with β-turns and 57% α-helix (Fen-nifu *et al.* 1994).

Immunoglobulins are glycoproteins with a molecular mass of 150-1000 kDa (Moore and Ha, 1993). These proteins have higher denaturation temperatures than β-lactoglobulin and α-lactalbumin, but in the presence of BSA, immunoglobulins have been shown to be very sensitive to heat. It was suggested that it might be due to the interaction with the free thiol group (Moore and Ha, 1993). Proteose peptones are also found in whey, and considered as a minor protein component. They are composed of proteolytic fragments of β-casein and contains surface-active peptides (Andrews 1978 and Andrews 1979), and play an important role in the functional properties of whey proteins.

There are two types of whey: (1) acid whey and (2) soft whey. Acid whey is recovered after acid coagulation of milk during the production of fresh soft cheeses like cream cheese or Camembert. Soft whey is obtained after rennet coagulation of milk at pH 6.6 (e.g. Cheddar or emmental manufacturing). Soft whey has a minimum pH of 5.6 and acid whey has a pH maximum of 5.1 (International Dairy Federation, 1978). Table 2.3 shows the relative proportions of total protein in the whey fractions. It should be noted that the relative ratios of the major protein fractions within the two types of whey are the same.

Table 2.3 Protein composition of Cheese Whey and Acid Whey

	Average composition (g/liter)	
	Cheese Whey	Acid Whey
Whole whey protein	6.7	5.5
β-Lactoglobulin	3.5	3.5
α-Lactalbumin	1.3	1.3
Serum albumin	0.1	0.1
Imunoglobulin	0.4	0.4
Lipoprotein	0.2	
Proteose Peptones	0.2	0.2
Glycomacropeptide	1.0	

Adapted from Damodaran and Paraf (Food Proteins and Their Applications, 1997 p. 136)

2.3 Methods of extracting WPI from whey solutions

Whey proteins have different net charge depending on pH. In general the charge on the protein is positive when the pH is lower than its isoelectric pH (pH ~4.6), and it is negative when the pH is higher than its isoelectric point. Proteins behave like cations when they have a net positive charge, and thus they can be absorbed on cation exchangers, and they can be absorbed by anion exchangers when they have a net negative charge.

Ion exchange fractionation processes have been commercialized for the manufacture of WPI: One such process known as, "Vistec" process, uses a cellulose-based exchanger in a stirred tank reactor (Palmer 1982). The process involves a series of steps that are performed as a fractionation cycle. 1) Whey is adjusted to a pH lower than 4.6 by acid and pumped into a reactor where it is stirred to allow the proteins to be absorbed by the ion exchanger, 2) all unabsorbed material such as lactose is filtered out, 3) the resin is then resuspended in water, and the pH is adjusted to a value higher than 5.5

by an alkali solution to release the proteins from the ion exchanger, 4) separation of the protein solution from the resin is achieved by filtration inside the tank reactor. The protein is concentrated in two steps, first ultrafiltration, followed by spray drying to yield WPI containing ~95% protein.

2.4 Characteristics of Protein Gels

"Gels are differentiated from other structured, network systems in which small proportions of solid are dispersed in a relatively large proportion of liquid by property of mechanical rigidity or the ability to support shearing stress at rest" (Ferry, 1948). Gels, which consist mostly of fluid, have the remarkable ability to behave as a solid while retaining many properties characteristics of the fluid component.

Flory (1974) and Hermansson (1979) define protein gels as three-dimensional matrices or networks in which polymer-polymer and polymer-solvent interactions occur in an ordered manner, resulting in the immobilization of large amounts of water by a small portion of protein. Gelation is generated by the action of heat, pressure and by divalent cations, and it is a physical manifestation of protein denaturation processes which depend on the type and concentration of protein, on the processing conditions used to induce gelation, and on the pH and ionic environment (Hermansson. 1979; Schmidt, 1981). All these agents induce formation of a network structure; however, the types of covalent and non-covalent interactions involved and the mechanism of network formation can differ considerably. The process by which proteins form a thermally induced gel matrix consists of the following three sequential events (Edwards *et al.*, 1987):

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). It can result in 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. Gels have relatively high viscosities, plasticity, and elasticity (Kinsella, 1976).

2.5 Gelation Process

Most food protein gels are prepared in the process of heating. In this mode of gelation, the protein in a sol state is first transformed into a "progel" state by denaturation. The progel state is usually a viscous liquid state in which some degree of protein polymerization has already occurred. This step causes unfolding of the protein and exposure of a critical number of functional groups, as well as hydrophobic groups, which leads to the formation of protein networks. Creation of the progel is irreversible, because many intermolecular protein interactions occur between the unfolded molecules. When the progel is cooled to ambient or refrigerated temperature, the decrease in the thermal kinetic energy facilitates formation of more stable non-covalent bonds among exposed functional groups of the various molecules. This constitutes gelation.

In some cases gel networks that are sustained primarily by non-covalent interactions are thermally reversible; that is, upon reheating they will melt to a progel state, as is commonly observed with gelatin gels. This is especially true when hydrogen bonds are the major contributors to the network (Damodaran, 1996). In other cases gels

formed as a result of hydrophobic interactions at elevated temperatures. Gel networks formed by hydrophobic interactions such as egg-white gels are not thermally reversible. Proteins that contain cysteine and cystine groups can undergo polymerization via sulfhydryl-disulfide interchange reactions during heating and form a continuous covalent network upon cooling. Such gels are usually thermally irreversible. Examples of gels of this type are ovalbumin, β-lactoglobulin, and whey protein gels.

Proteins form two types of gels, namely coagulum (opaque) gels and translucent gels. The type of gel formed by a protein is dictated by its molecular properties and solution conditions. Proteins containing large amounts of non-polar amino acid residues undergo hydrophobic aggregation upon denaturation.

2.5.1 Protein-Protein Interactions

As the conformation of protein is altered under the influence of heat, protein-protein interactions become more prevalent. These can involve covalent and non-covalent bonding. Friedman (1977) defined crosslinking as a "durable combination of (usually large) distinct elements at specific places to create a new entity that has different properties as a result of the union." With respect to heated proteins, cross-links can form among protein units or between proteins and non-protein components (Table 2.4)

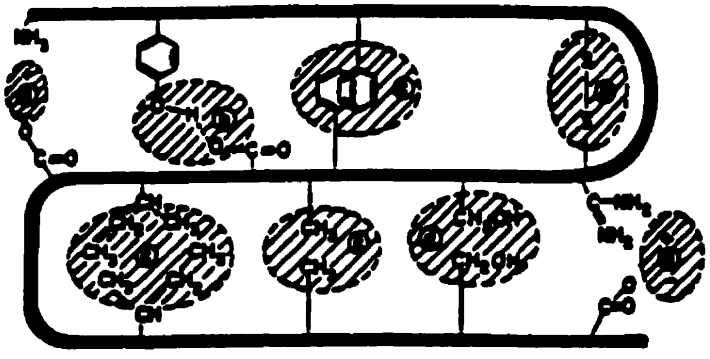
Table 2.4 Proposed cross-link bonding of protein gel structures and their properties (Adapted from Schmidt, 1981)

Type of bond or interaction	Energy (Kcal/mol)	Interaction Distance	Groups involved	Role in Gel Matrix
Covalent bonding	80 - 90	1 – 2 Å	-S-S-	Bridging; ordering
Hydrogen bonding	2 - 10	2 – 3 Å	-NH···O=C- -OH···O=C-	Bridging; Stabalizing
Hydrophobic and Related Interactions	1 - 3	3 – 5 Å	Nonspecific	Strand thickening; Stabilizing
Ionic bonding and Interactions	10 – 20	2 – 3 Å	$-NH_3^+$; $-COO^-$; etc..	Solvent interaction; Salt linking

The native state of a protein is maintained through 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 and the thermodynamic conditions are optimal for the formation of the tertiary matrix (Schmidt, 1981). Disulfide bonds, hydrogen bonding, and ionic interactions are involved in the crosslinking of protein gels (Catsimpoolas & Meyers, 1970; Morrisey *et al.*, 1987). A high degree of crosslinking results in undesirable gel structure (Schmidt, 1981).

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



The ability of a protein to form intermolecular disulfide bridges during heat treatment may be a prerequisite for gelation of the protein (Huggins *et al.* 1951, Nakamura *et al.* 1984; Utsumi and Kensella, 1985). Heat treatment results in the cleavage of existing disulfide bridges 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).

The role of disulfide bridging in protein gel systems has been primarily approached using thiol-reducing agents or sulfhydryl blocking agent. Reducing agents have been used to modify the gelation of proteins through modification of sulfhydryl interchange reactions (Pour-El *et al.* 1976; Schmidt, 1979). Direct analysis of protein gels for sulfhydryl and disulfide groups has been used to demonstrate their involvement in milk gels (Kalab, 1970).

Ionic bonding has been suggested to be of primary importance to the protein-solvent interface for 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, resulting in the formation of more aggregated gels (Hermansson, 1979; Schmidt, 1981). The increase in viscosity which proceeds the onset of gelation and gel structure stabilization is induced by hydrogen bonding. This type of crosslink allows for the open orientation necessary for water immobilization (Schmidt, 1981).

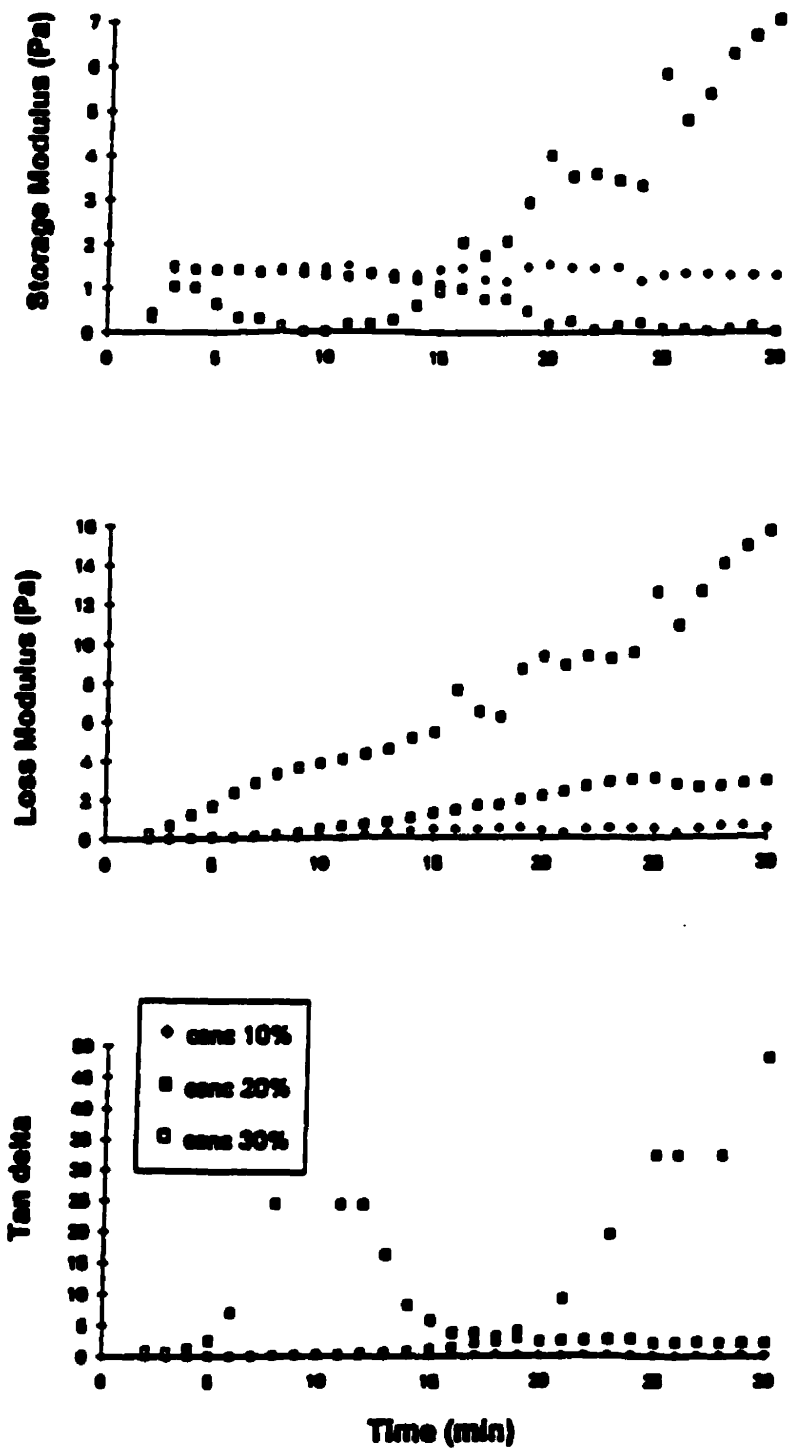
2.6 Viscoelastic Properties

Two viscoelastic properties of gels are measured: the storage (G') and the 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 (tan Δ G"/G') is a measure of the dynamic character of the protein-protein bonds in the gel network; a higher tan delta value means that a gel reacts to a stress in a relatively more viscous and less elastic manner or vice versa (Van Vliet *et al.* 1989). Boye and coworkers, (1997a) determined the effect of protein concentration on the storage, loss moduli and tan delta of WPC dispersions (pH 7, 0.8M NaCl) during heat treatment (Fig 2.2). At 10% (w/v) WPC the sample was heated at 80°C for 30 min and showed no change in storage modulus, but it did show a slight change in loss modulus and tan delta, which is indicative of an increase in viscosity without gelation. At 20% (w/v) WPC dispersion heated at 80°C showed a marked decrease in storage modulus between 15 and 20 min of heating; however, loss modulus and tan delta were markedly increased. 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) WPC both storage and loss moduli increased with heating time, suggesting an increase in both gel elasticity and viscosity.

Tan delta reached 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 (0-12min), the WPC gels were more viscous and less elastic. After 12 min of heating (second phase), gel elasticity increased and dominated over gel viscosity Boye *et al.* (1997b).

Fig 2.2 Effect of protein concentration on the storage, loss moduli and tan delta of WPC (pH 7.0, 0.8 M NaCl) heated at 80°C, for 30 min. (Adapted from Boye *et al.*, 1996a).



2.7 Structural Measurements of Protein gels

Infrared spectroscopy is a widely used technique for the analysis of protein secondary structure. With the advent of Fourier transform methods for data acquisition came the ability to examine dilute aqueous solutions via signal averaging and digital subtraction of background absorptions. Common features of the infrared spectra of proteins are the so-called "amide" bands, which arise from the delocalized vibrations of the peptide linkage (Susi, 1969). Of these, the amide I band (1700-1600 cm^{-1}) is the most useful for the analysis of protein secondary structure (Susi *et al.*, 1967; Timasheff *et al.*, 1967; Susi, 1969). The secondary structure of proteins has been studied in the solid state (Krimm, 1962), in H_2O (Koenig and Tabb, 1980), 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, and Byler, 1983).

The broad amide I bands of proteins are composed of several overlapping bands due to the various protein segments with different secondary structures (Surwicz and Mantsch, 1988). Each type of substructure, such as 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, known as Fourier self-deconvolution, results in narrowing of the amide I band to reveal the component peaks which can then be qualitatively and quantitatively characterized. This technique not only enriches the qualitative interpretation of protein infrared spectra,

but also provides a basis for the quantitative estimation of protein secondary structure.

Aggregation of proteins during gelation could be monitored by infrared spectroscopy. The gel texture is affected by the particular changes of the secondary structure of the protein and the manner in which the networks build from individual molecules during heat treatment (Kinsella, and Whitehead, 1989). Clark and coworkers, (1981) suggested that protein aggregation and the formation of hydrogen-bonded β-sheet structure are correlated to a band formation at 1620 cm^{-1}. Yu *et al.* (1973), Anderle *et al.* (1978), Painter and Koeing (1976), Kirsch *et al.* (1986), Ismail *et al.* (1992) and Boye *et al.* (1996b) referred to 1618 cm^{-1} and 1683 cm^{-1} bands as aggregation bands which are associated with antiparallel intermolecular β-sheet.

2.8 Gelation of Whey Protein Concentrates and Isolates

Gelation of whey protein products and constituent proteins has been reviewed many times in the past, denoting that it is a topic of enormous relevance both scientifically and commercially (Paulsson *et al.*, 1986; Mulvihill and Kinsella, 1987; Katsuta, 1990; Mangino *et al.*, 1988). 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). Boye *et al.* (1997a, b) 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 and Foegeding (1993) 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 strain values in 7.5mM $CaCl_2$. Schmidt and coworkers (1979) demonstrated that free sulfhydryl groups have a significant effect on properties of whey protein gels; at low concentrations, gel strength increased as the number of free sulfhydryl groups increased until a maximal concentration beyond which gel strength decreased with further increase in the number of free sulfhydryls, this depending on pH, ionic strength and calcium content of the system.

2.8.1 Gelation Mechanism of Whey Proteins

The "lowest concentration to form a gel," sometimes called least concentration endpoint (Trautman, 1966), is one measure of gelation; however, it must be viewed in association with other factors such as heating method and criteria used to evaluate whether the suspension has gelled. For example, a loosely associated aggregate may have the required yield stress to flow and be scored as a gel when it has no elastic structure.

Sternberg *et al.* (1976) heated WPC in a boiling water bath to determine the lowest concentration for gelation and found it to be 1.0-1.2% protein at pH 5.5 and 6.0. Visual scoring of gels made at pH 7.0 (100°C, 10 min) indicated that a concentration of between 2.5 and 5.0% was required for gelation (Schmidt *et al.*, 1978). From these studies it would seem likely that the minimum concentration for gelation is between 1 and 2.5%, depending on how gelation is defined.

