Comparative study of the effects of added calcium on the heat-induced changes in three complex whey protein systems

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ABSTRACT

Because of their superior functional properties and nutritional quality, whey proteins are widely used in the food industry. Some of the important functional properties of whey proteins include gelation, water-binding, emulsification and foaming. Heat-induced gelation of whey proteins is particularly important in many food applications, and it involves a series of complex changes to protein structures (denaturation, aggregation). However, recent clinical studies on health promoting properties of whey proteins (e.g. weight management, muscle mass retention) has prompted the food industry to develop foods with high levels of whey proteins, such as high protein beverages. In these products, the heat-induced aggregation and gelation functionality of whey proteins becomes a major limiting factor.

The main objective of the present study was to determine the effects of added calcium on heat-induced denaturation, aggregation and gelation of whey proteins in three different whey protein products: whey protein isolate (WPI), acid whey protein concentrate (AWPC) and cheese whey protein concentrate (CWPC). The results were interpreted to assess the suitability of different whey protein systems as influenced by the effects of added calcium on their properties for making new denatured whey protein products.

The effects of added calcium chloride on heat-induced changes in whey protein solutions prepared from WPI, AWPC and CWPC were investigated using polyacrylamide gel electrophoresis (PAGE), high-performance liquid chromatography (HPLC), differential scanning calorimetry (DSC), circular dichroism (CD), nuclear magnetic resonance (NMR), small deformation oscillatory rheometry, large deformation compression testing and transmission electron microscopy (TEM). The loss of native proteins in 4% (w/w) protein solutions increased with increase in added calcium levels up to an optimum level (varying between 20 – 110 mM depending on the whey protein product), but then decreased with further increase in added calcium levels. The firmness of gels was maximal at 4 mM added calcium for WPI solutions, 20 mM for AWPC, and 80 mM for CWPC. These results showed that a certain level of added calcium maximally enhanced the heat-induced aggregation and gelation of whey proteins, and

these levels were different for the different whey protein systems. The effects of added calcium appeared to be related to the initial calcium contents of the three systems (2.1, 8.4 and 11.2 mM for 4.8% w/w protein solutions of WPI, AWPC and CWPC). It was considered that the addition of calcium changed the types of protein interactions leading to the formation of protein aggregates during heating. Increasing levels of calcium caused dramatic decreases in the fracture stress of whey protein gels due to the formation of increasingly larger protein aggregates; the gels became softer and, to an extent, mushier depending on the whey protein system. The TEM micrographs showed that on addition of calcium, the gels became coarser. WPI (12%, w/w protein) gels without the addition of calcium had a very fine structure (< 0.1 μm). With 60 mM of added calcium, 0.5 μm bead-like aggregates formed, and with further increase in added calcium levels, the aggregate size increased to 2 μm. AWPC (12%, w/w protein) gels without addition of calcium also showed a relatively fine structure (< 0.2 μm), and with the addition of 60 mM of calcium, the aggregate size increased to 0.1 – 0.2 μm. In the case of CWPC (12%, w/w protein) gels, the aggregate size increased from 0.05 to 0.3 μm on the addition of 60 mM of calcium.

The kinetics study showed that the mechanism of denaturation and aggregation of whey proteins in AWPC (but not in WPI or CWPC) was not affected by protein concentration in the range 4 – 28% (w/w). The orders of reaction were found to be 1.7 for β-lactoglobulin and 1 for α-lactalbumin at all protein concentrations. Without addition of calcium, the transition temperature decreased from 85 to 80ºC with increasing total solids for both proteins, whereas with 20 mM added calcium the transition temperature remained constant (~ 80ºC) over the total solids range (5 – 35%, w/w) for β-lactoglobulin and α-lactalbumin. The effects of added calcium on the aggregation kinetics appeared to be related to the calcium to protein ratio. The addition of 20 mM of calcium to low total solids solutions (5 – 10%, w/w) increased the rate constants, whereas addition of 20 mM of calcium to high total solids solutions (25 – 35%, w/w) decreased the rate constants.

These findings contribute to knowledge of the effects of added calcium on changes in whey proteins during heat treatments, and the relevance of the initial mineral content of whey protein products. AWPC appeared to be potentially the most suitable of the three systems studied for use as a feed material for manufacturing denatured whey proteins

with the aid of added calcium. The addition of calcium to AWPC solutions decreased the fracture stress and fracture strain of the gels formed, making them softer and mushier, and possibly more "processable". Further, at high protein concentrations (20 – 28%, w/w), which correspond to desired feed material concentrations in a processing plant, the addition of 20 mM of calcium to AWPC solutions optimally slowed down the aggregation rate, which might help to decrease plant fouling during the manufacture of denatured whey protein products.

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LIST OF ABBREVIATIONS

ºC	Degree(s) Celsius
%	Per cent
2D	Second dimension
α-La	α-Lactalbumin
Asn	Asparagine
Asp	Aspartic acid
AWPC	Acid whey protein concentrate(s)
β-Lg	β-Lactoglobulin
BSA	Bovine serum albumin
Ca^{2+}	Calcium ion
$[Ca^{2+}]$	Concentration of ionic calcium (mM)
$CaCl_2$	Calcium chloride
CD	Circular dichroism
Cl^-	Chloride ion
cm	Centimeter(s)
CWPC	Cheese whey protein concentrate(s)
Cys	Cysteine
DF	Diafiltration
DSC	Differential scanning calorimetry
E_a	Activation energy
F_p	Fracture point
F_{sn}	Fracture strain
F_{ss}	Fracture stress
g	Gram(s) or centrifugal force
G'	Storage modulus
G'_{final}	Final storage modulus
G'_{max}	Maximum storage modulus
h	Hour(s)
H^+	Hydrogen ion
HCl	Hydrochloric acid
HPLC	High performance liquid chromatography

Hz	Hertz
IE	Ion-exchange
Ig	Immunoglobulin
Ile	Isoleucine
K^+	Potassium ion
kDa	Kilodalton(s)
kg	Kilogram(s)
kHz	Kilohertz
kJ	Kilojoule(s)
k_n	Rate constant(s) at the order of reaction n
kV	Kilovolt(s)
L	Litre(s)
Leu	Leucine
Lys	Lysine
μL	Microlitre(s)
μm	Micrometre(s)
M	Molar (mol L^{-1})
mA	Milliampere(s)
mdeg	Millidegree(s)
MeCN	Acetonitrile
Met	Methionine
MF	Microfiltration
mg	Milligram(s)
Mg^{2+}	Magnesium ion
MHz	Megahertz
min	Minute(s)
mL	Millilitre(s)
mm	Millimetre(s)
mM	Millimolar (mmol L^{-1})
mmol	Millimole(s)
mol	Mole(s)
ms	Millisecond(s)
mV	Millivolt(s)
MW	Molecular weight

n	Order of reaction or number of replicates
N	Newton(s)
Na^+	Sodium ion
NaCl	Sodium chloride
NaOH	Sodium hydroxide
NEM	N-ethylmaleimide
nm	Nanometre(s)
NMR	Nuclear magnetic resonance
P	Significance level
Pa	Pascal(s)
PAGE	Polyacrylamide gel electrophoresis
Phe	Phenylalanine
PO_4^{3-}	Phosphate ion
PP	Proteose peptones
ppm	Part(s) per million
RP-HPLC	Reverse-phase high performance liquid chromatography
s	Second(s)
SDS	Sodium dodecyl sulphate
Ser	Serine
TEM	Transmission electron microscopy
TFA	Trifluoroacetic acid
Thr	Threonine
TOCSY	Total correlation spectroscopy
Tris	Tris(hydroxymethyl)methylamine
Trp	Tryptophan
ts	Total solids
T_t	Transition temperature
UDP	Uranyl diphosphate
UF	Ultrafiltration
UV	Ultraviolet
V	Volt(s)
Val	Valine
vs.	Versus
v/v	Volume/volume

w/v	Weight/volume
w/w	Weight/weight
WPC	Whey protein concentrate(s)
WPI	Whey protein isolate(s)

CHAPTER 1   Introduction

Whey is a by-product of cheese and casein manufacture. Two types of whey, i.e. sweet (using rennet) and acid (using acid coagulation) whey are produced. These whey sources are used to manufacture a wide range of commercial whey protein products including whey protein concentrates, isolates, fractions, hydrolysates, etc. Whey proteins have been used in food products for their excellent functional properties, i.e. hydration properties, interfacial properties and gelling properties, but also for their nutritional properties; they are the best nutritive proteins for human consumption (El-Salam, El-Shibiny, & Salem, 2009).

To date, the key functional property of whey proteins that has attracted much research and commercial attention is their ability to form strong elastic gels on heating. Gelling is a consequence of protein denaturation followed by aggregation involving the formation of covalent disulphide bonds and non-covalent associations. Most of the current whey protein products produced by the dairy industry are designed to perform well as gelling agents, as this suits functional applications in many food systems. However, gelling behaviour is a limiting property in many other food systems, especially beverages. In these applications, high levels of whey protein are required for nutritional reasons, but aggregation and gelation are not wanted; indeed, the whey proteins are required to be functionally "inert" with respect to gelation.

A significant market demand has been created for a whey protein product that is still nutritionally superior but lacks the ability to form heat-induced gels. The only known way of achieving this is to pre-denature the whey proteins by means of chemical or heat modifications (Morr & Josephson, 1968; Mulvihill & Donovan, 1987). Chemical modification involves major technical and nutritional challenges making it very difficult to implement. Therefore, heat modification is the only practicable option for modification of whey protein functionality. However, heat modification of whey proteins can still be a hurdle; when whey proteins are heated they will undergo denaturation, aggregation and gelation making them very difficult to handle in a commercial process. Therefore, one of the challenges for manufacturers is to produce nutritional but not functional (gelling) whey proteins.

Many processes have been proposed to produce heat-denatured whey protein products (information on this is reviewed in Section 2.8). They include producing micro-particulated whey proteins by combining heat and mechanical action, producing micro-particulated whey proteins from diluted whey protein solutions with little or no shear applied, using acid or enzyme hydrolysis, etc. While these processes provide technically feasible ways of producing heat-denatured whey protein products, they are not commercially feasible (cost-effective), hence the lack of denatured whey protein products on the market. These processes are not cost-effective for two main reasons: whey proteins are heated at low protein concentrations (e.g. 1 to 10% w/w) and high energy demand equipment is used (e.g. homogenizer). It is desirable to establish a cost-effective process for making denatured whey protein products that can be used in high protein food applications.

McSwiney et al. (1994) showed that when whey proteins were heated, protein molecules denatured almost completely before a gel network was formed (Figure 1.1).
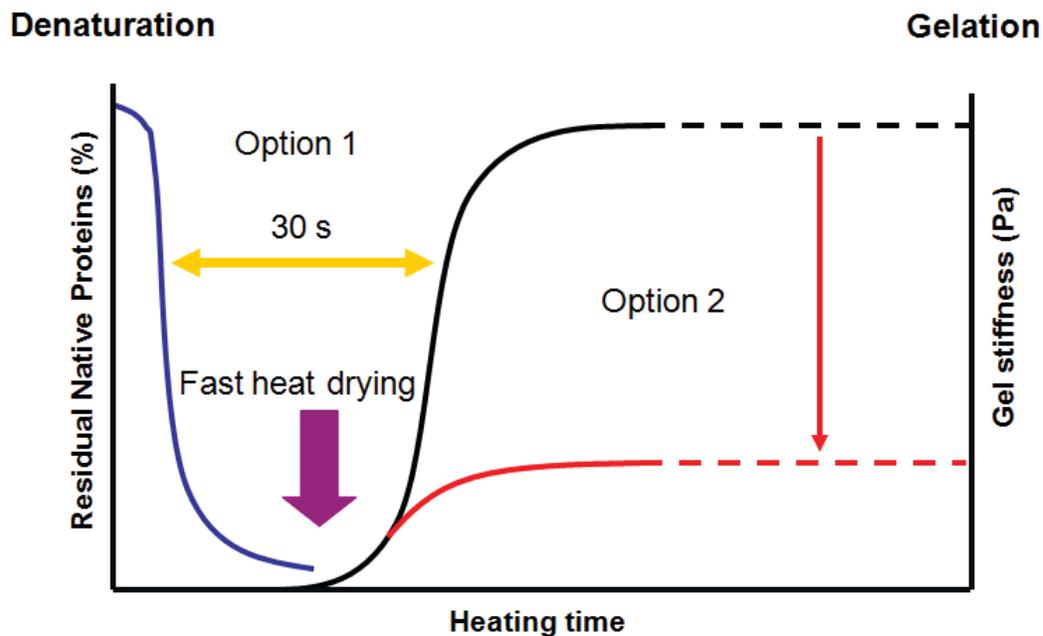
Figure 1.1: Relationship between the loss of native proteins and the gel strength during heating; —— loss of native proteins curve; —— gelation curve; —— weaker gelation curve (modified plot from McSwiney et al. (1994))

This figure highlights two possible technically feasible options for producing whey protein products that are functionally inert. The first is to quickly heat-denature whey proteins and then dry them before they start gelling as shown in option 1 (Figure 1.1).

The second is to manipulate process conditions so that the whey proteins form a weak gel that can easily be further processed as shown in option 2 (Figure 1.1), and this provides the impetus for the research presented in this thesis.

The effects of calcium on the heat-induced gelation of whey proteins have been extensively studied over the last two decades – but mainly in model systems (Hongsprabhas, Barbut, & Marangoni, 1999; Matsudomi, Rector, & Kinsella, 1991; Mercade-Prieto, Paterson, & Wilson, 2008; O'Kennedy & Mounsey, 2009; O'Kennedy, Mounsey, Murphy, Pesquera, & Mehra, 2006; Patocka & Jelen, 1991; Simons, Kosters, Visschers, & de Jongh, 2002; Xiong, Dawson, & Wan, 1993). Model systems are completely controlled in terms of whey protein composition, whey protein concentration, salt concentration, ionic strength, pH, etc. These studies were carried out with the purpose of understanding how calcium affects the functional properties of whey proteins. The work reported here focuses on understanding the effects of added calcium on the heat-induced behaviour of whey proteins as they are heated in a commercial processing environment. Can calcium be added into a feed stream of whey protein concentrate to modify the gelling behaviour of whey proteins so the process can be continued without blocking the commercial plant?

The objectives of the work were:

1) to determine the effects of added calcium on the denaturation and aggregation of heated whey proteins in three different whey protein systems;

2) to determine the effects of added calcium on the gelation of heated whey proteins from three different whey protein systems;

3) to determine the effect of the added calcium to protein concentration ratio on the kinetics of heat-induced whey protein aggregation;

4) to make recommendations on: i) the suitability of different whey protein systems for use as a feed material for making denatured whey protein products; ii) the usefulness of adding calcium as tool for making denatured whey protein products; iii) future study.

CHAPTER 2   Literature Review

2.1 Introduction

Whey is a by-product of cheese and casein manufacture and is defined as the liquid, or serum, remaining after removal of casein from milk. Because of a variety of ways to separate casein from milk, different types of whey are produced, divided into two principal ones: sweet and acid wheys. Sweet whey is produced during cheese or rennet casein manufacture, after removal of casein from milk by the addition of rennet, and acid whey during acid casein manufacture, after removal of casein from milk by acidification. There are mainly two ways for acidification, addition of mineral acid or in situ production of lactic acid by added starter bacteria. Because of various methods to obtain wheys, there is an inconsistency in product quality and composition which limits the industrial uses of whey products.

For decades, whey had been regarded as a waste product of cheese manufacture, but some studies revealed that wheys contain valuable nutrients: proteins, lactose and salts. Whey proteins especially are widely used as a food product because they are highly nutritious and possess useful functional properties. The best known and thus used in processed food are the gelling properties. Other functional properties of whey proteins that are important in the food industry are water binding, emulsification, foaming and whipping. Whey proteins are commercially available as ingredients such as whey powders, whey protein concentrates, whey protein isolates, lactalbumin and isolated whey proteins such as α-lactalbumin and β-lactoglobulin. Their applications are found in dairy products, meat products, beverages, baked products, dietary products, convenience foods, surimi and restructured seafood products, and edible films and coatings (Mulvihill & Ennis, 2003).

Under denaturing conditions, whey proteins denature, aggregate and form gels. Disulphide bonds and non-covalent interactions (hydrophobic and electrostatic interactions, and hydrogen bonds) are involved in this process. Parameters that have been found to have an influence on the interactions and thus behaviour of whey proteins, are heating temperature and time, pressure, protein concentration and purity, pH, ionic strength, minerals and other chemical denaturants. The mechanism of denaturation,

aggregation and gelation of whey proteins is still not fully understood, as well as how they are affected by the protein environment (minerals, ionic strength, pH, organic compounds, etc.). Because of their functional properties, whey proteins are widely used in the food industry and understanding how properties are linked to functionality is a real challenge. Due to their commercial importance, a large literature on the heat-induced denaturation, aggregation and gelation of whey proteins is available but the results of studies on mechanisms are still in conflict. Because of a number of different types of whey protein systems (pure protein mixtures or commercial products) and the use of different conditions and methods, meaningful comparisons are not possible (Mulvihill & Donovan, 1987; L. Sawyer, 2003a).

Understanding the linkage of whey protein properties to their functionality in food products is even more important since their high nutritional value has been emphasised. Indeed, whey proteins are now an ingredient of choice to be added to functional foods such as nutritional bars and baked goods, high protein waters and beverages and medical foods, but their functionality is often the limiting factor for their use in some functional foods. For nutritional products, whey proteins need to be pre-denatured in a way that prevents them from gelling in these types of products. There is a real demand for commercial denatured whey protein products and it is important to find solutions for the manufacture of such products.

In this review of the literature, the focus will be on the bovine whey proteins and their changes under heat treatment. Firstly, the whey proteins and the manufacture of commercial whey protein products, including their compositions, will be covered. Secondly, the heat-induced denaturation, aggregation and gelation of whey proteins in pure form in model systems and as components of commercial products will be reviewed, followed by the factors affecting these heat-induced changes. Thirdly, the different processes for manufacturing denatured whey protein products will be covered as well as the reasons why they are not always commercially feasible. And finally, there will be concluding remarks. This review covers publications up to 2007, the time at which the present study started. Current (2008-2010) literature is cited in the results and discussion sections of the relevant chapters.

2.2 Whey proteins

About 20% of the total protein of bovine milk belongs to a group of proteins generally referred to as whey or serum proteins. Basically, whey proteins are the proteins remaining soluble after isoelectric precipitation of casein at pH 4.6 at 20ºC or coagulation of casein by limited proteolysis with rennet. More properly, the group should be called non-casein nitrogen but this term also is inaccurate because acid whey contains casein-derived phosphopeptides soluble at pH 4.6 at 20°C, the proteose peptones, produced by plasmin mainly from β-casein. And cheese whey also contains (glyco)macropeptides produced by rennets from κ-casein (Fox & McSweeney, 1998).

Therefore, whey proteins are mainly four gene products: β-lactoglobulin, α-lactalbumin, bovine serum albumin, immunoglobulins. Whey proteins are globular proteins in solution and susceptible to heat denaturation. This thermolability, a consequence of the marginal stability of their three-dimensional structures, is a major functional property.

2.2.1 β-Lactoglobulin (β-Lg)

β-Lactoglobulin is a major protein in bovine milk, ~ 12% of total milk proteins, and the most abundant protein in bovine whey, ~ 50% of total whey proteins. It was among the first proteins to be crystallised and being a typical globular protein, β-lactoglobulin has been extensively studied over the years and is nowadays well characterised and understood (Fox & McSweeney, 1998; McKenzie, 1971; L. Sawyer, 2003b). It is member of the lipocalin family of proteins, so called because of their ability to bind small hydrophobic molecules into a hydrophobic cavity. Ten genetic variants have been identified in bovine milk (Eigel, Butler, Ernstrom, Farrell, Harwalkar, Jenness, et al., 1984; Farrell, Jimenez-Flores, Bleck, Brown, Butler, Creamer, et al., 2004; L. Sawyer, 2003b), the two most common A and B are occurring at an almost equal frequency.

A β-lactoglobulin monomer contains 162 amino acids, with all 20 amino acids in relative amounts that make it valuable nutritionally, and has a molecular weight of 18.3 kDa. It contains two cystine residues (two disulphide bonds) and one cysteine residue (one free thiol group) per monomer, which has important consequences for both structural and functional behaviours. The isoionic point is pH 5.2. Recent study of crystal structures show clearly that the two disulphide bridges link residues 106 – 119 and 66 – 106, respectively (Brownlow, Cabral, Cooper, Flower, Yewdall, Polikarpov, et

al., 1997; Papiz, Sawyer, Eliopoulos, North, Findlay, Sivaprasadarao, et al., 1986; Qin, Bewley, Creamer, Baker, Baker, & Jameson, 1998).

X-ray crystallography is probably the method giving the most complete structural description of a molecule. The main chain conformation (secondary structure) and configuration (tertiary structure) as well as the amino acid sequence (primary structure) and the subunit arrangement (quaternary structure) emerge from the crystallography. Due to β-lactoglobulin's importance and its propensity to form crystals, these crystals and X-ray diffraction data have been much reported (Aschaffenburg & Drewry, 1957; Aschaffenburg, Green, & Simmons, 1965; Crowfoot & Riley, 1938; Green, North, & Aschaffenburg, 1956; Palmer, 1934; Qin, Bewley, Creamer, Baker, Baker, & Jameson, 1998; Rocha, Brownlow, Saddler, Fothergill-Gilmore, & Sawyer, 1996). But β-lactoglobulin has not been the easiest protein to analyse (Green, Aschaffenburg, Camerman, Coppola, Dunnill, Simmons, et al., 1979) partly because of its multiple crystal forms. However, Brownlow, et al. (1997) provided the first high resolution demonstration of structure which provided an independent view of the dimer. β-Lactoglobulin is a highly structured protein. Optical rotary dispersion, circular dichroism, infrared, Fourier-transformation infrared and Raman spectroscopy measurements show that in the pH range 2 – 6, the monomer consists of ~ 10% α-helix, ~ 45% β-sheet and ~ 47% unordered structure (Byler & Susi, 1986; Casal, Kohler, & Mantsch, 1988; Dong, Matsuura, Allison, Chrisman, Manning, & Carpenter, 1996; Frushour & Koenig, 1975; Ruegg, Moor, & Blanc, 1975; Timasheff, Mescanti, Basch, & Townend, 1966; Timasheff, Townend, & Mescanti, 1966; Townend, Kumosinski, & Timasheff, 1967).

The tertiary structure of β-lactoglobulin is of a very compact globular form; each monomer exists almost as a sphere with a diameter of about 3.6 nm. The monomer consists of nine strands of anti-parallel β-sheet, eight of which wrap round to create a flattened conical barrel, or calyx, closed at one end by Trp19 (Figure 2.1). The β-barrel is formed by two β-sheets, where strands A to D form one sheet and strands E to H form the other (with some participation from strand A, facilitated by a 90° bend at Ser21). Two disulphide bonds link Cys66 on loop CD with Cys160 near the C-terminus, and Cys106 on strand G with Cys119 on strand H, leaving Cys121 as a free but unexposed

thiol. The ninth strand I is on the outside, on the opposite side of strand A to strand H, and so is able to form part of the dimer interface.

Figure 2.1: Schematic representation of the tertiary structure of bovine β-lactoglobulin.

β-Lactoglobulin may exist as a monomer, a dimer or an octamer depending on the conditions of the medium (pH, ionic strength and temperature). At pHs between 5.5 and 7.5, β-lactoglobulin exists mainly as a dimer of molecular mass 36 kDa, which dissociates due to strong electrostatic repulsions below pH 3.5 (Townend, Weinberger, & Timasheff, 1960). Between pH 3.5 and 5.2, especially at pH 4.6, dimers form octomers of molecular mass 144 kDa (Townend & Timasheff, 1960). Above pH 7.5, β-lactoglobulin undergoes a reversible conformational change (referred to as the N ⇆ R transition). It dissociates to monomers, and the thiol group becomes exposed and active and capable of sulphydryl-disulphide interchange. In this pH range β-lactoglobulin is unstable and forms aggregates of denatured proteins (Lyster, 1972). Fuller discussions on the association/dissociation for β-lactoglobulin can be found in Verheul, Pedersen, Roefs, and de Kruif (1999). The association/dissociation of β-lactoglobulin is summarised in Figure 2.2.

Octamer
(3.5 ≤ pH ≤ 5.5)

Dimer
(5.5 ≤ pH ≤ 7.5)

Monomer
(3.5 < pH)

Monomer
(pH > 7.5)
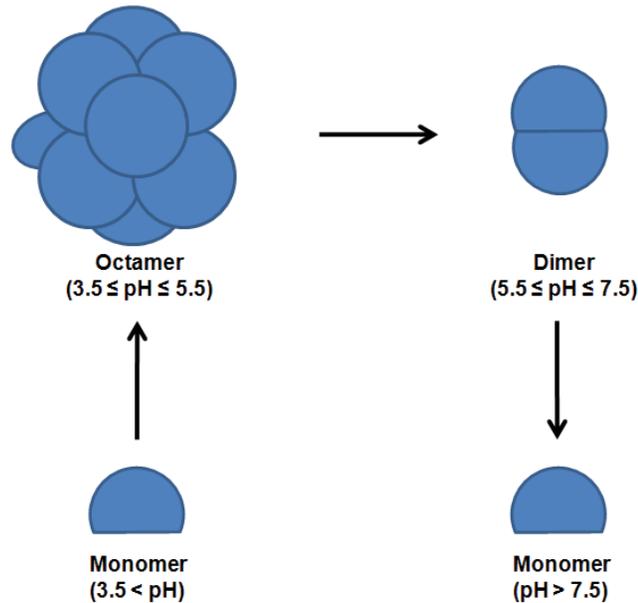
Figure 2.2: Effect of pH on the quaternary structure of β-lactoglobulin; modified from Fox and McSweeney (1998).

The biological function of β-lactoglobulin is still unknown. Its stability to acidic conditions and gastric proteolysis suggests that it is a carrier of retinol from maternal milk to the neonate via specific receptors in the intestine (Papiz, et al., 1986), and possibly facilitates vitamin A esterification. Other studies have indicated that retinol and fatty acids are bound to β-lactoglobulin in milk (Puyol, Perez, Ena, & Calvo, 1991), and that their uptake in the intestinal tract is enhanced by this association (Said, Ong, & Shingleton, 1989).

2.2.2 α-Lactalbumin (α-La)

α-Lactalbumin is the second major bovine whey protein, representing ~ 20% of total whey proteins and ~ 3.5% of total milk proteins. It is a small protein with a molecular weight of 14 kDa and three genetic variants are known, α-lactalbumin A, B and C. Interest in α-lactalbumin was enhanced in the late 1960s by the discovery of its biochemical function as a specificity regulator of lactose synthesis (Brew, Vanaman, & Hill, 1968; Brodbeck, Denton, Tanahashi, & Ebner, 1967; Brodbeck & Ebner, 1966), and the observation that it is homologous with the well-characterised C-type lysozymes (Brew, Vanaman, & Hill, 1967). It was not until 1980 that α-lactalbumin was found to be a calcium metalloprotein (Hiraoka, Segawa, Kuwajima, Sugai, & Murai, 1980) in which the calcium ion has been found to have a crucial role in folding and structure.

A α-lactalbumin monomer contains 123 amino acids. It is relatively rich in tryptophan (four residues per monomer), rich in sulphur present in form of cystine (four intramolecular disulphide bonds per monomer) and methionine. It does not contain cysteine (free sulphydryl group). Similarly to β-lactoglobulin, α-lactalbumin is also a compact globular protein, and consists of ~ 26% α-helix, ~ 14% β-structure and ~ 60% unordered structure. Crystallisation of bovine α-lactalbumin in a form suitable for X-ray crystallography has been difficult but work on the detailed structure is at an advanced stage. The tertiary structure of α-lactalbumin is very similar to that of lysozyme, as expected from their similarity in primary structure. It contains three regular α-helices, two regions of 3_{10} helix, and a small 3-stranded anti-parallel β-pleated sheet separated by irregular β-turns (Figure 2.3). The bilobal structure is formed by segregation of the α-helices in one lobe and the small β-sheet and irregular structures in the other. The cleft that separates the lobes is not open at both ends and the calcium binding elbow (residues 79 – 88) is located at the junction of the lobes. Two of the disulphide bonds, those linking Cys6 to Cys120 and Cys28 to Cys111, are located in the α-lobe, one disulphide bond is located in the β-lobe (Cys61 to Cys77), and the fourth disulphide bond is between Cys73 of the β-lobe and Cys90 of the α-lobe.
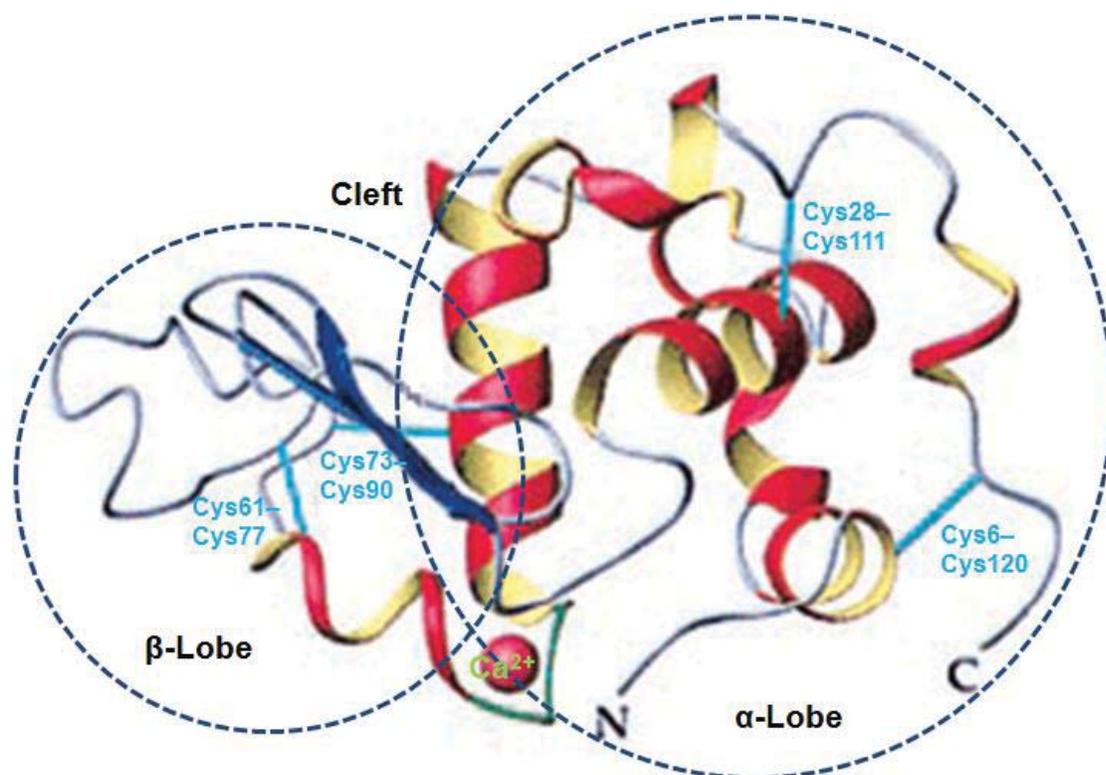
Figure 2.3: Schematic representation of the tertiary structure of bovine α-lactalbumin; modified from Fox and McSweeney (1998).

The presence of four disulphide bonds without a single sulphydryl group confers a stable conformation between pH 5.4 and 9.0. Below pH 4.8, its isoelectric point, α-lactalbumin associates quickly and in a reversible way into dimers and trimers. Above pH 9.0, conformational changes occur with a reversible aggregation (Lyster, 1972; Shukla, 1973). Under heating (80°C) at neutral pH, α-lactalbumin undergoes conformational changes without precipitation (Baer, Oroz, & Blanc, 1976). On the contrary, under more severe heating conditions (100°C) at neutral pH, disulphide bonds between α-lactalbumin molecules are formed (Chaplin & Lyster, 1986).