The first stages of gelation can be detected by some indicator of aggregation, such as changes in viscosity or light scattering. The viscosity transition temperature associated with unfolding/aggregation of whey protein is pH dependent (Hermansson, 1979). A 10% protein dispersion of WPC in distilled water shows a sharp increase in viscosity at 72-

75°C and the transition temperature decreases as the pH increases, occurring at 41-42°C in a pH 11.5 suspension. The addition of 0.2 M NaCl causes a general decrease in transition temperatures while maintaining the pH-dependent shift observed without added salts. Another method of detecting protein aggregation is loss of solubility. Heating WPC (80°C) in 0.05 M NaCl or in the absence of added salt causes a decrease in solubility, The solubility is maximum when the pH of the suspension is 5.5; adjusting the pH values above or below 5.5 causes a less severe loss of solubility (Varunsatian *et al.*, 1983). When calcium or magnesium chloride is added to the suspension, the solubility below pH 5.5 is similar to that in the suspension containing NaCl; however, at and above pH 5.5 the solubility decreases dramatically. The effect of ions is therefore strongly associated with the pH of the suspension, and a pH of around 5.5 appears to be optimal for protein-protein interactions. Results from aggregation studies that were not concerned with gelation should be interpreted in light of the fact that all protein-protein associations do not produce gels (Ziegler and Foegeding, 1990). Therefore, optimum conditions for association may not be optimum for gelation.

The transition from a viscous to a viscoelastic structure during heating is one way to detect gelation temperature. This is shown for WPI in Fig 2.3 as a transition in delta (the phase angle between the viscous and elastic stress components of the gel).

The transition is time/temperature (Beveridge *et al.*, 1984) and protein concentration dependent (Paulsson *et al.*, 1986), with slower heating and higher concentrations shifting the transition to a lower temperature. The gelling time, which is the time required for a self-supporting or non-flowing gel to form, is another means of detecting transition points. Gelling time is increased when sulfhydryl groups are blocked

with *p*-chloromercurobenzoic acid (Hillier *et al.*, 1980) or when calcium is replaced with sodium (Johns and Ennis, 1981). An increase in the amounts of lipids in WPC will cause an increase in gelation time (Sternberg *et al.*, 1976). Once an elastic structure is formed during heating, subsequent cooling causes an increase in storage modulus (elasticity) (Beveridge *et al.*, 1984). This rheological transition is reversible in one (Fig. 2.4) and three heating/cooling cycles (Beveridge *et al.*, 1984). This illustrates the irreversible nature of gelation and shows that rheological properties are temperature dependent.

Fig 2.3 Gelation of Whey Protein Isolate (10% protein, pH 7.0, 0.1 NaCl) at 80°C. Insert shows G' and delta transition on an expanded time scale.

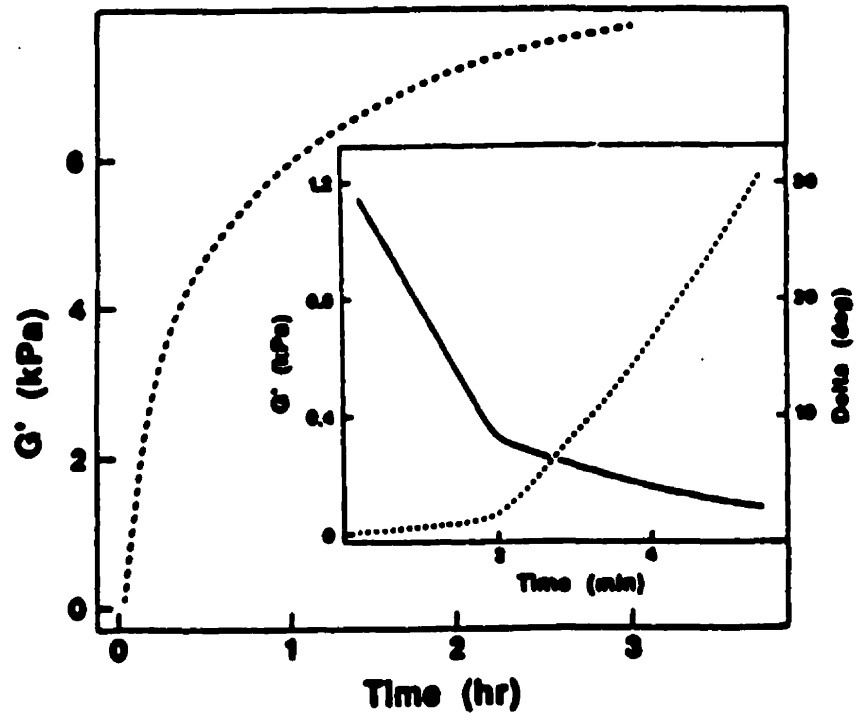
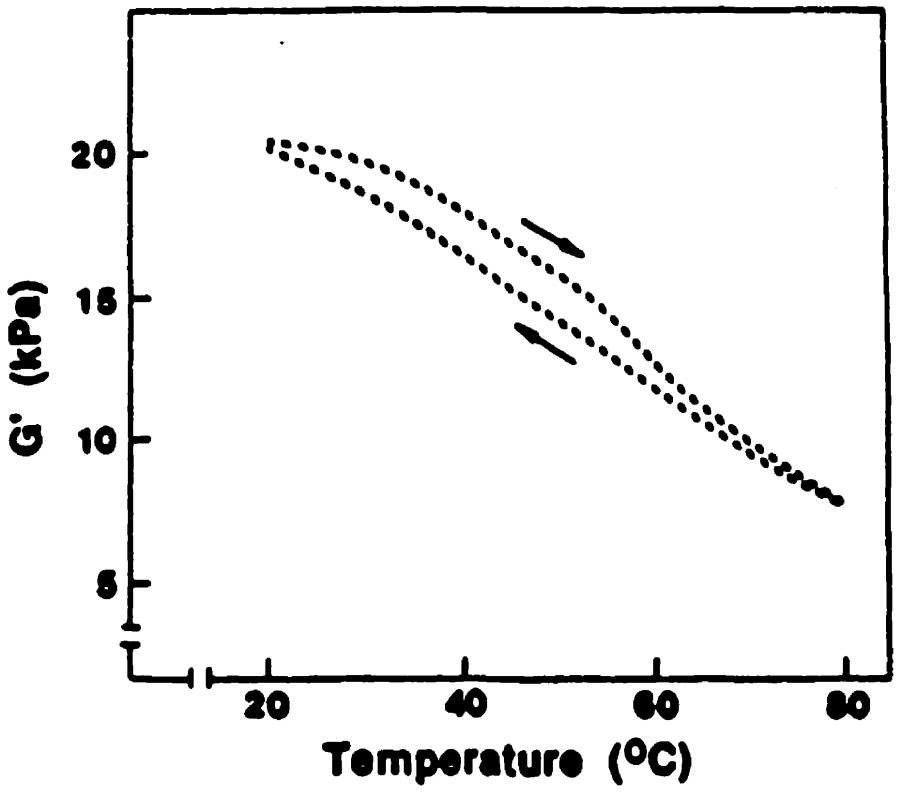


Fig 2.4 Changes in G' with cooling and reheating of a whey protein gel (pH 7.0, 0.1M NaCl) formed by heating at 80°C for 1 hr. (Ziegler and Foegeding, 1990).



2.9 β-Lactoglobulin

The gelling proteins found in whey are β-lactoglobulin (β-lg) and bovine serum albumin (BSA). Since β-lg is present in much higher concentrations than albumin, it is considered to be the primary gelling protein. β-lg has a molecular weight of 18,362 and contains two disulfide bonds and one free sulfhydryl group (Swaisgood, 1982). At pH values between 3.8 and 5.2, β-lg forms a dimer and will further associate to an octamer (Swaisgood, 1982). Over the pH range 8.5-9.5, 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. Circular dichroism and infrared studies suggest a secondary structure consisting of an α-helix content of 10-15%, and a β-structure content of ~50%, with 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 and Byler, 1986; Casal *et al.* 1988). Processing conditions such as pH and the concentrations of salt, sugar and protein affect the textural characteristics of β-lactoglobulin through their effects on the confirmation of the protein during heat treatment (de Wit and Klarenbeek, 1981; Harwalker and Ma, 1988; Arntfield *et al.* 1990; Boye *et al.*, 1996b). Although the inter-relationship between the molecular and functional properties of food proteins is qualitatively understood, quantitative prediction of the functional behavior of protein in food systems from a knowledge of their molecular properties has not been achieved (Damodaran, 1994).

Mulvihill and Kinsella (1988) investigated the effect of sodium chloride on gelation of β-lg. Maximum gel hardness (stress at 70% compression/initial gel area) is

obtained in a single protein gels at 200 mM NaCl and 10 mM $CaCl_2$. Higher mineral concentrations were shown by electron microscopy to produce a less ordered, randomly aggregated structure, which contributed to a decrease in hardness. Recently Boye and coworkers (1996b) studied the structural changes that occur when β-lactoglobulin is heated under different environmental conditions, by employing Fourier transform infrared (FTIR) spectroscopy and differential scanning calorimetry (DSC). β-Lactoglobulin showed maximum thermal stability at pH 3 and was easily denatured at pH 9. Upon denaturation, the protein unfolded into more extensive random coil structures at pH 9 than at pH 3 (Boye *et al.* 1996b). At all pH values studied (3, 5, 7 and 9), two new bands in the FTIR spectrum at 1618 cm^{-1} and 1684 cm^{-1}, characteristic of intermolecular β-sheet structure and associated with aggregation, were observed after the initial denaturation. DSC studies showed that the thermal stability of β-lactoglobulin was enhanced in the presence of sugars. At 59°C the amide I band began to broaden in a 100g glucose/l solution and at 63°C in a 500g glucose/l solution. This indicated the loss of native structure. The aggregation band (1618 cm^{-1}) became apparent at 67°C in 100g glucose/l and at 76°C in 500g glucose/l. The effect of 500g sucrose/l was similar to that of glucose, but the broadening of the amide I band started at 55°C and aggregation was not observed until 80°C. Thus, sucrose 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 (Boye *et al.* 1996b).

2.9.1 Gelation Mechanism

Surprisingly, gelation of isolated β-lg has not been the subject of many investigations. In studies of the gelation of β-lg A at pH 8.0, it was found that a self-supporting gel does not form when there are no salts present (de Wit et al. 1988; Mulvihill and Kinsella, 1988), but gels of maximum compressive strength were obtained with sodium chloride and calcium chloride at concentrations of 200 and 10mM respectively. It is not clear if this is due to unfolding or to association mechanisms. While it was not the main object of their investigation, Paulsson *et al.* (1986) indicated that an intermediate concentration of ~5% β-lg was required to measure elastic properties in complex modules during heating. Hegg (1982) evaluated gelation of β-lg by determining if the gel matrix would hold water during centrifugation. Hegg (1982) showed that heat induced gels occur at different combinations with specific salt concentration and pH. At concentration level of 85mM of sodium chloride, gelation can only occur in the pH range of 4-7.5, and at a concentration of 340mM of sodium chloride, gelation can occur at pH range of 3.5-11 Hegg (1982). Excess salt produces a protein precipitate and too little salt inhibits protein-protein interactions.

2.10 α-Lactalbumin

α-Lactalbumin (α-lac) is the second major globular protein in whey. It is a compact metalloprotein that accounts for 20% of the proteins in bovine whey. α-Lac has a low molecular weight of 14.2 kDa with eight cysteine residues which exist as four intramolecular disulfides. The protein consists of 123 amino acid residues with a single bound calcium ion and has the ability to bind zinc as well as other metals (Stuart *et al.*,

1986 and Kronman, 1989). α-Lac is considered to be the most heat-stable whey protein. Reduction in the stability of its native tertiary structure occurs on the removal of the bound Ca^{++} (Hiroaka *et al.*, 1980).

α-Lac undergoes intermolecular interactions, in solution, leading to varying degrees of polymerization on both sides of its isoelectric point (pH ~4.8) (Shukla, 1973). At acidic pH values, the protein undergoes a rapid reversible association and slow aggregation (Kronman and Andreotti, 1964). In the range of pH 6-8.5 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^{++} from the protein (McKenzie and White, 1991); this occurs regardless of whether the protein is at pH values below 4.0 or above 9.0, 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 structure (Lala and 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. EDTA) 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.

Using DSC, Boye *et al.* (1997a) observed two reversible transitions at 39.6°C (A) and 64.8°C (B) when α-lac was heated. At pH 3, transition A showed a reversibility of 61% while transition B was completely reversible. It was observed that there was a decrease in enthalpy with a decreasing pH (Boye *et al.* 1997a). This decrease may be attributed to partial unfolding of α-lac at acidic pH, since a partially unfolded protein would require less heat energy to denature completely than a native protein (Ma and Harwalkar, 1988). The decrease in enthalpy may also be due to protein aggregation, which is considered an exothermic reaction (Jackson and Brandts, 1970).

FTIR spectroscopic studies at 26°C revealed a band at 1627 cm^{-1} at pH 3 that was absent at pH 9. This observation suggests an increase in extended β-sheet formation at low pH (Boye *et al.* 1997a). At a higher temperature (97°C) and pH 3 and 5, two bands in the spectra of α-lac (1684 cm^{-1} and 1616 cm^{-1}) were missing. These bands were present in the spectra taken at pH's 7 and 9. These bands are attributed to intermolecular β-sheet formation resulting from aggregation of the protein. It was suggested that α-lac formed aggregates at pH 7 and 9, but not at pH 3 and 5.

Clark and Lee-Tuffnell (1986) visually observed the formation of α-lac gels. They reported that α-lac aggregates at pH 3 and 5 without forming a firm gel. At pH 7 no gel or aggregate formation was observed. A firm translucent gel formed at pH 9 that did not revert to a solution state on cooling. The formation of an opaque coagulum-type gel normally occurs as a result of hydrophobic interactions (Damodaran, 1994). The formation of a translucent gel at pH 9 suggests 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. This is supported

by the absence of the 1684 cm^{-1} and 1616 cm^{-1} bands, associated with hydrogen-bonded aggregate structures, in the IR spectra at pH 9 (Boye *et al.*, 1997a).

2.11 BSA Gelation Profile

Bovine serum albumin (BSA) accounts for approximately 10% of the proteins in whey (Fox, 1989); although it is relatively minor protein component, it is considered as an important gelling protein of whey (Hines and Foegeding, 1993). BSA has a molecular weight of 66,000 Da. It is composed of a single polypeptide chain consisting of 580 amino acids with 17 intrachain disulfide bonds and one free thiol group at residue 34 (Kinsella and Whithead, 1989). Its secondary structure consists of 57% α-helix and 43% β-structure (β-sheet + β-turns) (Fen-nifu *et al.*, 1994).

The β-sheet content of native BSA is relatively low in comparison to that of 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 and Purcel, 1989). This led to the proposition that β-sheet hydrogen bonding may be important for aggregate formation during the gelation of BSA. Bands at 1683 and 1618 cm^{-1} were observed when BSA was heated above its transition temperature by infrared spectroscopy, in this case 65°C. These bands are referred to as aggregation bands and are associated with antiparallel intermolecular β-sheet structure (Kirsch *et al.*, 1986; Ismail *et al.*, 1992; Boye *et al.*, 1997b). Using differential scanning calorimetry (DSC) and Fourier transform infrared (FTIR) spectroscopy, Boye *et al.* (1997b) studied the effect of pH

and varying concentrations of NaCl, lactose, sucrose, and glucose in the presence of cysteine, urea, *N*-ethylmaleimide (NEM), and sodium dodecyl sulfate (SDS), on whey protein concentrates. At pH 3, the peak temperature of denaturation (T_d) was 62.7°C. The maximum T_d increased to 65°C at pH 5 and at pH 7 was found to be 63.4°C. Therefore, this suggests that BSA has maximum thermal stability at pH 5. In the presence of cysteine and urea (50mM), T_d values of BSA were 60.1 and 61.0°C respectively. In the presence of NEM (50m*M*) T_d was 63.1°C, whereas in the presence of SDS, T_d was 83.9°C. In this study it was shown that SDS increases the thermal stability of BSA. It has been suggested 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 explain its stabilizing effect on thermal denaturation (Hegg and Löfqvist, 1974; Markus *et al.*, 1964).

2.12 Rheological Properties

The protein concentration and heating conditions used to form a gel have a major influence on the rheological properties of the gel. An increase in heating temperature (75-125°C) causes an increase in gel strength of WPC gels (Schmidt *et al.*, 1978) and firmness (force at 40% compression) (Shimada and Cheftel, 1988) of WPI gels. Increasing the heating temperature from 75 to 125°C has no effect on the water-holding and elasticity (force remaining after a 5min relaxation) of WPI gels (Shimada and Cheftel, 1988).

pH is one of the main factors that regulates the rheological properties of WPC/WPI gels. WPC forms a very weak gel at pH 4.0 and strong gels at pH 6.0 and 7.0

(de Wit *et al.*, 1988). Changing the pH from 6.5 to 9.5 decreases firmness and increases elasticity of WPI gels (Shimada and Cheftel, 1988). The decrease in firmness at elevated pH values is associated with an increase in protein solubility, suggesting that, at pH values which are favorable to disulfide formation, a decrease in protein-protein interaction (via electrostatic repulsion) lowers the effective protein concentration of the gel matrix. The effect of electrostatic repulsion can be decreased by addition of NaCl (0.1M), which causes an increase in firmness and decreases in solubility and elasticity (Shimada and Cheftel, 1988). Increasing pH from 6.0 to 8.0 decreases hardness (70% compression) of WPI gels formed at 80°C or 90°C; however, gels formed at 70°C increase in hardness as the pH is increased (Zirbel and Kinsella, 1988).

The water-holding ability of gels that are made in the pH range of 7.5-9.5 with 0.1M NaCl increases with pH; however, it remains constant in the absence of NaCl, even when sulfhydryl groups are blocked with *N*-ethylmaleimide (Shimada and Cheftel, 1988). Noncovalent bonding appears to regulate water-holding properties in this pH range. WPI gels formed at pH 3.5 and 2.5, below the isoelectric points of the major whey proteins, are inelastic and contain 75-80% soluble protein (Shimada and Cheftel, 1988). Shimada and Cheftel (1988) concluded that gels formed at alkaline pHs (above p*I*) are stabilized by hydrophobic and disulfide bonds, whereas gels formed at acid pHs depend on hydrogen bonds.

The hydrophobic properties of whey proteins have been correlated with gelation. Mangino *et al.* (1988) made a variety of WPCs by subjecting whey retentate to one of the following heat treatments: ambient, 64°C for 15 sec, and 72°C for 15sec. The WPC surface hydrophobicity was correlated with gel strength, but another measure of

hydrophobicity, alkane binding, was not. However, the retentate (WPC prior to drying) alkane binding was correlated with gel strength. These findings did not agree with previous WPC results which indicated a strong correlation between heptane binding and gel strength (Kohnhorst and Mangino, 1985). The two methods appear to be measuring different aspects of hydrophobicity that may not be consistently correlated with functional properties.

The contribution of disulfide bonding to the stability of WPC/WPI gels has been addressed in several investigations. For example, isothermal studies at 85 and 90°C indicate that the concentration of sulfhydryl groups decreases when gels are formed at pH 6.6-6.7 (Beveridge *et al.*, 1984). Shimada and Cheftel (1989) were able to investigate interchange reactions in β-lactoglobulin by assuming that the reactivity of SH^{121} is low compared to SH^{106} and SH^{119} formed from SH^{121}/S^{106}-S^{119} interchange reaction. Their investigations showed that at pH 7.5, the total sulfhydryl group content decreases only slightly during heating at 85°C, whereas the slow-reacting SH^{121} group decreases rapidly. This sulfhydryl/disulfide interchange coincides with an increase in elasticity and firmness. Disulfide-linked β-lactoglobulin dimers, trimers, and tetramers are detected during the initial phases of association and progression to high molecular-weight aggregates. At pH 2.5, there is a slow rate of sulfhydryl/disulfide interchange and no high-molecular-weight disulfide linked aggregates are formed (Shimada and Cheftel, 1989). The gel formed at pH 2.5 is firmer than that formed at pH 7.5; however, the low-pH gel is less elastic. Intermolecular disulfides appear to be essential to elasticity of WPI gels.

Chapter 3 Research Protocol

3.1 Preparation of Protein Solutions

Four different whey protein isolate (WPI) samples (designated A, B, C and D) were supplied by Avonmore (Richfield, Idaho). Protein content was approximately 90% and lactose content was <0.05%. A 10% solution of each sample was prepared in D_2O at different pHs as described in Table 3.1. The pH of the solution was adjusted by addition of either 1N NaOD or DCl.

The infrared spectra of the protein solutions were recorded on a Nicolet 8210E FTIR spectrometer equipped with a deuterated triglycine sulfate detector. All spectra were collected by coadding 512 scans at a resolution of 4 cm^{-1} and were ratioed against an open-beam background. The spectrometer was purged with dry air (Balston Inc., Boston, MA) to eliminate the interference resulting from infrared absorption from water vapor. Seven microliters of solution was loaded in a transmission IR cell between two CaF_2 windows, separated by a 25μm Teflon spacer. The temperature of the IR cell was controlled (within 0.1°C) by an Omega temperature controller. The temperature was increased gradually from 25 to 95°C at 5°C intervals allowing a 10-minute delay for temperature equilibration prior to recording the infrared spectrum. The temperature of the cell was then decreased back to 25°C with spectra recorded at 5°C intervals.

Table 3.1 Variable parameters of WPI solutions for gelation

WPI/pH	3	4	7	10
A	x	x	x	x
B	x	x	x	x
C	x	x	x	x
D	x	x	x	x

3.2 Preparation of WPI Gels

Aqueous suspensions of WPI (20% w/v) were prepared in a large beaker. The WPI powder was solubilized in water (pH unadjusted) by using a spatula. The solution was transferred to another beaker, to remove most of the foam that accumulated during solubilization of the WPI. The sample was stirred until a clear solution was obtained. The clear solution was then transferred to an Erlenmeyer flask and placed under a low vacuum, in order to remove air bubbles prior to heating. Thirty polypropylene vials (0.9 x 10cm OD) were each filled with 5ml of solution. The vials were closed with a punctured plastic cap, to be able to release the pressure that is accumulated from the vapor at higher temperatures. A water bath was set to a specified temperature, above the denaturation temperature of WPI. All the vials were transferred at the same time to the water bath and left for different time intervals (Table 3.2). Each treatment was carried out in triplicate. The samples were then cooled to room temperature by blowing ambient air with a fan for 30 minutes. The samples were placed on a shelf at room temperature for 90 minutes prior to measuring the gel strength as described below.

To study the changes in gel strength on as a function of cooling time, 30 vials were heated at 75°C for 45 minutes and allowed to cool to room temperature. Gel

strength measurements were then performed every 2 hours, three vials at a time, for a period of 20 hrs.

3.2.1 Measurement of gel strength

The gels were removed from their vials by cutting one end of the vial and sliding them out from the other end. The gel was cut in three identical pieces of 9mm length x 9mm diameter (Fig 3.1), by using a modified egg cutter (Fig 3.2). The three pieces of gels were each subjected to a 70% compression test (i.e. the gel was compressed to 70% of its original height) employing an Instron® Universal Testing Machine. The test procedure was the same as that described by Montejano et al. (1984) and Rejaei et al. (1996) with some minor changes. To perform the compression test, the plunger of the Instron® was fitted to a 50N compression load cell. A downward force of 10mm/min was applied to the samples to produce a constant peak-to-peak deformation in the cylindrical samples (9.0 mm in height x 9.0 mm in diameter). The gel strength was calculated as force (N) divided by initial area of the gel (cm^2).

Table 3.2 Temperature and heating time for gel formation employing 20% WPI in H_2O at pH 7

Time/Temperature	70°C	75°C	77°C	80°C
15 min	**N/A**	**X**	**X**	**X**
30 min	**X**	**X**	**X**	**X**
45 min	**X**	**X**	**X**	**X**
60 min	**X**	**X**	**X**	**X**
75 min	**X**	**X**	**X**	**X**
90 min	**X**	**X**	**X**	**X**

N/A Not Applicable (very weak gel/could not be measured by the Instron®)

Fig 3.1 Gel dimensions

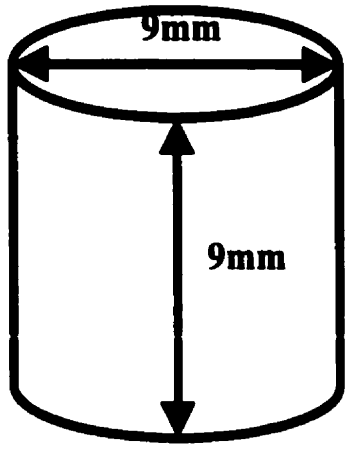
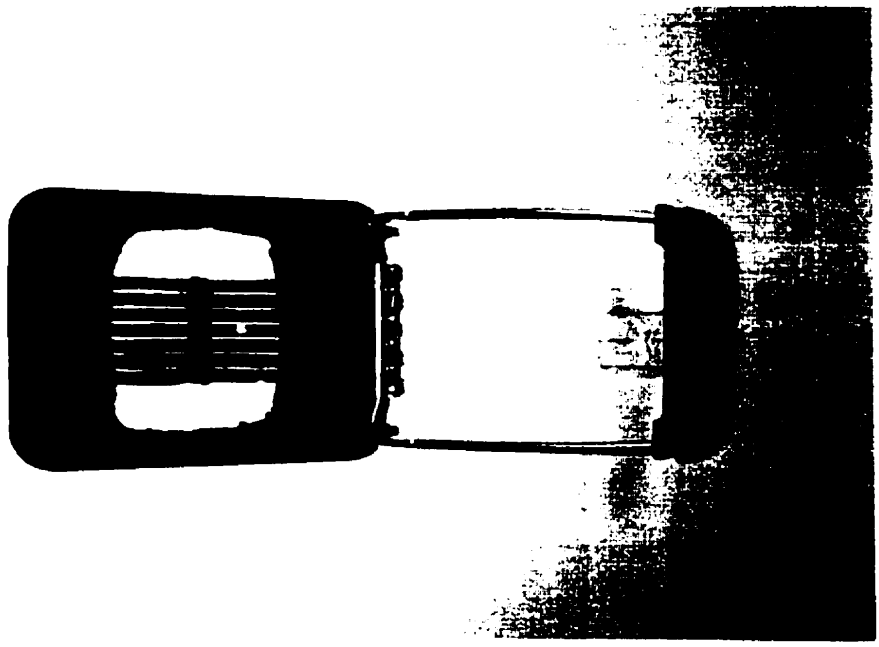


Fig 3.2 Modified egg-cutter



3.2.2 FTIR Measurements of WPI Gels

Aliquots of the unheated solutions of 20% WPI prepared in D_2O were placed between two CaF_2 windows and heated for the same time periods as outlined in Table 3.2. The solutions were prepared in D_2O to avoid interference caused by the water absorption in the amide I region of the infrared spectrum. All samples were heated to the specified temperature and heating time (Table 3.2), then cooled down to 25°C. The IR spectra were recorded just after the sample had cooled to 25°C (~30min) and at 30-minute intervals for 20 hours, to observe if any changes occurred as a function of cooling time.

Chapter 4 FTIR Spectroscopic Investigation of the Gelation Behavior of Whey Protein Isolates

4.1 Introduction

Whey protein isolate and concentrate solutions form viscoelastic gels after sufficient heating (Schmidt 1981; Kinsella and Whitehead 1989; Ziegler and Foegeding 1990). The nature of the gel is determined by three major conditions: (1) concentration and composition of the protein; (2) processing conditions, for instance, heating and cooling rate; and (3) environmental conditions, like ionic strength, pH, and mineral content (Kinsella and Whitehead 1989; Paulsson *et al.* 1990; Ziegler and Foegeding 1990)

In this chapter, a systematic investigation of the thermal denaturation of four whey protein isolates (WPIs) by FTIR spectroscopy is described. The objective of this study was to investigate the differences in the gelation behavior among these four different WPIs by examining the changes in the secondary structure of the whey proteins that occur during gelation of the WPI solutions. This study was then extended to examine the behavior of these WPIs at acidic, basic and neutral pH (Table 4.1.1) and to correlate the changes in rheological properties of gels prepared under varying physico-chemical conditions to the FTIR data.

Table 4.1.1 Experimental design

Sample/pH	3	4	7	10
A	X	X	X	X
B	X	X	X	X
C	X	X	X	X
D	X	X	X	X

4.2 Examination of the Secondary Structure of WPIs (A, B C and D) as a Function of Increasing Temperature and pH by FTIR Spectroscopy

4.2.1 FTIR Band Assignments

Many authors have demonstrated the usefulness of FTIR spectroscopy for studying the secondary structure of proteins (Susi and Byler 1982; Ismail et al. 1992; Hines and Foegeding 1993, Boye *et al.* 1997b). Table 4.2.1 summarizes the amide I band assignments in the infrared spectrum between 1600 cm^{-1} and 1700 cm^{-1}. A total of eight bands can be discriminated, and these bands may be assigned as follows (assignments are based on Clark *et al.* 1981; Yang *et al.* 1987; Casal *et al.* 1988; Prestrelski *et al.* 1991a,b; Ismail *et al.* 1992; and Boye *et al.* 1997a). Between 1600 cm^{-1} and 1614 cm^{-1} a minor band is observed due to side-chain vibrations; a band between 1616 cm^{-1} and 1620 cm^{-1} is observed at high temperatures and is attributed to intermolecular β-sheet formation upon aggregation; a band assigned to intramolecular β-sheet structure is positioned between 1625 cm^{-1} and 1639 cm^{-1}; the random coil band is positioned between 1640 cm^{-1} and 1650 cm^{-1}; whereas α-helical structure absorbs between 1651 cm^{-1} and 1655 cm^{-1}; in some cases a band is found at 1639 cm^{-1} that might be due to turns or 3_{10}-helix; a band between 1671 cm^{-1} and 1673 cm^{-1} can also be attributed to turns; and between 1680 cm^{-1} and 1688 cm^{-1} the high-energy band of β-sheet structures is observed.

Table 4.2.1 Band Assignments

Bands (cm^{-1})	Assignments
1600-1614	Side-chain vibrations
1616-1620	Intermolecular β-sheet
1632-1639	Intramolecular β-sheet
1640-1650	Random coil
1651-1655	α-Helix
1662	3_{10}-Helix
1671-1673	Turns
1680-1688	Intermolecular β-sheet

4.2.2 Effect of heat treatment on the secondary structure of WPI-A at pH 3

A 10% (w/v) solution of WPI-A in D_2O was prepared and the pH adjusted to pH 3 by addition of very small amounts of DCl. An 8 µl aliquot was transferred to a temperature controlled IR cell. Examination of the amide I region in the infrared spectrum of the WPI-A (Figure 4.2.1) at 25°C revealed six discernable bands at 1692cm^{-1}, 1678 cm^{-1}, 1666 cm^{-1}, 1649 cm^{-1}, 1636 cm^{-1} and 1626 cm^{-1}. The 1692 cm^{-1} band stems primarily from β-lg and has been recently assigned to β-structure deep within the protein and inaccessible to solvent at room temperature (Boye *et al.* 1996b). The 1678 cm^{-1} and 1666 cm^{-1} have been assigned to turns and are present in the spectra of all proteins found in whey. The 1649 cm^{-1} band can be assigned to helical structure predominately from β-lg with some significant contribution from α-lac and a very minor contribution from BSA and IgG. The 1636 cm^{-1} band is due to intramolecular β-sheet with a contribution from the 3_{10} helical structure from α-lac. The 1626 cm^{-1} band has been assigned to antiparallel β-structure extensively

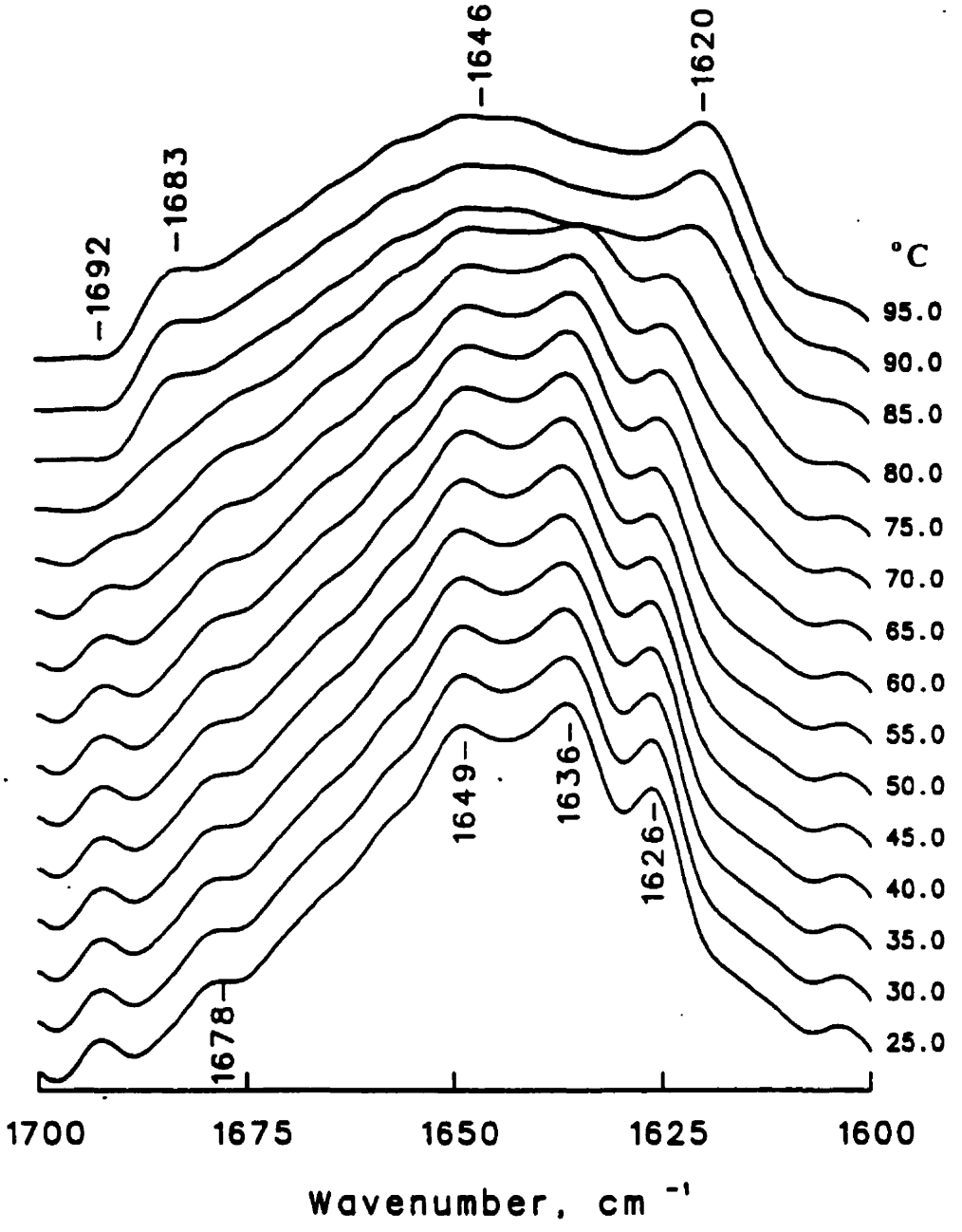


Figure 4.2.1: Stacked spectra of 10% WPI-A at pH 3 in the amide I region at different temperatures

hydrogen-bonded to solvent molecules and stemming primarily from β-lg (Ismail *et al.* 1992).

A gradual increase in temperature (between 25°C and 55°C) resulted in no discernable changes in the secondary structure of the whey proteins. Heating the sample between 55°C to 75°C resulted in small changes in the amide I bands. The 1649 cm^{-1} band increased slightly in intensity relative to the 1636 cm^{-1} band. Furthermore, the 1636 cm^{-1} became broader with increasing temperature. The band intensity at 1692 cm^{-1} remained unchanged. Above 75°C the 1692 cm^{-1} band disappeared and the other amide I bands coalesced, yielding a very broad band centered at 1646 cm^{-1} resulting from significant unfolding or denaturation of the protein. The disappearance of the discrete amide I bands is accompanied by the appearance of two new bands, a weak band at 1683 cm^{-1} and a strong band at 1620cm^{-1}. The relative intensity of the two bands and their respective absorption frequencies are suggestive of the formation of antiparallel intermolecular β-sheet structure, which forms concurrently with the denaturation of the protein (Ismail *et al.* 1992, Boye *et al.* 1996b). The relative intensity of the broad band at 1646 cm^{-1} to the intensity of the band at 1620 cm^{-1} is suggestive of partial rather than total aggregation of the WPI-A proteins Boye *et al.* 1996b.