α-Lactalbumin shows interesting metal-binding properties, mainly of calcium but also of zinc, magnesium, lead and other metals (Veprintsev, Permyakov, Kalinichenko, & Berliner, 1996). One α-lactalbumin monomer binds one calcium ion, co-ordinated by five oxygen atoms: side chain carboxyl oxygens of Asp82, Asp87 and Asp88 and peptide carbonyl oxygens of Lys79 and Asp84. The binding site has been described as an elbow and is shown in Figure 2.4.
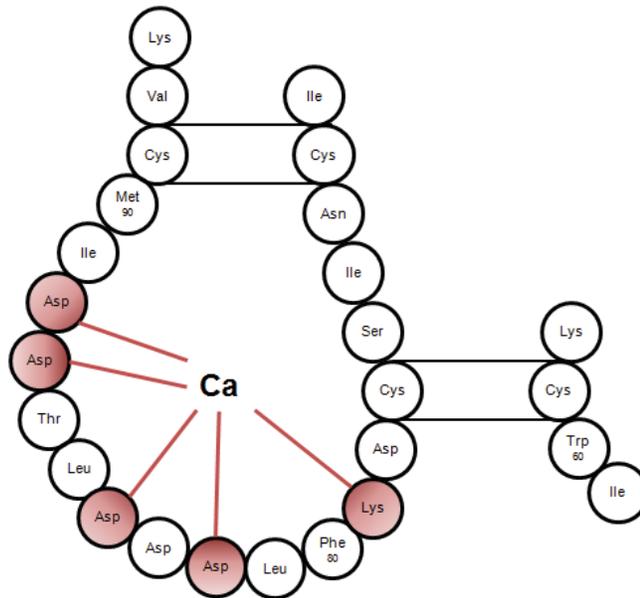
Figure 2.4: Calcium-binding loop in bovine α-lactalbumin; modified from Berliner, Meinholtz, Hirai, Musci, and Thompson (1991).

This binding of calcium by native α-lactalbumin is very strong and confers heat stability, which makes α-lactalbumin the most heat-resistant whey protein; more correctly, the protein renatures following heat denaturation (Walstra & Jenness, 1984). In the absence of a calcium ion, there is likely to be a strong charge repulsion between the carboxyl

groups in the binding site. This partly explains the stabilising effect of calcium on α-lactalbumin and the tendency of the tertiary structure of the apo-protein (the protein without Ca^{2+}) to be destabilised at low ionic strength, forming a molten globule (Kuwajima, 1996; Kuwajima, Mitani, & Sugai, 1989). The bound calcium ion has a pronounced effect on interactions at the interface of the two lobes of the protein (Chrysina, Brew, & Acharya, 2000; Forge, Wijesinha, Balbach, Brew, Robinson, Redfield, et al., 1999), which may be the basis for the large influence of calcium on the folding kinetics of α-lactalbumin. When the pH is reduced to below ~ 5, the carboxyl and carbonyl oxygens become protonated and lose their ability to bind calcium. The metal-free protein is denatured at quite low temperature and does not renature on cooling.

Most proteins exist as only two alternative states, native and unfolded due to the co-operative nature of protein folding. However, a stable partially folded state has been found by circular dichroism for α-lactalbumin, the A state (Kronman, 1989), where the secondary structure content is similar to that of the native state but the tertiary structure is not fixed. This is now recognised as a classic example of a third structural state found in some proteins under mild destabilising conditions and is called the molten globule state (Kuwajima, 1996). Depending on the conditions (typically elevated temperature, alkaline pH values and low concentrations of denaturants) the molten globule state can be more stable than the native or unfolded state. The molten globule state is of much interest with regard to folding processes, and therefore its structure and properties have been extensively investigated.

The main biological function of α-lactalbumin is its role in lactose synthesis:

$$\text{UDP-D-galactose} + \text{D-glucose} \xrightarrow{\textit{lactose synthetase}} \text{lactose} + \text{UDP}$$
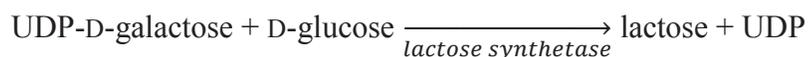
Lactose synthetase is the enzyme catalysing the final step of lactose synthesis and consists of two dissimilar protein subunits: UDP-galactosyl and α-lactalbumin. UDP-galactosyl is a non-specific galactosyl transferase but in the presence of α-lactalbumin it becomes highly specific and transfers galactose only to glucose to form lactose. The concentration of lactose in milk is directly related to the concentration of α-lactalbumin; milks of marine mammals contain no α-lactalbumin and thus no lactose (Fox &

McSweeney, 1998). Recent papers have reported α-lactalbumin anti-tumor action, and these results are discussed and reviewed by Brew (2003).

2.2.3 Bovine serum albumin (BSA)

Bovine serum albumin is identical to blood serum albumin. It constitutes the smallest proportion of the whey proteins, and is probably present as a result of leakage from blood. Bovine serum albumin is the biggest single chain whey protein, as opposed to immunoglobulins (four-chain molecules), with a molecular weight of 66 kDa. It contains 582 amino acids and 17 disulphide bonds for only one sulphydryl group per monomer (Fox & McSweeney, 1998). The cysteines containing the disulphide bonds are close together in the polypeptide chain, leading to a series of relatively short loops of different length. The molecule is elliptical in shape and is divided into three major domains, each of them dissimilar in hydrophobicity, net charge and ligand biding sites (Eigel, et al., 1984). Although bovine serum albumin is a monomer, there is a considerable micro-heterogeneity in its structure.

Various biological functions are known for bovine serum albumin in blood but they are probably of little significance in milk. However, bovine serum albumin binds metals and fatty acids.

2.2.4 Immunoglobulins (Ig)

Immunoglobulins are a complex mixture of large glycoproteins which possess antibody activity. For this very reason they are present in larger amounts in colostrum than in post-colostrum milk to protect the neonate. They are also present in blood. Four distinct classes of immunoglobulins occur in bovine milk, IgM, IgA, IgE and IgG (Eigel, et al., 1984), the last class subdivided into IgG_1 and IgG_2. They all exist as either monomers or polymers of a basic unit composed of four polypeptide chains, being two identical light chains (MW 22 kDa) and two identical heavy chains (MW 50-70 kDa) linked by disulphide bridges. Immunoglobulins are the most heat labile whey proteins.

2.2.5 Minor whey proteins

Milk contains numerous minor whey proteins, mainly transferrin and lactoferrin which are both iron-binding proteins, and several indigenous enzymes. Most of the minor proteins have biological functions and probably play significant roles.

2.2.6 Proteose peptones (PP) and non-protein nitrogen (NPN)

Proteose peptones are peptides derived from casein which remain soluble at pH 4.6 at 20°C and following heating at 95 - 100ºC for 30 min, but are insoluble in 8 - 12% trichloroacetic acid. They are fragments of β-casein from plasmin action and do not have any disulphide or sulphydryl residues. In accordance with their electrophoretic mobility, they are named PP3, PP5, PP8 slow and PP8 fast (Mulvihill & Donovan, 1987).

Non-protein nitrogen is soluble in 12% trichloroacetic acid and represents 5 – 6% of total milk nitrogen. It is a very heterogeneous fraction containing ammonia, urea creatine, creatinine, uric acid, α-amino nitrogen and some phospholipids, amino sugars, nucleotides, hippuric acid and orotic acid (Fox & McSweeney, 1998).

2.3 Manufacture of commercial whey protein products and their typical compositions

Commercial whey protein products include various whey powders, whey protein concentrates (WPC), whey protein isolates (WPI), lactalbumin and individual proteins, α-lactalbumin, β-lactoglobulin, immunoglobulins, lactoperoxidase, lactotransferrin and casein macropeptide, obtained by fractionation. Because of their high functional and nutritional value as food ingredients, WPC (up to ~ 85%, w/w protein) and WPI (at least 90%, w/w protein) are the most important whey protein products. They contain the principal whey proteins β-lactoglobulin, α-lactalbumin, bovine serum albumin and immunoglobulins, and minor whey proteins, but also lipids, lactose and minerals. Only the manufacture of WPC and WPI will be treated in this section owing to its relevance to the present study.

2.3.1 Whey protein concentrates (WPC)

Commercial WPC are manufactured by ultrafiltration/diafiltration of acid or rennet whey to remove varying amounts of lactose, and spray-drying. Ultrafiltration (UF) is a physico-chemical separation technique using an asymmetric microporous membrane to selectively separate, under mild conditions of temperature and pH, the small molecules from the large molecules contained in a solution (3A_Business_Consulting, 2008; Fox & McSweeney, 1998). A pressurised solution flows over the membrane, which allows the passage of the relatively small molecules. The solution retained by the membrane is called the retentate and the solution passing through the membrane is called the

permeate. The diameters of the pores of the membrane are chosen on the basis of the size of the molecules to be separated. In the case of the WPC manufacture, the solution is either sweet or acid whey and the retentate contains the whey proteins, fat globules and suspended solids (large molecules), while the permeate contains the minerals, lactose and water (small molecules) (Figure 2.5).
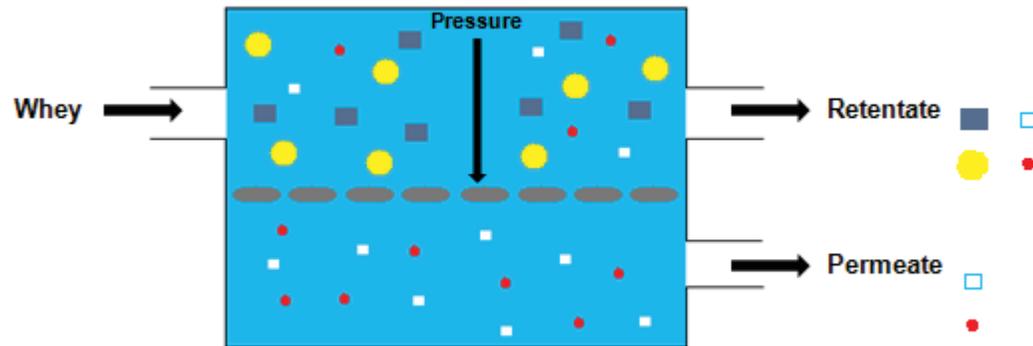
Figure 2.5: Ultrafiltration principle; ■ proteins, ⬤ fat globules and suspended solids, ☐ lactose, • minerals; modified from Fox and McSweeney (1998).

Diafiltration (DF) is the repeated ultrafiltration of a previously produced retentate diluted with water. This extra step is necessary to remove more extensively the small molecules, lactose and minerals from the retentate. In modern plants, the protein:total solids ratio limit for whey concentration by ultrafiltration is ~ 0.72:1 and total solids content limit is ~ 24%. With diafiltration, a higher protein:total solids ratio of ~ 0.80:1 and a total solids content of ~ 28% are achieved. Then the retentate is evaporated and spray-dried to produce WPC (Fox & McSweeney, 1998).

As often, there are problems associated with the manufacture of WPC including:

1) high capital and operating costs;
2) membrane fouling and concomitant loss of permeate flux rate;
3) cleaning, sanitation and related microbial problems;
4) disposal of large volumes of permeate.

Usually, whey is pre-treated before processing to dissolve colloidal calcium phosphate and/or to remove insoluble cheese curd or casein fines, milkfat and calcium lipophosphoprotein complexes. Pre-treatments involve pH and/or temperature

adjustments, addition of calcium or calcium complexing agents, and centrifugation or microfiltration. These pre-treatments are also beneficial in terms of increasing the flux rate during ultrafiltration by preventing membrane fouling, and reducing the lipid content and modifying the properties of the WPC (Mulvihill & Ennis, 2003).

Several WPC products with different protein contents, 35 to 85%, are available on the market. The most common WPC are WPC 35, WPC 60, WPC 75, WPC 80, with the number indicating the protein content. Table 2.1 shows typical compositions of commercial WPC manufactured from cheese whey.

Table 2.1: Typical compositions of commercial WPC manufactured from cheese whey; from 3A Business Consulting report (2008).

Component		WPC 35	WPC 60	WPC 75	WPC 80
Protein	**(%, w/w)**	34.0 – 36.0	60.0 – 62.0	75.0 – 78.0	80.0 – 82.0
Lactose	**(%, w/w)**	48.0 – 52.0	25.0 – 30.0	10.0 – 15.0	4.0 – 8.0
Fat	**(%, w/w)**	3.0 – 4.5	1.0 – 7.0	4.0 – 9.0	4.0 – 8.0
Ash	**(%, w/w)**	6.5 – 8.0	4.0 – 6.0	4.0 – 6.0	3.0 – 4.0
Moisture	**(%, w/w)**	3.0 – 4.5	3.0 – 5.0	3.0 – 5.0	3.5 – 4.5

Because of their different compositions, WPC products have different functional properties. WPC 35 is mainly used as a partial or full skim milk powder replacer. WPC 60 is used for protein supplementation. These products are soluble in acidic solutions and have good emulsifying and fat-binding properties. WPC 75 and 80 are also used for protein supplementation. These products are soluble and form stable colloidal dispersions at ambient temperature and neutral pH. They have similar properties to those of WPC 60 and additionally they have good water-binding, thickening and gelling properties.

2.3.2 Whey protein isolates (WPI)

Two different methods are utilised to manufacture commercial WPI. One is the microfiltration/ultrafiltration (MF/UF) process, with microfiltration being a pre-ultrafiltration membrane separation step, and the other is ion exchange (IE) chromatography, which exploits the electric properties of whey proteins. Whey proteins have a net positive surface charge at pH values lower than their isoelectric point (pH ~

4.6) and they have a net negative surface charge at pH values higher than their isoelectric point. Therefore, at pH < 4.6 whey proteins behave like cations, and at pH > 4.6 whey proteins behave like anions. WPI are manufactured by ion-exchange chromatography: proteins are adsorbed on an ion exchanger, washed free of lactose and salts and then eluted by pH adjustment. The eluate is further freed of salts by ultrafiltration and then spray-dried (Figure 2.6). Under ideal operating conditions, these adsorption processes recover ~ 85% of the proteins. Once concentrated and dried, the recovered products contain high protein and low lactose and lipid concentrations. They also have good functionality.
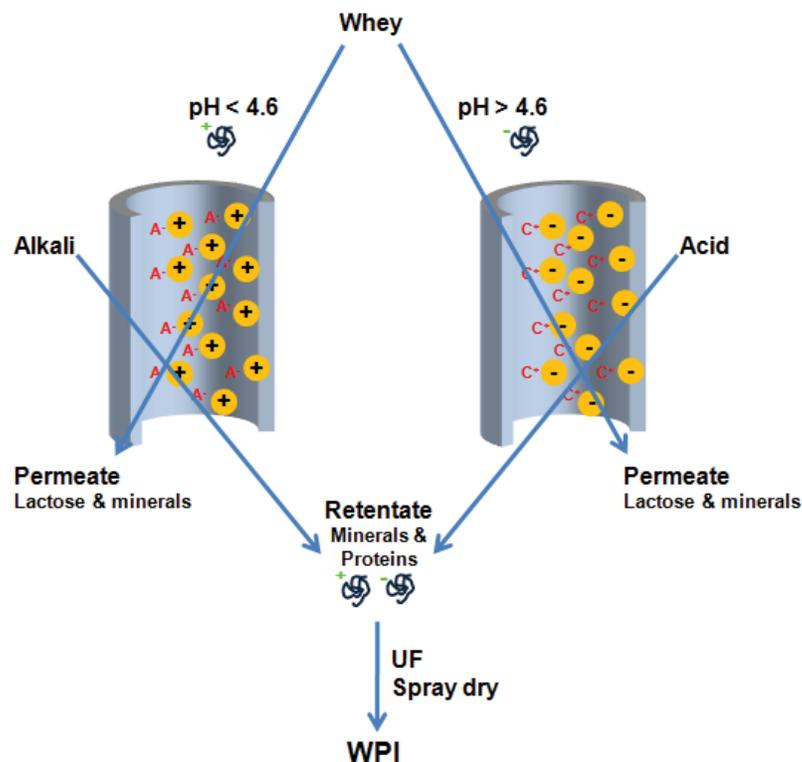
Figure 2.6: Cation and anion exchange adsorption principles; A^- anion, C^+ cation, protein with a net negative surface charge, protein with a net positive surface charge, molecule negatively charged, molecule positively charged; modified from Fox and McSweeney (1998).

The manufacture of WPI also has its problems including (Mulvihill & Ennis, 2003):

1) production of large volumes of rinse water, chemical solutions and deproteinised whey that must be processed or disposed of;

2) concentration and purification of the protein-containing retentate by UF and spray drying;

3) long time required for each fractionation cycle;

4) microbial contamination of the reactor.

Table 2.2 shows the typical composition of commercial WPI manufactured from cheese whey.

Table 2.2: Typical composition of commercial WPI manufactured from cheese whey; from 3A Business Consulting report (2008).

Component		WPI
Protein	**(%,w/w)**	90.0 – 92.0
Lactose	**(%, w/w)**	0.5 – 1.0
Fat	**(%, w/w)**	0.5 – 1.0
Ash	**(%, w/w)**	2.0 – 3.0
Moisture	**(%, w/w)**	4.5

WPI is a very concentrated source of protein and is used as protein supplement. But because its manufacture involves high costs, it is mainly used to add value to premium products, e.g. sports nutrition and beverages. The proteins have similar properties to those of WPC 75 and 80, with a major difference being the very low fat and lactose contents which results in excellent whipping properties. And the almost lactose-free composition makes it suitable for products for lactose intolerant people.

The two manufacturing methods give different protein and mineral compositions. The main differences are the glycomacropeptides and proteose peptones contents (these are absent from the ion exchange chromatography-produced WPI) and the calcium phosphate level (which is higher in microfiltration/ultrafiltration-produced WPI) (Table 2.3).

Table 2.3: Protein and mineral compositions of WPI manufactured from cheese whey; from 3A Business Consulting report (2008).

Component		MF/UF	IE chromatography
α-Lactalbumin	(%, w/w)	14.9	25.6
β-Lactoglobulin	(%, w/w)	55.7	66.0
Bovine serum albumin	(%, w/w)	1.4	5.9
Immunoglobulins	(%, w/w)	2.5	2.5
Glycomacropeptides	(%, w/w)	20.6	0.0
Lactoferrin	(%, w/w)	0.1	0.0
Proteose peptones	(%, w/w)	4.8	0.0
Calcium	(%, w/w)	0.492	0.081
Magnesium	(%, w/w)	0.059	0.010
Potassium	(%, w/w)	0.592	0.080
Phosphorous	(%, w/w)	0.242	0.025
Sodium	(%, w/w)	0.228	0.467

2.4 Thermal denaturation and aggregation of whey proteins

Literally, denaturation means "away from the native state" (Mulvihill & Donovan, 1987). But this definition is not accurate enough and Colvin (1964) defined it more precisely as "any major alteration in the original native structure without hydrolysis of primary covalent bonds [peptide bonds], changes are restricted to those occurring in secondary or higher structure". The three-dimensional structure of proteins is a resultant of disulphide bonds and various attractive and repulsive interactions of the protein chain with itself and with the surrounding solvent. These interactions are hydrogen bonding, and hydrophobic and electrostatic interactions that lead to the formation of rather labile structures. Thus, denaturation is unfolding of the compact globular protein molecule into a less organised structure. It consists of the exposure of the non-polar groups and the free thiol groups, which become available to react, the former via hydrophobic interactions, the latter via thiol oxidation and thiol/disulphide exchange reactions. This leads to the irreversible aggregation of the denatured protein molecules via non-covalent interactions and covalent bonds (Verheul & Roefs, 1998a). Some degree of conformational changes in the structure is necessary to facilitate intermolecular interactions by exposing parts of the molecule, but complete unfolding of the globular protein is not required and does not occur, except under very high heating temperature

and alkali conditions. Protein unfolding is different from, and precedes, protein aggregation (de Wit, 1981).

Denaturation of proteins can be induced by several physical and chemical agents such as heat, high pressure, salts, organic solutes and solvents (ethanol, mercaptoethanol), detergents (sodium dodecyl sulphate SDS) and extremes of pH (Mulvihill & Donovan, 1987). Heating is regarded as the most important of the chemical and physical denaturants because of its ubiquity in food processing, and thus studies of heat-induced denaturation and aggregation of whey proteins have been heavily published. From an industrial point of view, heat is a cost-effective denaturant as it is easy to implement heat treatment capability to an existing plant if not already present. Therefore, this review focuses on the thermal denaturation and aggregation of whey proteins.

2.4.1 Whey protein interactions

2.4.1.1 Sulphydryl-disulphide interchange reactions

In the native state of the proteins, hydrophobic groups, cysteine and cystine included, are buried to avoid interactions with the solvent, generally water in food processing. During food processing operations involving heating, high pressure, mechanical shear and exposure to air or oil-water interfaces, the tertiary structure of the proteins may change in a such way that free sulphydryl (cysteine) and disulphide bridges (cystine) groups are exposed to the solvent and become chemically reactive. The actual reactivity and the biological functionality of the free sulphydryl groups are precisely regulated by the tertiary folding of the protein and also delicately depend on the redox state of the environment and the protonation state of the cysteine residue. The rate of disulphide bond formation in most native proteins is low but is increased by the unfolding of the native structure caused by food processing operations.

Under heating, two possible aggregation mechanisms are possible, depending on the protein and the composition of protein mixtures. The first mechanism is the formation of intermolecular cross-links by thiol/thiol oxidation reactions and thiol/disulphide interchange, the free sulphydryl groups and disulphide bridges being made available to react by the unfolding of the native proteins. The second mechanism is based on the physical principle that an aggregation process counters the increased exposure of

hydrophobic patches, caused by unfolding, by lowering the unfavourable exposure via the formation of patch-to-patch hydrophobic bonds (see Section 2.4.1.2).

In pure protein solutions, the aggregation process can be described as a radical chain polymerization, with radical initiation, elongation and termination reactions of which the relative rates determine the average size and polydispersity of the heat-induced aggregates. In agreement with this mechanism, replacement of the single cysteine in β-lactoglobulin with a serine residue completely blocks the heat-induced irreversible aggregation (Jayat, Gaudin, Chobert, Burova, Holt, McNae, et al., 2004). In protein mixtures like whey protein isolates, the formation of disulphide bonds dominates the aggregation process, and both homo- and heteropolymers of the different proteins are usually formed.

Differences in free thiol group and disulphide group reactivity play a role in the subtle differences that exist in the ways that unfolding and aggregation of proteins during food processing. And the molecular properties of free sulphydryl groups and disulphide bonds have important consequences for the properties of food gels based on protein aggregates (Visschers & de Jongh, 2005).

2.4.1.2   Non-covalent interactions

Proteins are composed of amino acid residues, some being hydrophobic and others being negatively or positively charged. Therefore, several distinct types of non-covalent interactions are involved, including hydrogen bonding, electrostatic and hydrophobic interactions and van der Waals forces. Although these forces have been known for some time to be involved in the formation of heat-induced protein aggregates and then gels, their respective roles remain unclear (Havea, Carr, & Creamer, 2004).

2.4.2 Model systems

The mechanism of denaturation and aggregation is complex, especially in whole systems such as milk or whey protein products. Therefore, individual whey proteins and mixtures of purified proteins have been studied as "model" systems to help elucidate the likely mechanisms that might govern whey protein interactions during heating. β-Lactoglobulin especially has been studied in model systems as it is the most abundant whey protein and dominates the heat-induced behaviour of whey protein systems.

Because of the heterogeneity of whey protein systems, and because individual proteins exhibit different behaviour under heating, it is difficult to relate the findings from model systems directly to those occurring in whey protein systems where other components such as lipids, lactose, minerals are also present and affect the protein thermal behaviour. The thermal denaturation and aggregation of the total whey proteins in such systems reflects the collective response of the component proteins (de Wit & Klarenbeek, 1984). Whey protein denaturation and aggregation have been extensively studied in a wide range of whey protein systems such as milk, skim milk, and cheese whey, but for the purpose of this study the following section will focus on the thermal denaturation and aggregation of whey proteins in model systems and in the complex whey protein systems, WPI and WPC.

2.4.2.1 β-Lactoglobulin

McKenzie (1971) concluded that β-lactoglobulin denaturation and aggregation at neutral pH involved several steps with a number of intermediate species. Mulvihill and Donovan (1987) reviewed earlier work and reported the mechanism of denaturation and aggregation of β-lactoglobulin being as below:

- dimer - monomer dissociation $N_2 \leftrightarrows 2N$

- reversible denaturation $2N \leftrightarrows 2D$

- irreversible aggregation $2D \xrightarrow{type\ I} A_1 \xrightarrow{type\ II} A_n$ or $2D \xrightarrow{type\ III} A_x$

At room temperature, β-lactoglobulin in solution exists in a dynamic equilibrium between its dimeric (N_2) and monomeric ($2N$) forms. Under heating, there is dissociation of the native dimer to native monomer, then conformational changes of the native monomer ($2N \leftrightarrows 2D$), which is the denaturation step (unfolding). This step involves the exposure of apolar residues to the solvent and disruption of hydrogen and hydrophobic bonding with loss of tertiary and secondary structures (Dupont, 1965b). Unmasking of thiol groups also occurs (Mulvihill & Donovan, 1987). The denaturation is reversible under mild conditions and on restoration of the original environmental conditions.

At higher temperatures, interactions occur via a series of irreversible associations to form a polydisperse set of aggregates. Two distinct aggregations have been discovered, Type I and Type II aggregations, resulting in a series of small (A_1) and large aggregates

(A_n) (McKenzie, 1971). Type I aggregation is the tetramerization of four monomers (Pantaloni, 1964b), involving intermolecular disulphide bond formation (Sawyer, 1968). Then, the Type II aggregation is the conversion of these small aggregates (A_1) into larger aggregates (A_n), involving non-covalent bonds. It is a "non-specific" aggregation without involvement of thiol groups (Sawyer, 1968). When free thiol groups are alkylated with N-ethylmaleimide (NEM), a thiol group blocking agent, prior to heating, Type I aggregation cannot occur (and so neither can Type II aggregation), and a third type of aggregation of denatured β-lactoglobulin molecules (2D) takes place without involving disulphide bonds (A_x) (Sawyer, 1968).

However, it would be wrong to assume that the thermal denaturation and aggregation phenomena are principally the result of any one of the following: disulphide interchange reactions, hydrophobic interactions or ionic effects.

2.4.2.2   α-Lactalbumin

α-Lactalbumin does not contain a free thiol group, and when heated under mild conditions (80°C, pH ~ 6.7) α-lactalbumin does not form aggregates (Hines & Foegeding, 1993; Matsudomi, Oshita, Kobayashi, & Kinsella, 1993; Matsudomi, Oshita, Sasaki, & Kobayashi, 1992; Schokker, Singh, & Creamer, 2000). However, when heated under severe conditions (100°C, 10 – 30 min), α-lactalbumin formed polymers linked by disulphide bonds and modified monomers (Chaplin & Lyster, 1986; Hong & Creamer, 2002). Kuwajima (1996) suggested that the latter were probably in the molten globule state or in other words that the disulphide bonds were not in their native state (Hong & Creamer, 2002). Ruegg, Moor, and Blanc (1977) demonstrated by DSC that α-lactalbumin undergoes a reversible transition at 64°C.

2.4.2.3   Bovine serum albumin

Little attention has been paid to the thermal behaviour of bovine serum albumin on its own from a food science point of view, but de Wit and Klarenbeek (1984) found that under a range of heating conditions at near neutral pH, bovine serum albumin is the most heat sensitive of the whey proteins. It has been suggested that bovine serum albumin thermal interactions take place in a similar way to those previously reported for β-lactoglobulin (Gezimati, Singh, & Creamer, 1996a, 1996b). Similarly to β-lactoglobulin, bovine serum albumin contains one free thiol group, and, on this basis, similar behaviour can be expected. Electrophoretic studies showed that bovine serum

albumin aggregates were held together by disulphide bonds, and also by hydrophobic interactions. The major difference with β-lactoglobulin is that bovine serum albumin aggregation began at a lower temperature.

2.4.2.4 Mixtures of β-lactoglobulin and α-lactalbumin

When heated together, β-lactoglobulin and α-lactalbumin aggregated mainly through disulphide-linkages and to a lesser extent by non-covalent interactions (Dalgleish, Senaratne, & Francois, 1997; Gezimati, Creamer, & Singh, 1997; Havea, Singh, & Creamer, 2001; Hines & Foegeding, 1993; Matsudomi, Oshita, Kobayashi, & Kinsella, 1993; Matsudomi, Oshita, Sasaki, & Kobayashi, 1992; Schokker, Singh, & Creamer, 2000). Havea, Singh, and Creamer (2001) observed the formation of intermediate disulphide-linked homopolymers (especially dimers and trimers) of β-lactoglobulin and α-lactalbumin, and in addition Hong and Creamer (2002) found the presence of the heterodimer 1:1 α-lactalbumin – β-lactoglobulin. When heated in the presence of β-lactoglobulin, at β-lactoglobulin to α-lactalbumin ratios varying from 1:1 to 20:1, native α-lactalbumin disappeared faster than when heated alone, whereas native β-lactoglobulin disappearance was not affected by the presence of α-lactalbumin (Hines & Foegeding, 1993; Hong & Creamer, 2002; Matsudomi, Oshita, Sasaki, & Kobayashi, 1992; Schokker, Singh, & Creamer, 2000). The fact that β-lactoglobulin disappearance was not affected by α-lactalbumin indicated that the initial heat-induced intramolecular reorganisation of the β-lactoglobulin disulphide bonds happened before α-lactalbumin induced any modification to the aggregation mechanism. However, at a β-lactoglobulin to α-lactalbumin ratio of 3:1, β-lactoglobulin aggregates were markedly reduced by the presence of α-lactalbumin. Dalgleish, Senaratne, and Francois (1997) found that during the early stages of heating, the aggregates formed contained more β-lactoglobulin than α-lactalbumin, whereas in the later stages, they contained equal amounts of both proteins.

2.4.2.5 Mixtures of β-lactoglobulin and bovine serum albumin

The intermediate aggregates formed in heated mixtures of bovine serum albumin and β-lactoglobulin were mostly homopolymers of each protein (Gezimati, Singh, & Creamer, 1996a; Havea, Singh, & Creamer, 2001) probably due to the lower thermal transition temperature of bovine serum albumin (Ruegg, Moor, & Blanc, 1977), allowing the majority of the bovine serum albumin molecules to denature and aggregate in the early stage of heating. Havea, Singh, and Creamer (2001) observed very small quantities of

bovine serum albumin and β-lactoglobulin complexes after heating at 75ºC for 10 min, fine and lightly stained bands were observed in 2D PAGE gels. Gezimati, Singh, and Creamer (1996b) suggested that bovine serum albumin formed polymers prior to the unfolding of β-lactoglobulin at moderate (75ºC) heat-treatment temperatures. Hines and Foegeding (1993) found that the rate of aggregation of bovine serum albumin was much greater than that of β-lactoglobulin when the proteins were mixed in an equimolar ratio (22:78 w/w β-lactoglobulin: bovine serum albumin) in the presence of 100 mM NaCl. Under these conditions, the addition of bovine serum albumin increased the rate of aggregation of β-lactoglobulin. Kehoe, Morris, and Brodkorb (2007) also found that bovine serum albumin increased the rate of denaturation of β-lactoglobulin.

2.4.2.6   Mixtures of α-lactalbumin and bovine serum albumin

As observed with mixtures of β-lactoglobulin and bovine serum albumin, Gezimati, Singh, and Creamer (1996b) suggested that bovine serum albumin formed polymers prior to the unfolding of α-lactalbumin at moderate (75ºC) heat-treatment temperatures. In a mixed bovine serum albumin – α-lactalbumin system, they also suggested that α-lactalbumin then probably unfolded and formed some sort of adduct with the bovine serum albumin polymers. Havea, Singh, and Creamer (2000) studied bovine serum albumin – α-lactalbumin 1:1 mixtures (heated at 75ºC) and suggested a possible mechanism for the formation of α-lactalbumin polymers and hydrophobically associated α-lactalbumin.

2.4.2.7   Mixtures of β-lactoglobulin, α-lactalbumin and bovine serum albumin

Havea, Singh, and Creamer (2001) showed that when heated at 75ºC a mixture of the three major whey proteins formed various disulphide homopolymers of each protein as well as various adducts of the three proteins. Initial aggregates were formed predominantly by polymerisation of bovine serum albumin with itself while the aggregates involving β-lactoglobulin and α-lactalbumin, homopolymers and mixed aggregates, were generated at a later stage.

2.4.3 WPI

A few studies investigated the thermal denaturation and aggregation of WPI solutions using various analytical techniques: size exclusion chromatography, mass spectroscopy, gel filtration chromatography, differential scanning calorimetry, nuclear magnetic resonance, electrophoresis and dynamic light scattering (Fitzsimons, Mulvihill, & Morris, 2007; Ju, Hettiarachchy, & Kilara, 1999; Ju & Kilara, 1998b, 1998e;

Kazmierski & Corredig, 2003; Lambelet, Berrocal, & Renevey, 1992; Mahmoudi, Mehalebi, & Nicolai, 2007). All of these workers agreed that 0.5 to 10% (w/v) WPI solutions (heated at 60 to 85°C for various times) formed soluble aggregates with the disappearance of native α-lactalbumin and β-lactoglobulin. As seen for isolated β-lactoglobulin, the extent and the size of the aggregates increased with increasing WPI concentration, temperature and heating time. At low temperature, native α-lactalbumin disappeared faster than native β-lactoglobulin (Kazmierski & Corredig, 2003; Mahmoudi, Mehalebi, & Nicolai, 2007). A differential scanning calorimetric study of the denaturation and aggregation of WPI solutions (Fitzsimons, Mulvihill, & Morris, 2007) showed that the main peak of the WPI thermogram was centred at ∼ 75°C, corresponding to the β-lactoglobulin denaturation peak, with a shoulder at ∼ 62°C, corresponding to the α-lactalbumin denaturation peak.