4.2.3 Effect of heat treatment on the secondary structure of WPI-B at pH 3

The FTIR spectrum of a 10% (w/v) solution of WPI-B in D_2O at pH 3 is shown in Figure 4.2.2. Five discrete bands can be discerned at 1692 cm^{-1}, 1679 cm^{-1}, 1649 cm^{-1}, 1637 cm^{-1}, and 1627 cm^{-1}. The relative intensities of these peaks appear to be very similar to those in the spectrum of WPI-A at pH 3. A gradual increase in temperature (between 25 and 55°C) resulted in minor changes in the secondary structure of the whey proteins. The bands at 1626 cm^{-1}, 1637 cm^{-1}, and 1678 cm^{-1} became broader with increasing temperature. The band at 1692 cm^{-1} showed no discernable change. Above 75°C the band at 1692 cm^{-1} disappeared and the remaining amide I bands coalesced yielding a broad band centered at 1645 cm^{-1}. The disappearance of the 1692 cm^{-1} band is accompanied by the appearance of two new bands at 1682 cm^{-1} and 1620 cm^{-1}. This transition occurred at a higher temperature with WPI-A at pH 3 than with WPI-B, showing that WPI-B is more heat sensitive than WPI-A. The relative intensity of the two new bands (1683 cm^{-1} and 1620 cm^{-1}) in the spectrum of WPI-A at pH 3 is slightly higher than that observed in the case of WPI-B, suggesting that the proteins in WPI-A aggregated more than the proteins in WPI-B.

WPI-A contains the naturally occurring amounts of calcium, and WPI-B is calcium enriched. Boye *et al.* (1995) reported that the denaturation of WPC proteins involves dissociation of calcium, and thus a higher calcium content would be expected to make the protein less heat labile. The reason for the greater heat sensitivity of WPI-B described above is therefore not clear.

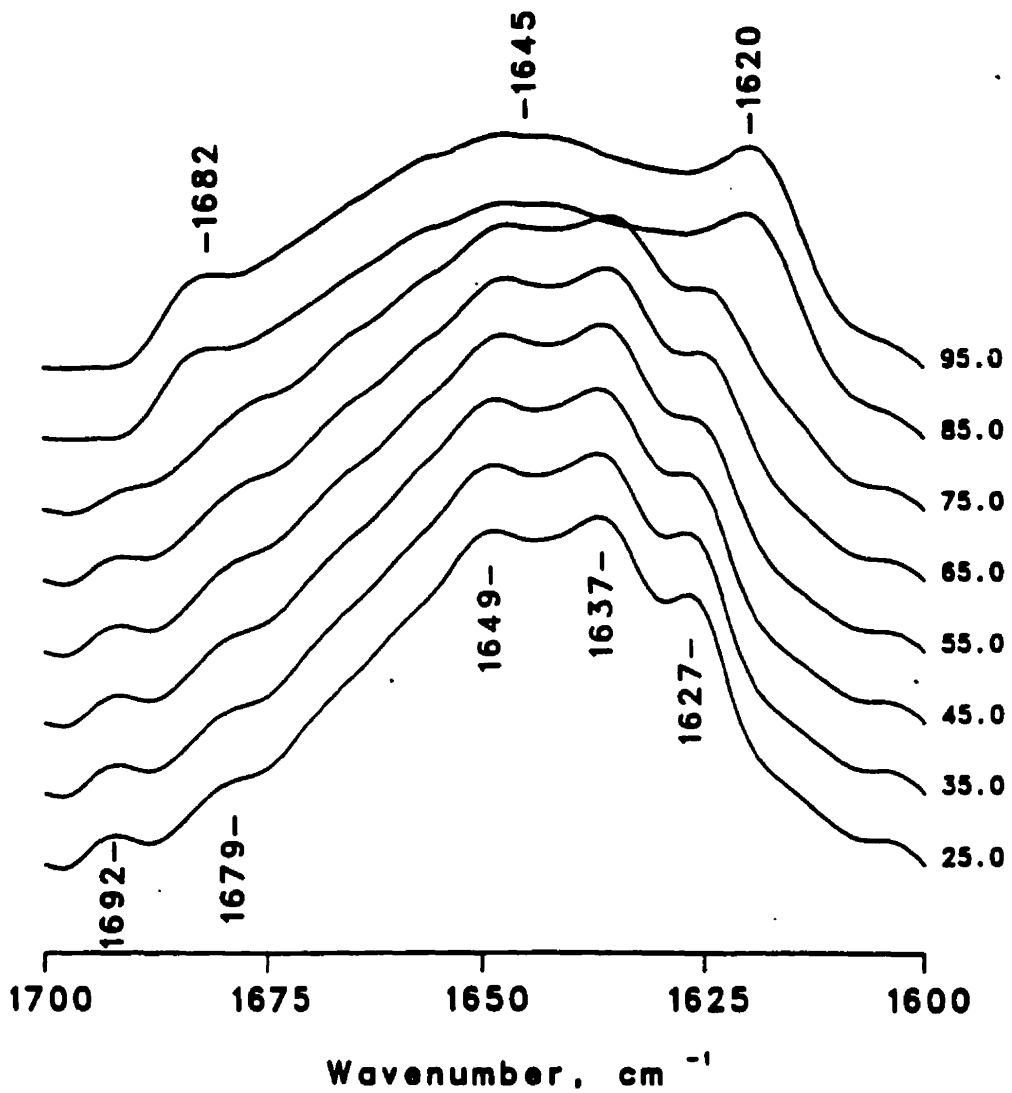


Figure 4.2.2 : Stacked spectra of 10% WPI-B at pH 3 in the amide I region at different temperatures

4.2.4 Effect of heat treatment on the secondary structure of WPI-C at pH 3

The infrared spectrum in the amide I region of a 10% (w/v) solution of WPI-C at pH 3 is shown in Figure 4.2.3. Five discrete bands can be discerned at 1692 cm^{-1}, 1679 cm^{-1}, 1650 cm^{-1}, 1636 cm^{-1}, and 1627 cm^{-1}. The relative intensities of these bands are noticeably different from those observed in the spectra of the WPI-A and WPI-B samples. In general, the amide I bands are sharper and the 1636 cm^{-1} band appears to be more intense relative to the 1650 cm^{-1} band. The 1627/1636 cm^{-1} intensity ratio also appears to be higher than both the 1627/1637 cm^{-1} intensity ratio in the infrared spectrum of WPI-A and the 1626/1637 cm^{-1} intensity ratio in the infrared spectrum of WPI-B. Gradual heating of the sample from 25°C to 55°C did not result in any apparent changes. Between 55°C and 75°C the 1650/1636 cm^{-1} and 1627/1636 cm^{-1} intensity ratios increase, accompanied by broadening of the 1627cm^{-1} band. Above 75°C, the 1692 cm^{-1} band completely disappears, the remaining amide I bands coalesce completely, resulting in a dramatic reduction in the total amide de I band intensity, and a new band appears at 1619 cm^{-1}. It is of interest to note that the intensity of the 1619 cm^{-1} band, assigned to intermolecular β-sheet formation, is significantly higher, relative to the broad band at 1641 cm^{-1}, than in the case of WPI-A and WPI-B, indicating that a greater proportion of the proteins in WPI-C have aggregated.

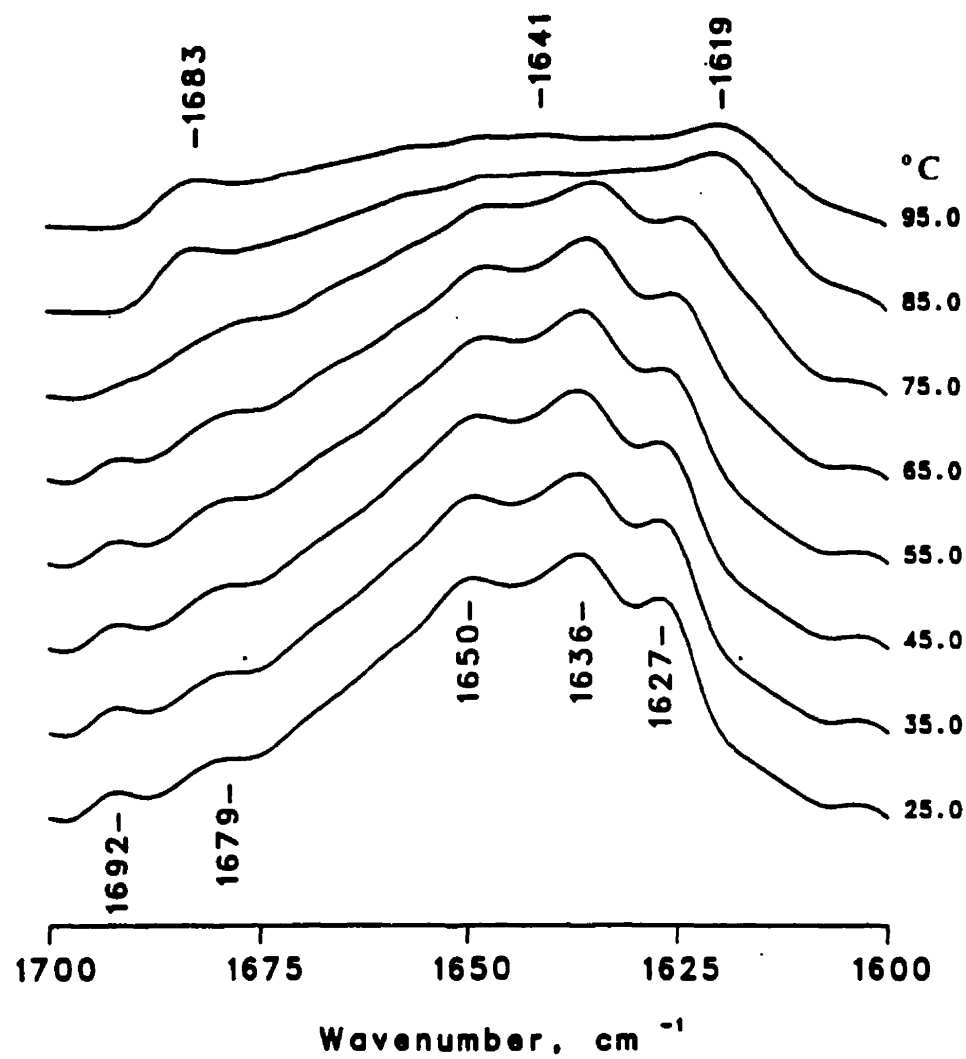


Figure 4.2.3: Stacked spectra of 10% WPI-C at pH 3 in the amide I region at different temperatures

4.2.5 Effect of heat treatment on the secondary structure of WPI-D at pH 3

The amide I band in the infrared spectrum of WPI-D at ambient temperature shows five bands at 1693 cm^{-1}, 1682 cm^{-1}, 1651 cm^{-1}, 1637 cm^{-1}, and 1628 cm^{-1}. The relative intensities of the three strong bands (1651 cm^{-1}, 1637 cm^{-1} and 1628 cm^{-1}) are significantly different from those observed for samples of WPI-A, WPI-B, and WPI-C. The observation that the bands are broader and the relative intensities are different (Figure 4.2.4) may indicate that the relative proportions of the whey proteins in WPI-D are different or that one or more of the protein components within the sample is partially denatured. Gradual heating of the sample to 55°C did not result in any noticeable change in the amide I bands. Heating to 65°C resulted in a significant drop in the intensity of the 1693 cm^{-1} band along with a drop in the 1628/1636 cm^{-1} intensity ratio. The 1693 cm^{-1} and 1628 cm^{-1} bands stem primarily from β-lg, and these results may indicate that the structure of β-lg in the WPI-D sample is slightly more labile than in the samples of WPI-A, WPI-B, and WPI-C. At 75°C the relative intensities of the 1651, 1636, and 1628 cm^{-1} bands are also markedly different from those found in the spectrum of WPI-C but are similar to those found in the spectra of WPI-A and WPI B. Furthermore, the 1693 cm^{-1} band disappears completely. Above 75°C the amide I bands coalesce, with the appearance of two bands at 1619 cm^{-1} and 1683 cm^{-1}. The 1647/1619 cm^{-1} intensity ratio is intermediate between that for WPI-A/B and that for WPI-C. The shift of the amide I band to 1619 cm^{-1} in the spectrum of WPI-D and WPI-C relative to 1620 cm^{-1} in the spectra of WPI-A and WPI-B is suggestive of the formation of stronger H-bonds between the protein aggregates in WPI-D and WPI-C (Ismail *et al.* 1992)

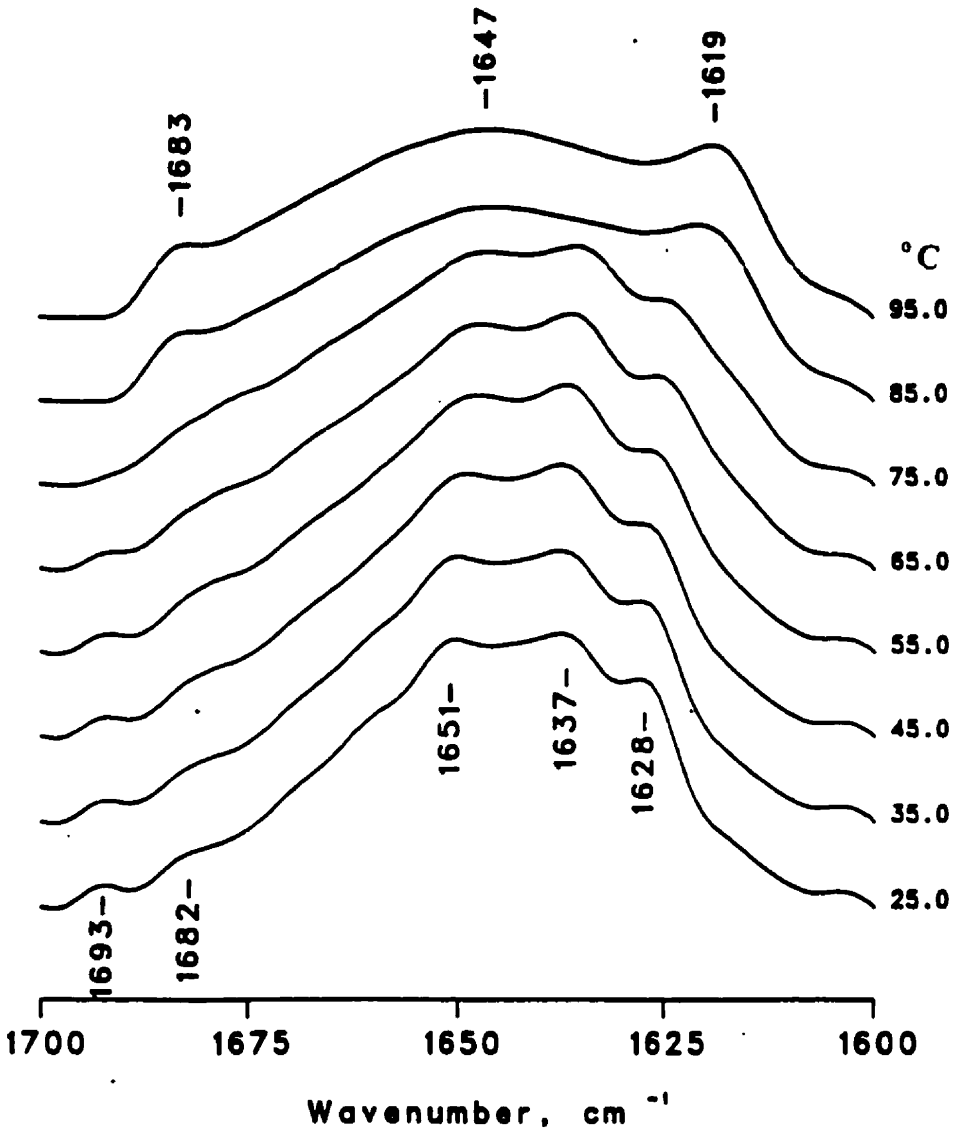


Figure 4.2.4: Stacked spectra of 10% WPI-D at pH 3 in the amide I region at different temperatures

4.2.6 Effect of heat treatment on the secondary structure of WPI-A at pH 4

WPI-A at pH 4 (Fig 4.2.5) was prepared and measured in the same way as WPI-A at pH 3. The infrared spectrum of WPI-A at ambient temperature showed seven distinct peaks at 1691 cm^{-1} 1678 cm^{-1}, 1657 cm^{-1}, 1649 cm^{-1}, 1640 cm^{-1}, 1627 cm^{-1}, and 1615 cm^{-1}. The amide I bands are generally sharper at pH 3 (Fig 4.2.1) than at pH 4, but their relative ratios are very similar. Continuous heating of the sample between 25°C and 55°C did not result in any apparent changes. Between 55°C and 75°C the amide I bands at 1691 cm^{-1} and 1627 cm^{-1} showed a very slight decrease in intensity and there was some subtle broadening of the 1678 cm^{-1}, 1657 cm^{-1}, and 1640 cm^{-1} bands. Furthermore, the 1649 cm^{-1} band increased in intensity in relation to the 1640 cm^{-1} band. Total disappearance of the 1691 cm^{-1} band was observed at 80°C. The band at 1678 cm^{-1} showed a dramatic decrease in intensity, and at the same time the other amide I band components coalesced, yielding a broad band centered at 1649 cm^{-1}. Above 85°C, two weak bands appeared at 1682 cm^{-1} and 1621 cm^{-1}. Comparison of the relative intensities of the 1649 cm^{-1} and 1621 cm^{-1} bands at pH 4 with the relative intensities of the 1646 cm^{-1} and 1620 cm^{-1} bands at pH 3 suggests that WPI-A aggregates more at pH 3 than at pH 4.

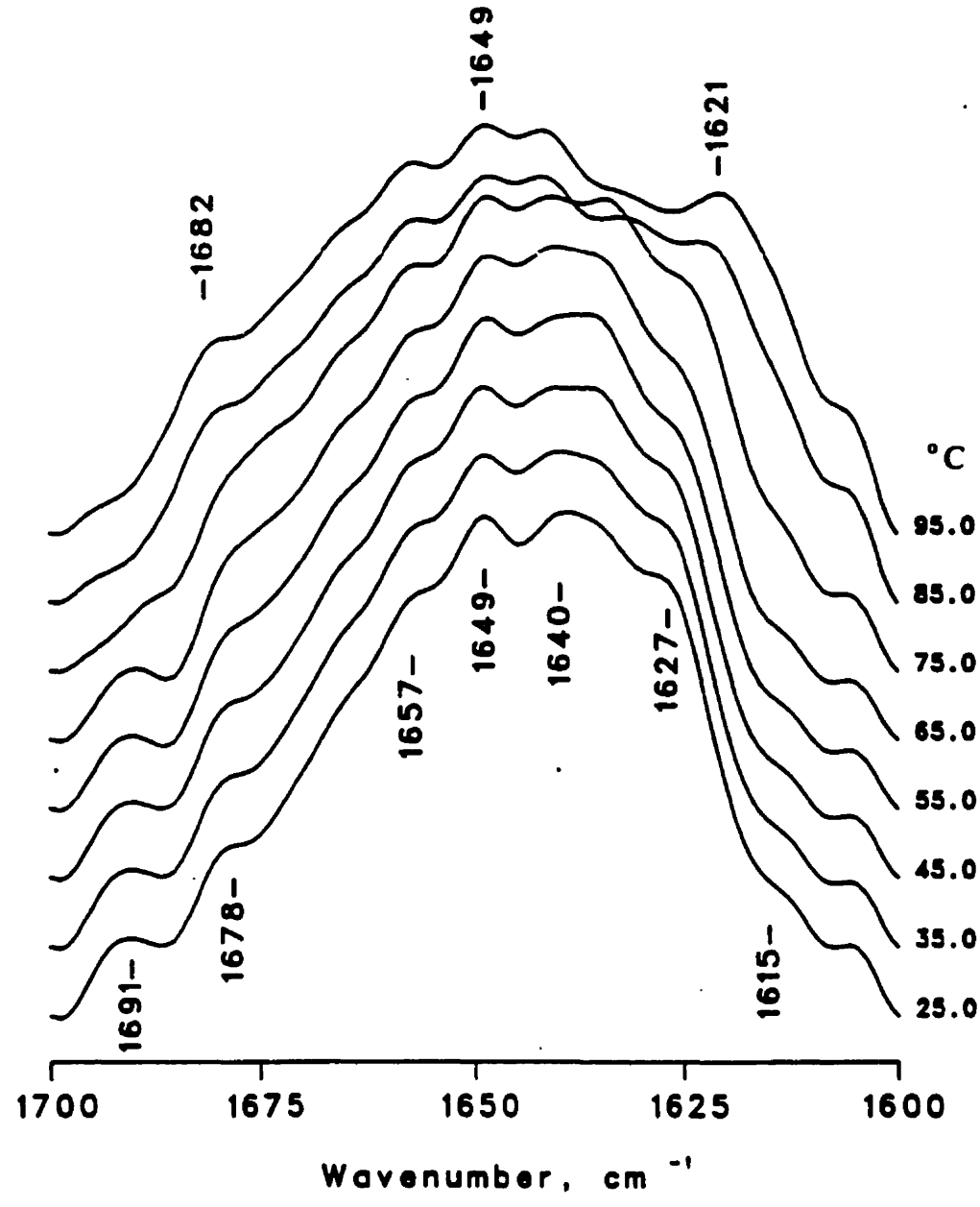


Figure 4.2.5: Stacked spectra of 10% WPI-A at pH 4 in the amide I region at different temperatures

4.2.7 Effect of heat treatment on the secondary structure of WPI-B at pH 4

The amide I band in the infrared spectrum of WPI-B at pH 4 (Fig 4.2.6) reveals many similarities to WPI-B at pH 3. However, the intensity of the 1649 cm^{-1} relative to that of the 1637 cm^{-1} band is much weaker at pH 3. This indicates that at pH 4 intramolecular β-sheet structures predominate over α-helical structures whereas at pH 3 the proportions of β-sheet and α-helix structures are quite similar. The 1692 cm^{-1} and 1627 cm^{-1} bands seem to be sharper at pH 4. The 1679 cm^{-1} band shows no significant difference between pH 4 and pH 3. Above 65°C, the 1692 cm^{-1} band totally disappears, and the remaining amide I bands coalesce, with the appearance of two new bands at 1619 cm^{-1} and 1682 cm^{-1}. As mentioned above, at pH 3 the 1692 cm^{-1} band disappeared above 75°C. This suggests that WPI-B is more heat labile at pH 4 than at pH 3. Furthermore, the 1619/1646 cm^{-1} intensity ratio at pH 4 is lower than the 1620/1645 cm^{-1} intensity ratio at pH 3, which is indicative of a lower amount of aggregation at pH 4.

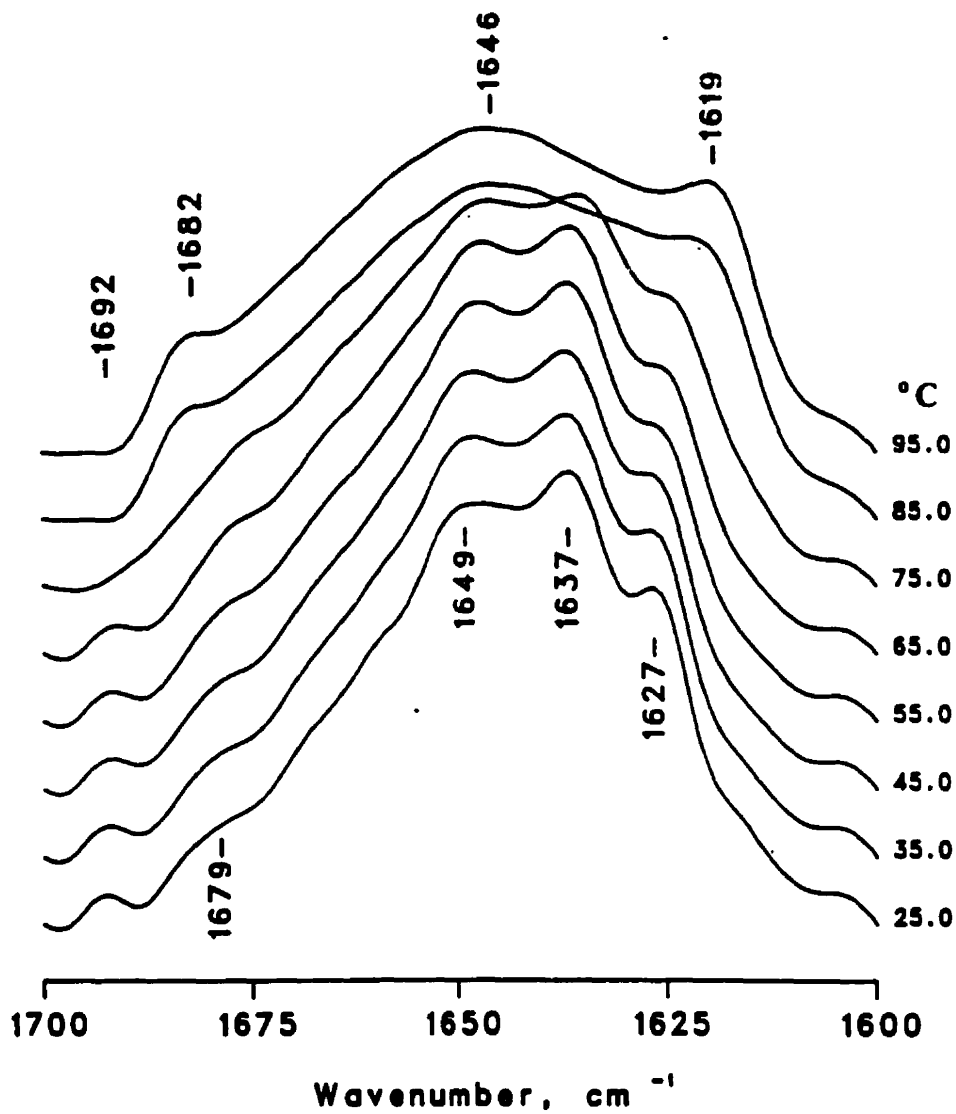


Figure 4.2.6: Stacked spectra of 10% WPI-B at pH 4 in the amide I region at different temperatures

4.2.8 Effect of heat treatment on the secondary structure of WPI-C at pH 4

The infrared spectrum in the amide I region of WPI-C at pH 4 is shown in Figure 4.2.7. At room temperature the protein reveals five discrete peaks at 1692 cm^{-1}, 1679 cm^{-1}, 1648 cm^{-1}, 1636 cm^{-1}, and 1627 cm^{-1}. The same bands were observed in the spectrum of WPI-C at pH 3, but with different relative intensities. The 1648/1636 cm^{-1} intensity ratio at pH 4 is higher than the 1650/1636 cm^{-1} intensity ratio at pH 3. The rest of the peaks appear to be sharper at pH 4 than at pH 3. As the temperature was gradually increased, the 1692 cm^{-1} band disappeared at 75°C, while the rest of the bands coalesced into a broad band centered at 1641 cm^{-1}, along with the appearance of two bands at 1683 cm^{-1} and 1619 cm^{-1}. At pH 3 the same phenomenon occurred, but the 1692 cm^{-1} band disappeared above 75°C and the intensity of the 1619 cm^{-1} band relative to the intensity of the 1641 cm^{-1} band (Fig 4.2.3) is weaker than at pH 4. This suggests that WPI-C aggregates more at pH 4 than at pH 3 and is more heat sensitive.

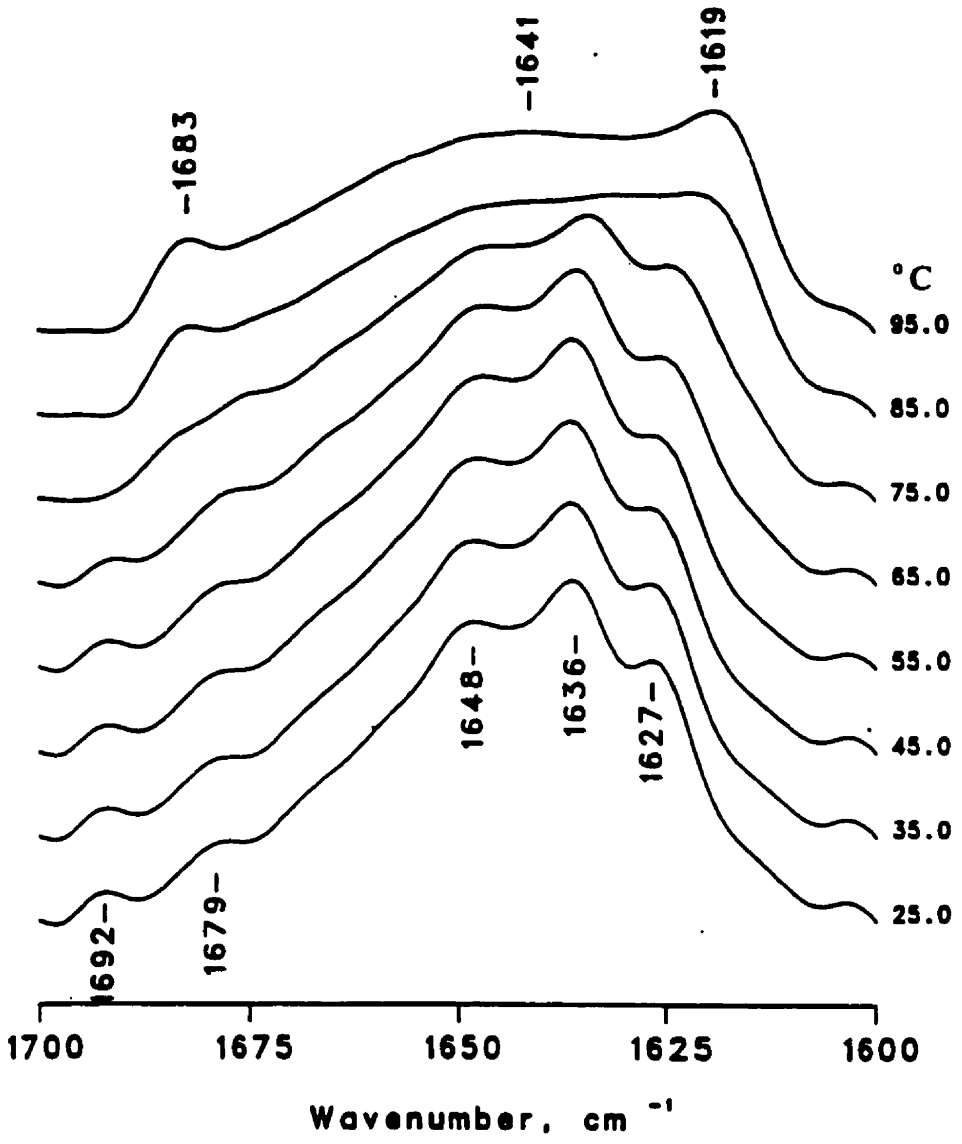


Figure 4.2.7: Stacked spectra of 10% WPI-C at pH 4 in the amide I region at different temperatures

4.2.9 Effect of heat treatment on the secondary structure of WPI-D at pH 4

The spectra of WPI-D at pH 4 in D_2O as a function of increasing temperature are shown in Figure 4.2.8. Eight discrete bands can be discerned at 1693 cm^{-1}, 1683 cm^{-1}, 1675 cm^{-1}, 1669cm^{-1}, 1652 cm^{-1}, 1645 cm^{-1}, 1636cm^{-1}, and 1626 cm^{-1}. The amide I bands appear sharper at pH 4 than at pH 3. With an increase in the temperature by 10 °C above ambient temperature, most of the bands between 1692 cm^{-1} and 1652 cm^{-1} disappeared. The 1652/1636 cm^{-1} intensity ratio at pH 4 is higher than the 1651/1637 cm^{-1} intensity ratio at pH 3 (Fig 4.2.4), suggesting that at pH 4 there are more intramolecular β-sheet structures relative to α-helix structures. Total disappearance of the 1692 cm^{-1} band was observed at 75°C, along with coalescence of the rest of the bands, forming a broad band centered at 1648 cm^{-1}. Above 75°C two new bands appeared at 1683 cm^{-1} and 1620 cm^{-1}. The shift of the aggregation band from 1619 cm^{-1} band at pH 3 to 1620 cm^{-1} at pH 4 suggests that the intermolecular H-bonding is weaker at pH 4 than at pH 3 (Ismail *et al.* 1992).

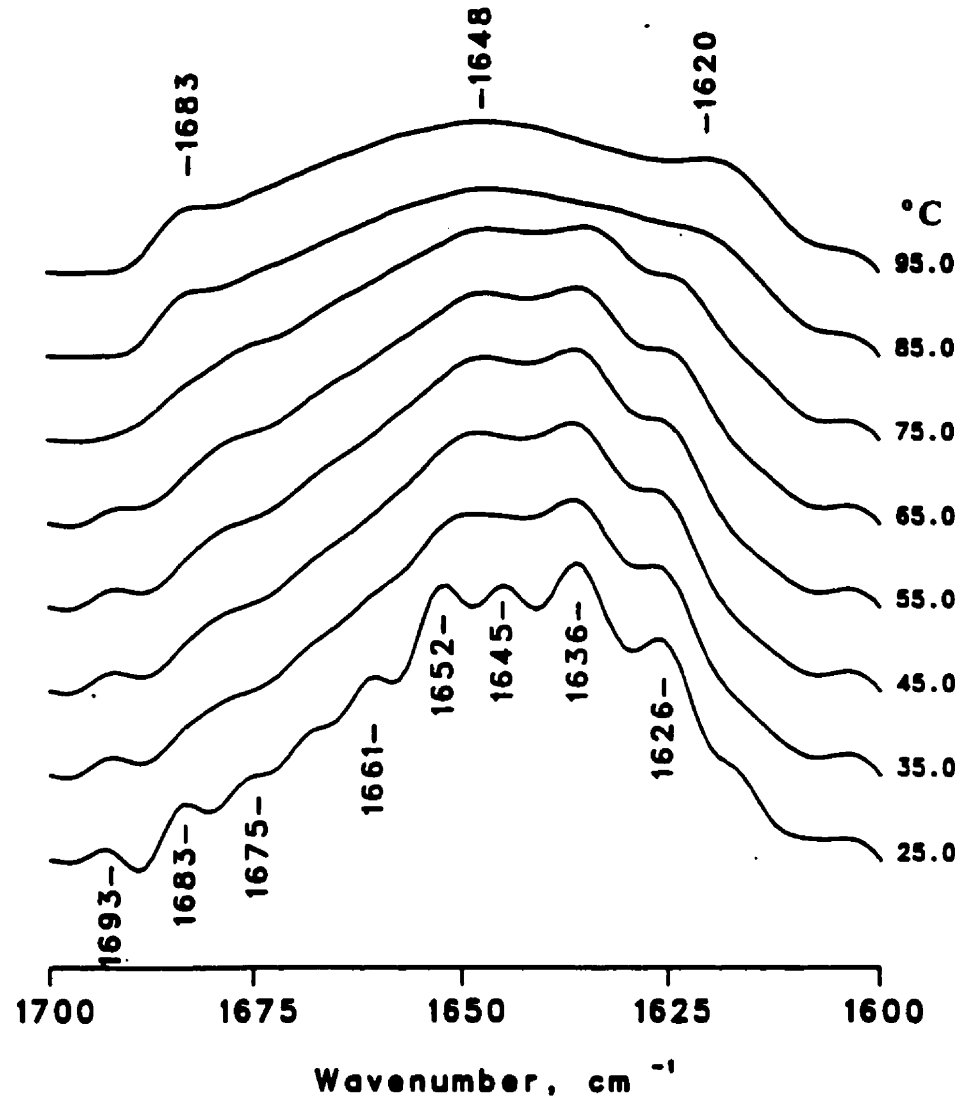


Figure 4.2.8: Stacked spectra of 10% WPI-D at pH 4 in the amide I region at different temperatures

4.2.10 Effect of heat treatment on the secondary structure of WPI-A at pH 7

The amide I band in the infrared spectrum of WPI-A at pH 7 is illustrated in Figure 4.2.9. The spectrum recorded at ambient temperature resembles that of WPI-A at pH 4, but the peaks are sharper and the intensity of the 1652 cm^{-1} band relative to the 1636 cm^{-1} band is higher. As the temperature was gradually increased to 35°C, four of the nine bands remained discernable: the 1693 cm^{-1}, the 1652 cm^{-1}, the 1636 cm^{-1}, and the 1625 cm^{-1} band. Between 35°C and 55°C some subtle changes were observed. The 1693 cm^{-1} band showed a slight decrease in intensity followed by broadening of the 1652 cm^{-1}, 1636 cm^{-1}, and 1625 cm^{-1} bands. Above 55°C the band at 1693 cm^{-1} disappeared, the other amide I bands coalesced, forming a broad band centered at 1647 cm^{-1}, and two strong bands appeared at 1616 cm^{-1} and 1683 cm^{-1}. These bands are assigned to intermolecularly hydrogen-bonded structures (Ismail *et al.* 1992). A shift of the 1620 cm^{-1} band at pH 3 to 1616 cm^{-1} at pH 7 was observed. However, comparison of the 1616/1647 cm^{-1} intensity ratio at pH 7, the 1621/1649 cm^{-1} intensity ratio at pH 4, and the 1646/1620 cm^{-1} intensity ratio at pH 3 indicates that WPI-A is more heat sensitive at pH 7, that it aggregates less, and that the aggregates formed are very strongly H-bonded. At pH 7, the aggregation bands (1616 cm^{-1} and 1683 cm^{-1}) begin to appear at 75°C whereas at pH 3 and 4 they begin to appear above 80°C.