2.4.4 WPC

Li-Chan (1983) studied the heat-induced changes in the proteins of WPC. They found that WPC solutions formed aggregates during heating and that much of the aggregation was reversible by the addition of sodium dodecyl sulphate and/or β-mercaptoethanol at moderate temperatures (60 and 73°C), implying that the aggregates were held together by hydrophobic interactions and disulphide bonds. At high temperature (95°C) and neutral pH, the solubility of WPC solutions was low, revealing the importance of other bonding forces, which could have been isoelectric aggregation and/or the formation of isopeptide bonds. Puyol, Cotter, and Mulvihill (1999) showed by differential scanning calorimetry that the pH 4.6 insoluble aggregates in WPC solutions were composed of denatured protein, whereas the proteins present in the supernatant were undenatured. Havea, Singh, Creamer, and Campanella (1998) characterised the protein interactions involved in aggregation of WPC solutions by electrophoretic analysis. WPC solutions were heated at 75°C for 6 min and 1D- and 2D-PAGE patterns showed that both hydrophobic and disulphide interactions were present in aggregates formed by α-lactalbumin, β-lactoglobulin, bovine serum albumin and other minor proteins. Several intermediates were also found, being β-lactoglobulin dimers and trimers, α-lactalbumin dimers and 1:1 β-lactoglobulin and α-lactalbumin complexes. Havea et al. also found that at low WPC concentrations, the aggregates were mainly of low molecular weight intermediate species (dimers, trimers, etc.) predominantly linked by disulphide bonds

whereas at high WPC concentrations, larger aggregates formed involving mainly non-covalent interactions (ionic bonding and hydrophobic interactions).

2.5 Kinetics of thermal denaturation and aggregation of whey proteins

Reaction kinetics is a mathematical tool which can be used to model the denaturation and aggregation of whey proteins. Studies on denaturation and aggregation kinetics show a wide variation in both the reaction order and the kinetic constants. One reason is because these studies have been carried out in a broad range of media, and have used a variety of analytical techniques, including solubility at pH 4.6, quantitative electrophoresis, differential scanning calorimetry (DSC) and reverse-phase high performance liquid chromatography (RP-HPLC). The two major whey proteins, β-lactoglobulin and α-lactalbumin, have been extensively studied, while immunoglobulin and bovine serum albumin have received only minor attention.

2.5.1 Order of reactions

The reaction order can be determined by the integral method starting from the general rate equation (Equation 2.1).

$$-dC/dt = k_n C_t^n \tag{2.1}$$

C = concentration of native protein (mol L^{-1})

t = time (s)

k_n = rate constant ((mol L^{-1})$^{(1-n)}$ s^{-1})

n = reaction order

Integration of equation 2.1 with $n = 1$ gives

$$\ln C_0 - \ln C_t = k_1 t \tag{2.2}$$

C_0 = initial concentration of native protein (mol L^{-1})

C_t = concentration of native protein (mol L^{-1}) at time t

k_1 = rate constant (s^{-1})

t = time (s)

and with $n \neq 1$ gives

$$C_t^{(1-n)} - C_0^{(1-n)}/(n-1) = k_n t \tag{2.3}$$

C_t = concentration of native protein (mol L^{-1})

n = reaction order

C_0 = initial concentration of native protein (mol L^{-1})

k_n = rate constant $((\text{mol L}^{-1})^{(1-n)} \text{s}^{-1})$

t = time (s)

The value of n is found as that value which results in the best fit of experimental concentration-time data.

There is no clear agreement on the reaction order for β-lactoglobulin denaturation: n varies between 1 and 2. There is better agreement on the denaturation of α-lactalbumin which is found to follow first order reaction kinetics. The denaturation of immunoglobulins follows a second order reaction in whole and skim milk (Resmini, Pellegrino, Hogenboom, & Andreini, 1989) but the denaturation of bovine serum albumin cannot be described by either simple first or second order kinetics (Hillier & Lyster, 1979).

Protein concentration could be important in determining the reaction order. In distilled water (Harwalkar, 1980b) and phosphate buffer (de Wit & Swinkels, 1980; Gough & Jenness, 1962) at β-lactoglobulin concentrations below 1% the order is 1. At 3.5% β-lactoglobulin, denaturation in distilled water is second order. Further concentration increases up to 24% have no effect on the order (Relkin & Launay, 1990). Park and Lund (1984) found a reaction order of 2 for 10% β-lactoglobulin in phosphate buffer. In the pH range 6.0 to 9.0, β-lactoglobulin denaturation can be described as second order and below pH 5.0 as third order.

Given the varied reported reaction orders of β-lactoglobulin, it is apparent that the mechanism of denaturation is complex. According to Arrhenius plots (see section 2.4.2.2), it seems more plausible to treat β-lactoglobulin denaturation as two consecutive reactions (Hillier & Lyster, 1979). It should be noted that kinetic equations are only a convenient means of describing a highly complex reaction. Though the reaction orders are reasonably constant, a greater variation is seen in the kinetic parameters, such as the activation energy and the rate constant.

2.5.2 Temperature dependency

Once the order is established, the kinetic parameters can be determined so that the reaction can be modelled. The Arrhenius equation is the most commonly used way of defining the temperature dependence of the rate constant k_n (Equation 2.4).

$$k_n = k_0 exp(-E_a/RT) \tag{2.4}$$

k_0 = frequency factor ($(\text{mol L}^{-1})^{(1-n)} \text{ s}^{-1}$)

E_a = activation energy (kJ mol^{-1})

R = universal gas constant ($8.314 \text{ J mol}^{-1} \text{ K}^{-1}$)

T = absolute temperature (K)

The natural logarithm of k_n plotted against the reciprocal of the absolute heating temperature yields a straight line, i.e. the Arrhenius plot. The activation energy can be calculated from the slope of the straight line. In milk, for β-lactoglobulin and α-lactalbumin, there are two distinct temperature ranges characterised by straight lines with different slopes and therefore different activation energies (Dannenberg & Kessler, 1988; Spiegel, 1999). The temperature at which the straight lines of both ranges intersect is the kinetic transition temperature (T_t), the temperature at which the reaction mechanism changes. Resmini, Pellegrino, Hogenboom, and Andreini (1989) reported no break in the Arrhenius plot for immunoglobulin denaturation in skim milk. Hillier, Lyster, and Cheeseman (1979) showed that two-fold dilution or up to three-fold concentration of the total solids content of milk and cheese whey has no effect on the kinetic transition temperature, although the reaction rates change.

Activation energies reported in the literature for the lower temperature range ($<T_t$) are typical for an unfolding reaction of globular proteins. In the upper temperature range, activation energies are typical for chemical reactions. This means that the overall reaction is determined by the protein denaturation at low temperatures, whereas the aggregation reaction is rate-limiting at high temperatures (Dannenberg & Kessler, 1988).

2.6 Thermal gelation of whey proteins

Gelation is the formation of a three-dimensional self-supporting network and in the case of whey protein gelation, it is based on protein-protein interactions. Whey protein thermal gelation happens as a continuity of whey protein thermal denaturation and aggregation when the extent of aggregation exceeds some critical level. When this critical level is not reached, a precipitate or soluble aggregates will be formed. Similarly to denaturation and aggregation, gelation is affected by the type of proteins involved, the protein concentration, temperature, pH, ionic strength and the presence of minerals and other chemical molecules such as lactose. Therefore, the structure and thus

properties of whey protein gels vary widely. If the protein-protein interactions are too weak, the viscosity will increase due to the presence of aggregates, but the protein dispersion will remain a liquid and no gel will be formed. In contrast, if protein-protein interactions are too strong, the network will collapse expelling the water from the structure, coagulation occurs. Attractive forces and repulsive forces between proteins need to be well balanced to lead to gel formation (Mangino, 1992).

The gelation mechanism has long been described as a two step mechanism, starting with unfolding of the protein molecules followed by associative reactions leading to formation of a gel network (Schmidt & Illingworth, 1978). Aguilera (1995) has suggested a more detailed mechanism for the thermal gelation of whey proteins:

- denaturation (unfolding) of native proteins $N \rightarrow D(U)$
- aggregation of unfolded molecules $D(U) \rightarrow A$
- strand formation of aggregates $A \rightarrow S$
- association of strands and network formation $S \rightarrow G$

As said previously for the denaturation and aggregation mechanism, proteins initially in their native state (N) denature (D) or unfold (U) under the action of a denaturant (heat being of greatest interest in the present study). Then the unfolded (U) molecules interact with each other and form soluble aggregates (A) which in turn keep interacting with each other to form strands of aggregates (S). Further interactions lead to the association of the strands (S) to form a gel network (G).

The gelation behaviour of commercial whey protein products tends to be rather unpredictable, due to inconsistency of product quality and composition. Further, there is a lack of understanding of the nature of the proteins in industrial whey protein products and how they behave under different heating conditions. A better understanding of this behaviour can lead to better control of the industrial processing to make whey protein products, and development of new processes, or products with predictable or tailor-made functional properties (Singh & Havea, 2003). This is why the thermal gelation of whey proteins has been studied in model systems as well as in complex whey protein systems. For the purpose of this study the following sections will focus on the thermal gelation of whey proteins in the complex whey protein systems, WPI and WPC.

2.6.1 Model systems

2.6.1.1 β-Lactoglobulin

Gelation of β-lactoglobulin has been extensively studied, as β-lactoglobulin is the major whey protein. The gelation mechanism of β-lactoglobulin has been described as being a two-step process, the first being partial unfolding of the protein molecules, which allow them to interact with each other via sulphydryl/disulphide exchange and hydrophobic interactions leading to the formation of soluble aggregates, and the second step being the association of these aggregates to form a gel network (Ferry, 1948). McSwiney, Singh, and Campanella (1994) demonstrated that before any gel network development was detected, ~ 80% of the β-lactoglobulin molecules had aggregated. Boye, Kalab, Alli, and Ma (2000) studied the microstructural properties of heat-set whey protein gels by transmission electron microscopy. When heated at 90°C for 30 min at a concentration of 20% (w/v) β-lactoglobulin formed, at pH 3, a firm and opaque gel made of compact spherical bead-like globular aggregates (~ 1 µm) that coalesced to form larger aggregates. In contrast, at pH 8.6, under the same heating conditions, β-lactoglobulin formed a firm translucent gel, consisting of small fluffy aggregates (nanometre scale) that were loosely packed into clusters linked in a chain-like pattern. Void spaces were very large (> 1 µm). Ikeda and Morris (2002) observed β-lactoglobulin aggregates by atomic force microscopy. When heated at 80°C for 60 min at pH 7, heat-denatured β-lactoglobulin formed polydisperse granular aggregates as well as secondary aggregates of these primary granules. They suggested that the primary granules could aggregate into larger clusters before being fully developed; therefore simple granule-granule aggregation might not be a good model of β-lactoglobulin aggregation and gelation mechanism.

2.6.1.2 α-lactalbumin

Hines and Foegeding (1993) found that α-lactalbumin alone was slow to aggregate and gel. Boye, Kalab, Alli, and Ma (2000) studied heat-set gels of whey proteins by transmission electron microscopy and they found that α-lactalbumin formed large visible white particulate aggregates without gelling; when heated at 90 °C for 30 min, at 20% (w/v) and pH 3, α-lactalbumin formed very large compact spherical bead-like globules (~ 4 µm) that, similarly to β-lactoglobulin, coalesced to form larger aggregates. On the contrary, at pH 8.6 (under the same heating conditions), α-lactalbumin formed a firm translucent gel consisting of two phase-separated network matrices, the main

matrix being very fine strands of very small aggregates with very small void spaces and the other matrix comprising clusters of larger aggregates.

2.6.1.3   Bovine serum albumin

Wang and Damodaran (1991) studied the thermal gelation of bovine serum albumin and the influence of the protein conformation on gel strength. When heated at 80°C for 30 min in 20 mM phosphate buffer, pH 8.0, bovine serum albumin formed stiffer gels with 0.5 M NaCl than those with no salts which latter were stiffer gels than those with 0.5 M NaClO$_4$. When they related these results with the protein conformation studied by circular dichroism, they demonstrated that β-sheet structure was involved in protein aggregation and gelation. The more β-sheet structure that was present in the protein conformation during aggregation, the stiffer the gel that formed. On the basis of infrared and Raman spectroscopy results, they suggested that the β-sheet structure might be essential for protein-protein interactions and gel network formation, with a critical minimum level of β-sheet structure of about 25%. When heated at 80°C for 30 min in 100 mM sodium phosphate buffer, pH 6.8, bovine serum albumin formed self-supporting gels at a minimum concentration of 4% (w/v) (Matsudomi, Oshita, Kobayashi, & Kinsella, 1993). In similar conditions, neither α-lactalbumin nor β-lactoglobulin can form self-supporting gels. In addition, bovine serum albumin began to form aggregates and then a gel network at lower temperature than α-lactalbumin and β-lactoglobulin did. Boye, Kalab, Alli, and Ma (2000) studied whey protein heat-set gels by transmission electron microscopy and found that bovine serum albumin formed a firm and opaque gel when heated at 90°C for 30 min, 20% (w/v) and pH 3. The gel consisted of small fluffy aggregates (nanometre scale) connected together in a chain-like filamentous shape with large void spaces (~ 0.3 μm) evenly distributed through the matrix. At pH 8.6 (under the same heating conditions), bovine serum albumin form a firm translucent gel made of very small aggregates forming a dense homogeneous network with very small void spaces (nanometre scale).

2.6.1.4   Mixtures of β-lactoglobulin and α-lactalbumin

A few studies showed that α-lactalbumin increased the rigidity of β-lactoglobulin gels (Gezimati, Creamer, & Singh, 1997; Hines & Foegeding, 1993; Matsudomi, Oshita, Sasaki, & Kobayashi, 1992) but no clear explanation on the protein interactions involved in the enhancement of gel rigidity was propounded. When α-lactalbumin was heated with β-lactoglobulin in an equimolar ratio (55:46 w/w ratio β-lactoglobulin: α-

lactalbumin) in the presence of 100 mM NaCl, both proteins had similar aggregation rates. Even though it was apparent that α-lactalbumin interacted with β-lactoglobulin, mixed gels of both proteins appeared to be rheologically similar to gels made of β-lactoglobulin alone, and initial gelation rates were similar (Hines & Foegeding, 1993). Gezimati, Singh, and Creamer (1996b) suggested that the α-lactalbumin – β-lactoglobulin interactions were more extensively disulphide cross-linkages in aggregates formed from both proteins than the β-lactoglobulin – β-lactoglobulin interactions in β-lactoglobulin aggregates alone. In addition, the aggregates in α-lactalbumin and β-lactoglobulin mixtures seemed to be linked by a larger number of disulphide bonds. Boye, Kalab, Alli, and Ma (2000) studied heat-set gels of whey protein mixtures by transmission electron microscopy. When heated at 90°C for 30 min, a 1:1 mixture of β-lactoglobulin and α-lactalbumin (20%, w/v) formed at pH 3 a firm opaque gel whereas at pH 8.6, it formed a translucent gel. The gel formed at pH 3 was made of compact spherical bead-like globules, as observed for each individual protein, with intermediate diameters of ~ 1.6 – 2 μm and that also coalesced into larger aggregates. At pH 8.6, similarly to the gel observed for α-lactalbumin alone, the mixture gel comprised two matrices, the main one being a less dense (than for α-lactalbumin alone) network of very small interlinked aggregates, and the other being clusters of larger and denser aggregates, larger and denser than for α-lactalbumin alone.

2.6.1.5   Mixtures of β-lactoglobulin and bovine serum albumin

Various studies have shown that the presence of bovine serum albumin increases the gel stiffness of heat-set β-lactoglobulin gels (Hines & Foegeding, 1993; Matsudomi, Rector, & Kinsella, 1991). Tobitani and Ross-Murphy (1997) studied the interaction between bovine serum albumin and β-lactoglobulin in heated-induced gels and found that the gelation behaviour was affected by the ratio of the two proteins to each other, with a higher proportion of bovine serum albumin accelerating the formation of the heat-set gels. The heating temperature and rate were also found to determine the ratio of β-lactoglobulin molecules to bovine serum albumin molecules in the aggregates and gels (Gezimati, Singh, & Creamer, 1996a, 1996b). When β-lactoglobulin and bovine serum albumin mixtures were heated at low temperature (≤ 70°C), less β-lactoglobulin molecules underwent thermal transition than bovine serum albumin molecules leading to bovine serum albumin being predominant in the aggregates and gels. When β-lactoglobulin and bovine serum albumin mixtures were heated at higher temperature (>

75°C), each protein aggregated, at similar rates, leading to aggregates and gels formed from comparable amounts of both proteins. Boye, Kalab, Alli, and Ma (2000) studied heat-set whey protein gels by transmission electron microscopy and found that a 1:1 mixture of β-lactoglobulin and bovine serum albumin, when heated at 90°C for 30 min at a total concentration of 20% (w/v), formed at pH 3 a firm and opaque gel made of a bi-continuous matrix. One aggregate structure consisted of compact globules (~ 50 μm) coalescing into larger aggregates (~ 1 μm). The other aggregate structure consisted of a network of smaller aggregates (~ 100 nm) and surrounded the first aggregate structure. At pH 8.6, the mixture formed a translucent gel made of a homogeneous network of very small aggregates, similar to that observed for bovine serum albumin alone, but with larger aggregates and greater porosity than for bovine serum albumin-only gels.

2.6.1.6 Mixtures of α-lactalbumin and bovine serum albumin

There have been limited studies on the effect of α-lactalbumin on the gelation of bovine serum albumin. Matsudomi, Oshita, Kobayashi, and Kinsella (1993) demonstrated that the addition of ≥ 3% α-lactalbumin to 6% bovine serum albumin solutions (heated at 80°C for 30 min in 100 mM sodium phosphate buffer, pH 6.8) enhanced the gel stiffness significantly through the formation of a finer and more uniform gel matrix. They found that during gel formation, α-lactalbumin and bovine serum albumin formed aggregates via disulphide bonds, whereas under thiol blocking conditions α-lactalbumin was not incorporated in hydrophobically associated aggregates formed by bovine serum albumin alone. Boye, Kalab, Alli, and Ma (2000) studied the microstructural properties of whey protein heat-set gels by transmission electron microscopy. They found that when heated at 90°C for 30 min, with a 20% (w/v) total protein concentration, a 1:1 mixture of α-lactalbumin and bovine serum albumin formed gels at pH 3 and pH 8.6 similar to those of the β-lactoglobulin – bovine serum albumin mixture (see Section 2.5.1.5 for description of those gels), but the clusters of aggregates at pH 3 were smaller than in the β-lactoglobulin and bovine serum albumin gel.

2.6.1.7 Mixtures of β-lactoglobulin, α-lactalbumin and bovine serum albumin

There appears to have been no investigation of the thermal gelation of mixtures of β-lactoglobulin, α-lactalbumin and bovine serum albumin in a model system reported in the literature. However, one would expect that the interactions observed in the binary mixtures discussed above would be observed in mixtures of the three proteins. On the basis of the different denaturation temperatures, bovine serum albumin would be

expected to aggregate first, followed by α-lactalbumin and β-lactoglobulin. Homopolymers and heteropolymers of all sorts would be formed but no clear understanding of mechanisms is available. Further studies in this area would be of great interest.

2.6.2 WPI

The gelation mechanism of WPI is similar to that of β-lactoglobulin: it is a two-step process (Mahmoudi, Mehalebi, & Nicolai, 2007; Vardhanabhuti & Foegeding, 1999; Verheul & Roefs, 1998a). The first step is the formation of the initial spatial structure: whey protein monomers aggregating via disulphide bonds and non-covalent interactions. The second step is the association of these aggregates via non-covalent interactions into large self-similar aggregates forming the gel network without changing the spatial structure. Vardhanabhuti and Foegeding (1999) demonstrated that the size of the initial aggregates depended on the initial protein concentration, but that the extent of aggregation in the second step depended on the heating time. Ikeda and Morris (2002) showed by atomic force microscopy that, when heated at 80°C for 60 min (at either 2 or 11%, w/w protein), the initial aggregates were granular at neutral pH which then associated into fine network strands. While investigating cold-set gelation mechanism, Ju and Kilara (1998e) found that the gel hardness of cold-set WPI gels was correlated to the size and content of soluble aggregates produced during the pre-heating stage. The more and the larger were the aggregates, the stiffest were the gels formed.

2.6.3 WPC

As WPC are made of the same material as WPI, whey proteins, the gelation mechanism is the same. However, the differences in composition of the main components lead to differences in functional properties. This is also true within the WPC product range; variations in manufacturing methods and compositions of WPC products, as well as treatment prior to gelation, lead to variations in WPC gel properties (Karleskind, Laye, Mei, & Morr, 1995; Puyol, Cotter, & Mulvihill, 1999). Several factors affect thermal changes in WPC solutions, and many studies on WPC have investigated these effects. These will be reviewed in the following section, Section 2.7. The correlation between the free thiol group content and the strength of the gels confirmed the predominant role of disulphide bonding during gelation. Strong WPC gel networks were obtained by adequate combinations of hydrophobicity, free thiol groups and ionic strength (Karleskind, Laye, Mei, & Morr, 1995). The results of Mangino, Kim, Dunkerley, and

Zadow (1987) results were in agreement with the latter; they found that the calcium content (affecting the ionic strength) and protein hydrophobicity were the two most important compositional factors affecting gel strength at low pH (pH = 6.5). On the contrary, at higher pH (pH = 8), the two most important factors affecting gel strength were the free thiol group content and the concentration of soluble protein. Other studies demonstrated that the development of gel structure and gel hardness were affected by the formation (greater at pH 4.6 than at pH 7) of soluble aggregates which subsequently became part of the gel network (Li-Chan, 1983; Puyol, Cotter, & Mulvihill, 1999). The presence of insoluble material formed under heating at pH 4.6 enhanced the gel hardness, whereas gelation at pH 7 led to weaker gels. Several workers studied the microstructure of WPC gels using light microscopy, transmission electron microscopy and scanning electron microscopy (Karleskind, Laye, Mei, & Morr, 1995; Langton & Hermansson, 1996; Puyol, Cotter, & Mulvihill, 1999). They found that stronger gels were made of a fine stranded structure formed by small aggregates evenly distributed in the matrix resulting in a uniform distribution of particles and void spaces. The visual appearance of WPC gels varies from grey translucent to white opaque depending on the degree of network crosslinking and hydration (Schmidt & Illingworth, 1978).

2.7 Factors affecting heat-induced changes in whey proteins

The susceptibility of whey proteins to heat-induced denaturation, aggregation and gelation is influenced by such factors as pH, $[Ca^{2+}]$, ionic strength, protein concentration and protein-modifying agents but the data are conflicting. The functional properties of whey protein products depends additionally on various processing treatments applied during manufacture.

2.7.1 Effects of heating temperature

Several workers demonstrated by means of various techniques that the heating temperature influenced the denaturation and aggregation mechanism. With increase in heating temperature, protein molecules underwent larger conformational changes exposing reactive groups (Bonomo, Minim, Coimbra, Fontan, da Silva, & Minim, 2006; Wada, Fujita, & Kitabatake, 2006), the protein solubility decreased (Pelegrine & Gasparetto, 2005), the aggregation rate increased (Verheul & Roefs, 1998b) and the extent of aggregation increased (Elshereef, Budman, Moresoli, & Legge, 2007). The heating temperature also affected the aggregate structure and size (Spiegel, 1999). At low heating temperature (75°C), the unfolding of the protein molecules was slow,

leading to the formation of loose aggregates with a porous structure and high serum binding capacity. As the heating temperature increased (up to 120°C), the rate of unfolding of the protein molecules increased, leading to the formation of very rigid aggregates with a compact structure and low serum binding capacity. The smallest aggregates were found at temperatures close to the kinetic transition temperature T_t (85°C) (see Section 2.5.2), and at temperatures above T_t, the aggregate size increased considerably.

Gel hardness increased with increasing heating temperature and time, and gelation time (being the time of heating required for the phase angle to drop to 45°) or gel point decreased with increasing temperature and time (Matsudomi, Rector, & Kinsella, 1991; Tang, McCarthy, & Munro, 1993; Verheul & Roefs, 1998b). However, Tang, McCarthy, and Munro (1995) found that for 25% (w/v) WPC solutions (pH 7), the gel hardness increased with increasing heating temperature up to 78°C, then decreased with further increase in heating temperature. They suggested that this decrease in gel stiffness was due to high temperature promoting protein-protein interactions at the expense of protein-solvent interactions, leading to precipitation of proteins and the formation of particulate aggregates leading to coarse and fragile gel network structures. Matsudomi, Rector, and Kinsella (1991) studied the gelation of β-lactoglobulin and bovine serum albumin and found that when heated at temperatures below their minimum denaturation temperatures, β-lactoglobulin and bovine serum albumin did not form a gel network. When 10% (w/v protein) solutions of β-lactoglobulin and bovine serum albumin were heated for 15 min between 70 and 80°C, β-lactoglobulin gels were stiffer than bovine serum albumin gels, whereas at temperatures above 85°C, the opposite was observed. They also found that when these protein solutions were heated at 90°C for variable times, below 15 min β-lactoglobulin gels were stiffer than bovine serum albumin gels, whereas above 15 min it was the opposite. Hongsprabhas and Barbut (1997a) studied the calcium-induced cold gelation of WPI and found that both gelation temperature and calcium concentrations affected the characteristics of cold set gels (pre-heating being at 80°C for 30 min, pH 7). Gelation temperature (1, 11 and 24°C) had a significant effect on opacity, force to fracture and distance to fracture. As gelation temperature increased, opacity increased, and force to fracture and distance to fracture decreased. A synergistic effect between gelation temperature and calcium concentration was also observed. The

combined effect of gelation temperature and calcium concentration on cold set gels will be reviewed in Section 2.7.4.

2.7.2 Effect of protein concentration

Hillier, Lyster, and Cheeseman (1979) assessed the effect of concentration on protein denaturation in cheese whey. They found that α-lactalbumin was more susceptible to denaturation at higher total solids levels. In contrast, β-lactoglobulin was less susceptible to denaturation with increase in total solids levels up to ~ 20% (w/v) where a maximum protective effect against denaturation was observed. Nielsen, Coulter, Morr, and Rosenau (1973) showed that minimum denaturation of total whey proteins in Colby and Cottage cheese wheys occurs at intermediate levels of total solids (~ 20%, w/v) although there was a specific protective effect against β-lactoglobulin denaturation at high levels of total solids (> 50%, w/v). These results are in agreement with Guy, Vettel, and Pallansch (1967) who observed maximum heat stability in whey concentrates containing 20% total solids. McKenna and O'sullivan (1971) reported that the extent of denaturation of whey proteins in skim milk concentrates decreased with increasing total solids and was more dependent on solids content than on temperature variation between 75 and 80ºC. However, at lower β-lactoglobulin AB concentration (0.5 to 8%, w/v) (Schokker, Singh, Pinder, & Creamer, 2000) and lower total α-lactalbumin and β-lactoglobulin concentration (0.3 to 1.6%, w/v) (Elshereef, Budman, Moresoli, & Legge, 2007), protein aggregation increased with increasing protein concentration. Results reported in literature are conflicting with regard to the effect of protein concentration on aggregate size. The latter was found not to be influenced by the protein concentration at pH 2.5 (Schokker, Singh, Pinder, & Creamer, 2000) and at neutral pH (Gimel, Durand, & Nicolai, 1994) whereas other studies at neutral pH (Hoffmann & van Mil, 1997) and at pH 6.80 and 7.95 (Boulet, Britten, & Lamarche, 2000) showed that aggregate size increased with increase in initial protein concentration.

As protein aggregation leads to protein gelation (if conditions allow gelation), protein concentration is expected to affect gelation and gel properties. Gel strength increased with protein concentration, suggesting that a minimal critical concentration is necessary for gelation (Fitzsimons, Mulvihill, & Morris, 2007; Matsudomi, Rector, & Kinsella, 1991; Tang, McCarthy, & Munro, 1993; Twomey, Keogh, Mehra, & O'Kennedy, 1997). The protein concentration also affected the gelation time (being the time of heating

required for the phase angle to drop to 45°), the latter decreasing as the protein concentration increased (Tang, McCarthy, & Munro, 1993). Hongsprabhas and Barbut (1997b) studied the calcium-induced cold gelation of WPI and found that both protein and calcium concentrations affected the characteristics of cold set gels (80°C for 30 min, pH 7). Protein concentration (6 – 10%, w/v) had a significant effect on opacity, fracture force, Young's modulus, distance to fracture and water holding capacity. As protein concentration increased, opacity decreased, fracture force, Young's modulus and distance to fracture increased, and water holding capacity increased. A synergistic effect between protein concentration and calcium concentration was also observed on opacity, fracture force and Young's modulus. The calcium concentration effect on cold set gels will be reviewed in Section 2.7.4.

2.7.3 Effect of pH

It is well known that during heating of whey protein solutions, pH has an influence on their thermal behaviour as it determines their net charge. When the pH is close to the isoelectric point of the proteins (~ 4.6 – 5.3 depending on what proteins are present), the net charge on the protein molecules is low or absent, decreasing intermolecular repulsion and allowing molecular attraction. However, weak net charge on the molecule leads to weak intramolecular repulsion, giving a conformational stability to the protein molecules and increasing their resistance to heat denaturation. Therefore, less and slower unfolding occurs and the protein molecules are less reactive in terms of the ability to form a network, but more prone to coagulation. As the pH moves away from the isoelectric pH range, the net charge increases leading to increasing repulsion of the protein molecules. Two regions are particularly interesting, where whey proteins are most and least sensitive to heat treatments, respectively. The isoelectric pH range is the most heat-sensitive region and is often used for the recovery of heat denatured whey proteins. The least heat-sensitive pH range of the whey proteins lies between pH 2.5 and 3.5, where these proteins retain their good solubility (de Wit, 1981). From a practical point of view, the pH range between 6 and 7 is more interesting because abrupt changes in the thermal behaviour and properties of whey proteins occur in this range.

The denaturation temperature of whey proteins is very dependent on pH, decreasing as pH increases (de Wit, 1981; Mulvihill & Donovan, 1987). Law and Leaver (2000) showed that either lowering the pH of milk to 5.2 or increasing it to pH 9.5 followed by

re-equilibration at pH 6.7 reduced significantly the rates of thermal denaturation of whey proteins. Similar results were found with 4.45% (w/v) WPI solutions (68.5°C) (Verheul & Roefs, 1998b) and 10% (w/) WPC solutions (80°C) (Spiegel & Huss, 2002); the rate of aggregation decreased with decreasing pH from 7.5 to 6 and from 6.7 to 4.3, respectively. In contrast, Elshereef, Budman, Moresoli, and Legge (2007) found that α-lactalbumin and β-lactoglobulin aggregated faster as pH increased from 3.7 to 5 when heated at 85°C for 90 min with a β-lactoglobulin to α-lactalbumin ratio of 3:1. WPI and β-lactoglobulin solutions (0.12% w/v, 25 to 96°C at 1.6°C min^{-1}) showed a large transition in optical density at pH 5.5, a sign of aggregation. An increase in pH to ≥ 6.5 suppressed this transition (Xiong, 1992; Xiong, Dawson, & Wan, 1993). Similarly, Patocka and Jelen (1991) showed by absorbance experiments that mixtures of α-lactalbumin and β-lactoglobulin (0.2% and 0.4%, respectively, 95°C for 30 min) were less heat stable at pH 4.5 than at pH 6 − 7. As expected, whey protein molecules were most sensitive to temperature at pHs closer to the isoelectric point.

As gelation occurs after protein denaturation and aggregation, it is also affected by the pH. At pH close to the isoelectric point of whey proteins (pH ~ 4.6 − 5.2 depending on protein composition), large attractive interactions due to low or absent net charges on the proteins led to the formation of gels with low rigidity and elasticity. Similar gel properties were found at extreme pHs, where the net charge on the proteins is very high leading to high repulsion. The maximal rigidity and elasticity were found in gels made at pH 4 and 7, where an optimal balance between attractive and repulsive forces existed (Tang, McCarthy, & Munro, 1995). Matsudomi, Rector, and Kinsella (1991) found that β-lactoglobulin and bovine serum albumin gels (10%, w/v, 90°C for 15 min) were the stiffest at pH 6.5 with a dramatic increase in gel hardness from pH 6. Below pH 6, the gels hardness was very low. Above pH 6.5, gel hardness decreased with further increase in pH. Langton and Hermansson (1992) studied the microstructure of β-lactoglobulin and WPC gels at varying pHs and showed that β-lactoglobulin formed particulate gels between pH 4 and 6, and fine-stranded gels below pH 4 and above pH 6. WPC formed similar gels at comparable pH, although the region of particulate gels was broader. Interestingly, Tang, McCarthy, and Munro (1993) found that the gelation time (being the time of heating required for the phase angle to drop to 45°) was lowest between pH 4 and 6.5, coinciding with the particulate gels, whereas outside this range, the gelation

time increased as pH moved away from this range, coinciding with fine-stranded gels. Similar results showed that at pH 5.7 to 6.9, the gelation time decreased with decreasing pH (Tobitani & Ross-Murphy, 1997). Tobitani and Ross-Murphy suggested that this decrease in gelation time as the pH decreased towards the isoelectric point was due to the lower net charges on the proteins allowing greater attraction and thus interactions leading to formation of a gel. In contrast, Verheul and Roefs (1998b) found that the gel point (being the time after which G' > 1 Pa) decreased with increasing pH (from 6.3 to 7.5).

2.7.4 Effect of minerals and ionic strength

The susceptibility of whey proteins to denaturation is largely determined by the pH of the solution, but the extent of aggregation seems to be dependent on the presence of minerals (de Wit, 1981). Havea, Singh, and Creamer (2002) compared the heat-induced aggregation of CWPC and AWPC solutions and showed that their thermal behaviour was very different. However, after AWPC and CWPC solutions were dialysed against each other, the thermal behaviour of AWPC changed and moved towards that of CWPC, and vice versa, demonstrating the relevance of mineral composition. Modification of the mineral composition by the removal or addition of minerals (or salts) to whey protein solutions implies variation in the ionic strength of the solutions. Therefore the effect of ionic strength has mainly been studied via the addition of minerals, sodium chloride and calcium chloride being the most popular salts. Similarly to the pH, the ionic strength influences the protein aggregation via two opposite effects. Increasing the ionic strength reduces the intermolecular repulsion between protein molecules by screening the charged groups, leading to protein aggregation. However, the screening of the charged groups by increasing the ionic strength also reduces the intramolecular repulsion, giving a conformational stability to the protein molecules which then become less sensitive to heat denaturation, slowing down protein aggregation. Another effect of adding salts to protein solutions is the dehydration of the protein molecules by the hydration of salts surrounding the protein molecules. In other words, water molecules are moving away from the protein molecules (dehydration) to interact with the salts (hydration). Therefore, the hydrophobic residues of the protein molecules will be gradually exposed with increasing salt concentration, enhancing hydrophobic interactions between protein molecules (Bonomo, Minim, Coimbra, Fontan, da Silva, & Minim, 2006; Tobitani & Ross-Murphy, 1997).

Several studies showed that increasing the ionic strength by increasing levels of added sodium chloride (0 – 200 mM) to β-lactoglobulin (2%, w/v) (Schokker, Singh, Pinder, & Creamer, 2000) and WPI solutions (1 – 3%, w/w) (Fitzsimons, Mulvihill, & Morris, 2007; Schmitt, Bovay, Rouvet, Shojaei-Rami, & Kolodziejczyk, 2007) increased the rate of aggregation and decreased the aggregation temperature. Not only increase in ionic strength but also the mineral species influences the heat-induced denaturation and aggregation of whey proteins. Sodium sulphate was found, by optical density measurements, to greatly suppress protein association (WPI, 0.12% w/v) whereas increasing sodium chloride (0 – 600 mM) and calcium chloride (0 – 50 mM) levels decreased the aggregation temperature to a minimum (74.7 and 70.8°C, respectively, 0.12%, w/v WPI, pH 6), which then increased with further increase in sodium or calcium chloride levels. In the case of β-lactoglobulin solutions (0.12% w/v, pH 6), increasing sodium chloride levels (0 – 1 M) increased the aggregation temperature (83 to 93.3°C), and increasing calcium chloride levels (0 – 200 mM) first decreased the aggregation temperature to 75°C and then increased it with further increase in calcium chloride levels (Xiong, 1992; Xiong, Dawson, & Wan, 1993). Calcium chloride was more effective than sodium chloride and magnesium chloride at enhancing whey protein thermal aggregation (Roefs & Peppelman, 2001). In contrast, α-lactalbumin alone was stabilised in its native form by both sodium and calcium ions (Relkin, Launay, & Eynard, 1993).

Removal of calcium from retentate mixtures of α-lactalbumin and β-lactoglobulin (Patocka & Jelen, 1991) and WPC (Spiegel & Huss, 2002) enhanced the heat stability of the proteins. In contrast, increase in calcium concentrations (0 – 9 mM) increased the insoluble precipitate in WPC solutions (12% w/w, pH 6.6, 71°C for up to 120 min) (Parris, Hollar, Hsieh, & Cockley, 1997). Ju and Kilara (1998a) suggested that pH-induced whey protein aggregation was a rapid process and dissimilar to calcium-induced whey protein aggregation (Ju & Kilara, 1998c; Zhu & Damodaran, 1994b). Three effects, probably in combination, have been suggested to explain calcium-induced aggregation: i) intermolecular cross-linking of adjacent anionic molecules by forming protein-Ca^{2+}-protein complexes, also called calcium bridges, ii) inter- and intramolecular electrostatic screening of negative charges on the protein, and/or iii) conformational changes due to ion binding and moderation of hydrophobic interactions

(Kinsella, Whitehead, Brady, & Bringe, 1989; Wang & Damodaran, 1991). Results obtained by Simons, Kosters, Visschers, and de Jongh (2002) demonstrated that calcium had a specific role in the aggregation process by screening the negative surface charges, rather than bridging two protein molecules, with an optimal amount of calcium ions per protein giving the fastest aggregation rate. Sherwin and Foegeding (1997) found that a maximum rate of whey protein aggregation existed at $CaCl_2$/protein (mM/%, w/v) ratios between 3.33 and 23.3. The presence of excess calcium in some way inhibits aggregation either by a singular mechanism or by the advent of a competing mechanism above some critical calcium-protein ratio. Ju and Kilara (1998a) confirmed the importance of the calcium-protein weight ratio; there is an optimal extent of aggregation at 6.7×10^{-3} w/w, which may be explained by the fact that the amount of Ca^{2+} that binds to β-lactoglobulin is stoichiometrically equivalent to the net charge of the protein (Zittle, Dellamonica, Rudd, & Custer, 1957).

As a consequence of protein aggregation, gelation is also influenced by this ratio, with the hardest gels obtained at a ratio of 3.3×10^{-3} w/w, slightly different from the optimal aggregation ratio owing to the importance of aggregate size in the gelation process (Ju & Kilara, 1998a). A study of β-lactoglobulin, WPC and WPI gels by Twomey, Keogh, Mehra, and O'Kennedy (1997) revealed the importance of ion levels and pH, as well as the protein concentration and the protein type, on gel characteristics. The type of gel and the ability to form a gel under heating depends on the balance between attractive and repulsive forces which in turn depends on pH, and salt concentration and salt type in the protein solution (Mulvihill & Kinsella, 1988). When heated at 80°C for 30 min, WPI gel hardness measured as storage modulus (3% w/w, pH 7) (Fitzsimons, Mulvihill, & Morris, 2007) and fracture stress (10% w/w, pH 8) (Glibowski, Mleko, Wasko, & Kristinsson, 2006) increased with increasing levels of sodium chloride (0 – 100 mM and 90 – 270 mM, respectively). The effect of sodium chloride can also be visually perceived; β-lactoglobulin gels (2.5 – 12% w/v, pH 7, 80°C for 24 h) and WPI gels (10% w/v, pH 7, 80°C for 30 min) varied from transparent at a sodium level of 50 mM to opaque at 200 mM due to increasing heterogeneity of the gels (Barbut, 1995a; Pouzot, Durand, & Nicolai, 2004).

Several studies compared the effect of sodium and calcium on gelation properties. Both calcium and sodium lowered the gel point of WPC gels (7.9 – 15% w/w, 80°C) and increased the gel stiffness to a maximum, probably by screening the repulsive negative charges, allowing protein-protein attractive association and gelation. However, further increasing the ionic strength (sodium and calcium levels) resulted in a decrease in gel stiffness, probably due to excessive attractive interactions allowing random aggregation, leading to the fall of gel stiffness. In addition, calcium had a greater effect than sodium, exerting its effect at much lower ionic strength (Tang, McCarthy, & Munro, 1993, 1995). Other workers investigated the effect of sodium and calcium on gel fracture properties at failure (Kuhn & Foegeding, 1991a; Mulvihill & Kinsella, 1988). They also found that calcium had a dramatic effect on gel strength compared with sodium, explained by a greater binding affinity of calcium to β-lactoglobulin. Roefs and Peppelman (2001) observed that the minimum concentration for WPI (5% w/w, pH 6.8) gel formation was lower for calcium gels (5 to 6 mM) than for sodium gels (30 to 35 mM). In addition, both calcium and sodium gels were coarse gels, but calcium gels appeared coarser than sodium gels, reflecting a different type of aggregation.

The texture of WPI (6 – 8% w/w) was markedly influenced by calcium chloride concentration (0 – 30 mM), with the stress at fracture increasing with increasing calcium concentration up to 15 mM, and then decreasing with further increase in calcium concentration (Glibowski, Mleko, & Wesolowska-Trojanowska, 2006). They suggested that at low calcium concentrations, the network was predominantly formed by charge sheilding and low calcium cross-linking, whereas at high calcium concentrations, calcium cross-linking became the predominant mechanism. Matsudomi, Rector, and Kinsella (1991) observed a similar effect of the addition of calcium. Up to a critical calcium concentration (2 mM for β-lactoglobulin, 5 mM for bovine serum albumin, 10% w/v protein, pH 8, 90°C for 15 min), β-lactoglobulin and bovine serum albumin gel hardness increased to maxima, then decreased with further increase in calcium concentration. Below this critical concentration, they suggested that calcium ions enhanced the protein-protein interactions by bridging two negatively charged protein molecules leading to stiffer gel matrix. However, above this critical concentration, excessive bridging led to collapse of the matrix, forming a coagulum. They also found that calcium was much more effective than sodium in enhancing gel stiffness.

Barbut and co-workers extensively studied the calcium-induced cold gelation of WPI solutions (Barbut, 1995b; Barbut & Foegeding, 1993; Hongsprabhas & Barbut, 1996, 1997a, 1997b, 1997c, 1997d, 1998; Hongsprabhas, Barbut, & Marangoni, 1999). They demonstrated that preheated WPI solutions would form gels at room temperature on the addition of calcium chloride. These gels were very different from thermally induced gels, with a fine stranded microstructure versus a particulate microstructure. At low calcium concentration, the structure was finer and more transparent. As the calcium concentration increased, the protein strand thickness increased till forming bead-like structures. They demonstrated that the gelation mechanism was dependent on the calcium concentration. The electrostatic forces appeared to be the primary forces in gel network formation, at the expense of disulphide bonds which were mainly involved in the pre-polymerisation of the protein and which helped to maintain the network structure. At low calcium levels, a cluster-cluster interaction mechanism governed the gelation process, and at high calcium concentration the growth of bead-like structures to fill up voids governed the gelation process. Finally, temperature also played a role in the properties of calcium-induced cold-set gels. Higher gel strength and water holding capacity could be obtained by increasing the pre-heating temperature, whereas increasing the gelation temperature decreased the gel strength and water holding capacity. And re-heating affected the WPI cold-set gel properties. To summarise, manipulating the temperature and calcium concentration of cold-set gels may be of great interest in designing gels with tailor-made properties by manipulating the microstructure and functional properties of the gels.

2.8 Manufacture of heat-denatured whey protein products

The most recent developments in whey protein manufacture have been driven by the market demand for cheaper, more nutritionally valuable proteins. Since the early 1980s, the world consumer market has shifted more towards health consciousness, demanding low calorie, low sugar, low fat, and/or low cholesterol products and giving rise to a higher demand for "health" proteins, especially whey proteins (UBIC_Consulting, 2004). Whey proteins are regarded as having high nutritional value, next to that of egg proteins. They are also known to be easily digested and to have excellent metabolic value (i.e. a high biological value) (Barth & Behnke, 1997). They have high absorbability by the digestive system and are an attractive protein source in the sports world (Sinha, Radha, Prakash, & Kaul, 2007). Whey proteins are also believed to have a

positive effect with respect to cancer prevention (Kent, Harper, & Bomser, 2003), and have been linked to enhancement of the nervous and immune systems (Boehm, Cervantes, Georgi, Jelinek, Sawatzki, Wermuth, et al., 1998; Tseng, Lin, Hsiao, Chen, Lee, Wu, et al., 2006).

These benefits have led to food manufacturers looking at ways of adding more whey proteins to their food products. Research findings of the past two decades have led to diversification of the functional properties of whey proteins, and the invention of various manufacturing processes for novel products that are suitable for a wider range of applications than traditional uses of standard whey products such as WPI and WPCs. The most significant development in whey protein manufacture is the introduction of various heat-denatured whey protein products.

The manufacture of denatured whey protein products requires means of heat denaturing the proteins in a way that retards or eliminates gel formation. The most common method for making this range of products is the heat treatment of a whey protein solution to denature the proteins under high shear rate conditions prior to concentrating (e.g. evaporation) and drying. The most common denatured whey protein is lactalbumin, with > 90 g protein 100 g^{-1}, on the market for more than 20 years (Robinson, Short, & Marshall, 1976). The heated protein is fully denatured and lacks the ability to form gels, but the process of making lactalbumin is cumbersome. An alternative, microparticulation process has been used for the manufacture of denatured whey proteins. It involves heating a whey protein concentrate under high shear conditions, the heat inducing protein denaturation and aggregation and the shear breaking up the aggregated protein to obtain fine particles. The microparticulation conditions must be well controlled to restrain the denatured whey proteins to a certain maximum degree of aggregation, and to control the particle size to specific ranges. The properties of the aggregates formed result from a dynamic balance between shear-controlled aggregate growth and shear-controlled aggregate break-up (Steventon, Donald, & Gladden, 1994).

Several microparticulation processes have been used for the manufacture of denatured whey proteins. One process is the heating of whey proteins under high shear in a special shear device where heat and mechanical action are combined (Singer, Yamamoto, & Latella, 1988). Other processes include the use of a scraped surface heat exchanger

(Thorsen, Koeningsfeldt, & Poulsen, 2007), homogenisation of a heated whey protein solution (Holst, Christensen, Albertsen, Jensen, Pedersen, Thomsen, et al., 1996; Kruesemann, Nordanger, & Krusemann, 2008; Oudeman, 1995; Paquin, Lebeuf, Richard, & Kalab, 1993; Wolfschoon-Pompo, Rose, Habermeier, Muxfeldt, & Eibel, 2006), hydrolysation (Hudson, Daubert, & Foegeding, 2001), and extrusion (Merrill, Blanton, Jaskulka, & Singh, 2006; Queguiner, Dumay, Saloucavalier, & Cheftel, 1992). All of these processes involve high shear action which is not cost-effective on a commercial scale, and thus the processes are not commercially feasible. In two other patents, the inventors disclosed low shear (resulting only from the pumping and stirring operations) processes (Huss & Spiegel, 2004; Visser & Bakker, 1994), but these processes involve low protein concentration, again making the processes non-cost-effective and thus not commercially feasible.

2.9 Concluding remarks

So far, many studies have been done on whey protein denaturation, aggregation and gelation. But there is still a lot to be understood because of the complexity of whey protein products. Most of the research has been carried out with model systems, and does not relate well to real systems in which all proteins are present and are mixed with other components of the whey protein products. As all the findings apply to low protein concentration systems (maximum 12 – 15%) and reveal an effect of protein concentration, they are inapplicable to high protein concentration systems (~ 30%). More studies in this field need to be done, especially where the objective is the improvement of industrial production.

A careful look at the data available in the literature reveals a lack of understanding of how protein interactions during manufacture affect final product properties. The native states of the whey proteins are well known, and gel formation under different sets of conditions has been well described, but there is a lack of understanding in the literature of how the protein-protein interactions during whey protein denaturation and aggregation affect the functionality of final products. An understanding of the mechanisms will allow manipulation of processes to obtain suitable whey protein products with specific desired functional properties.

CHAPTER 3   Effect of Added Calcium on the Heat-induced Denaturation and Aggregation of Proteins in Three Different Whey Protein Products: Study and Comparison

3.1 Introduction

Under heating, whey proteins denature, aggregate and then form gels. It is now well known that mineral ions (Ca^{2+}, Mg^{2+}, Na^{+}, Cl^{-}, PO_4^{3-}, etc.), ionic strength and pH affect the heat-induced denaturation, aggregation and gelation of whey proteins. The effect of calcium in particular has been studied extensively. At low concentrations, calcium enhances the heat-denaturation and aggregation of whey proteins but at high concentrations, the rate of denaturation and aggregation decreases. Both the concentration and the equilibrium state of calcium (i.e. ionic, soluble or colloidal) are important factors (Parris, Hollar, Hsieh, & Cockley, 1997). However, to date no clear and definite explanation has been given about how the minerals, calcium in particular, influence denaturation, aggregation and gelation of whey proteins. Moreover, studies are still conflicting about these effects due to the number of different types of whey protein systems examined and the different methods of analysing them (Mulvihill & Donovan, 1987). Because of their properties, whey proteins are much used in the food industry and understanding how properties are linked to functionality is an important challenge.

Sherwin and Foegeding (1997) found that the extent of denaturation and aggregation of whey proteins is dependent on the $CaCl_2$/protein ratio. They reported that the rate of aggregation at 40 and 50°C was maximal between 3.33 and 23.3 $CaCl_2$/protein (mM/%, w/v). This is an important step towards understanding the mechanism of the effect of calcium on the heat-induced denaturation and aggregation of whey proteins.

In this study, the effects of added calcium on the heat-denaturation and aggregation of whey proteins are reported for three different systems, whey protein isolate (WPI), acid whey protein concentrate (AWPC) and cheese whey protein concentrate (CWPC) under the same heat treatment. The results obtained were then compared with regard to the initial mineral composition. Denaturation and aggregation were analysed by several

methods, which are described in Section 3.2. Gelation properties are described in Chapter 4.

3.2 Materials and Methods

3.2.1 Composition of whey protein powders

The total protein content of the whey protein powders was determined using the Kjeldahl method (Williams, 1984) with a nitrogen conversion factor of 6.38. The fat content was determined using the Soxhlet extraction method, as described by Russell et al. (1980). The moisture content was determined by oven drying preweighed duplicate samples at 102°C for 5 h, cooling in a desiccator for 2 h, and reweighing. The lactose content was as reported by the manufacturer. The mineral analyses were carried out at the New Zealand Pastoral Agricultural Research Laboratory, Palmerston North, by inductively coupled argon-plasma emission spectrometry using the method described by Lee et al. (1986). All powders were provided by the Fonterra Co-operative Group Ltd, New Zealand and their composition is shown in Table 3.1.

Table 3.1: Compositions of the three whey protein products used in this study.

Component		WPI	AWPC	CWPC
Protein	%, w/w	93.1	80.4	83.0
Fat	%, w/w	0.45	5.43	5.90
Ash	%, w/w	1.62	3.81	2.78
Lactose	%, w/w	0.18	4.08	3.89
Moisture	%, w/w	4.93	4.96	5.28
Minerals	mmol kg^{-1} protein			
	Ca	18.775	74.375	111.25
	K	13.308	471.15	175.64
	Mg	2.4167	7.6667	27.875
	Na	259.43	14.043	147.30

3.2.2 Preparation of the whey protein solutions

Whey protein solutions were prepared by reconstituting appropriate quantities of whey protein powders (WPI, AWPC or CWPC) in milli-Q water so that the protein concentration was > 4% (w/w). Appropriate volumes of a 2 M calcium chloride ($CaCl_2$) or 2 M sodium chloride (NaCl) solution were added to give a range of different added calcium or sodium concentrations (see just below). Then, each solution was topped up

with milli-Q water so that the final protein concentration was 4% (w/w). The different ranges of final ion concentrations prepared depended on the analysis technique used:

- for polyacrylamide gel electrophoresis (PAGE) and reverse-phase high performance liquid chromatography (RP-HPLC), final added calcium concentrations were 0, 2, 4, 6, 8, 11, 14, 17, 20, 50, 80, 110, 140, 170, 200 and 230 mM, and final added sodium concentrations were 0, 6, 12, 18, 24, 33, 42, 51, 60, 150, 240, 330, 420, 600 and 690 mM;

- for circular dichroism (CD), final added calcium concentrations were 0, 4, 10, 20, 80, 140 and 200 mM;

- for 2D nuclear magnetic resonance (NMR), final added calcium concentrations were 0, 10, 20, 80, 140 and 200 mM;

- for differential scanning calorimetry (DSC), final added calcium concentrations were 0, 4, 6, 20, 50, 80, 140 and 200 mM.

When 0 to 230 mM calcium chloride was added to 4% (w/w protein) whey protein solutions the pH decreased. To investigate the effect of pH shifting due to added calcium chloride on whey protein aggregation, three sets of samples (4%, w/w protein) were prepared:

- samples with added calcium chloride but without pH adjustment;

- samples with added calcium chloride but with the pH re-adjusted to the original pH of the solutions without added calcium chloride by the addition of 1 M NaOH;

- samples without added calcium chloride but with the pH adjusted to the corresponding pHs of the samples with added calcium chloride using 1 M HCl.

Initially, the range of calcium concentration was chosen to be broad, 0 to 230 mM, with many intermediate points, to allow a good understanding of the effects of calcium on the whey protein behaviour under heat treatment. PAGE was the first analysis technique used, and applied to the whole range of calcium concentrations with all intermediate points. From the PAGE results, certain key intermediate calcium concentrations were selected to be analysed with the other techniques, while keeping some consistency to be able to correlate the results.

3.2.3 Heat treatment of the whey protein solutions

Aliquots (3 mL for PAGE and RP-HPLC or 5 mL for CD and 2D NMR) of each whey protein solution were heated, in glass test tubes (7.5 cm in length, 1.2 cm in outside diameter and 1.0 cm in inside diameter), at a given temperature for a given time (depending on the experiment) in a water bath. The samples were then immediately immersed in an ice-water bath (~ 0ºC) for 1 h to stop protein denaturation. The control was the unheated sample without any salt added.

The samples were then analysed by polyacrylamide gel electrophoresis (PAGE), reverse-phase high performance liquid chromatography (RP-HPLC), circular dichroism (CD), and 2D nuclear magnetic resonance (2D NMR).

The samples analysed by differential scanning calorimetry (DSC) were not heat-treated before analysis. The heat treatment was done during the analysis (see section 3.2.8).

3.2.4 PolyAcrylamide Gel Electrophoresis (PAGE)

After heat-treatment, a 50 μL sample of the control (unheated and with no added salt) and heat-treated solutions were mixed with 1 mL native or SDS (dissociating agent) sample buffer. The native sample buffer contained 0.03% (w/v) of bromophenol blue dissolved in 0.5 M Tris-HCl buffer. The SDS sample buffer contained 0.01% (w/v) of bromophenol blue and 2% (w/v) of SDS dissolved in 0.5 M Tris-HCl buffer. The control and heat-treated solutions were analysed using a Mini-Protean II dual cell system (Bio-Rad Laboratories, Richmond, CA, USA) and the PAGE system for PAGE. The native resolving gel contained 12.5% (w/v) acrylamide dissolved in 1.5 M Tris-HCl buffer, pH 8.8, and the native stacking gel was composed of 4% (v/v) acrylamide made up in 0.5 M Tris-HCl buffer, pH 6.8. The SDS resolving and stacking gels were composed of the same chemicals as the native resolving and stacking gels, respectively, but small quantities (a few μL) of a 10% (w/v) SDS solution were added. 10 μL of the samples mixed with the sample buffer were injected into the wells of the gels.

The gels were run at 200 V and 70 mA for approximately 1 h 15 min (until the tracking dye seeped out of the bottom of the gel) and then stained with Coomassie Brilliant Blue (R-250) in 25% (v/v) acetic acid for 1 h. This was followed by two destaining steps using a 10% (v/v) acetic acid/2-propanol solution for a total of 20 h. Immediately after

destaining, the gels were scanned using a computing laser densitometer (Molecular Dynamics model P.D., Sunnyvale, CA, USA) and the integrated intensities of the α-lactalbumin, β-lactoglobulin and bovine serum albumin bands were determined using the Molecular Dynamics ImageQuant software (Anema & McKenna, 1996).

3.2.5 Reverse-Phase High Performance Liquid Chromatography (RP-HPLC)

After heat treatment, the control (unheated and with no added salt) and heat-treated samples were centrifuged at 16,000 g for 3 minutes to remove any insoluble materials. The supernatants were diluted in milli Q water to make 1% (w/w) protein concentration solutions and analysed by RP-HPLC. The RP-HPLC system consisted of a Waters 2690 Alliance Separation Module (Waters, Milford, MA, USA) interfaced with a Waters 486 MS tunable absorbance detector and a Waters Millenium 32 data acquisition and manipulation system. Queued samples were refrigerated at 5ºC.

Acetonitrile (MeCN; far UV grade) and trifluoroacetic acid (TFA; HPLC grade) were from BDH (Poole, UK). Q Sepharose Fast Flow and Sephadex G-75 were from Pharmacia Biotech (Uppsala, Sweden). All other buffers and reagents were analytical grade or better. Aqueous buffers (eluents) were filtered through 0.45 μm cellulose acetate membranes (Millipore, Bedford, MA, USA) and degassed prior to use.

A 1 mL Resource RPC column (Pharmacia Biotech) was operated at room temperature and at a flow-rate of 1 mL min^{-1}. The column was equilibrated in 80% solvent A (0.1%, v/v, TFA in milli-Q water) and after sample injection a 1 min isocratic period was applied followed by a series of linear gradients to 100% solvent B (0.09%, v/v, TFA, 90%, v/v, MeCN in milli-Q water) (Elgar, Norris, Ayers, Pritchard, Otter, & Palmano, 2000).

3.2.6 Circular Dichroism (CD)

After heat treatment, the control (unheated and with no added salt) and heat-treated samples were centrifuged at 16,000 g for 3 minutes to remove any insoluble material. The supernatants were diluted 10-fold in milli Q water and then scanned from 250 to 400 nm in a 10 mm quartz cell with a Jasco Model J-720 spectropolarimeter (Jasco, Hachioji City, Tokyo, Japan) to obtain near-UV CD spectra. The samples were scanned at 50 nm/ min, using a 2 s time constant, a 0.2 nm step resolution, a 1 nm bandwidth,

and a sensitivity of 10 mdeg. Five scans were accumulated, and the average spectrum was saved. The same solutions were diluted another 10-fold in milli-Q water then scanned using a 0.5 mm quartz cell from 185 to 250 nm, and 10 scans were averaged and saved as the far-UV spectrum. The sample compartment of the instrument was flushed with oxygen-free dry nitrogen prior to and during measurements (Considine, Patel, Singh, & Creamer, 2007).

3.2.7 2D Nuclear Magnetic Resonance (2D NMR)

After heat treatment, the pH of the control (unheated and with no added salt) and heat-treated samples was adjusted to 2.5 with a 1 M HCL solution and the samples were then centrifuged at 16,000 g for 3 minutes to remove any insoluble material. The supernatants were analysed by NMR. The spectra were recorded on a Bruker Avance 700 MHz spectrometer (Germany). The 2D TOCSY spectra were recorded with a mixing time of 60 ms and a special width of 9.1 kHz centred at the water frequency. Each spectrum was recorded using a data matrix of 2048 x 256 points. Phase discrimination in the indirect dimension was achieved using the States-TPPI method. Excitation sculpting was used to suppress the water signal. Spectra were processed using Bruker's Topspin software (v. 2.1) using standard parameters. Spectra were referenced using the residual water peak at 4.7 ppm.

3.2.8 Differential Scanning Calorimetry (DSC)

All DSC scans were made on a Perkin Elmer (Norwalk, CT, USA) DSC 7 differential scanning calorimeter equipped with an Intracooler II mechanical refrigeration unit. The DSC 7 was controlled, and data were collected, using Perkin Elmer Pyris DSC software, version 2.04. A 20 mg sample of each solution was put into an aluminium volatile-sample pan (Pan-sell kit 0319-1525, Cover-sell kit 0319-1526, O-ring set 0319-1535, all QTY-1000). The pan was sealed and placed in the sample holder of the DSC 7. An empty pan was used as a reference. The samples were heated from 20 to 100ºC at a heating rate of 5ºC/min. The control was the sample with no added salt.

The DSC curves obtained were analysed using the Pyris software by the following method. Two anchor points were inserted at the start and the end of the major peak and a baseline was drawn between them. The following parameters were calculated:

- the onset temperature, calculated from the intersection of the baseline with the extrapolated tangent to the inflection point of the leading edge of the major peak;

- the peak temperature, the highest temperature point of the major peak;

- the area, the calculated area under the thermogram between the two anchor points.

3.3 Results and Discussion

3.3.1 Composition of whey protein powders

The compositional analyses of the whey protein powders showed that WPI had a significantly higher protein content (93.1%, w/w) and lower ash (1.6%, w/w), fat (5%, w/w) and lactose (2%, w/w) contents than AWPC or CWPC, which had similar compositions (Table 3.1). The main differences between AWPC and CWPC were that: (1) CWPC had ~ 15% (w/w) glycomacropeptide (based on total solids), which was also measured as part of the total protein content; and (2) AWPC contained more (485 mmol kg^{-1} protein) total monovalent cations (K^+ and Na^+) but a third less (82 mmol kg^{-1} protein) total divalent cations (Ca^{2+} and Mg^{2+}) than CWPC (323 and 139 mmol kg^{-1} protein, respectively).

Using RP-HPLC (Elgar, Norris, Ayers, Pritchard, Otter, & Palmano, 2000), it was estimated that, for 4.8% (w/w) protein solutions prepared from WPI, AWPC and CWPC powders, the β-lactoglobulin contents were 3.4, 2.5 and 1.8% (w/w) and the α-lactalbumin contents were 0.76, 0.74 and 0.78% (w/w). Thus, the whey protein products had similar α-lactalbumin contents but very different β-lactoglobulin contents. These results also indicated that the WPI, AWPC and CWPC powders had β-lactoglobulin to α-lactalbumin ratios of 4.4, 3.3 and 2.3.

3.3.2 Whey protein isolate (WPI)

3.3.2.1   PAGE

The effect of calcium on the heat-induced aggregation of whey proteins in WPI was investigated using 4% (w/w) protein solutions (a common protein concentration used in many food applications) when heated at 85ºC for 5 min. To demonstrate the specific effect of added calcium, the effects of pH and ionic strength were also studied at 85ºC

for 5 min. Lastly, the effect of temperature combined with the effect of added calcium was determined at 75 and 80ºC.

Loss of alkaline-monomeric whey proteins in WPI during heating

The PAGE patterns for WPI solutions with added calcium chloride heated at 85ºC are shown in Figure 3.1.
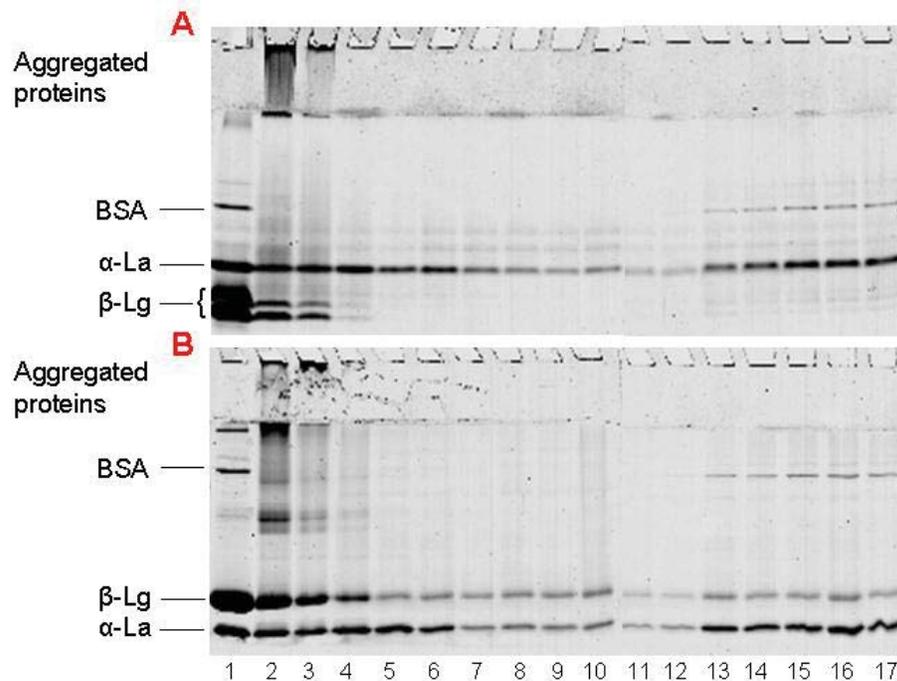
Figure 3.1: Alkaline- (**A**) and SDS- (**B**) PAGE patterns of heated (85ºC, 5 min) WPI solutions (4%, w/w protein); lane 1: unheated, no added CaCl$_2$; lane 2: heated, no added CaCl$_2$; lanes 3–17: heated with 2, 4, 6, 8, 11, 14, 17, 20, 50, 80, 110, 140, 170, 200 and 230 mM added CaCl$_2$.