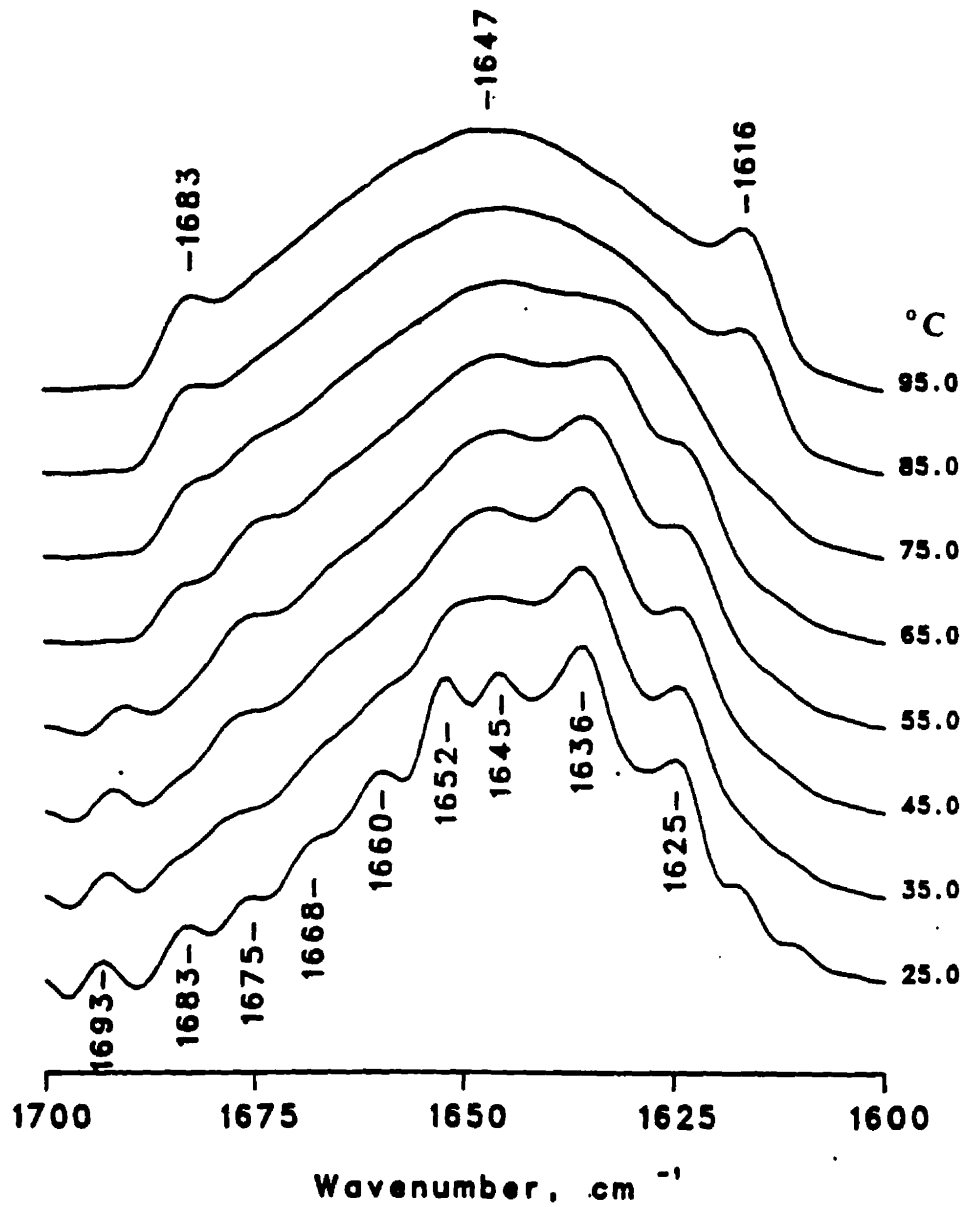


Figure 4.2.9: Stacked spectra of 10% WPI-A at pH 7 in the amide I region at different temperatures

4.2.11 Effect of heat treatment on the secondary structure of WPI-B at pH 7

The infrared spectrum in the amide I region of a 10% (w/v) solution of WPI-B in D_2O at pH 7 is shown in Figure 4.2.10. The spectrum is very similar to that of WPI-B at pH 3 and pH 4. Five distinct bands can be discerned at 1693 cm^{-1}, 1678 cm^{-1}, 1649cm^{-1}, 1635 cm^{-1}, and 1624 cm^{-1}. The bands at 1635 cm^{-1} and 1624 cm^{-1} are slightly shifted to lower frequencies in comparison with the corresponding bands in the spectra of WPI-B at pH 4 and 3 at ambient temperature, indicating strong H-bonding. As the temperature was gradually increased from 25°C to 45°C, no significant changes were observed. At 55°C all the bands with the exception of the 1693 cm^{-1} band showed significant broadening. Above 65°C the band at 1693 cm^{-1} disappeared and the peaks at 1649 cm^{-1}, 1635 cm^{-1} and 1624 cm^{-1} coalesced to form a broad band centered at 1649cm^{-1}. The bands at 1683 cm^{-1} and 1618 cm^{-1} appeared at 75°C, whereas at pH 4 and pH 3 the two bands appeared above 80°C, suggesting that WPI-B is more susceptible to heat at pH 7 than at pH 4 and pH 3. However, comparison of the 1618/1649 cm^{-1} intensity ratio to the 1620/1645 cm^{-1} intensity ratio at pH 3 and the 1619/1646 cm^{-1} intensity ratio at pH 4 shows that the intensity ratio is the lowest at pH 7 and the highest at pH 3, indicating that the extent of aggregation is the highest at pH 3.

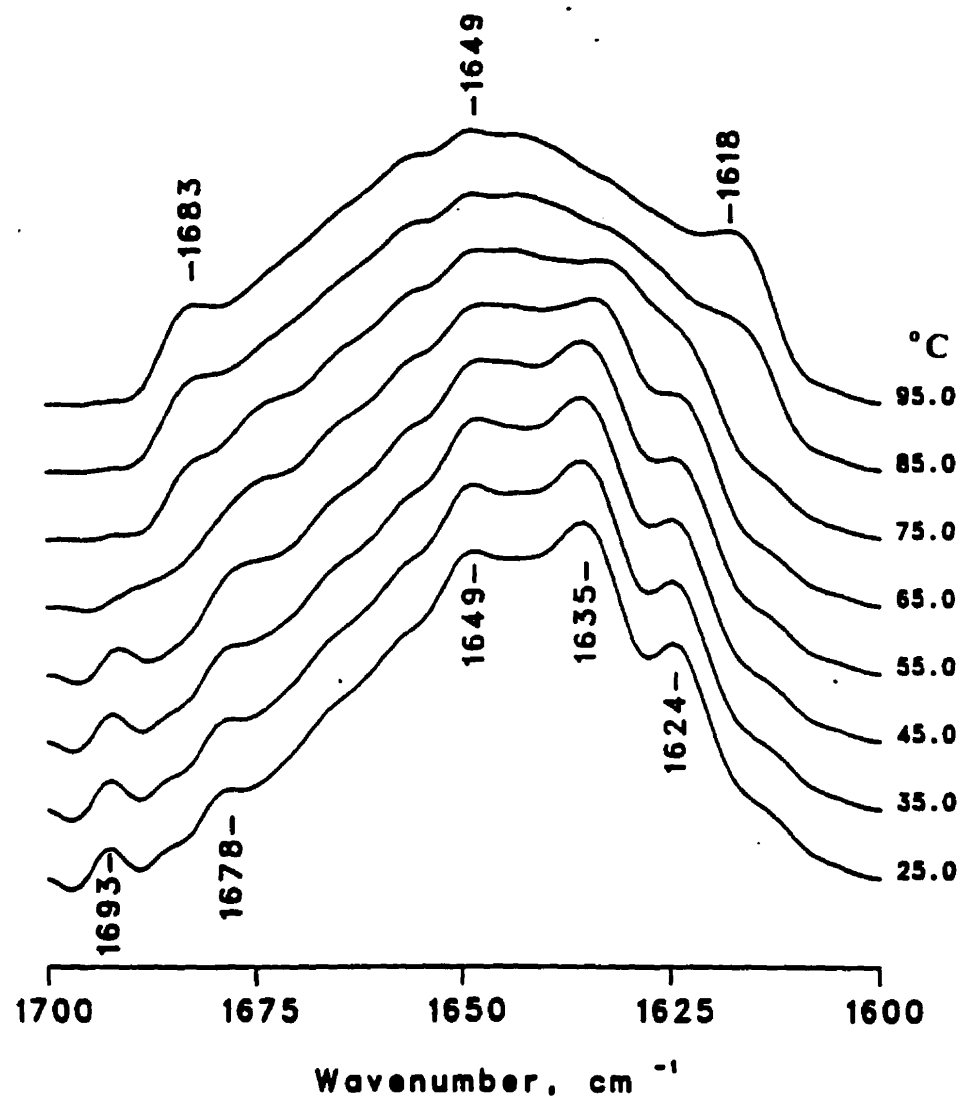


Figure 4.2.10: Stacked spectra of 10% WPI-B at pH 7 in the amide I region at different temperatures

4.2.12 Effect of heat treatment on the secondary structure of WPI-C at pH 7

The amide I band in the infrared spectrum of WPI-C at pH 7 is shown in Figure 4.2.11. At 25°C five discrete peaks at 1691 cm^{-1}, 1676 cm^{-1}, 1648 cm^{-1}, 1635 cm^{-1} and 1624 cm^{-1} are observed. Thus, the amide I band components shift to lower frequency as the pH increases from 4 to 7. As the temperature was gradually increased to 45°C, no changes were observed. However, in the spectrum recorded at 55°C the band at 1691cm^{-1} has totally disappeared, with some broadening of the rest of the peaks. Above 65°C, two new peaks (1683 cm^{-1} and 1617 cm^{-1}) started to be visible. In contrast, at pH 4 and pH 3 these aggregation bands did not appear below 80°C. This means that WPI-C at pH 7 aggregates at milder temperatures than at pH 4 and 3. Despite the fact that WPI-C at pH 7 starts to aggregate at an earlier stage in heating than at pH 3 and 4, the relative intensity of the 1645 cm^{-1} band to the 1617 cm^{-1} band is the lowest at pH 7 among the three pHs studied. It should be noted that WPI-C shows the strongest H-bonding at pH 7 among the three pHs studied, based on the shift of the aggregation band from 1620 cm^{-1} at pH 3 to 1617 cm^{-1} at pH 7.

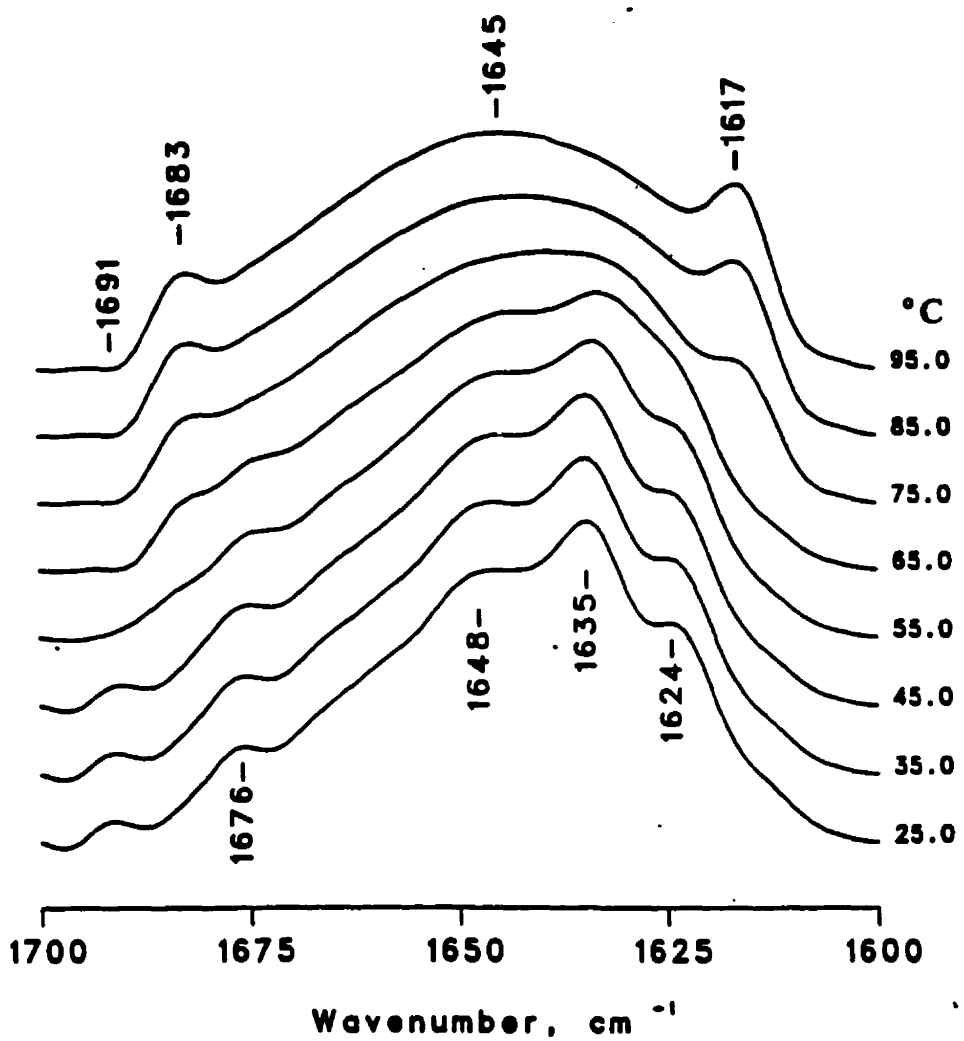


Figure 4.2.11 : Stacked spectra of 10% WPI-C at pH 7 in the amide I region at different temperatures

4.2.13 Effect of heat treatment on the secondary structure of WPI-D at pH 7

Figure 4.2.12 illustrates the changes in the amide I region in the spectrum of WPI-D at pH 7 during heat treatment. At 25°C, the spectrum resembles that of WPI-D at pH 3. Five discrete bands at 1691 cm^{-1}, 1676 cm^{-1}, 1647 cm^{-1}, 1635 cm^{-1}, and 1625 cm^{-1} can be seen. The intensity of the 1647 cm^{-1} band relative to that of the 1635 cm^{-1} band is higher than the corresponding relative intensities at lower pH (1652/1636 cm^{-1} at pH 4 and 1651/1635 cm^{-1} at pH 3). This indicates that the proportion of β-sheet structure increases with increasing pH. Above 55°C the band at 1691 cm^{-1} disappears, while the remaining bands coalesce to a broad band at 1648 cm^{-1}. The aggregation bands (1683 cm^{-1} and 1615 cm^{-1}) start appearing above 65°C. The 1615/1648 cm^{-1} intensity ratio is much lower than the 1619/1647 cm^{-1} intensity ratio in the spectrum of WPI-D at pH 3 and the 1620/1648 cm^{-1} intensity ratio in the spectrum of WPI-D at pH 4, indicating that the protein aggregates at pH 7. The infrared data also show that as the pH increases, the WPI becomes more heat labile and the resulting aggregates are strongly H-bonded.