Alkaline-monomeric β-lactoglobulin disappeared markedly in the heated (85ºC) sample (4%, w/w) with 4 mM added calcium chloride (Figure 3.1**A**, lane 4), and completely disappeared with further increase in the level of added calcium up to 80 mM (Figure 3.1**A**, lane 12). Faint bands corresponding to β-lactoglobulin appeared in the heated sample with 110 mM added calcium chloride (Figure 3.1**A**, lane 13), and the intensities of these bands appeared to increase with further increase in the level of added calcium. These results suggest that all levels of added calcium chloride (4–230 mM) enhanced the aggregation of β-lactoglobulin as compared with the control (lane 2), but some retardation of aggregation took place at higher levels ($\geq$ 110 mM) of added calcium. The enhancement by calcium of the denaturation and aggregation of β-lactoglobulin

during heating has been reported previously (O'Kennedy & Mounsey, 2009; Sherwin & Foegeding, 1997; Simons, Kosters, Visschers, & de Jongh, 2002; Xiong, Dawson, & Wan, 1993). It appears that added calcium prevents the unfolded regions from refolding into the native state, hence promoting aggregation, subsequent to unfolding, during heating.

After heating the control solution (Figure 3.1**A**, lane 2), a considerable amount of alkaline-monomeric α-lactalbumin remained (compare lanes 1 and 2 in Figure 3.1**A**). The loss of α-lactalbumin increased with an increase in the level of added calcium chloride to a maximum at around 50-80 mM (Figure 3.1**A**, lanes 11 and 12), and then decreased with further increase in the level of added calcium. These results suggest that addition of calcium chloride to the protein solutions enhanced the aggregation of α-lactalbumin, the effect being optimal ~ 80 mM added calcium chloride. It appeared that high levels of added calcium (≥ 110 mM) stabilized the α-lactalbumin structure.

Alkaline-monomeric bovine serum albumin disappeared completely after heating for 5 min, even in the heated control sample without any added calcium chloride (compare lanes 1 and 2 in Figure 3.1**A**). However, in the sample with 110 mM added calcium chloride, the bovine serum albumin band started to reappear (Figure 3.1**A**, lane 13) and continued to increase in intensity with increases in the level of added calcium chloride up to 230 mM. The results indicated that bovine serum albumin is sensitive to heat treatment, as reported previously (Havea, Singh, Creamer, & Campanella, 1998), but that its aggregation is somewhat retarded in the presence of added calcium chloride at ≥ 110 mM.

Loss of SDS-monomeric whey proteins in WPI during heating

The losses of SDS-monomeric proteins (Figure 3.1**B**) followed similar patterns to those observed for alkaline-monomeric proteins (Figure 3.1**A**). There appeared to be some faint bands corresponding to bovine serum albumin in the heated control, suggesting that some non-covalently associated bovine serum albumin aggregates may have been dissociated under SDS conditions (Figure 3.1**B**, lane 2). Otherwise, the loss of SDS-monomeric bovine serum albumin in the samples with added calcium chloride was very similar to the loss of alkaline-monomeric bovine serum albumin. Likewise, the loss of SDS-monomeric α-lactalbumin (Figure 3.1**B**) was similar to the loss of alkaline-

monomeric α-lactalbumin (Figure 3.1**A**); the α-lactalbumin band intensities in Figures 3.1**A** and **B** were similar. These results suggest that disulphide bonding of bovine serum albumin and α-lactalbumin was predominantly involved in aggregate formation during heat treatment. The possible involvement of bovine serum albumin and α-lactalbumin in non-covalent interactions was not apparent from these results.

Although the trend for the loss of SDS-monomeric β-lactoglobulin was similar to that of alkaline-monomeric β-lactoglobulin, it was clear that considerable amounts of non-covalently-linked aggregates dissociated under SDS conditions to give more intense bands (Figure 3.1**B**). The intensity of the β-lactoglobulin bands decreased to a minimum at ~ 20-80 mM added calcium chloride and then increased with further increases in added calcium chloride.

α-Lactalbumin is widely known for its calcium binding properties (Bernal & Jelen, 1984; Brew, 2003). The role of bound calcium ions appears to be stabilization of the tertiary structure (Relkin, 1996). The added calcium chloride probably limited the extent of unfolding of α-lactalbumin molecules, suppressing the exposure of some of the hydrophobic residues, and hence hydrophobic aggregations. The results suggest that the aggregation of any denatured α-lactalbumin was predominantly by disulphide linkage.

The effect of calcium on the aggregation of bovine serum albumin is not fully understood. Because bovine serum albumin contains more disulphide bonds than β-lactoglobulin and contains a free thiol group, its denatured form will be more susceptible to the formation of intermolecular disulphide-linked aggregates than to non-covalently-linked aggregates (Figure 3.1).

Effect of pH shifting on the aggregation of whey proteins in WPI
When 0 to 230 mM calcium chloride was added to 4% (w/w) WPI solutions the pH shifted from 6.70 to 6.08. To investigate the effect of pH shifting due to added calcium chloride on whey protein aggregation, three sets of samples (4%, w/w protein) were prepared: i) samples with added calcium chloride but without pH adjustment (Figure 3.1); ii) samples with added calcium chloride but with the pH adjusted back to the original pH of the solutions without added calcium chloride by the addition of 1 M NaOH (Figure 3.2); iii) samples without added calcium chloride but with the pH

adjusted to the corresponding pHs of the samples with added calcium chloride using 1 M HCl (Figure 3.3). These sets were then heated (85ºC, 5 min) and the losses of proteins were determined using alkaline- and SDS-PAGE.
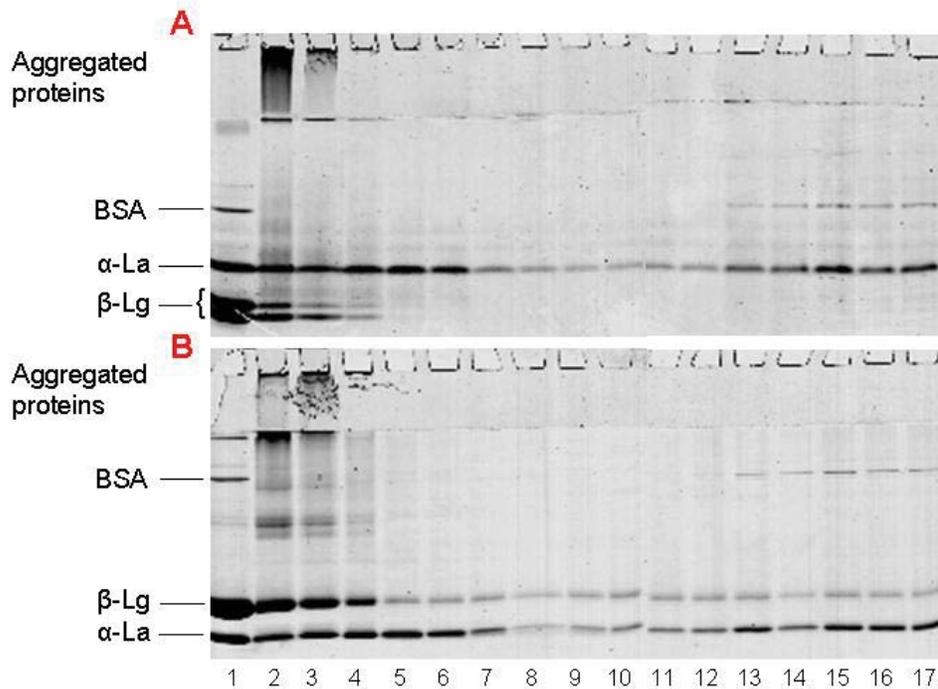
Figure 3.2: Alkaline- (**A**) and SDS- (**B**) PAGE patterns of heated (85ºC, 5 min) WPI solutions (4%, w/w protein) and with the pH adjusted to 6.70 with NaOH; lane 1: unheated, no added $CaCl_2$; lane 2: heated, no added $CaCl_2$; lanes 3–17: heated with 2, 4, 6, 8, 11, 14, 17, 20, 50, 80, 110, 140, 170, 200 and 230 mM added $CaCl_2$ and pH adjusted to 6.70 with NaOH.

The results demonstrated that heat induced losses of protein in the samples of set (i) and that in the samples of set (ii) were the same. It was also clear that the heat-induced losses of proteins in the samples of set (iii) did not vary with decreasing pH over the pH range (6.08 – 6.61) relevant to this study. To conclude, the shifts in pH in these samples due to the addition of calcium chloride did not significantly alter the calcium chloride-induced trends. Therefore, further experiments were conducted without adjusting the pH of solutions after the addition of calcium chloride.
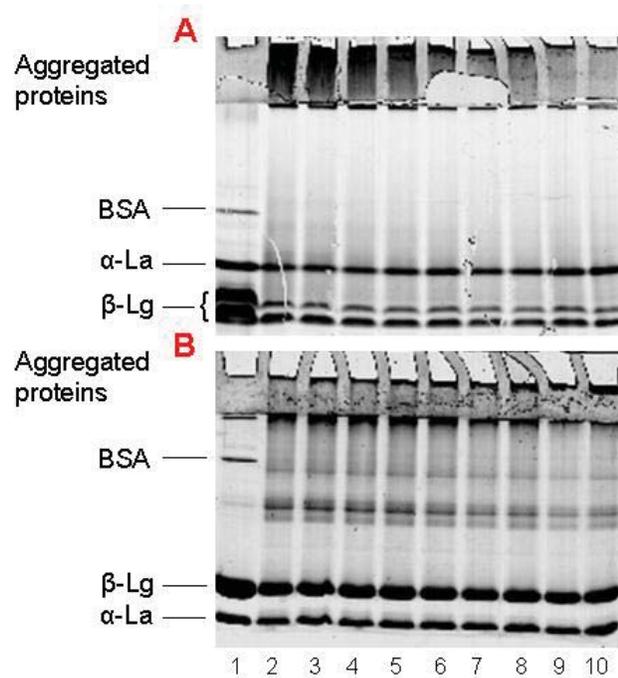
Figure 3.3: Alkaline- (**A**) and SDS- (**B**) PAGE patterns of heated (85ºC, 5 min) WPI solutions (4%, w/w protein); lane 1: unheated, no added HCl; lane 2: heated, no added HCl; lanes 3–10: heated with HCl added to adjust the pH to 6.61, 6.54, 6.48, 6.37, 6.30, 6.23, 6.16 and 6.08.

Effect of sodium chloride on the aggregation of whey proteins in WPI

In the experiments concerned, samples (4%, w/w protein solutions, pH 6.7) were heated under the same conditions as before but with sodium chloride (NaCl) added at levels that simulated the ionic strength of each of the samples with added calcium chloride for which results are shown in Figure 3.1.
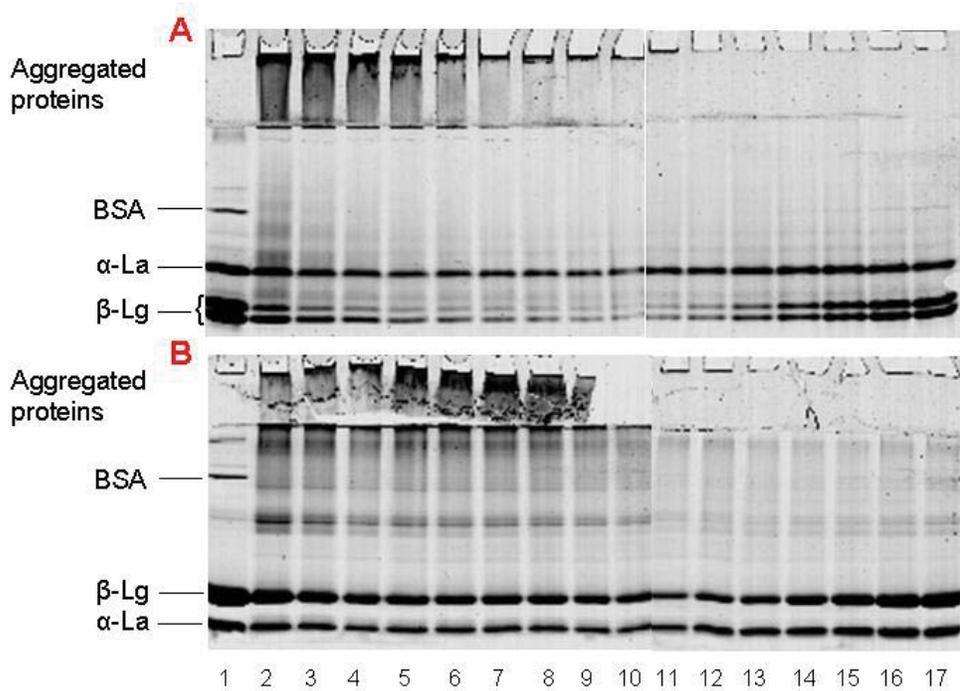
Figure 3.4: Alkaline- (**A**) and SDS- (**B**) PAGE patterns of heated (85ºC, 5 min) WPI solutions (4%, w/w protein); lane 1: unheated, no added NaCl; lane 2: heated, no added NaCl; lanes 3–17: heated with 6, 12, 18, 24, 33, 42, 51, 60, 150, 240, 330, 420, 510, 600 and 690 mM added NaCl.

Figure 3.4 shows the effect of added sodium chloride on the aggregation of whey proteins. The trends observed in Figure 3.1 were also observed in Figure 3.4. However, the effect of added sodium (Figure 3.4) on the loss of alkaline-monomeric and SDS-monomeric proteins was much less than that of added calcium (Figure 3.1). For example, after heating (85ºC, 5 min) the sample with 6 mM added calcium chloride no alkaline-monomeric β-lactoglobulin remained (Figure 3.1**A**, lane 5). However, in the heated sample (85ºC, 5 min) with added sodium chloride of equivalent ionic strength, 26% of the original alkaline-monomeric β-lactoglobulin remained after heating (Figure 3.1**A**, lane 5). This indicated that, although there may be an ionic strength effect, the effect of added calcium chloride has more specific effects. Veerman et al. (2003) reported that both monovalent and divalent cations screen electrostatic interactions but that the effect is greater with divalent cations.

Effect of temperature on the aggregation of whey proteins in WPI
Figures 3.5 and 3.6 show the effect of added calcium chloride on WPI solutions (4%, w/w protein) when heated at 80 and 75°C respectively. Similar trends to those described

at 85°C were observed also at 80 and 75°C. However, comparing Figures 3.1, 3.5 and 3.6 show that the aggregation of whey proteins increased with temperature at all levels of added calcium chloride.
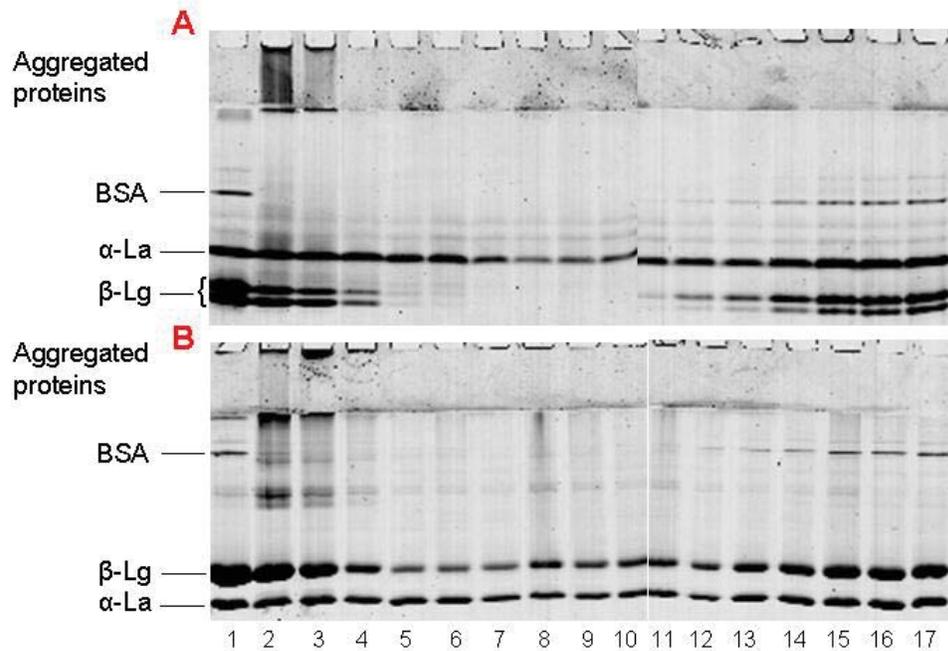
Figure 3.5: Alkaline- (**A**) and SDS- (**B**) PAGE patterns of heated (80°C, 5 min) WPI solutions (4%, w/w protein); lane 1: unheated, no added CaCl$_2$; lane 2: heated, no added CaCl$_2$; lanes 3–17: heated with 2, 4, 6, 8, 11, 14, 17, 20, 50, 80, 110, 140, 170, 200 and 230 mM added CaCl$_2$.

Alkaline-monomeric β-lactoglobulin disappeared markedly when heated at 80°C with 6 mM added calcium (Figure 3.5**A**, lane 5) and completely with further increase of the level of added calcium up to 20 mM (Figure 3.5**A**, lane 10). Faint bands corresponding to β-lactoglobulin appeared in the heated sample with 50 mM added calcium (Figure 3.5**A**, lane 11) and their intensities increased with further increase in the level of added calcium. The range of added calcium concentrations when β-lactoglobulin is the most aggregated was narrower when heated at 80°C, 6 to 50 mM (Figure 3.5**A**, lanes 5 to 10) than when heated at 85°C, 4 to 80 mM (Figure 3.1**A**, lanes 4 to 12). Alkaline-monomeric α-lactalbumin did not disappear completely over the range of levels of added calcium; less intense bands were observed for the heated (80°C) samples with 11 to 20 mM added calcium (Figure 3.5**A**, lanes 7 to 10). Compared with the PAGE pattern when samples where heated at 85°C, the loss of alkaline-monomeric α-lactalbumin when heated at 80°C was much less over the range of levels of added calcium studied.

After heating the control solution without addition of calcium (Figure 3.5**A**, lane 2), alkaline-monomeric bovine serum albumin disappeared completely as well as in the heated samples with low levels of added calcium. Faint bands appeared in the heated sample with 80 mM added calcium and the intensities of these bands increased with further increase in the level of added calcium. Again, compared with the samples heated at 85ºC, the loss of alkaline-monomeric bovine serum albumin at high added calcium concentrations was less in the samples heated at 80ºC.
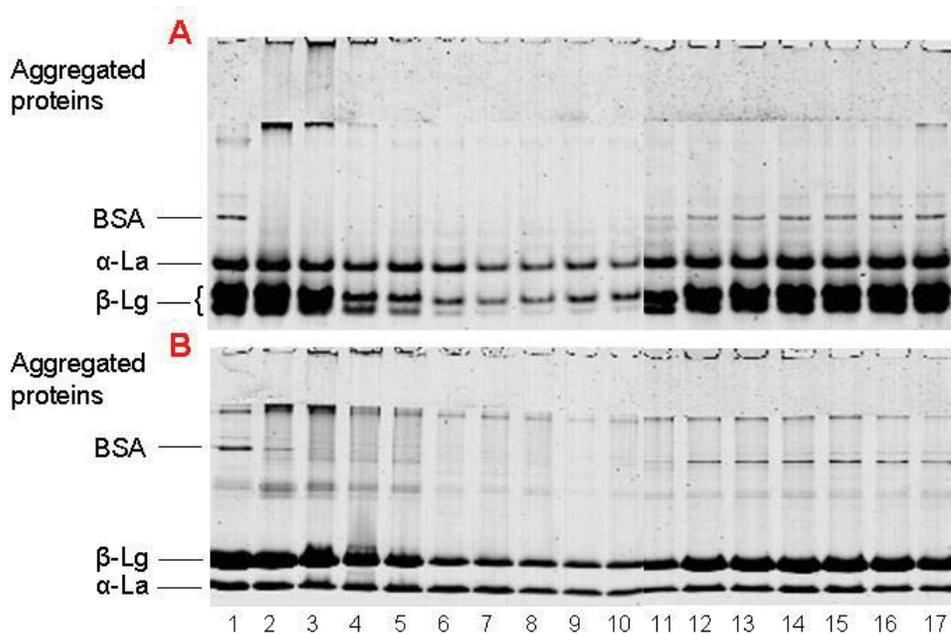
Figure 3.6: Alkaline- (**A**) and SDS- (**B**) PAGE patterns of heated (75ºC, 5 min) WPI solutions (4%, w/w protein); lane 1: unheated, no added CaCl$_2$; lane 2: heated, no added CaCl$_2$; lanes 3–17: heated with 2, 4, 6, 8, 11, 14, 17, 20, 50, 80, 110, 140, 170, 200 and 230 mM added CaCl$_2$.

When heated at 75ºC with added calcium, alkaline-monomeric β-lactoglobulin never disappeared completely as it did when heated at 80 and 85ºC in the presence of added calcium (Figure 3.1**A**, 3.5**A** and 3.6**A**). In the case of alkaline-monomeric α-lactalbumin, the added calcium concentration ranges where the bands were the faintest were 8 to 80 mM when heated at 85ºC (Figure 3.1**A**), and 11 to 50 mM when heated at 80 (Figure 3.5**A**) and 75ºC (Figure 3.6**A**). For alkaline-monomeric bovine serum albumin, bands disappeared between 0 and 80 mM added calcium when heated at 85ºC (Figure 3.1**A**), between 0 and 50 mM added calcium when heated at 80ºC (Figure 3.5**A**) and between 0 and 20 mM added calcium when heated at 75ºC (Figure 3.6**A**).

Looking at the SDS-PAGE patterns (Figures 3.1**B**, 3.5**B** and 3.6**B**), the trends for β-lactoglobulin, α-lactalbumin and bovine serum albumin were similar to the corresponding alkaline- PAGE patterns at each heating temperature (Figures 3.1**A**, 3.5**A** and 3.6**A**).

Quantification of the PAGE band intensities provided the typical loss profiles for the native proteins (β-lactoglobulin, α-lactalbumin, or bovine serum albumin) during heating (at 75, 80 or 85ºC) as affected by increasing levels of added calcium chloride (results not shown). It is clear that at concentrations ~ 20 – 50 mM of added calcium chloride aggregation of these proteins was greatest, and beyond these levels denaturation decreased with further increases in added calcium chloride concentration.

Sherwin and Foegeding (1997) reported that the stoichiometric relationship between the concentrations of calcium and β-lactoglobulin is the important factor in determining the rate of protein aggregation and that a maximum aggregation rate existed between calcium chloride (mM)-to-protein (%, w/v) ratios of 3.33 and 23.3, and that excess calcium chloride appeared to have an inhibitory effect on protein aggregation. A concentration of 3 mM added calcium chloride was reported to induce maximal aggregation of denatured β-lactoglobulin (1%, w/w) when heated at 78ºC for 10 min (Mounsey & O'Kennedy, 2007; O'Kennedy & Mounsey, 2009). In these studies (which were conducted predominantly on pure protein model systems) observations were interpreted on the basis of the effect of added calcium chloride on interactions (e.g. the formation of calcium bridges) giving rise to the formation of protein aggregates. The current results demonstrate that the aggregation of whey proteins (Figures 3.1 to 3.6) is also affected by added calcium. Sherwin and Foegeding (1997) found that an excess of added calcium inhibited aggregation either by a singular mechanism or by a switch to a competing mechanism after some critical calcium-protein ratio.

The enhancement of the aggregation of the whey proteins in WPI due to the addition of calcium was observed at all temperatures studied, with the greatest effect for the samples with 14 – 20 mM added calcium. However, the degree of this enhancement was dependent on the heating temperature. If the solutions were not heated at a high enough temperature to induce the unfolding of the whey proteins, the effect of added calcium was not as great as at higher heating temperature.

3.3.2.2   RP-HPLC

Another analytical technique used to analyse the effect of added calcium on the heat-induced aggregation of the whey proteins and quantify the loss of soluble proteins was reverse-phase high performance liquid chromatography (RP-HPLC). Basically, the principle of HPLC is to separate a mixture of soluble compounds as they pass through the column, separation being based on the polar/non-polar equilibrium. In the case of RP-HPLC, the stationary phase in the column comprises silica particles surface-modified to become non-polar, and the solvent is a polar. The polar constituents of the mixture analysed will preferably interact with the solvent and pass through the column quickly, without interacting much with the polar stationary phase. The non-polar constituents of the mixture will preferably interact with the stationary phase and take longer to pass through the column. Often, the solvent is a mixture of water and alcohol, the ratio of which varies over time to create a gradient from very polar to less polar solvent. Depending on the polarity of the constituents of the mixture analysed and the polarity of the solvent over the gradient, each constituent will interact and remain on the stationary phase for a different length of time, called the retention time. The more polar constituents will pass through the column first, followed by less polar constituents, and lastly the least polar constituents, leading to the separation of the constituents. A UV detector is usually utilised for the detection of the constituents leaving the column. A chromatogram is then obtained, showing peaks at different retention times, specific to the different constituents of the mixture analysed, for the specific experimental conditions (stationary phase and solvent used). Because of the possible relationship between UV absorbance and constituent concentration, it is possible to quantify the amount of each constituent passing through the column.

In our present study, the method of Elgar et al. (2000) was used and by measuring the area under the specific peak of each protein, the amount of soluble protein remaining in solution was quantifiable. Therefore, it was possible to determine the extent of aggregation of the whey proteins, individually or in total. The typical chromatograms of whey proteins, individually or as a mixture, are shown in Figure 3.7
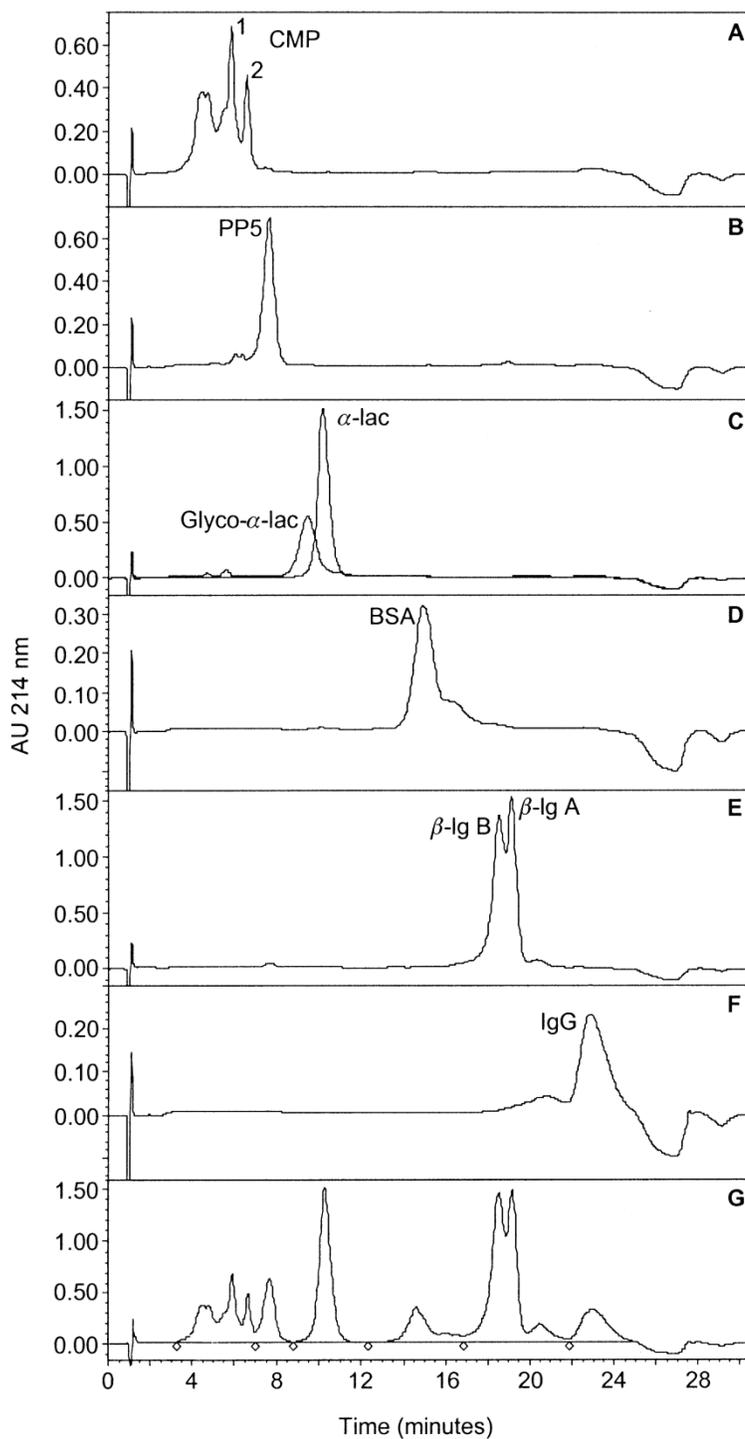
Figure 3.7: RP-HPLC chromatograms of individual (A – F) and mixed (G) whey protein standards; from Elgar, Norris, Ayers, Pritchard, Otter, and Palmano (2000).

Figure 3.8 shows the effect of added calcium on the heat-induced aggregation of α-lactalbumin and β-lactoglobulin individually in WPI solutions at three different temperatures.
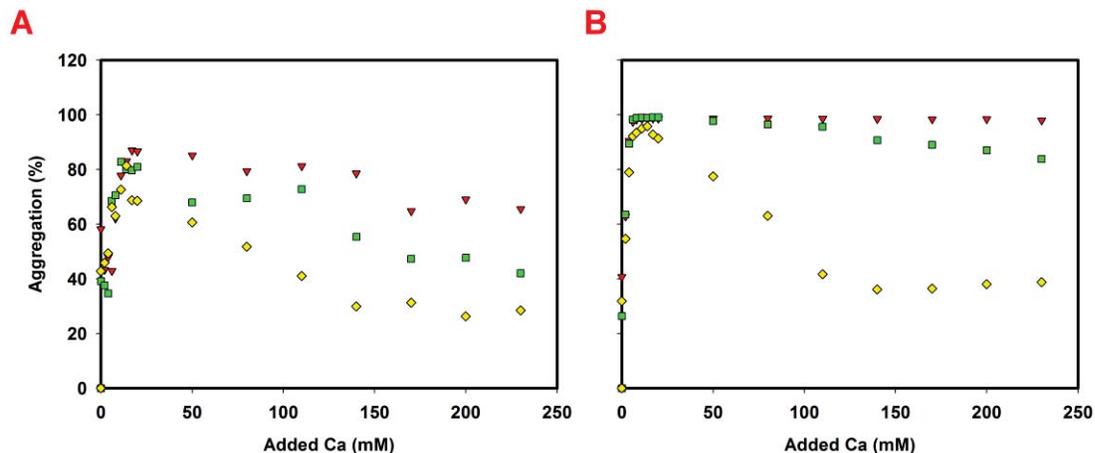
Figure 3.8: Aggregation of α-lactalbumin (**A**) and β-lactoglobulin (**B**) in heated (5 min) WPI solutions (4%, w/w protein) quantified by RP-HPLC; ▼ 85°C, ■ 80°C, and ◇ 75°C.

The effect of added calcium on the heat-induced denaturation and aggregation of α-lactalbumin and β-lactoglobulin shown by HPLC was similar to that observed by PAGE previously. The extent of aggregation increased rapidly with increasing added calcium concentration to a maximum at about 20 mM added calcium, and then decreased with further increase in added calcium level. The rate of decrease in the extent of aggregation was gradual for α-lactalbumin at all three temperatures whereas for β-lactoglobulin the rate was affected dramatically by temperature.

At all three temperatures, the extent of aggregation was significantly less for α-lactalbumin than for β-lactoglobulin, which was expected as α-lactalbumin is more heat stable than β-lactoglobulin. However, for both α-lactalbumin and β-lactoglobulin, the extent of aggregation at 85ºC was higher than at 80ºC, which was higher than at 75ºC. But in the case of β-lactoglobulin, the extent of aggregation at 75ºC was markedly less than at 80 and 85ºC whereas in the case of α-lactalbumin, the decrease in the extent of aggregation due to the decrease in temperature was gradual.