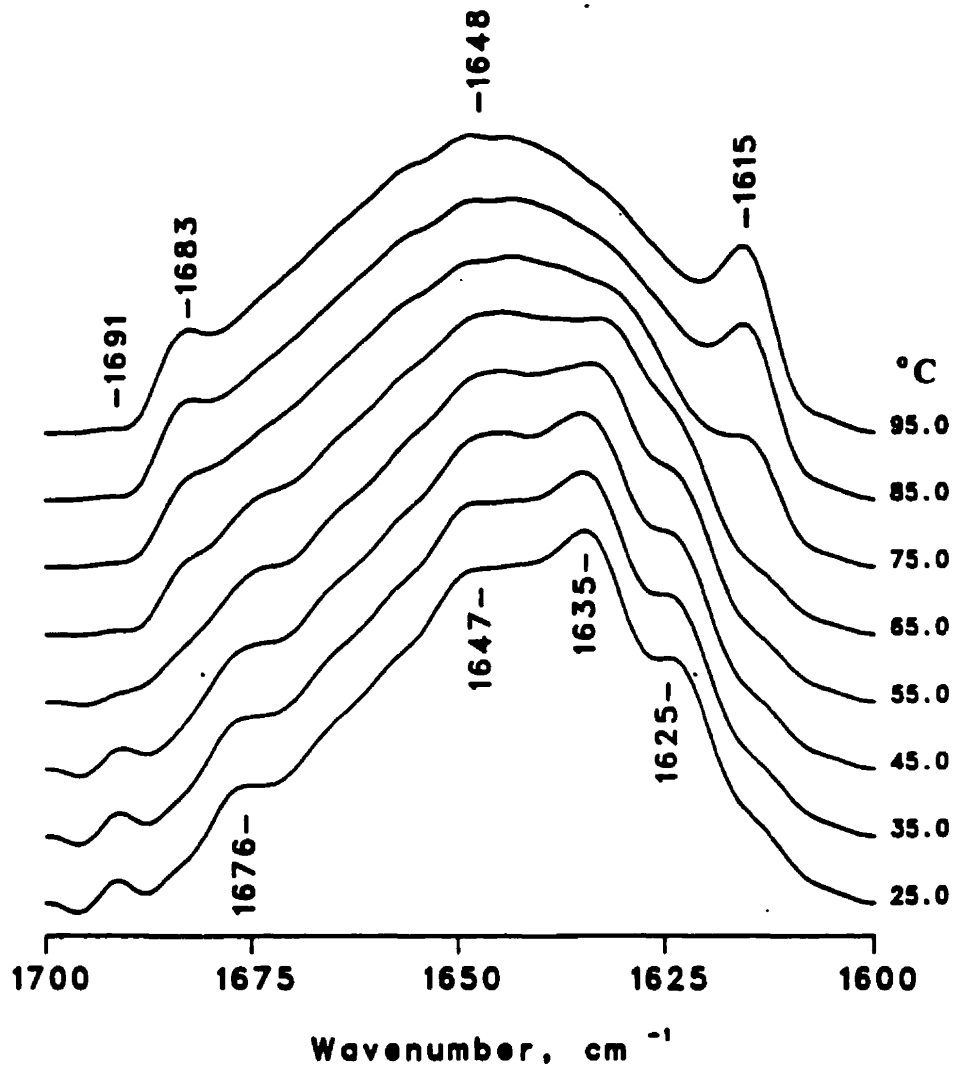


Figure 4.2.12: Stacked spectra of 10% WPI-D at pH 7 in the amide I region at different temperatures

4.2.14 Effect of heat treatment on the secondary structure of WPI-A & WPI-C at pH 10

Figures 4.2.13 and 4.2.14 illustrate the changes in the amide I region of the spectra of WPI-A and WPI-C, respectively, at pH 10. At ambient temperature WPI-A shows four very broad bands at 1674 cm^{-1}, 1647 cm^{-1}, 1632 cm^{-1} and 1622 cm^{-1}. It was observed that the bands were shifted to lower frequency in comparison to their positions in the spectra recorded at pH 3, 4 and 7. The broadness of the bands could be explained by partial unfolding of the protein in basic solutions. Four discrete bands are also observed in the ambient temperature spectrum of WPI-C (Figure 4.2.14), with peak positions at 1676 cm^{-1}, 1651 cm^{-1}, 1645 cm^{-1}, and 1637 cm^{-1}. The 1637 cm^{-1} and 1651 cm^{-1} bands are slightly shifted to higher frequency compared to pHs 7. 4, and 3. At pH 10, WPI-C had very broad bands, which might be attributed to partial unfolding of the proteins in basic solution. As the temperature was gradually increased to 45°C, the amide I peaks coalesced in the spectra of both samples (WPI-A and WPI-C), yielding a broad band centered at 1648 cm^{-1}, and the aggregation bands that appeared are shifted to lower frequency, suggestive of stronger H-bonding between the β-sheets. Comparison of the1616/1649 cm^{-1} (WPI-A) and 1615/1648 cm^{-1} (WPI-C) intensity ratios at pH 10 with the corresponding intensity ratios at pH 7, 4 and 3 shows that the WPI samples aggregate the least at pH 10.

The behavior of WPI-B and WPI-D at pH 10 was similar to that of WPI-A (results not shown).

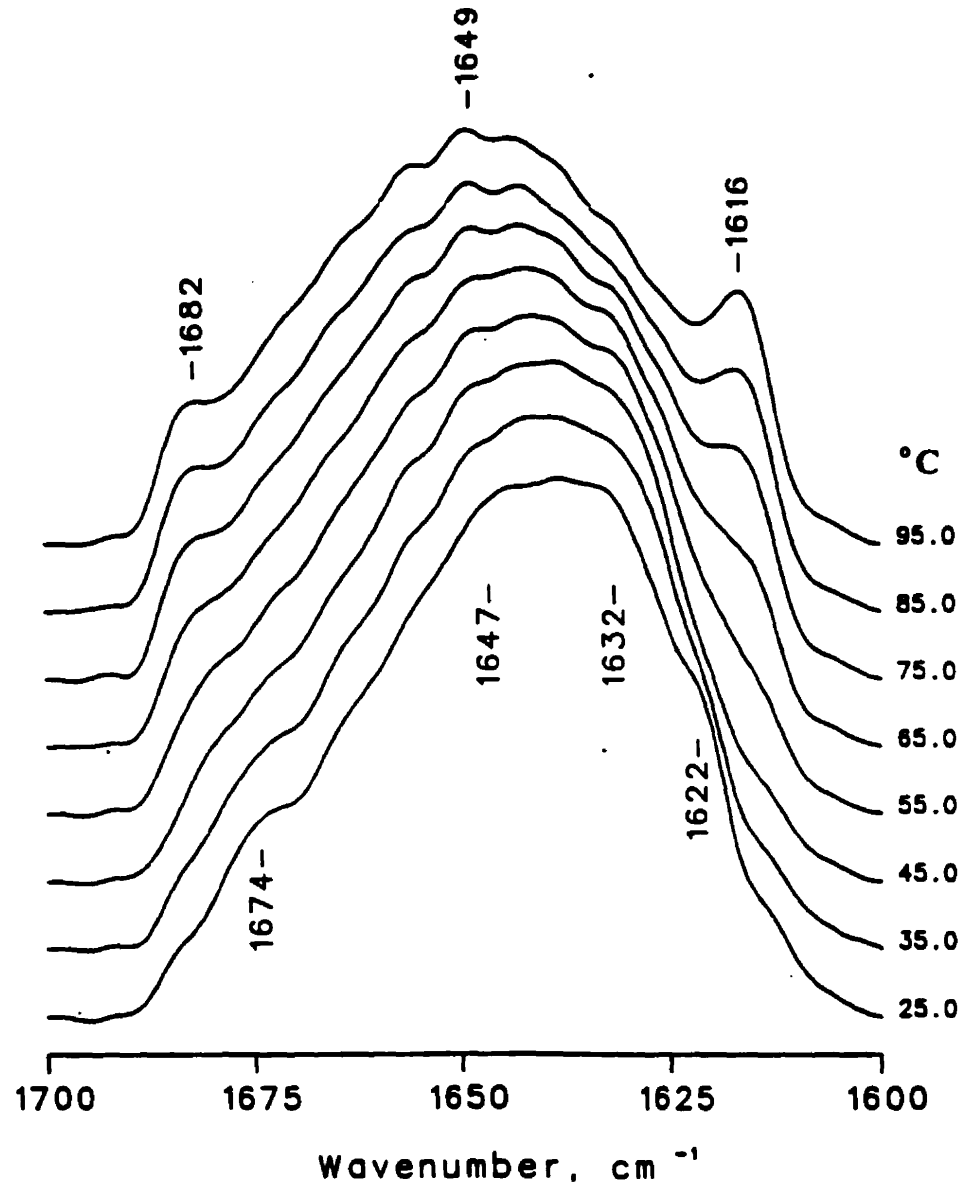


Figure 4.2.13: Stacked spectra of 10% WPI-A at pH 10 in the amide I region at different temperatures

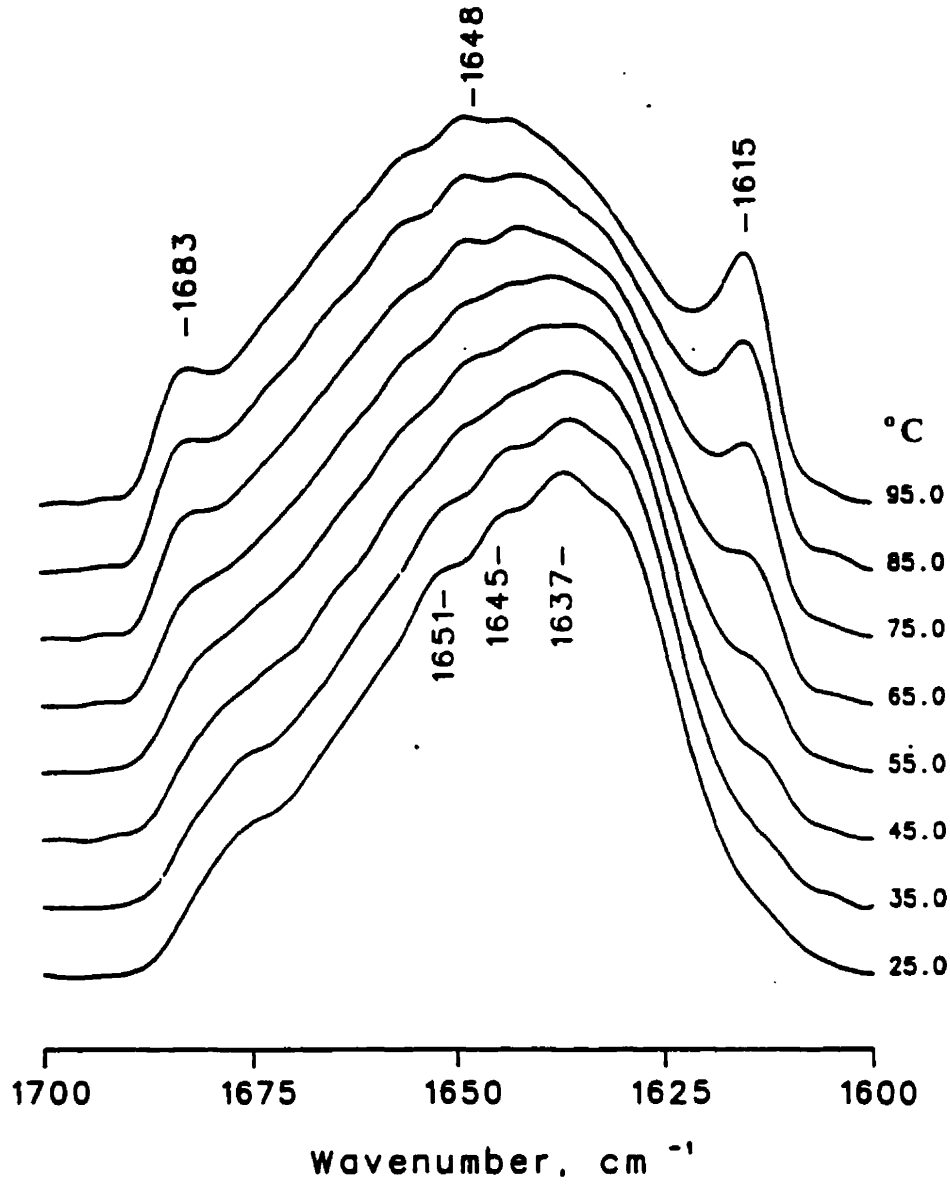


Figure 4.2.14: Stacked spectra of 10% WPI-C at pH 10 in the amide I region at different temperatures

4.3 Summary

Comparison of the FTIR spectra of the four WPIs studied in this work demonstrates that all four samples follow similar trends with changes in pH. In all cases, the temperature at which individual amide I band components in the spectrum of the native proteins begin to coalesce, corresponding to partial unfolding of one or more WPI components, decreased with increasing pH over the pH range investigated (pH 3-10). The extent of aggregation, as measured by the height of the lower-frequency association band relative to the overall amide I band intensity, also decreased with increasing pH. Furthermore, the shifts in the position of this aggregation band toward lower frequency with increasing pH indicate that intermolecular hydrogen bonding is stronger at higher pH in all four WPI samples.

Despite these overall similarities, differences in the aggregation behavior of the four WPIs are apparent when the FTIR spectra recorded at a particular pH are compared. At pH 3, all four WPI samples began to aggregate at the same temperature. However, the extent of aggregation varied in the order WPI-D < WPI-A ≈ WPI-B < WPI-C. Comparison of the effect of temperature on the spectra of the WPI samples at pH 4 reveals that the temperature at which partial denaturation was first observed was 10°C lower for WPI-C and WPI-D than for WPI-A and WPI-B. Although all four WPIs lost most of their secondary structure at the highest temperature examined (95°C), they all underwent some aggregate formation beginning at 85°C. As at pH 3, the extent of aggregation differs among the four samples, increasing in the order WPI-A < WPI-B < WPI-D < WPI-C.

At pH7, all four WPIs showed similar secondary structure profiles at ambient temperature. However, as the temperature was increased gradually, the extent of unfolding of the WPI samples differed. As at pH 4, the temperature at which partial denaturation was first observed was 10°C lower for WPI-C and WPI-D than for WPI-A and WPI-B. Extensive loss of secondary structure occurred at 65°C for WPI-C and WPI-D but not until 75°C for WPI-A and WPI-B. Furthermore, WPI-C and WPI-D began to aggregate at 75°C, as compared to 85°C in the case of WPI-A and WPI-B. Again, the extent of aggregate formation was greatest for WPI-C.

At pH 10, all the samples exhibited partial unfolding at ambient temperature, WPI-A being unfolded to a lesser degree than WPI-C. The onset of aggregate formation was observed at a lower temperature for WPI-C, with slightly stronger intermolecular hydrogen bonding. The extent of aggregation at 95°C was found to be similar for all four WPI samples.

The results obtained in this study demonstrate that careful examination of the temperature-induced changes in the FTIR spectra of solutions of WPIs can be employed to detect subtle differences in thermal denaturation and aggregation of the component proteins, both of which play important roles in gelation. In the following section, the results of FTIR and rheological measurements on WPI gels will be described. It is of interest to note here that the work reported in the next section was conducted with WPI-C because this sample yielded the stronger self-supporting gels than WPI-A, WPI-B, and WPI-D. The latter observation is consistent with the FTIR findings reported above that the extent of aggregation at high temperatures was greatest for WPI-C at both acidic and neutral pH.

4.4 Correlation of Changes in the Gel Strength of WPI Gels to Changes in the FTIR Spectra of the Gels

WPI-C gels were prepared by heating solutions of the same concentration at various temperatures and for various amounts of time. When protein solutions gel, they are converted from a viscoelastic fluid to a viscoelastic gel (Kinsella and Whitehead 1989; Ziegler and Foegeding 1990). The gel strength of the gels was measured with a Universal Testing Machine (Instron®). The changes in the FTIR spectra upon gelation were monitored in parallel experiments under identical conditions, except that the protein solutions were prepared in deuterium oxide instead of water to eliminate interference from water absorption in the amide I region (1600-1700 cm^{-1}).

To measure gel strength, gels were prepared in triplicate (3 tubes) and cut in three identical pieces per tube, for a total of nine gel samples for each experiment. WPI-C showed an increase in gel strength as a function of heating time at 75°C, from 7.95 N/cm^2 after 15 min of heating to 16.35 N/cm^2 after 90 min of heating (Fig 4.4.1). WPI-C samples that were heated at 75, 77, and 80°C for less than 15 min gave very weak gels (<0.8N/cm^2). Gel strength increased as a function of increasing temperature; gels prepared by heating at 75°C and at 80°C for 15 min yielded gel strengths of 7.95N/cm^2 and 13.65 N/cm^2, respectively. These results confirm the findings that were reported in the previous study of Boye *et al.* (1997b).

As mentioned above, parallel experiments were carried out using FTIR spectroscopy. Figure 4.4.2 shows two spectra of WPI gels prepared by heating the protein solution at 75°C. One spectrum was recorded after 15 min, and the second

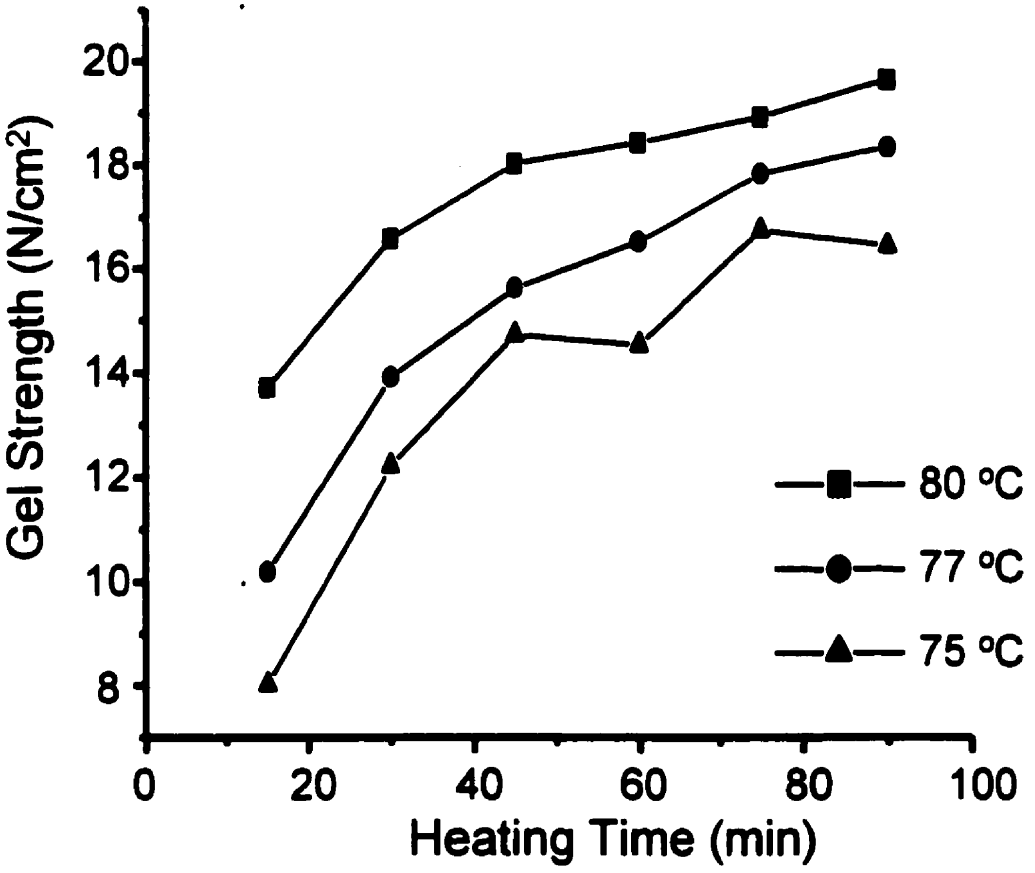
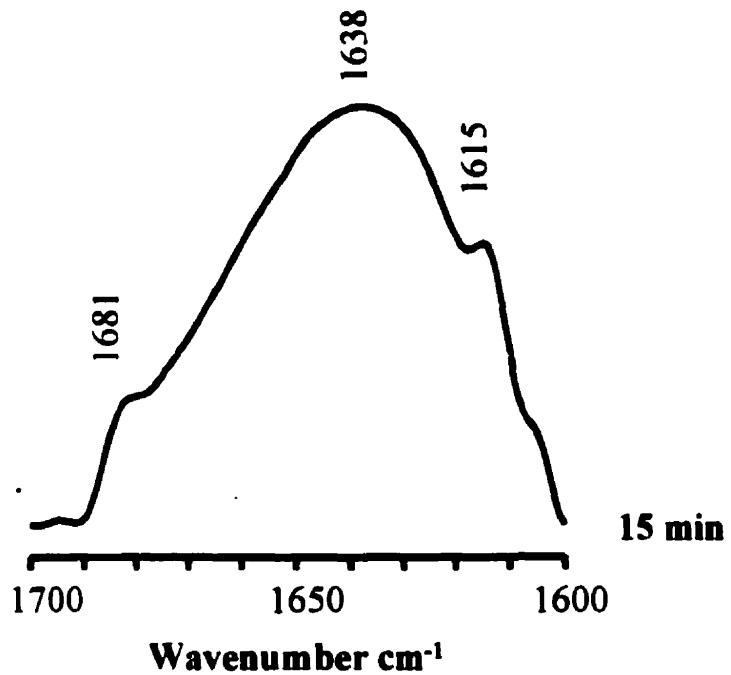