Figure 3.9 shows the effect of added calcium on the aggregation of the total whey proteins in WPI heated at three different temperatures. The total extent of whey protein aggregation was calculated by summing the percentages of the individual proteins remaining in solution, the percentages being weighted according to the protein proportions in WPI.
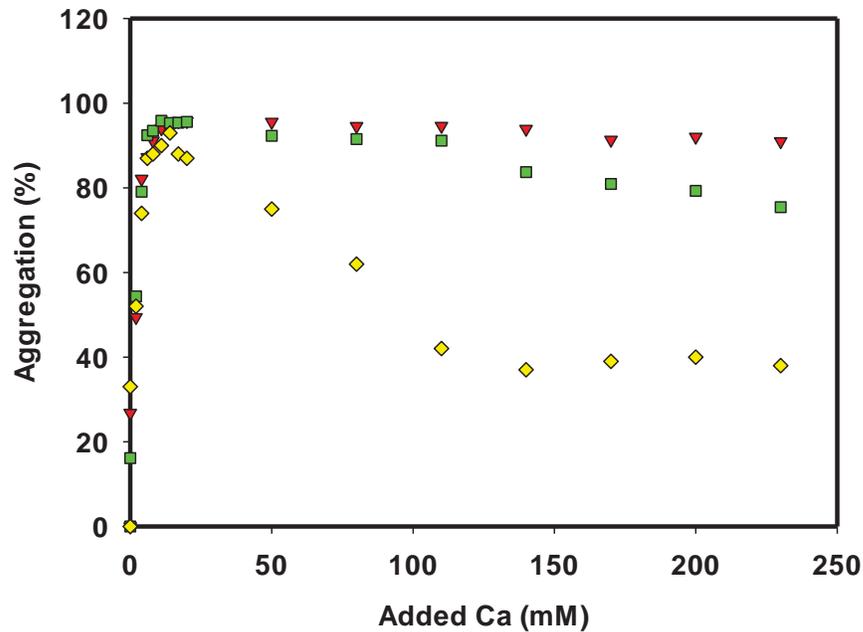
Figure 3.9: Aggregation of total whey proteins in heated (5 min) WPI solutions (4%, w/w protein) quantified by RP-HPLC; ▼ 85°C, ■ 80°C, and ◇ 75°C.

As expected, the extent of aggregation of total whey proteins (Figure 3.9) was very similar to the extent of aggregation of β-lactoglobulin alone at all three temperatures and along the range of added calcium concentration studied (Figure 3.8**B**). However, it was slightly less for the total whey proteins than for β-lactoglobulin alone. This was due to the fact that the extent of aggregation of α-lactalbumin, the second main whey protein, was less than that of β-lactoglobulin.

Whey proteins were detected and identified by their UV absorbance which is a tool for analysing denaturation. The shape of the UV absorbance peak was related to the state of the spatial configuration of the whey proteins. The absorbance peak of the native proteins was different from that of the denatured proteins. Figure 3.10 shows the chromatograms of the WPI solutions heated at 85, 80 and 75ºC for 5 min with different levels of added calcium. To allow a better reading of the data, only four chromatograms have been overlaid: the unheated solution without added calcium (control), the heated solution without added calcium and two solutions heated with added calcium at two different levels, 20 and 230 mM, carefully chosen. The solution heated with 20 mM added calcium was chosen because the maximum extent of aggregation was obtained at around that level of added calcium. And the solution heated with 230 mM added

calcium was chosen because less aggregation was observed at this high level of added calcium.
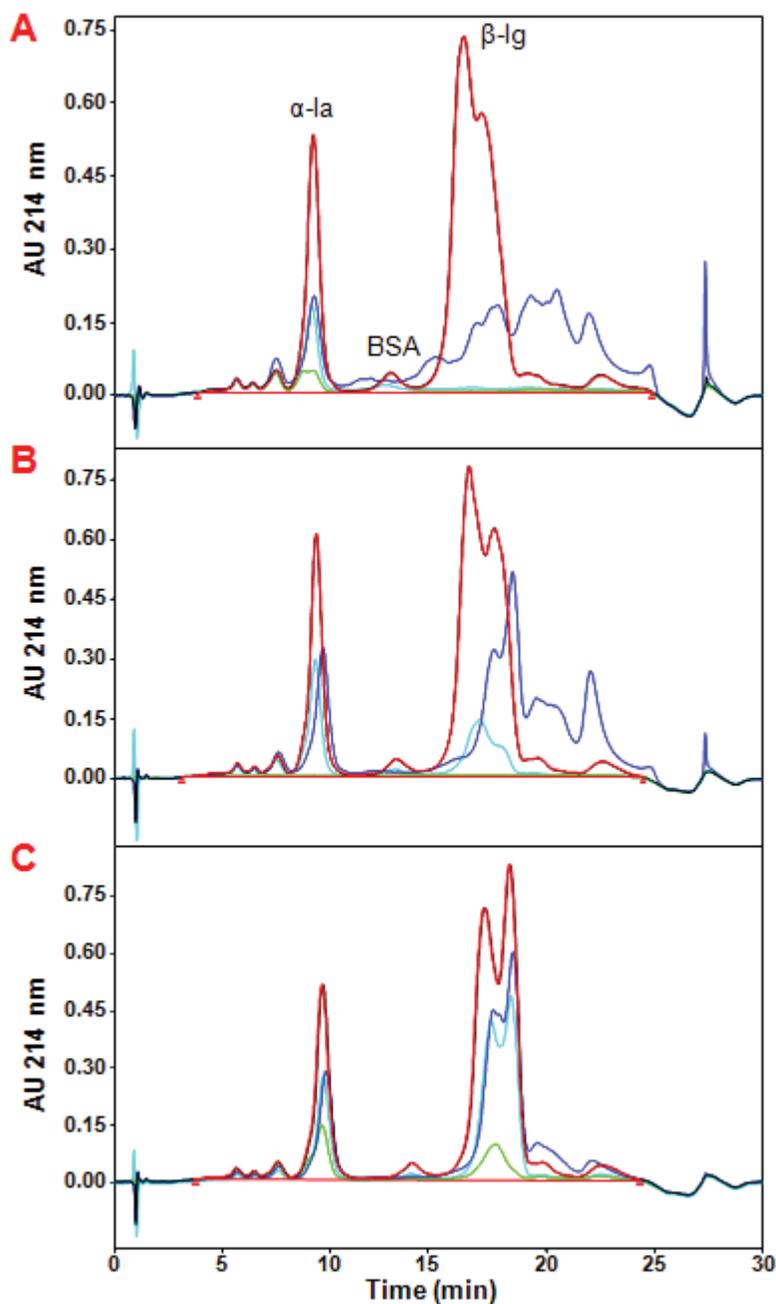
Figure 3.10: Chromatograms of heated (5 min) WPI solutions (4%, w/w protein) at 85ºC (**A**), 80ºC (**B**) and 75ºC (**C**); — unheated, no added CaCl₂; — heated, no added CaCl₂; — heated with 20 mM added CaCl₂; — heated with 230 mM added CaCl₂.

At 85ºC (Figure 3.10**A**), the chromatogram of the heated solution without addition of calcium showed that the shape of the absorbance peak was dramatically different from the one for the unheated sample without added calcium. With addition of calcium, for

both levels, the β-lactoglobulin absorbance peak was almost non-existent; only a very small amount of soluble β-lactoglobulin molecules remained in solution. In the case of α-lactalbumin, the shape of the absorbance peak for all heated solutions remained the same as that of the unheated solution, but was smaller. It seemed that the soluble α-lactalbumin molecules were still in their native state. However, it is interesting to note that the α-lactalbumin absorbance peak for the heated solutions without added calcium and with 230 mM of added calcium are almost similar. The extent of aggregation of the 230 mM added calcium solution was almost the same to that of the solution without added calcium.

At lower heating temperature, 80 and 75ºC (Figures 3.10**B** and **C**), the effect of the concentration of added calcium on the heat-induced denaturation and aggregation of the whey proteins was more noticeable as the effect of the temperature was less dramatic, especially in the case of β-lactoglobulin. Similarly to the observation at 85ºC, α-lactalbumin and β-lactoglobulin underwent some degree of denaturation and aggregation in the heated solutions without added calcium. In the heated solution with 20 mM of added calcium, the β-lactoglobulin absorbance peak completely disappeared at 80ºC and was small at 75ºC. In the heated solution with 230 mM of added calcium, the β-lactoglobulin absorbance peak was detected at 80 and 75ºC, and at 75ºC it almost overlaid that of the heated solution without added calcium. This suggested that at low temperature, high levels of added calcium may protect β-lactoglobulin molecules from heat-induced aggregation instead of enhancing heat-induced aggregation as observed at low levels of added calcium. For α-lactalbumin, in the heated solution with 20 mM of added calcium, the absorbance peak completely disappeared at 80°C. At 75°C, the α-lactalbumin absorbance peak was bigger than at 85°C. In the heated solution with 230 mM at all three temperatures, the α-lactalbumin absorbance peak almost overlaid that for the heated solution without added calcium. This suggested that the high level of added calcium may have had a stabilising effect against heat-induced aggregation instead of enhancing the effect of temperature as observed at lower added calcium levels (e.g. 20 mM).

RP-HPLC was a useful in quantifying the extent of aggregation of whey proteins, and also gave some indication on the heat-induced denaturation. Indeed, peak size relates to aggregation, and by measuring the area under the peak the software calculates the

amount of soluble molecules in solution. Peak shape relates to denaturation, as the peak shape is the specific UV signal of the molecule in a specific spatial configuration. When this configuration changes (denaturation) the UV signal changes, and thus the peak shape. The shape of the α-lactalbumin absorbance peak was not altered when WPI solutions were heated, with or without added calcium, at all three heating temperatures. However, the shape of the β-lactoglobulin absorbance peak was altered by heat treatment and addition of calcium at 80 and 85°C, but not so much at 75°C.

3.3.2.3 DSC

Knowing that added calcium had an effect on the heat-induced aggregation of the whey proteins in WPI, it was of interest to determine the effect of added calcium on the heat-induced denaturation of these proteins. Differential scanning calorimetry (DSC) is well described in the literature as being useful for studying the heat-induced denaturation of the whey proteins. When the system being analysed changes state due to temperature, e.g. change of conformation, or the creation or cleavage of chemical bonding, energy will be required (endothermic reaction) or released (exothermic reaction). DSC subjects the samples to temperature gradients, and measures the heat flow required to maintain equality of temperature between the sample and a non-reactive blank, and records a thermogram. From this thermogram, thermodynamic parameters can be calculated. In the case of heat-induced denaturation and aggregation of whey proteins, unfolding is followed by cleavage of chemical bonding and the formation of new bonds. These phenomena are endothermic. In this study, the effect of added calcium on the onset temperature and the temperature of the endothermic peak during the heating gradient was investigated.

Figure 3.11 shows the effect of added calcium on the onset and peak temperatures for heat-induced denaturation of the whey proteins in WPI.
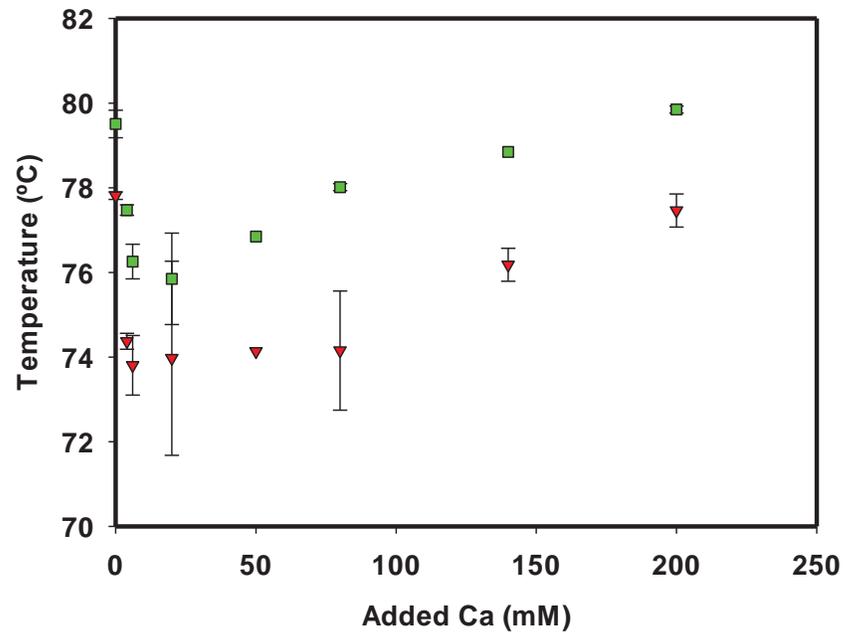
Figure 3.11: Differential scanning calorimetry, analysis of WPI solutions (4%, w/w protein); ▼ onset temperature; ■ peak temperature.

Both onset and peak temperatures followed the same trend, decreasing with increase of added calcium concentration up to 20 mM, then increasing with increasing added calcium concentration. Similar results were observed by Xiong (1992) when adding calcium chloride to 0.12% (w/v) WPI solution (pH 6). The peak temperature decreased with increases in the level of added calcium chloride up to 5 – 10 mM then increased with further increase in the level of added calcium chloride (up to 50 mM). Interestingly, the onset and peak temperatures of the sample with 200 mM added calcium were similar to the ones of the sample without added calcium (Figure 3.11). As suggested by Xiong (1992), the whey protein conformations were less and less destabilised by increasing added calcium concentrations to the point, 200 mM added calcium, where the destabilisation of the whey protein conformations was similar to that of the WPI solution with no added calcium. With 20 mM added calcium, the peak temperature was 4°C less than that of the sample without added calcium. These results directly supported the previous results obtained using PAGE and RP-HPLC, where maximum aggregation was found to occur in the sample with 20 mM added calcium.

During the DSC experiments, the variation of the sample enthalpy with added calcium was also measured. Figure 3.12 shows the effect of added calcium on the sample enthalpy when the whey proteins in WPI denatured and aggregated.
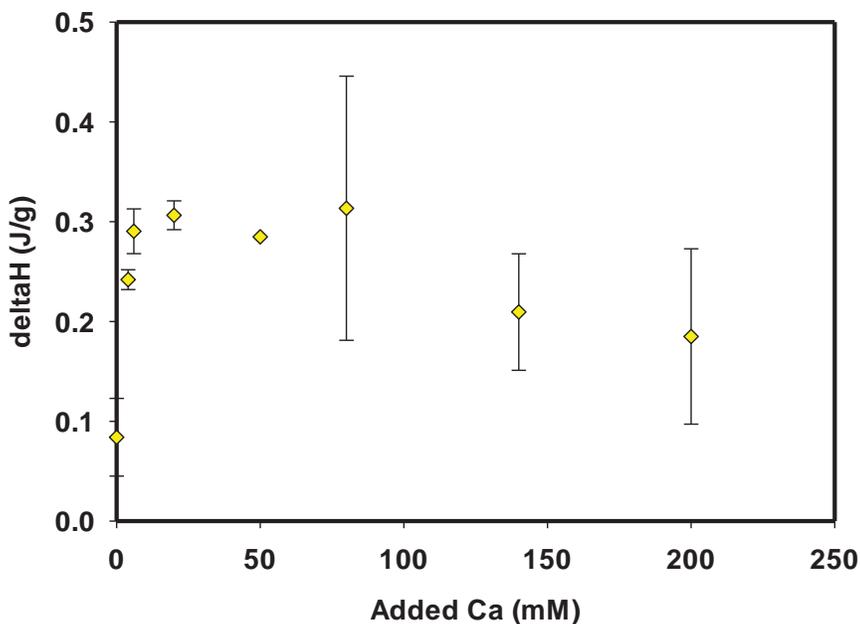
Figure 3.12: Enthalpy spectrum of WPI solutions (4%, w/w protein), obtained using differential scanning calorimetry.

No specific trend was detectable in the variation of the enthalpy when heated with different levels of added calcium. The difficulty of interpreting the results was due to very small values of the enthalpy and the large error bars.

3.3.2.4   CD

RP-HPLC and DSC results showed there was an effect of the added calcium on the heat-induced denaturation of the whey proteins, through the alteration of the shape of the RP-HPLC absorbance peak and variation in the denaturation temperature (DSC). Thus, it was of interest to investigate the effect of added calcium on the spatial structures of the whey proteins during heat-induced denaturation. Circular dichroism (CD) has often been described in the literature as being useful for studying the structure of the whey proteins, based on the UV emission of the aromatic amino acids of the proteins. However, it has usually been applied to model systems comprising single-protein solutions. In this investigation, complex whey protein systems were studied.

Figure 3.13 shows the circular dichroism spectra of WPI solutions heated at 85ºC for 5 min with added calcium chloride.

It is well documented in the literature that the tryptophan 19 of the β-lactoglobulin molecule, in its native environment, gives a characteristic band for β-lactoglobulin which is easy to follow to investigate changes in conformation. When β-lactoglobulin molecules unfold and denature, the environment of the tryptophan 19 (Trp19) changes and the characteristic band disappears. The α-lactalbumin molecule has not been studied as extensively as the β-lactoglobulin molecule, but it has a different UV emission spectrum.
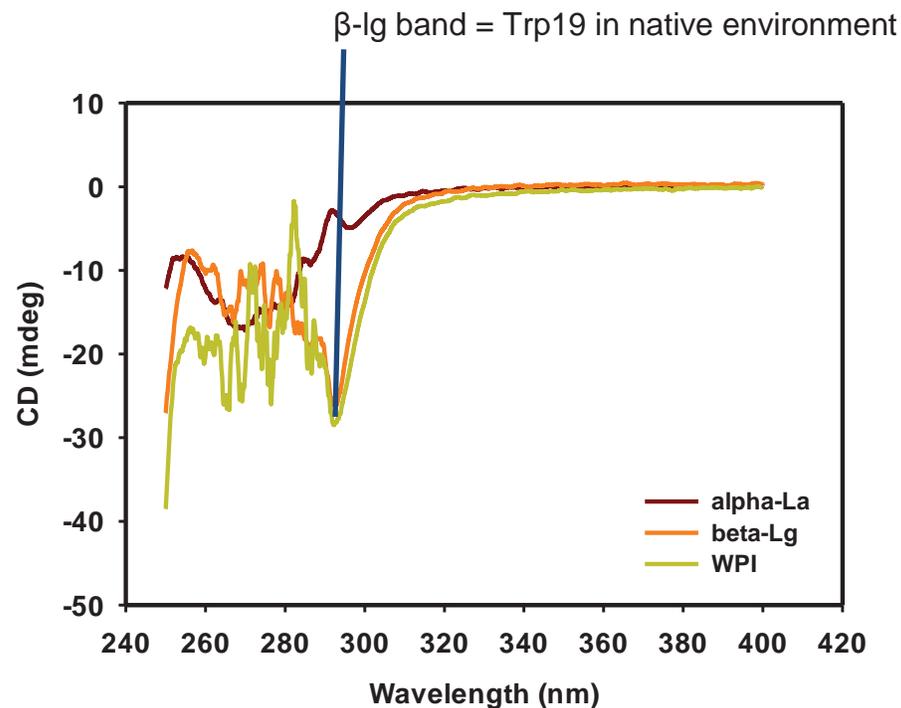
Figure 3.13: Near-UV circular dichroism spectra of ⎯ native α-lactalbumin; ⎯ native β-lactoglobulin; ⎯ unheated WPI solution without added calcium.

When heated without addition of calcium chloride, a partial loss of structure appeared on the near-UV spectrum (Figure 3.14). With the addition of calcium, the loss of structure was greater, almost complete for the solution heated with 20 mM of added calcium. With further addition of calcium chloride, the loss of structure became less, but still greater than for the heated sample without addition of calcium. When comparing the WPI spectra obtained for the solutions heated with added calcium with the native α-lactalbumin spectrum, there was little difference between the spectra (results not shown). When heating the WPI solutions in presence of calcium at 85°C for 5 min, most of the

β-lactoglobulin molecules aggregated. However, the RP-HPLC chromatograms showed that the remaining soluble α-lactalbumin molecules in solution were in their native state. The CD spectra supported these results, as the WPI spectra when heated with added calcium were similar to the native α-lactalbumin spectrum.
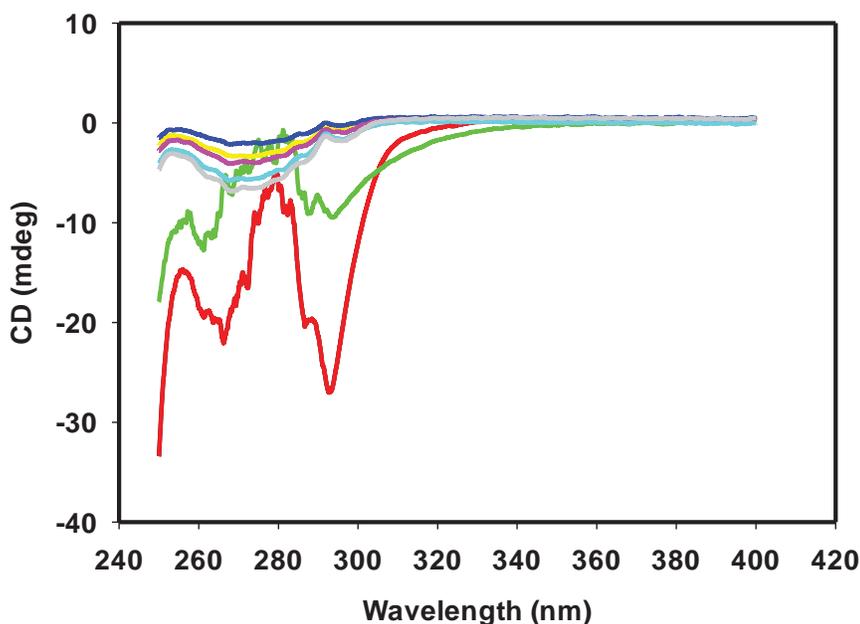
Figure 3.14: Near-UV circular dichroism spectrum of heated (85ºC, 5 min) WPI solutions (4%, w/w protein); — unheated, no added CaCl₂; — heated, no added CaCl₂; — heated with 10 mM added CaCl₂; — heated with 20 mM added CaCl₂; — heated with 80 mM added CaCl₂; — heated with 140 mM added CaCl₂; — heated with 200 mM added CaCl₂.

Far-UV spectra were measured as well, but as the results were inconclusive (data not shown).

3.3.2.5   2D NMR

2D nuclear magnetic resonance (2D NMR) was also used to study the effect of added calcium on the spatial structure of the whey proteins during heat-induced aggregation. The effect of 20 mM added calcium on the 2D NMR spectrum of WPI solutions heated at 85ºC for 5 min is shown in Figure 3.15. The spectrum of unheated β-lactoglobulin is also shown on Figure 3.15 as a reference. Unheated WPI is shown as a control.
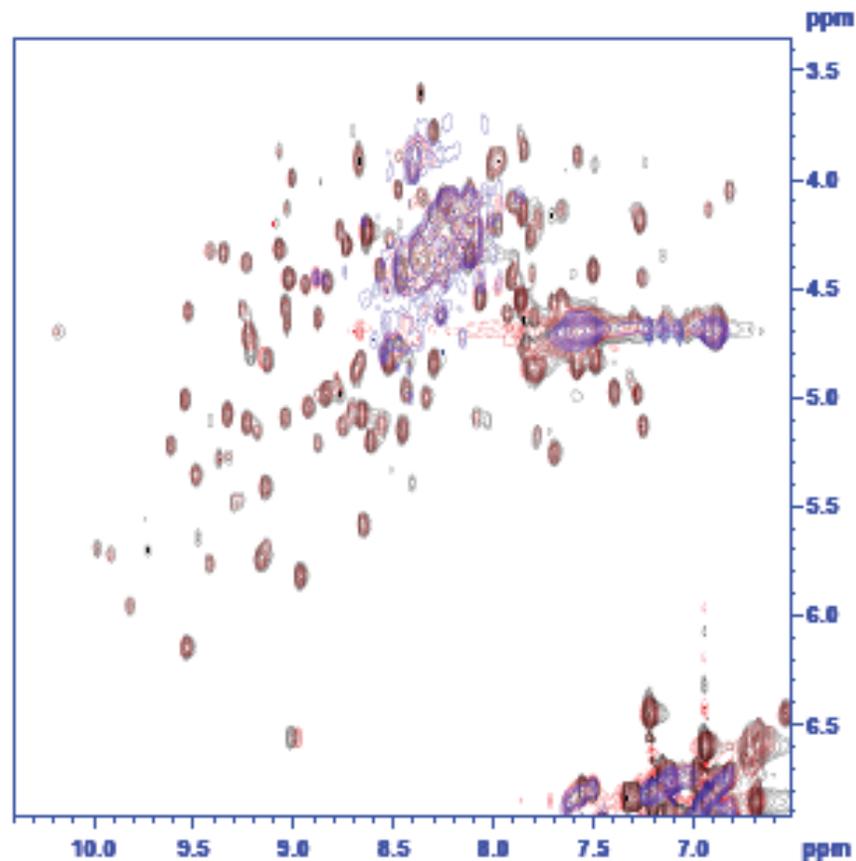
Figure 3.15: 2D Nuclear magnetic resonance spectra of heated (85ºC, 5 min) WPI solutions (4%, w/w protein) with 20 mM added CaCl$_2$; ■ unheated WPI, no added CaCl$_2$; ■ unheated β-lg, no added CaCl$_2$; ■ heated WPI with 20 mM added CaCl$_2$.

The unheated WPI spectrum almost completely overlaid the unheated β-lactoglobulin spectrum. This was expected as β-lactoglobulin is the main whey protein in WPI. When heated at 85ºC for 5 min with 20 mM of added calcium, the WPI spectrum did not overlay the unheated WPI spectrum; many "dots" disappeared. A 2D NMR spectrum is like the fingerprint of a molecule; it is unique to the spatial structure of the molecule, i.e. to its configuration. The "dots" represent the interactions between the atoms in this specific configuration. When the spatial structure of the molecule is changed the spectrum (fingerprint) is changed as well; some interactions disappear and others appear. When WPI solutions were heated with added calcium, the spectrum did not overlay the spectrum of the unheated WPI anymore. Most of the interactions disappeared and the ones that were still visible on the spectrum were not well defined; they were represented by quite noisy aggregates of "dots". This was related to the loss of sensitivity of the NMR due to the presence of aggregates. When molecules form an aggregate, obviously

the interactions between atoms are different from those between non-aggregated molecules, but, as well, some molecules are buried inside the aggregates and the signal coming from each individual atom of these molecules is not easily detected by the probe; the signal is thus noisy. Observation of the spectrum of heated WPI solution with 20 mM of added calcium leads to the conclusion that under heating in the presence of 20 mM of added calcium, the whey proteins lost their configuration, denatured and partly aggregated.

Figure 3.16 shows the effect of different levels of added calcium on the WPI 2D NMR spectra when heated at 85ºC for 5 min. Plot **A** shows the spectra of unheated β-lactoglobulin and unheated WPI. As already observed in Figure 3.15, these two spectra almost overlaid, due to the dominance of β-lactoglobulin in the whey protein systems. Plot **B** shows the spectra of unheated WPI and heated WPI. The spectrum of the heated WPI partly overlaid the spectrum of the unheated WPI. Under heating the whey proteins denatured and partly lost their native spatial structure. Plots **C** to **F** show the effect of different level of added calcium, 10, 80, 140 and 200 mM, on the denaturation and aggregation of the whey proteins. There were no major differences noticeable for 10 and 80 mM, the spectra being similar to that of WPI heated with 20 mM of added calcium (Figure 3.15). This leads to the same conclusion, that under heating in the presence of 20 mM of added calcium, the whey proteins lost their configuration, denatured and partly aggregated. However, at high added calcium concentration, 140 and 200 mM (Figures 3.16**E** and **F**), there was a loss of signal detection; another limitation of the NMR is that at high salt concentration, the probe is not as sensitive as at low salt concentration.
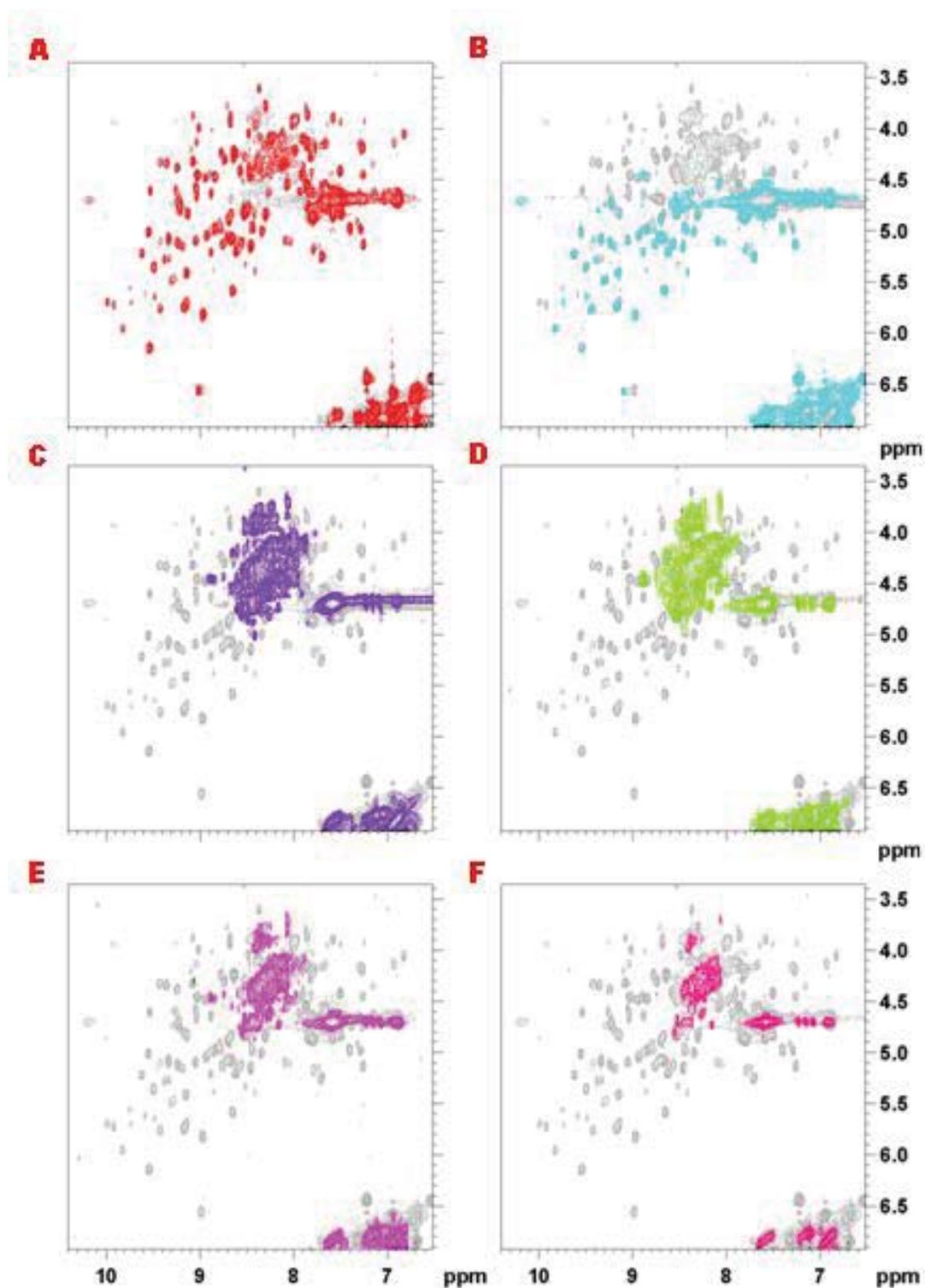
Figure 3.16: 2D Nuclear magnetic resonance spectra of heated (85ºC, 5 min) WPI solutions (4%, w/w protein); ▆ unheated WPI, no added $CaCl_2$; **A** ▆ unheated β-lg, no added $CaCl_2$; **B** ▆ heated WPI, no added $CaCl_2$; **C** ▆ heated WPI with 10 mM added $CaCl_2$; **D** ▆ heated WPI with 80 mM added $CaCl_2$; **E** ▆ heated WPI with 140 mM added $CaCl_2$; **F** ▆ heated WPI with 200 mM added $CaCl_2$.

3.3.2.6   Conclusions

The effect of added calcium on the heat-induced aggregation of whey proteins in WPI was studied using PAGE and RP-HPLC with 4% (w/w protein) WPI solutions heated for 5 min under several experimental conditions: with different amounts of added calcium chloride (0 to 230 mM) or sodium chloride (0 to 690 mM to give the same ionic strength as with calcium chloride), with or without adjustment of the pH (with NaOH or HCl), and at different temperatures (75, 80 and 85°C). The PAGE results showed that the loss of alkaline-monomeric and SDS-monomeric β-lactoglobulin, α-lactalbumin and BSA increased with increasing added calcium concentration up to a certain level (varying between 20 and 110 mM depending on the protein) and then decreased with further increase in added calcium levels. From the experiments without and with adjustment of the pH with NaOH or HCl (Figures 3.1, 3.2 and 3.3), it was concluded that the effect observed was due to the addition of calcium and not to the variation in pH. Similarly, the results from the experiments relating to the ionic strength (Figures 3.1 and 3.4) confirmed the specific effect of added calcium and the absence of an ionic strength effect. When comparing the results obtained for the three heating temperatures (Figures 3.1, 3.5 and 3.6), the loss of alkaline-monomeric and SDS-monomeric β-lactoglobulin, α-lactalbumin and bovine serum albumin decreased with decrease in this temperature (85 to 75ºC).