Figure 4.4.1 Plot of WPI-C Gel Strength Vs Heating Time at Different Temperatures



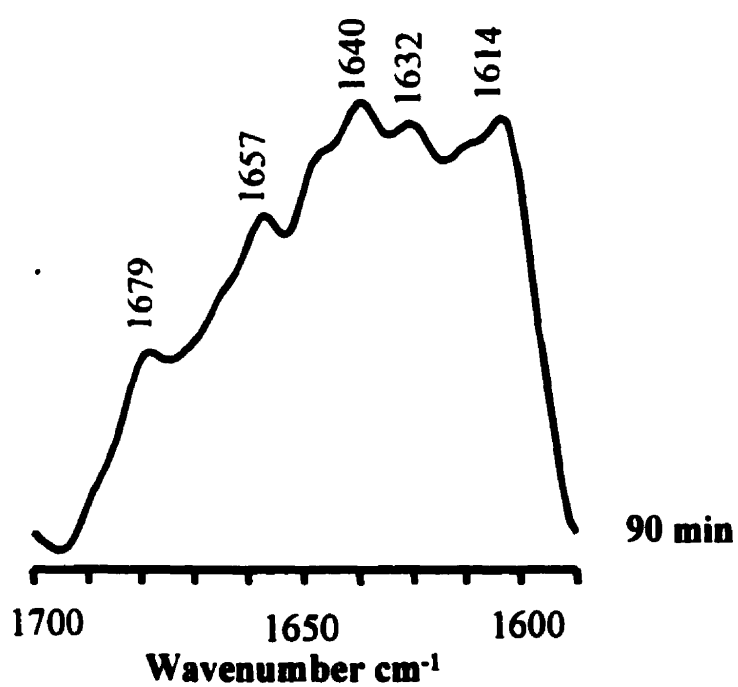


Figure 4.4.2 Spectra of 20% WPI-C Heated at 75°C at Different Heating Times

spectrum was recorded after 90 min. After 15 min of heating, the spectrum of the WPI showed changes indicative of protein aggregation. Two peaks at 1615 cm^{-1} and 1681cm^{-1} attributed to intermolecular β-sheet structures appeared, together with a broad band centered at 1638 cm^{-1}, which is attributed to random coil. After 90 min of heating, the 1681 cm^{-1} band shifted to 1679 cm^{-1} and the 1615 cm^{-1} band shifted to 1614 cm^{-1}, and three new bands became apparent between these bands. The observed band shifts suggest that after 90 min of heating, the structure of the protein became tighter, hence forming stronger intermolecular H-bonds, which might have caused syneresis in the gel. The new bands that appeared between the two aggregation bands have not been previously reported in the literature. The bands appeared at 1632 cm^{-1}, 1640 cm^{-1}, and 1657 cm^{-1} and are attributed to intramolecular β-sheet, random coil and α-helical structures, respectively. The appearance of these bands could be due to restructuring of the proteins, or these bands may have been hidden under the broad band centered at 1638 cm^{-1}. This suggests that during prolonged heating (90 min), more intramolecular β-strands were converted to intermolecular β-sheets, hence lowering the intensity of the broad band centered at 1638 cm^{-1} and revealing the weak bands that were beneath the broad band.

Figure 4.4.3 shows the superimposed spectra of two samples heated for the same period of time but at two different temperatures (75°C and 80°C). As discussed above, the higher the temperature is, the stronger the gel is. In Figure 4.4.3 the spectrum that was recorded at 80°C shows a slight increase in intensity at 1615 cm^{-1} by comparison with the spectrum that was recorded at 75°C. This suggests that the gel strength may be correlated to the increase in intensity of this aggregation band. Figure 4.4.4 shows the spectra

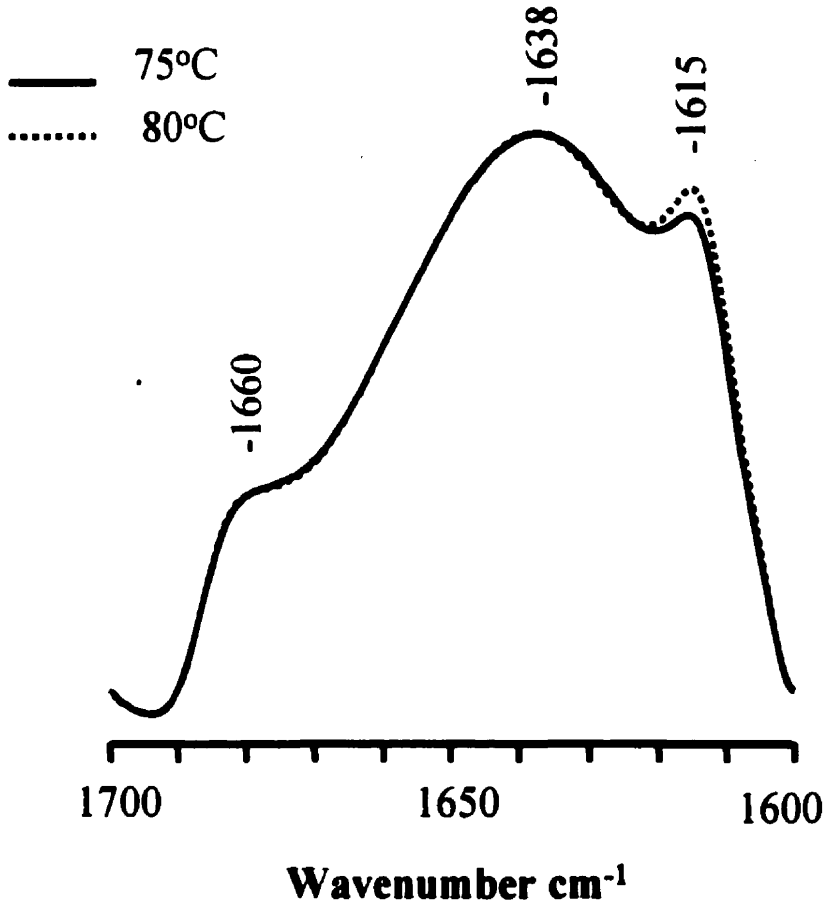


Figure 4.4.3 Overlapped spectra of 20% WPI-C in D_2O, After 15 min of Heating at Different Temperatures

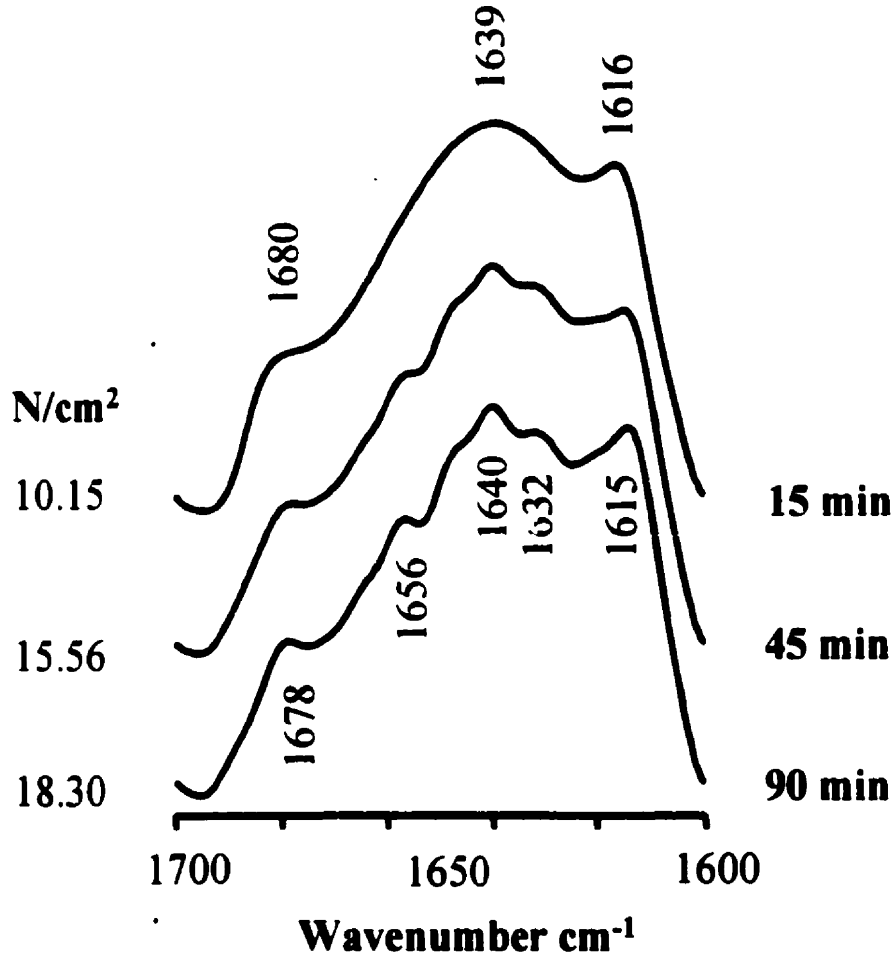


Figure 4.4.4 Stacked spectra of 20% WPI-C in D_2O, After 15 min 45 min and 90 min of Heating at 77°C, with their respective Gel Strength

recorded at 77°C after 15 min, 45 min, and 90 min of heating. The gel strengths (as measured by the Instron®) for gels prepared under the same conditions were 10.15 N/cm^2, 15.56 N/cm^2, and 18.30 N/cm^2, respectively. After 15 min of heating, the spectrum showed three discrete peaks at 1680 cm^{-1}, 1639 cm^{-1}, and 1616 cm^{-1}. After 45 min of heating, a shift from 1680 cm^{-1} to 1678 cm^{-1} and from 1616 cm^{-1} to 1615 cm^{-1} was observed, indicative of stronger intermolecular H-bonding. In addition, three new bands could be discerned at 1656 cm^{-1}, 1640 cm^{-1}, and 1632 cm^{-1}. These bands became more clearly discernable and intense with increasing time of heating. This suggests that after a certain period of time the molecules undergo rearrangement, forming new structures

The effect of cooling the gels was also examined. Figure 4.4.5 shows two spectra of WPI-C heated at 80°C for 15 min, but one spectrum was taken just after heating, whereas the second spectrum was taken after 20 hours of cooling. An increase in the intensity of the bands at 1639 cm^{-1} and 1616 cm^{-1} is observed in the second spectrum, suggesting that aggregation continues upon cooling.

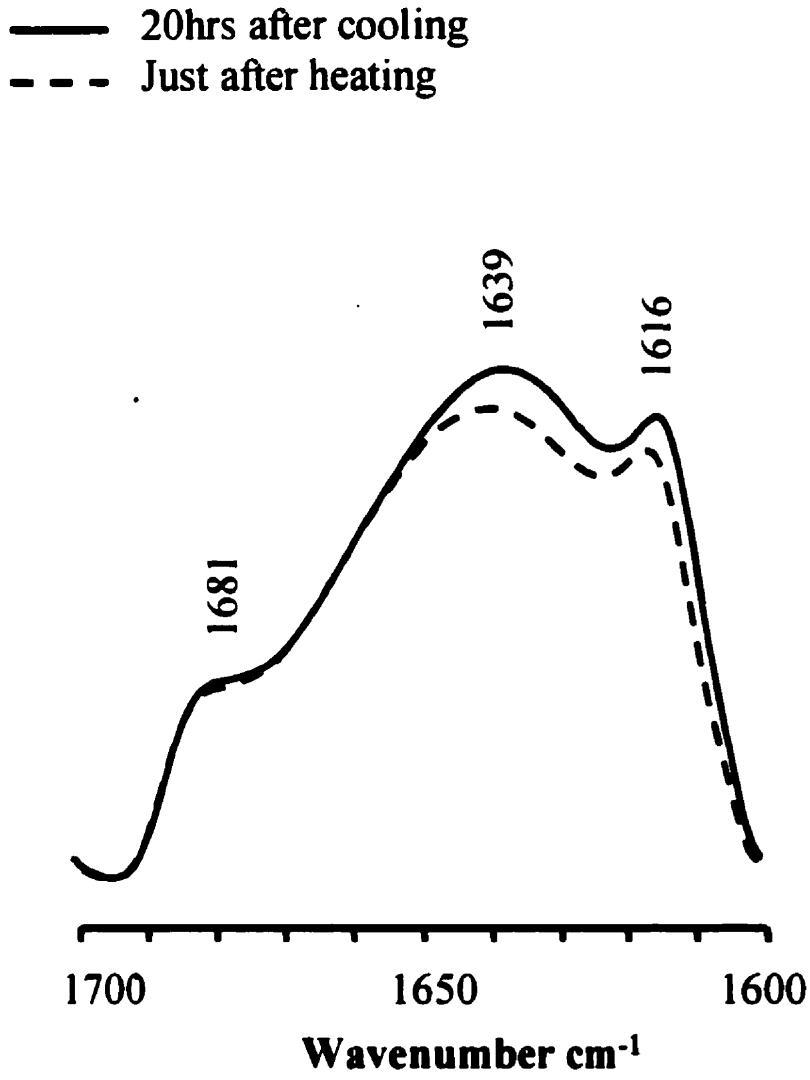


Figure 4.4.5 Overlapped spectra, of 20% WPI-C in D_2O, Heated at 80°C for 15min and measured at Different Cooling Times.

4.5 Conclusion

The major objective of the work described in this thesis was to investigate the variability in the gelation behavior of whey protein isolates obtained by different manufacturing processes. The primary technique employed in this investigation was variable-temperature Fourier transform infrared (VT/FTIR) spectroscopy. This technique was selected because it has been shown to be useful in the examination of the thermally induced changes in the secondary structure of whey proteins and can be applied to the study of both proteins in solution and protein gels. Thus, in order to gain a better insight, at the molecular level, into the variability in the gelation behavior of WPI, VT/FTIR spectroscopy was employed to examine solutions of four WPIs during heating. Because the gelation behavior of WPI is highly dependent on the pH of the solution, the thermally induced changes in the FTIR spectra of the WPI samples were also studied as a function of pH.

A systematic analysis of the spectra of the four WPIs examined in this work revealed differences in their thermal denaturation profiles. Specifically, the FTIR spectra recorded for each WPI at four different pHs showed differences in their thermal stability, in the extent of aggregation subsequent to denaturation, and in the strength of intermolecular hydrogen bonding in the aggregated proteins. The magnitude of these differences reflects the extent of variability in gelation behavior among WPIs produced by different processes.

Because the specific changes that take place in the secondary structure of a protein during gelation affect the gel texture (Kinsella and Whitehead, 1989), FTIR spectroscopy may also provide a means of examining the molecular basis for

differences in the viscoelastic properties of WPI gels. To investigate this possibility, the gel strengths of various WPI gels were measured with an Instron Universal Testing Machine and the FTIR spectra of gels prepared under identical conditions were recorded. The FTIR spectra of the gels showed marked differences in the amide I' region with changes in gel strength. Future work should be directed at employing multivariate analysis techniques such as partial-least-squares (PLS) regression to obtain calibration models for the quantitative prediction of gel strength by FTIR spectroscopy. If such an approach is successful, then FTIR spectroscopy could become a useful quality control tool in the manufacturing of WPIs.

Annex 1

Data of WPI-C Gel Strength at pH 7.0 Measured by Instron® Universal Testing Machine as a Function of Heating Time.

At 80°C

Time (min)	Mean (N/cm^2)	Standard deviation	% Error
15	13.65	0.071	0.52
30	16.55	0.071	0.43
45	17.95	0.071	0.39
60	18.35	0.495	2.70
75	18.85	0.636	3.38
90	19.55	0.354	1.81

At 77°C

Time (min)	Mean (N/cm^2)	Standard deviation	% Error
15	10.15	0.212	2.09
30	13.85	0.071	0.51
45	15.56	0.141	0.91
60	16.45	0.071	0.43
75	17.75	0.495	2.79
90	18.30	0.141	0.77

At 75°C

Time (min)	Mean (N/cm^2)	Standard deviation	% Error
15	7.95	0.212	2.67
30	12.20	0.072	0.59
45	14.65	0.071	0.48
60	14.50	0.084	0.58
75	16.65	0.071	0.42
90	16.35	0.212	1.30

Annex 2

Whey Protein Isolate Powders:
Provided by Avonmore

WPI Types	Amount of Calcium
WPI-A	Regular
WPI-B	High
WPI-C	Unknown
WPI-D	Low

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