The quantification obtained by means of RP-HPLC experiments supported the PAGE results. In addition, RP-HPLC chromatograms gave some picture of the heat-induced denaturation of α-lactalbumin and β-lactoglobulin molecules. More precisely, the shape of the peaks on the chromatograms indicated whether the remaining protein molecules in solution were in their native state or denatured. It appeared that the α-lactalbumin peak kept the same shape under heat treatment with and without added calcium at all three heating temperature studied (Figure 3.9). In the case of β-lactoglobulin molecules, at 75°C, the shape of the peak is similar to that of the unheated solution, but at 80 and 85°C, the shape is altered, a sign of denaturation.

Following these observations from RP-HPLC experiments, the effect of added calcium on the heat-induced denaturation of the whey proteins was investigated using several further analytical techniques: DSC, CD and 2D NMR. The DSC experiments showed that the denaturation temperature varied with the level of added calcium (Figure 3.11).

The denaturation temperature reached a minimum where the maximum extent of aggregation was observed by PAGE and RP-HPLC (Figures 3.1 and 3.9), that is when 20 mM of calcium was added to the WPI solution. At higher levels of added calcium, the denaturation temperature increased, reaching the value of the denaturation temperature of the WPI solution without added calcium (Figure 3.11). Other studies in the literature showed that calcium promoted the aggregation of β-lactoglobulin by decreasing its denaturation temperature. By extrapolation to the WPI system in the present study, a low level of added calcium promoted heat-induced aggregation by lowering the denaturation temperature, allowing the denaturation and subsequent aggregation to occur sooner in time. Therefore the aggregation process lasted longer over the time period investigated, leading to a greater extent of aggregation. As a higher level of added calcium did not lower the denaturation temperature as much as a low level of added calcium, the aggregation process did not benefit so much from "extra time" compared with the heated solution without added calcium, leading to a lower extent of aggregation than that at low level of added calcium.

CD and 2D NMR experiments (Figures 3.14, 3.15 and 3.16) did not provide satisfactory enough results to provide a basis for discussing the heat-induced denaturation of the whey proteins. These two analytical techniques are very powerful tools for studying the structures of molecules but have some limitations, especially when mixtures of proteins are studied.

3.3.3 Acid whey protein concentrate (AWPC)

The effect of added calcium on the heat-induced denaturation and aggregation of whey proteins in AWPC was studied with the same experimental techniques as those used with WPI.

3.3.3.1 PAGE

Similarly to the experiments with WPI, the effect of calcium on the heat-induced aggregation of whey proteins in AWPC was investigated with 4% (w/w) protein solutions heated at 85ºC for 5 min. To demonstrate the specific effect of added calcium, the effects of pH and ionic strength were also studied at 85ºC for 5 min. Lastly, the effect of temperature combined with the effect of added calcium was investigated at 75 and 80ºC.

Loss of alkaline-monomeric whey proteins in AWPC during heating

Figure 3.17 shows the PAGE patterns of AWPC solutions heated at 85ºC for 5 min in the presence of added calcium at different concentrations.
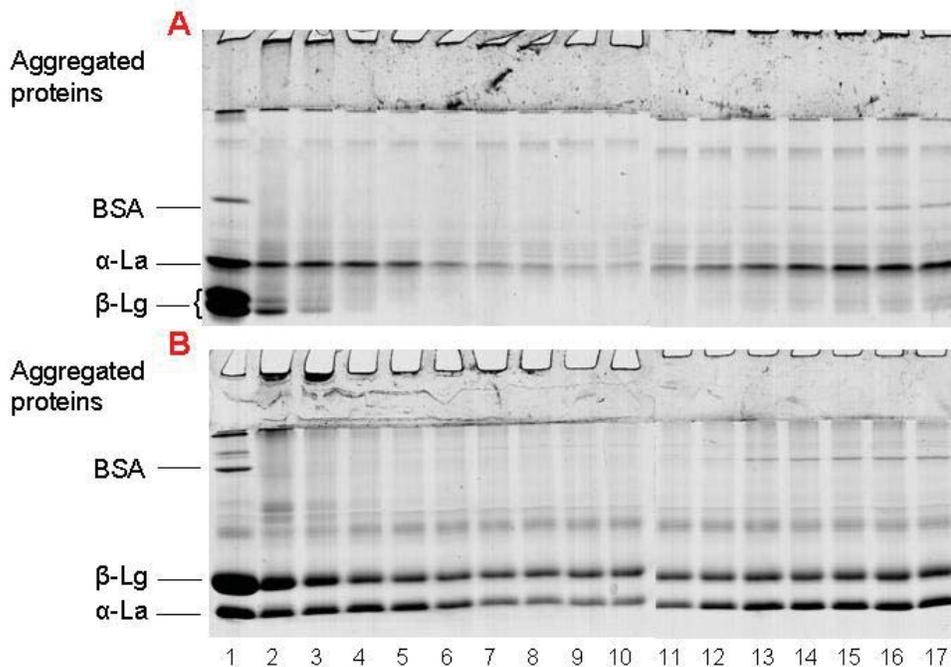
Figure 3.17: Alkaline- (**A**) and SDS- (**B**) PAGE patterns of heated (85ºC, 5 min) AWPC solutions (4%, w/w protein); lane 1: unheated, no added CaCl$_2$; lane 2: heated, no added CaCl$_2$; lanes 3–17: heated with 2, 4, 6, 8, 11, 14, 17, 20, 50, 80, 110, 140, 170, 200 and 230 mM added CaCl$_2$.

The trends observed for the three main whey proteins (β-lactoglobulin, α-lactalbumin and bovine serum albumin) in the alkaline-PAGE pattern (Figure 3.17**A**) were similar to those observed in the case of the WPI solutions (Figure 3.1**A**). Alkaline-monomeric β-lactoglobulin bands markedly disappeared in the heated sample with 4 mM added calcium and disappeared completely in the samples with added calcium levels up to 80 mM. The intensity of these bands increased with further increase in the level of added calcium. For alkaline-monomeric α-lactalbumin, band intensity decreased with increasing added calcium concentration up to 20 mM, and then the intensity increased with further increase in the added calcium concentration. Alkaline-monomeric bovine serum albumin bands disappeared from the heated control sample and in the heated samples with added calcium concentration up to 80 mM. Bands appeared in the heated sample with 110 mM added calcium, and the bands intensity increased slightly with further increase in the added calcium concentration.

Loss of SDS-monomeric whey proteins in AWPC during heating

SDS-monomeric β-lactoglobulin did not disappear markedly across the range of levels of added calcium (Figure 3.17**B**). The intensity of the bands relating to β-lactoglobulin decreased with increase in the level of added calcium from 0 to 6 mM, then the intensity reached a constant value for the heated samples with 8 to 20 mM added calcium, and then the band intensity increased with further increase in the level of added calcium. Comparison with the alkaline-monomeric β-lactoglobulin band intensities led to the conclusion that when β-lactoglobulin aggregated under these conditions, non-covalent interactions were mostly involved.

The intensity of the bands corresponding to SDS-monomeric α-lactalbumin decreased with increasing level of added calcium up to 20 mM, and then the intensity increased with further increase of the level of added calcium (Figure 3.17**B**). The SDS-bands were only slightly more intense than the alkaline-bands, which means that α-lactalbumin mostly aggregated through disulphide linkages but with small percentage of α-lactalbumin aggregating through non-covalent interactions.

The SDS-PAGE pattern for bovine serum albumin was similar to the alkaline-PAGE pattern (Figure 3.17). The bands relating to SDS-monomeric bovine serum albumin completely disappeared for the heated samples with 0 to 80 mM added calcium. At and above 110 mM added calcium, faint bands appeared, and their intensity increased slightly with increase in the level of added calcium. However the band intensity in the SDS-PAGE pattern was similar to that in the alkaline-PAGE pattern. Thus bovine serum albumin aggregated principally through disulphide linkages, which is expected as bovine serum albumin contains three free thiol groups capable of interacting with the disulphide bridges at intra- and intermolecular levels.

Effect of pH shifting on the aggregation of whey proteins in AWPC

Similarly to the WPI solutions, when calcium chloride was added to the AWPC solutions the pH decreased from 6.7 in the control sample (without addition of calcium chloride) to 6.11 in the sample with 230 mM added calcium. To prove that the effects observed were due to the addition of calcium, following the first set of samples with different levels of added calcium chloride (Figure 3.17), two further sets of sample were prepared. One was the same set of samples with different levels of added calcium chloride but with the pH of each sample adjusted to 6.7 (the pH of the control solution,

without addition of calcium chloride) with a 1 M NaOH solution (Figure 3.18). The second set comprised AWPC solutions, 4% (w/w) protein, without addition of calcium chloride but with the pH adjusted with a 1 M HCl solution to the pH of the samples with added calcium (Figure 3.19). Figure 3.17 shows the alkaline- and SDS-PAGE patterns of the effect of added calcium on the heat-induced aggregation of the whey proteins when the pH of each sample was adjusted to 6.7, the pH of the control sample (without addition of calcium).
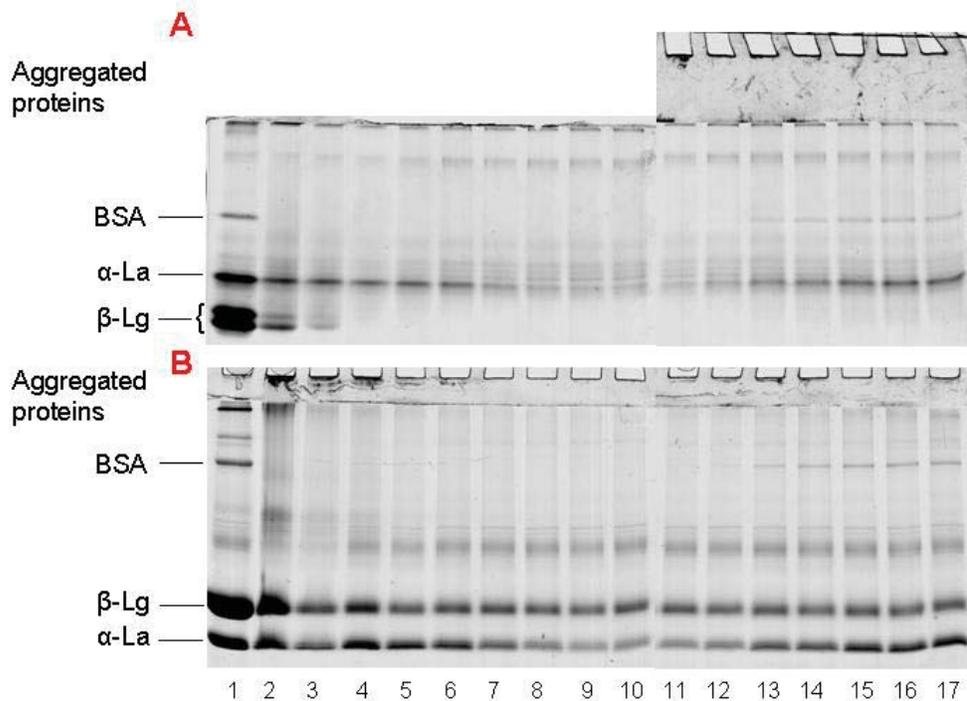
Figure 3.18: Alkaline- (**A**) and SDS- (**B**) PAGE patterns of heated (85ºC, 5 min) AWPC solutions (4%, w/w protein) and with the pH adjusted to 6.70 with NaOH; lane 1: unheated, no added $CaCl_2$; lane 2: heated, no added $CaCl_2$; lanes 3–17: heated with 2, 4, 6, 8, 11, 14, 17, 20, 50, 80, 110, 140, 170, 200 and 230 mM added $CaCl_2$ and pH adjusted to 6.70 with NaOH.

The alkaline- and SDS-PAGE patterns of the samples with the pH adjusted to 6.7 (Figure 3.18) were very similar to those for the samples without adjustment of pH (Figure 3.17). This was the case for the three main whey proteins, β-lactoglobulin, α-lactalbumin and bovine serum albumin.

Figure 3.19 shows the alkaline- and SDS-PAGE patterns of the effect of pH on the heat-induced aggregation of the whey proteins (no added calcium) when the pH in each sample was adjusted to the pH of the samples with different levels of added calcium.
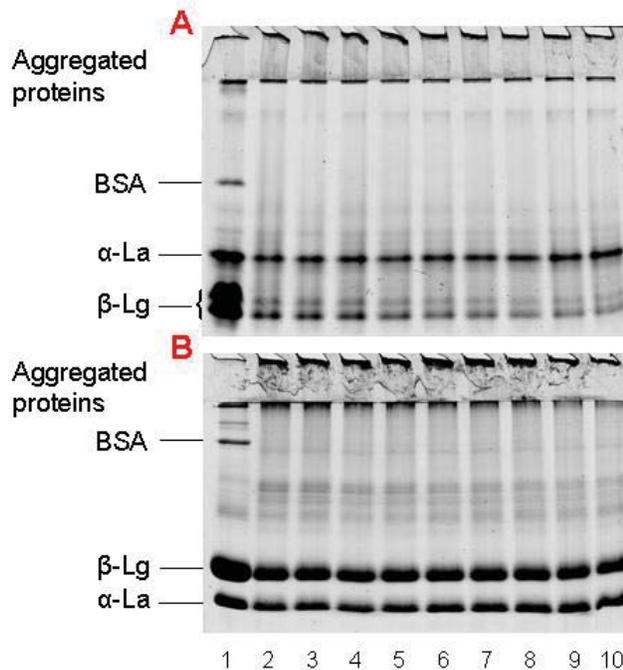
Figure 3.19: Alkaline- (**A**) and SDS- (**B**) PAGE patterns of heated (85ºC, 5 min) AWPC solutions (4%, w/w protein); lane 1: unheated, no added HCl; lane 2: heated, no added HCl; lanes 3–10: heated with added HCl to adjust pH to 6.64, 6.57, 6.52, 6.43, 6.35, 6.28, 6.20 and 6.11.

The alkaline- and SDS-PAGE patterns of the pH-adjusted samples without addition of calcium chloride (Figure 3.19) were completely different from those for the samples with added calcium (Figure 3.17). Alkaline-monomeric β-lactoglobulin bands disappeared with decreasing pH. For alkaline-monomeric α-lactalbumin, it seemed that the bands were of similar intensity across the pH range studied and the alkaline-monomeric bovine serum albumin bands completely disappeared in all samples as soon as they were heated (Figure 3.19**A**). SDS-monomeric bovine serum albumin bands also completely disappeared in all samples as soon as they were heated. SDS-monomeric α-lactalbumin and β-lactoglobulin bands seemed to be of constant intensity across the pH range studied (Figure 3.19**B**).

These results showed that the effects observed when calcium chloride was added to the AWPC solutions which were then heated at 85ºC for 5 min were specific to the added calcium and not to the decrease of pH.

Effect of sodium chloride on the aggregation of whey proteins in AWPC
Similarly to the WPI solutions, there was still uncertainty about whether the effect observed were really were due to the added calcium or due to the variation of ionic strength induced by the addition of calcium chloride. Therefore, AWPC solutions were made with different level of added sodium chloride to match the ionic strength induced by the addition of calcium chloride in the previous sets of samples, and then heated at 85ºC for 5 min. Alkaline- and SDS-PAGE patterns are shown in Figure 3.20.
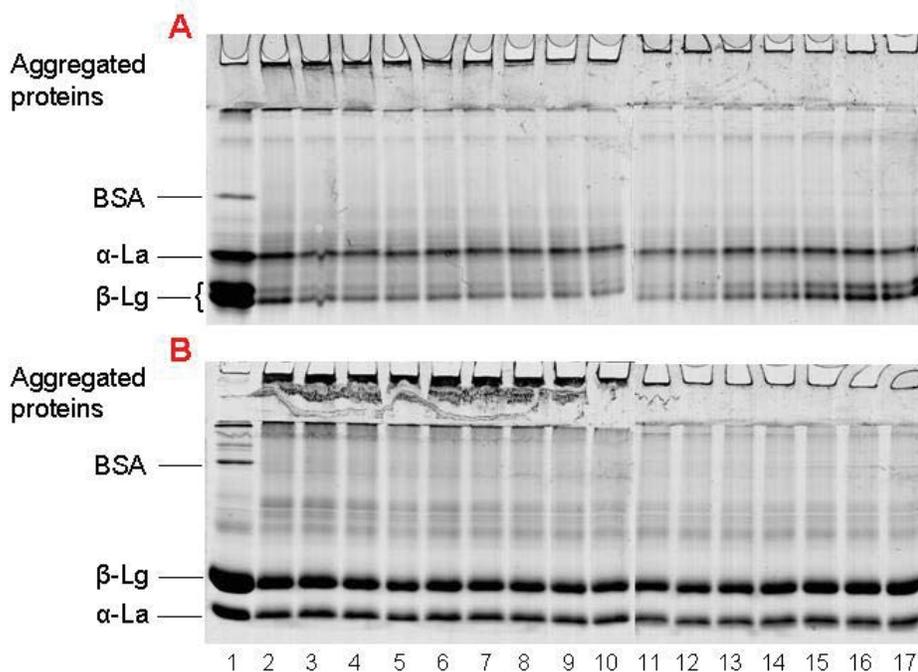
Figure 3.20: Alkaline- (**A**) and SDS- (**B**) PAGE patterns of heated (85ºC, 5 min) AWPC solutions (4%, w/w protein); lane 1: unheated, no added NaCl; lane 2: heated, no added NaCl; lanes 3–17: heated with 6, 12, 18, 24, 33, 42, 51, 60, 150, 240, 330, 420, 510, 600 and 690 mM added NaCl.

For alkaline-monomeric and SDS-monomeric α-lactalbumin and β-lactoglobulin, the alkaline-PAGE patterns with added sodium chloride (Figure 3.20) were similar to those with added calcium chloride (Figure 3.17) but the intensity of the bands was lower with added calcium than with added sodium; more aggregation took place with added calcium than with added sodium.

Interestingly, for alkaline-monomeric and SDS-monomeric bovine serum albumin it was the opposite; the bands were fainter with added sodium chloride than with added calcium chloride; more aggregation occurred with added sodium than added calcium.

Effect of temperature on the aggregation of whey proteins in AWPC

As for the WPI solutions, the effect of added calcium on the heat-induced aggregation of the whey proteins in AWPC was studied at two further heating temperatures, 75 and 80ºC, for 5 min. The alkaline- and SDS-PAGE patterns when heated at 80ºC are shown in Figure 3.21 and when heated at 75ºC in Figure 3.22.
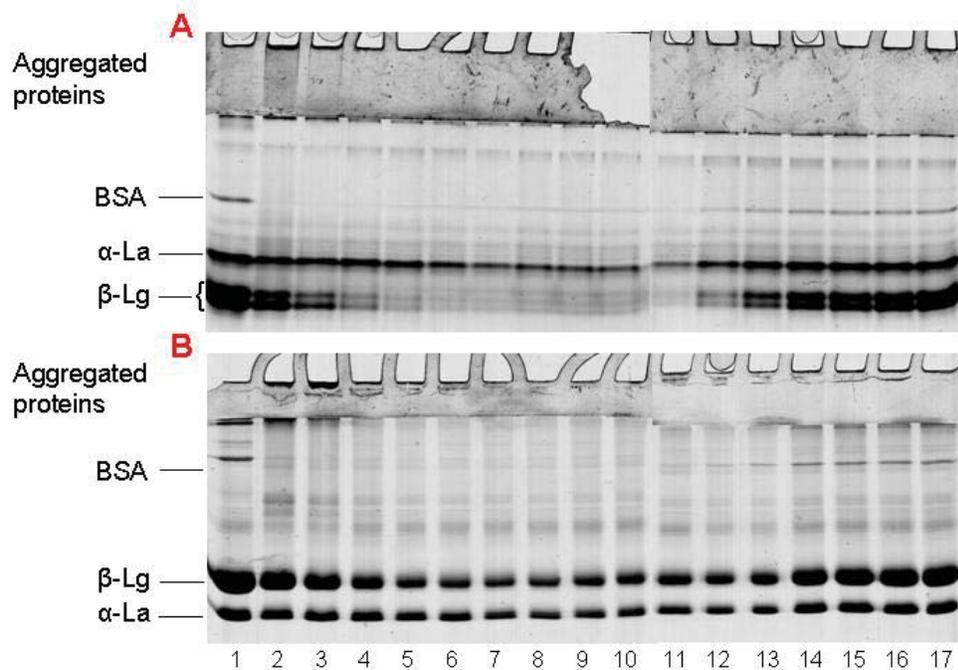
Figure 3.21: Alkaline- (**A**) and SDS- (**B**) PAGE patterns of heated (80ºC, 5 min) AWPC solutions (4%, w/w protein); lane 1: unheated, no added CaCl$_2$; lane 2: heated, no added CaCl$_2$; lanes 3–17: heated with 2, 4, 6, 8, 11, 14, 17, 20, 50, 80, 110, 140, 170, 200 and 230 mM added CaCl$_2$.

The alkaline- and SDS-PAGE patterns at 80 and 75ºC (Figures 3.21 and 3.22) were similar to those at 85ºC (Figure 3.16), but, as seen for the WPI solutions (Figures 3.1, 3.5 and 3.6), the band intensity for the α-lactalbumin, β-lactoglobulin and bovine serum albumin in AWPC were more intense at all levels of added calcium. Less aggregation took place at 80 and 75ºC than at 85ºC.
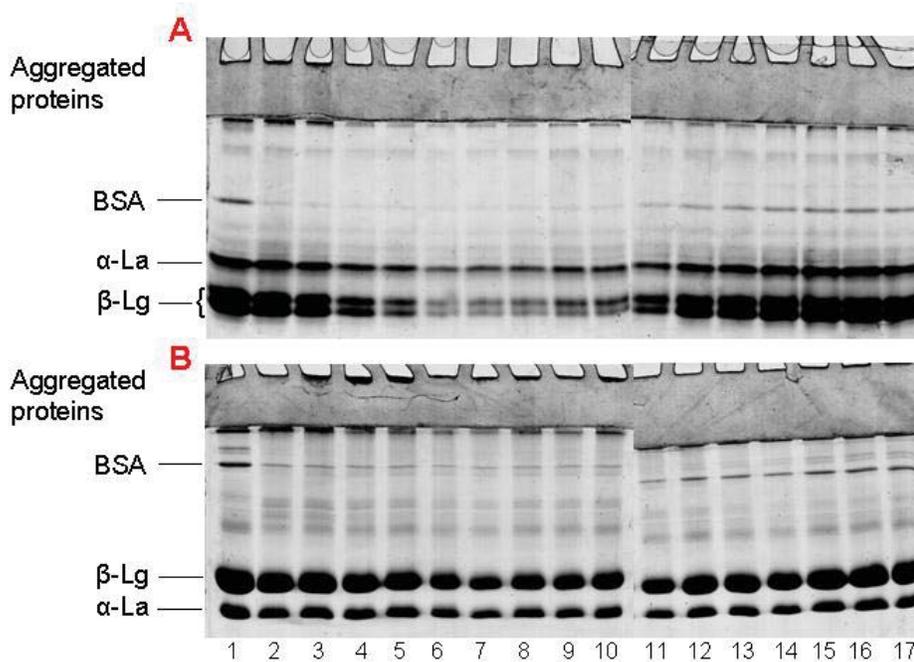
Figure 3.22: Alkaline- (**A**) and SDS- (**B**) PAGE patterns of heated (75ºC, 5 min) AWPC solutions (4%, w/w protein); lane 1: unheated, no added CaCl$_2$; lane 2: heated, no added CaCl$_2$; lanes 3–17: heated with 2, 4, 6, 8, 11, 14, 17, 20, 50, 80, 110, 140, 170, 200 and 230 mM added CaCl$_2$.

The bands for all three main whey proteins were more intense in the alkaline- and SDS-PAGE patterns at 75ºC than at 80ºC (Figures 3.22 and 3.21); again less aggregation occurred at 75ºC than at 80ºC.

Similarly to the WPI solutions, the aggregation of the whey proteins in AWPC was greater at higher temperature at all levels of added calcium.

3.3.3.2 RP-HPLC

Similarly to the investigation with WPI solutions, RP-HPLC was used to quantify the extent of aggregation of the whey proteins in AWPC. Results are shown in Figure 3.23, (**A**) α-lactalbumin only and (**B**) β-lactoglobulin only. Under heating in the presence of added calcium, α-lactalbumin and β-lactoglobulin did not aggregate at the same rate (Figure 3.23). At all three temperatures and all levels of added calcium, the extent of aggregation was higher for β-lactoglobulin than for α-lactalbumin. However, for both α-lactalbumin and β-lactoglobulin, at 75ºC, the extent of aggregation reached to a constant value (~ 10%) for the samples with 110 to 230 mM added calcium.

In the case of α-lactalbumin (Figure 3.23**A**), at all three temperatures the aggregation extent increased with increasing the levels of added calcium up to around 17- 20 mM then decreased with further increase in the addition level of calcium chloride. At all levels of added calcium, the extent of aggregation was greater at 85ºC than at 80ºC, which was itself greater than at 75ºC.
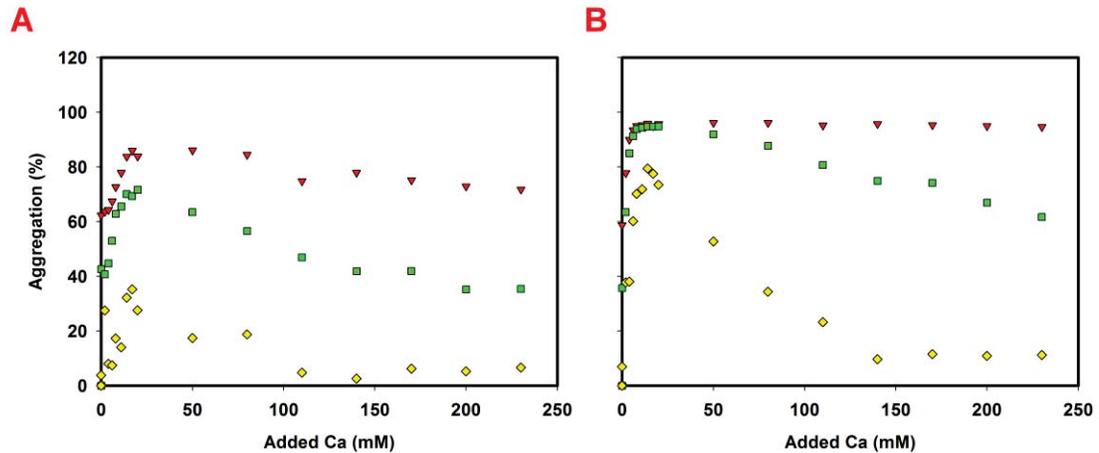
Figure 3.23: Aggregation of α-lactalbumin (**A**) and β-lactoglobulin (**B**) in heated (5 min) AWPC solutions (4%, w/w protein) quantified by RP-HPLC; ▼ 85°C, ■ 80°C, and ◇ 75°C.

For β-lactoglobulin (Figure 3.23**B**), at 85ºC, the extent of aggregation increased with increasing added calcium concentration until reaching ~ 95% aggregation (8 mM added calcium) and then plateaued at that value with further increase in the level of added calcium. At 80 and 75ºC, the extent of aggregation increased with increasing the levels of added calcium up to 20 and 14 mM added calcium respectively, and then decreased with further increase in the added calcium concentration. Similarly to α-lactalbumin, at all levels of added calcium, the extent of aggregation was greater at 85ºC than at 80ºC, which was itself greater than at 75ºC.

Figure 3.24 shows the effect of added calcium on the heat-induced aggregation of the total whey proteins in AWPC when heated for 5 min at three different temperatures, 75, 80 and 85ºC. As expected, the extents of aggregation of the whey proteins were very similar to those of β-lactoglobulin at all three temperatures and all levels of added calcium.
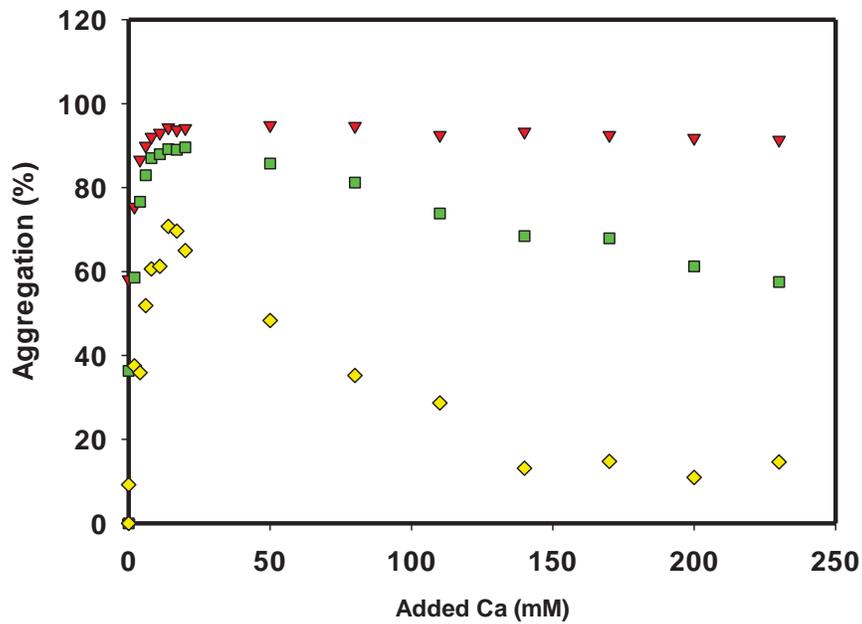
Figure 3.24: Aggregation of total whey proteins in heated (85ºC, 5 min) AWPC solutions (4%, w/w protein) quantified by RP-HPLC; ▼ 85°C, ■ 80°C, and ◆ 75°C.

Figure 3.25 shows the chromatograms obtained for AWPC solutions heated at 85, 80 and 75°C for 5 min without added calcium, and with 20 and 230 mM of added calcium, the controls being unheated solutions without added calcium.
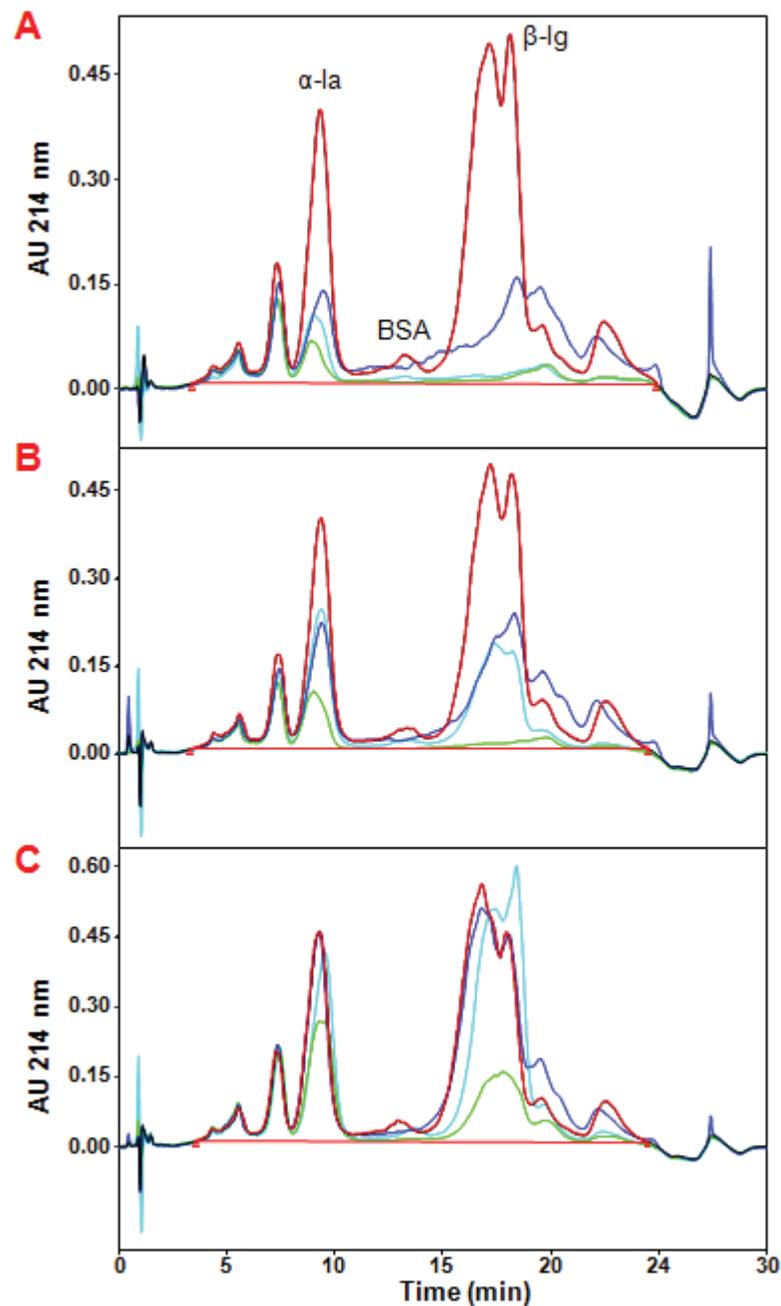
Figure 3.25: Chromatograms of heated (5 min) AWPC solutions (4%, w/w protein) at 85ºC (**A**), 80ºC (**B**) and 75ºC (**C**); — unheated, no added $CaCl_2$; — heated, no added $CaCl_2$; — heated with 20 mM added $CaCl_2$; — heated with 230 mM added $CaCl_2$.

Similarly to WPI solutions, at 85ºC (Figure 3.25**A**), the chromatogram of the heated solution without addition of calcium showed that the remaining soluble β-lactoglobulin molecules in solution underwent a lot of denaturation; the shape of the absorbance peak was dramatically different from that for the unheated sample without added calcium. With the addition of calcium, for both levels, the β-lactoglobulin absorbance peak was

almost non-existent; only a very small amount of soluble β-lactoglobulin molecules remained in solution. In the case of α-lactalbumin, the shape of the absorbance peak for all heated solutions remained the same as that of the unheated solution, but was much smaller. It seemed that the soluble α-lactalbumin molecules were still in their native state. However, it is interesting to note that the α-lactalbumin absorbance peak for the heated solution with 230 mM of added calcium was bigger than that for the heated solution with 20 mM of added calcium but smaller than that for the heated solution without added calcium.

At lower heating temperatures, 80 and 75ºC (Figures 3.25**B** and **C**), the effect of the concentration of added calcium on the heat-induced denaturation and aggregation of the whey proteins was more noticeable as the effect of the heating temperature was less dramatic, especially on β-lactoglobulin molecules. Similarly to the observations at 85ºC, α-lactalbumin and β-lactoglobulin molecules underwent some degree of denaturation and aggregation in the heated solutions without added calcium. In the heated solution with 20 mM of added calcium, the β-lactoglobulin absorbance peak mostly disappeared at 80ºC but remained reasonably prominent at 75ºC. In the heated solution with 230 mM of added calcium, the β-lactoglobulin absorbance peak at 80°C almost overlaid that of the heated solution without added calcium. Interestingly, at 75ºC the absorbance peak of the heated solutions without added calcium and with 230 mM of added calcium almost overlaid the absorbance peak of the unheated solution without added calcium. For α-lactalbumin, in the heated solution with 20 mM of added calcium, the absorbance peak was small at 80°C. At 75°C, the α-lactalbumin absorbance peak of the heated solution with 20 mM added calcium was almost half as the size of the absorbance peak of the unheated solution without added calcium. Similarly to the observation for the β-lactoglobulin absorbance peaks at 80 and 75°C, the α-lactalbumin absorbance peak of the heated solutions without added calcium and with 230 mM almost overlaid the one in the unheated solution without added calcium.

3.3.3.3 DSC

Similarly to the WPI solutions, as added calcium had an effect on the heat-induced aggregation of the whey proteins in AWPC (shown previously by PAGE and RP-HPLC), the effect of added calcium on the heat-induced denaturation was investigated

by DSC. Figure 3.26 shows the effect of added calcium on the onset and peak temperatures for the heat-induced denaturation of the whey proteins in AWPC.

The onset and peak temperatures decreased with increasing added calcium concentration up to 20 mM and then increased with further increase in the level of added calcium, but in the case of peak temperature, not to the same value as that for the control sample (without addition of calcium chloride). The peak temperature decreased by more than 6°C for the sample with 20 mM added calcium compared with the peak temperature of the control sample.
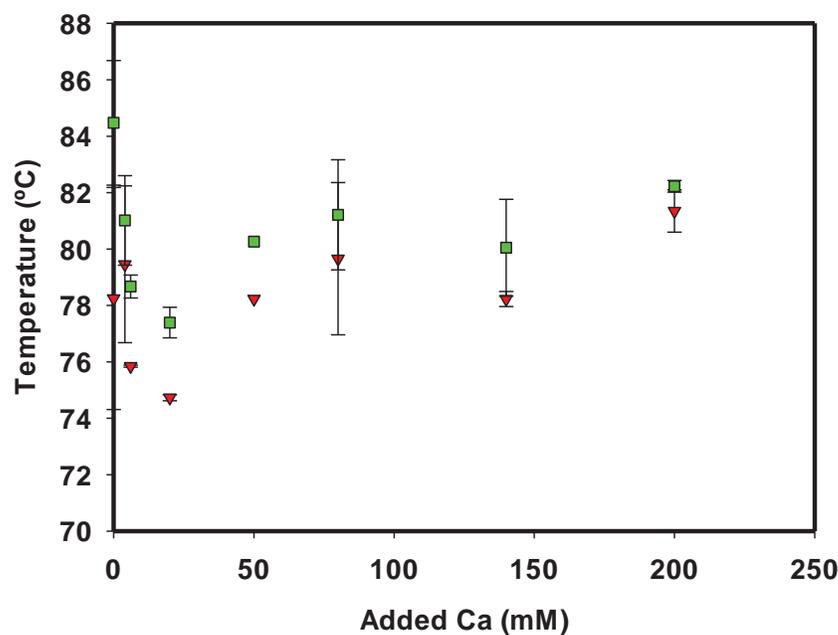
Figure 3.26: Differential scanning calorimetry temperature spectrum of AWPC solutions (4%, w/w protein) ; ▼ onset temperature; ■ peak temperature.

With regard to enthalpy of unfolding, the values were smaller and the error bars larger for AWPC solutions than for WPI solutions. The results were consequently more difficult to interpret and analyse, and therefore they are not shown and discussed in this section.

3.3.3.4 Conclusions

Same experiments were run with AWPC as with WPI solutions at the same protein concentration, 4% (w/w), and under the same experimental conditions. PAGE, RP-HPLC and DSC were used to investigate the effect of added calcium on the heat-

induced denaturation and aggregation of the whey proteins in AWPC. CD and 2D NMR experiments were not done with AWPC.

Similarly to WPI, PAGE results showed that the loss of alkaline-monomeric and SDS-monomeric β-lactoglobulin, α-lactalbumin and BSA increased with increasing added calcium concentration up to a certain level varying between 20 and 80 mM depending on the protein (whereas it was between 20 and 110 mM for WPI solutions) and then decreased with further increase in added calcium levels. The results of the experiments without and with adjustment of the pH with NaOH or HCl (Figures 3.17, 3.18 and 3.19) and those of the experiments with added calcium chloride and added sodium chloride (Figures 3.17 and 3.20) led to the conclusion that the effect observed was due only to the addition of calcium and not to the variation in pH and ionic strength. Similarly to WPI, when the temperature of heating decreased from 85 to 75ºC (Figures 3.17, 3.21 and 3.22), the loss of alkaline-monomeric and SDS-monomeric β-lactoglobulin, α-lactalbumin and bovine serum albumin in AWPC decreased. This suggested that the effect of high temperature (85°C) dominated the effect of added calcium.

In terms of quantification of the effect of added calcium on the extent of aggregation of whey proteins in AWPC, RP-HPLC experiments supported the PAGE results. In addition, RP-HPLC chromatograms also appeared to give some information on the denaturation state of the protein molecules remaining in solution; in other words, whether remaining α-lactalbumin and β-lactoglobulin molecules were in their denatured or native state was shown by the shape of the peaks on the chromatograms. Similarly to the WPI α-lactalbumin peak, the AWPC α-lactalbumin peak kept the same shape under heat treatment with and without added calcium at all three heating temperatures studied (Figure 3.25). In the case of β-lactoglobulin molecules, at 75°C, the shape of the peak is similar to that of the unheated solution. In addition, when heated without added calcium or with 230 mM of added calcium, the β-lactoglobulin peak was almost the same as the β-lactoglobulin peak in the control sample. At 80 and 85°C, the shape of the peak changed, a sign of denaturation of the β-lactoglobulin molecules remaining in solution. At all three temperatures, the profiles in the chromatograms were different from those of the WPI solutions.

Further experiments on the effect of added calcium on the heat-induced denaturation of the whey proteins were carried out using DSC. These experiments showed that the denaturation temperature varied with the level of added calcium (Figure 3.26), and the denaturation temperature reached a minimum at which the maximum extent of aggregation was observed by PAGE and RP-HPLC (Figures 3.17 and 3.25), ~ 20 mM of added calcium, as observed for WPI solutions. However, at higher levels of added calcium, the denaturation temperature increased to a value higher than that of the AWPC with 4 mM of added calcium, but not as high as the value of the denaturation temperature of the AWPC solution without added calcium (Figure 3.26), in contrast to WPI solutions (Figure 3.11).

When studying the effect of added calcium on the heat-induced denaturation and aggregation of whey proteins in different systems, some differences between AWPC and WPI were observed. These differences and their potential impact on mechanisms and discussed further in the Section 3.3.5.

3.3.4 Cheese whey protein concentrate (CWPC)

The effect of added calcium on the heat-induced denaturation and aggregation of whey proteins in CWPC was studied using the same experimental techniques as those used with WPI and AWPC.

3.3.4.1 PAGE

Similarly to the experiments with WPI and AWPC, the effect of calcium on the heat-induced aggregation of whey proteins in CWPC was investigated with 4% (w/w) protein solutions heated at 85ºC for 5 min. To demonstrate the specific effect of added calcium, the effect of pH and ionic strength were also studied at 85ºC for 5 min. Lastly, the effect of temperature combined with the effect of added calcium was investigated by repeating experiments at 75 and 80ºC.

Loss of alkaline-monomeric whey proteins in CWPC during heating

Figure 3.27 shows the alkaline- and SDS-PAGE patterns of CWPC solutions (4%, w/w protein) heated at 85ºC for 5 min in the presence of different levels of added calcium chloride.
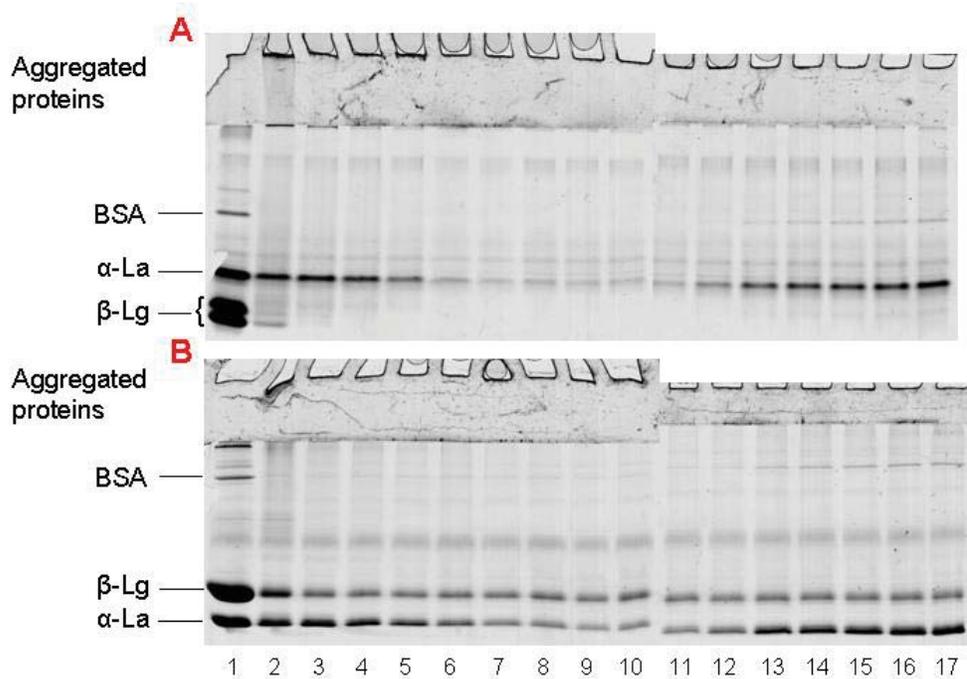
Figure 3.27: Alkaline- (**A**) and SDS- (**B**) PAGE patterns of heated (85°C, 5 min) CWPC solutions (4%, w/w protein); lane 1: unheated, no added $CaCl_2$; lane 2: heated, no added $CaCl_2$; lanes 3–17: heated with 2, 4, 6, 8, 11, 14, 17, 20, 50, 80, 110, 140, 170, 200 and 230 mM added $CaCl_2$.

Looking at the alkaline-monomeric bands (Figure 3.27**A**), the β-lactoglobulin band intensity decreased markedly in the heated sample without addition of calcium chloride (Figure 3.27**A**, lane 2). With the addition of calcium, the bands disappeared completely. Bovine serum albumin bands disappeared completely in the heated samples with 0 to 80 mM added calcium. A faint band appeared in the heated sample with 110 mM added calcium and its intensity increased with further increase in the levels of added calcium. For α-lactalbumin, the band intensity decreased with increasing levels of added calcium up to 20 mM and then increased with further increases in the added calcium concentration.

Loss of SDS-monomeric whey proteins in CWPC during heating
SDS-monomeric bovine serum albumin (Figure 3.27**B**) followed the same pattern as the alkaline-monomeric bovine serum albumin (Figure 3.27**A**). The bands disappeared completely in the heated samples with 0 to 80 mM added calcium and reappeared in the heated sample with 110 mM, added calcium with increases in their intensity with further increases in the level of added calcium. Similarly to bovine serum albumin, SDS-monomeric α-lactalbumin (Figure 3.27**B**) followed the same pattern as the

alkaline-monomeric α-lactalbumin (Figure 3.27**A**). The intensity of the bands decreased with increasing added calcium concentration up to 50 mM and then increased with further increases in the level of added calcium. As well, SDS-monomeric β-lactoglobulin (Figure 3.27**B**) followed the same pattern as the alkaline-monomeric β-lactoglobulin (Figure 3.27**A**). The band disappeared partly in the heated sample without addition of calcium chloride (Figure 3.27**B**, lane 2), and decreased markedly to an almost constant intensity in the heated samples with 2 to 230 mM added calcium (Figure 3.27**B**, lanes 3 to 17).

For α-lactalbumin and BSA, the intensity of the bands was slightly stronger for the SDS-monomeric proteins than for the alkaline-monomeric ones, comparing the samples at the same level of added calcium; bovine serum albumin and α-lactalbumin aggregation mainly involved covalent bonding. For β-lactoglobulin, the intensity of the bands was stronger in the SDS-PAGE pattern than in the alkaline-PAGE pattern; aggregation of β-lactoglobulin involved a mixture of non-covalent and covalent bonding.

Effect of pH shifting on the aggregation of whey proteins in CWPC

When calcium chloride was added to the CWPC solutions, the pH decreased, from 6.8 without addition of calcium chloride to 6.36 with 230 mM added calcium. To investigate if the effect on the heat-induced aggregation observed previously was due to the addition of calcium chloride as such or to the decrease in pH cause by the addition of the salt, two further sets of samples were prepared: the first was CWPC solutions with 0 to 230 mM added calcium and with the pH adjusted back to the control sample pH of 6.7 with 1 M NaOH solution, and the second CWPC solutions with no addition of calcium chloride but with the addition of 1 M HCl solution to adjust the pH of the different samples to the same pH as the samples with different levels of added calcium chloride. Both sets were then heated at 85°C for 5 min and analysed by alkaline- and SDS-PAGE. The results are shown in Figure 3.28 and Figure 3.29.
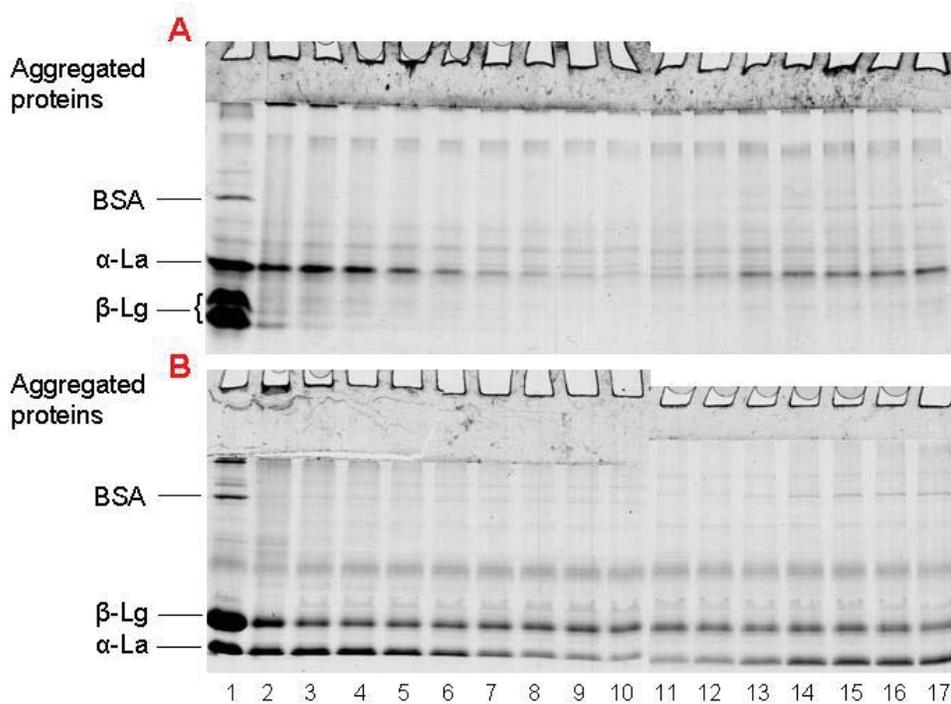
Figure 3.28: Alkaline- (**A**) and SDS- (**B**) PAGE patterns of heated (85ºC, 5 min) CWPC solutions (4%, w/w protein) and with the pH adjusted to 6.80 with NaOH; lane 1: unheated, no added $CaCl_2$; lane 2: heated, no added $CaCl_2$; lanes 3–17: heated with 2, 4, 6, 8, 11, 14, 17, 20, 50, 80, 110, 140, 170, 200 and 230 mM added $CaCl_2$ and pH adjusted to 6.70 with NaOH.

The alkaline- and SDS-PAGE patterns of the CWPC solutions with added calcium chloride and without (Figure 3.27) and with (Figure 3.28) adjustment of the pH to 6.8 were very similar. Hardly any difference was seen for the three main whey proteins, α-lactalbumin, β-lactoglobulin and bovine serum albumin. When looking at the alkaline- and SDS-PAGE patterns of the CWPC solutions with added HCl (Figure 3.29), they were very different from those of the CWPC solutions with added calcium chloride. The alkaline-monomeric β-lactoglobulin band disappeared markedly in the heated sample at pH 6.8 (Figure 3.29**A**, lane 2) and then the intensity decreased slightly when decreasing pH to pH 6.36 (Figure 3.29**A**, lane 10). The SDS-monomeric β-lactoglobulin band also disappeared significantly in the heated sample at pH 6.8 (Figure 3.29**B**, lane 2) but then the intensity remained the same for all heated samples even though the pH was decreasing (Figure 3.29**B**, lanes 3 to 10). The intensity of the alkaline and SDS-monomeric α-lactalbumin bands partly decreased in the heated samples at pH 6.8 (Figure 3.29, lane 2) and then increased with decreasing pH to pH 6.36 (Figure 3.29,

lane 10). Alkaline- and SDS-monomeric bovine serum albumin bands disappeared in all heated samples, regardless of the pH (Figure 3.29, lanes 2 to 10). The PAGE patterns for α-lactalbumin and bovine serum albumin in CWPC solutions with added HCl (Figure 3.29) were different from those with added calcium chloride (Figure 3.27).
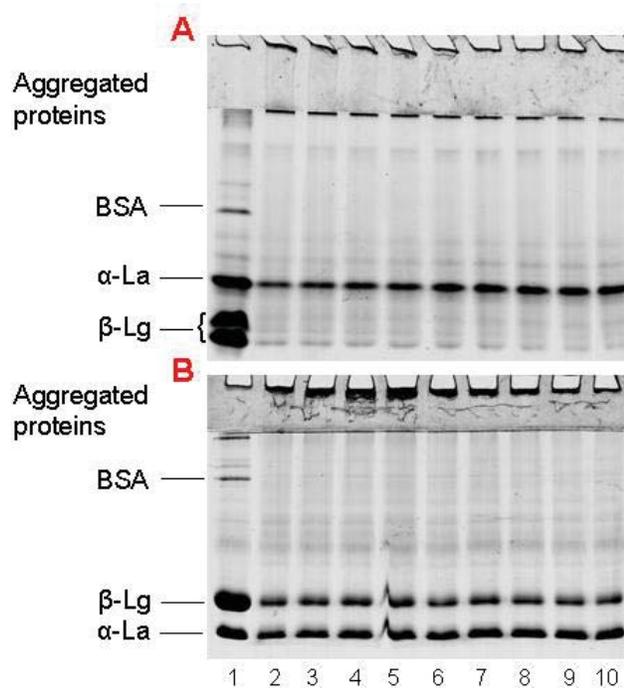
Figure 3.29: Alkaline- (**A**) and SDS- (**B**) PAGE patterns of heated (85ºC, 5 min) CWPC solutions (4%, w/w protein); lane 1: unheated, no added HCl; lane 2: heated, no added HCl; lanes 3–10: heated with added HCl to adjust pH to 6.76, 6.69, 6.65, 6.54, 6.49, 6.45, 6.40 and 6.36.

Comparing these three sets of CWPC solutions led to the conclusion that the effect on the heat-induced aggregation of the whey proteins was due to the addition of calcium ions and not to the shift in pH caused by this addition.

Effect of sodium chloride on the aggregation of whey proteins in CWPC
Similarly to the pH adjustment experiments, the hypothesis that the effect of added calcium on the heat-induced aggregation of the whey proteins in CWPC was due to the ionic strength change and not the calcium ions as such was assumed. To test this hypothesis, another set of CWPC solutions was made with the addition of sodium chloride at levels to match the ionic strength of the samples containing added calcium chloride. Figure 3.30 shows the alkaline- and SDS-PAGE patterns of the CWPC solutions heated at 85°C for 5 min in the presence of added sodium chloride.
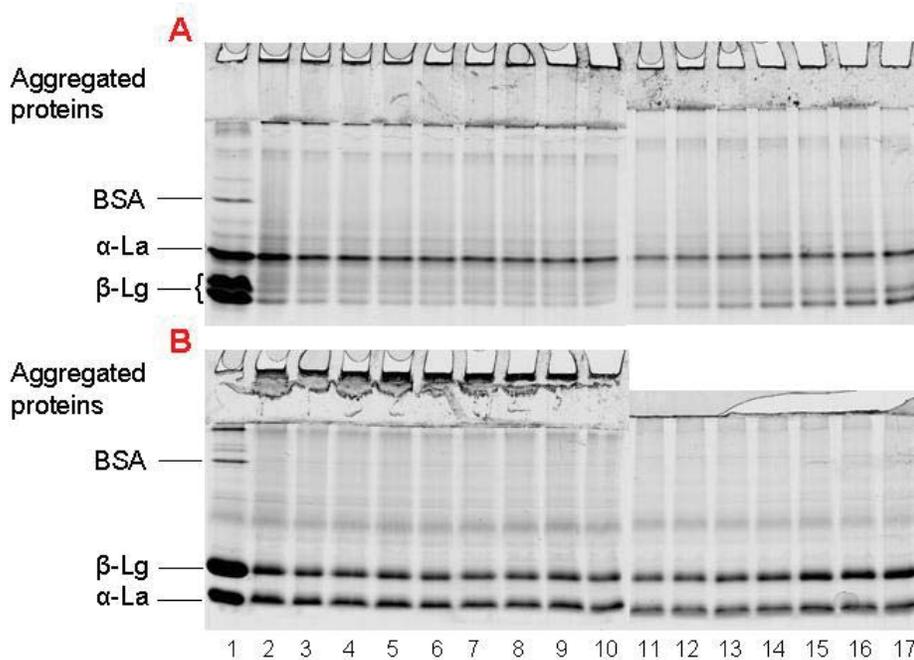
Figure 3.30: Alkaline- (**A**) and SDS- (**B**) PAGE patterns of heated (85°C, 5 min) CWPC solutions (4%, w/w protein); lane 1: unheated, no added NaCl; lane 2: heated, no added NaCl; lanes 3–17: heated with 6, 12, 18, 24, 33, 42, 51, 60, 150, 240, 330, 420, 510, 600 and 690 mM added NaCl.

Even at equivalent added ionic strength, the alkaline- and SDS-PAGE patterns of the CWPC solutions with added sodium were very different from those of the solutions with added calcium. Both alkaline- and SDS-monomeric bovine serum albumin bands disappeared in all heated samples (Figure 3.30, lanes 2 to 17). Alkaline- and SDS-PAGE α-lactalbumin and β-lactoglobulin band intensity decreased in the heated sample with added sodium (Figure 3.30, lane 2), then decreased with increasing levels of added sodium up to 60 - 150 mM, and then increased with further increase of the levels of added sodium. However, the intensity of the bands was greater than for the samples with added calcium.

The effect of added calcium on the heat-induced aggregation of the whey proteins in CWPC was specific to the calcium ions and not to the change in ionic strength in the solutions.

Effect of temperature on the aggregation of whey proteins in CWPC
Similarly to the WPI and AWPC solutions, the effect of the heating temperature on the heat-induced aggregation of the whey proteins in CWPC solutions in the presence of

different levels of added calcium chloride was investigated. Figure 3.31 and 3.32 show the alkaline- and SDS-PAGE patterns of the CWPC solutions with added calcium chloride heated at 80 and 75°C (5 min) respectively.
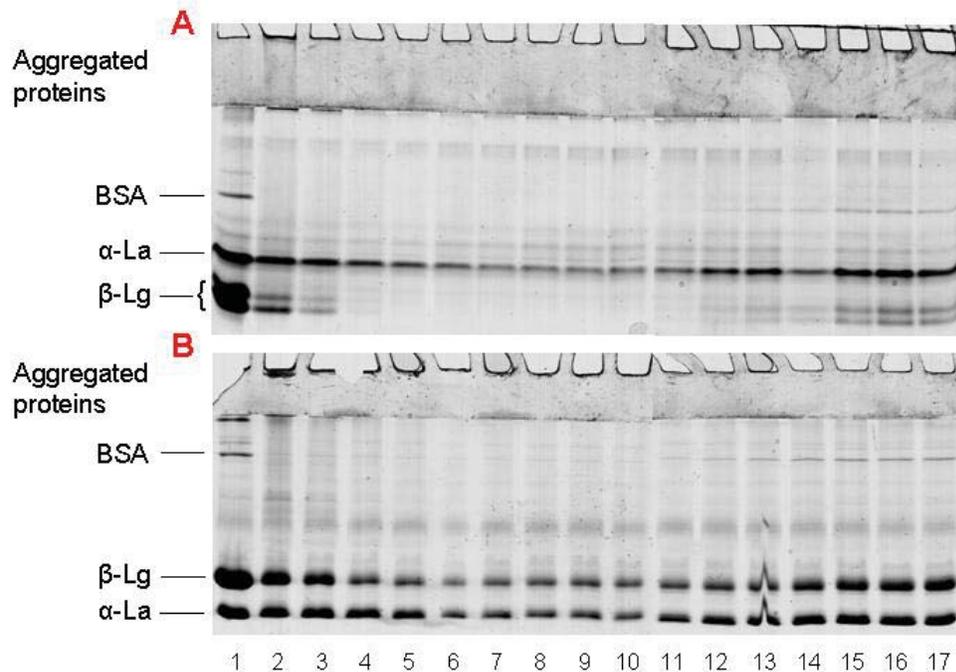
Figure 3.31: Alkaline- (**A**) and SDS- (**B**) PAGE patterns of heated (80°C, 5 min) CWPC solutions (4%, w/w protein); lane 1: unheated, no added $CaCl_2$; lane 2: heated, no added $CaCl_2$; lanes 3–17: heated with 2, 4, 6, 8, 11, 14, 17, 20, 50, 80, 110, 140, 170, 200 and 230 mM added $CaCl_2$.

The alkaline- and SDS-PAGE patterns were similar to those of the CWPC solutions heated at 85°C, but the intensity of the bands was greater at 75°C (Figure 3.32) than at 80°C (Figure 3.31), and that at 80°C greater than at 85°C (Figure 3.27).

At 80°C, the alkaline-monomeric bovine serum albumin bands completely disappeared in the heated samples with 0 to 50 mM added calcium chloride (Figure 3.31**A**, lanes 2 to 11), reappeared in the heated sample with 80 mM added calcium, and then band intensity increased with further addition of calcium chloride. There was a similar pattern for SDS-monomeric bovine serum albumin, except that the bands markedly disappeared in the heated samples with 0 to 20 mM added calcium (Figure 3.31**B**, lanes 2 to 10), and then the intensity increased with further increase in the levels of added calcium. Both alkaline- and SDS-monomeric α-lactalbumin bands intensity decreased with increasing the levels of added calcium up to 20 mM (Figure 3.31, lanes 2 to 10), and then the

intensity increased with further increase in the levels of added calcium. For both bovine serum albumin and α-lactalbumin, the bands were slightly more intense in the SDS-PAGE pattern than in the alkaline-PAGE one; bovine serum albumin and α-lactalbumin were mainly aggregated via covalent bonding. Alkaline-monomeric β-lactoglobulin bands markedly decreased in the heated samples with 0 and 2 mM added calcium (Figure 3.31**A**, lanes 2 and 3), completely disappeared in the heated samples with 4 to 20 mM added calcium (Figure 3.31**A**, lanes 4 to 10), and then their intensity increased with further increase in the levels of added calcium (Figure 3.31**A**, lanes 11 to 17). The SDS-monomeric pattern was similar, the band intensity of the heated samples decreasing with increasing levels of added calcium up to 20 mM (Figure 3.31**B**, lanes 2 to 10), and then increasing with further increase in the added calcium concentration (Figure 3.31**B**, lanes 11 to 17). The bands intensity was stronger for the SDS-monomeric β-lactoglobulin than for the alkaline-monomeric β-lactoglobulin at all levels of added calcium; β-lactoglobulin aggregated via a large amount of non-covalent bonding as well as covalent bonding.
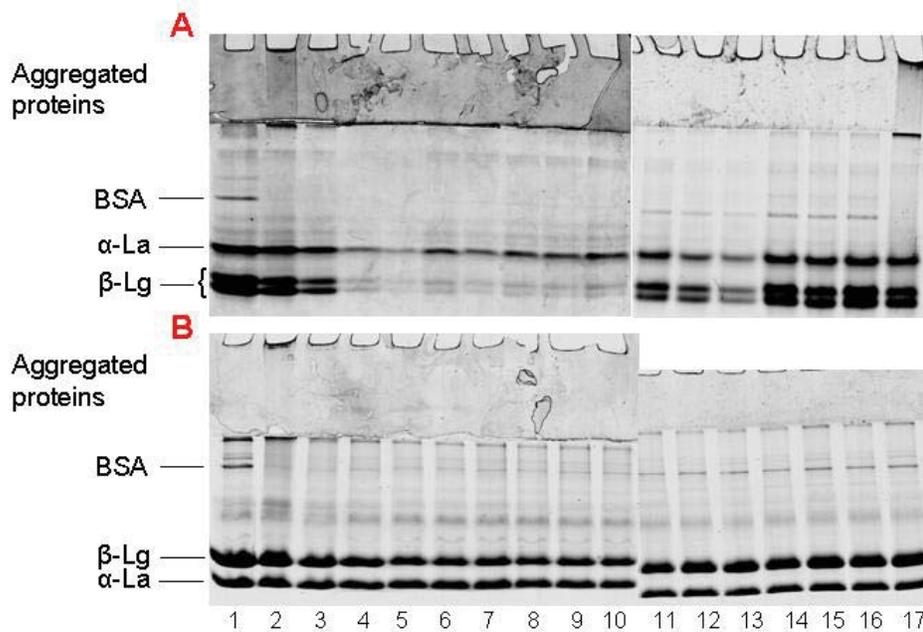
Figure 3.32: Alkaline- (**A**) and SDS- (**B**) PAGE patterns of heated (75ºC, 5 min) CWPC solutions (4%, w/w protein); lane 1: unheated, no added CaCl$_2$; lane 2: heated, no added CaCl$_2$; lanes 3–17: heated with 2, 4, 6, 8, 11, 14, 17, 20, 50, 80, 110, 140, 170, 200 and 230 mM added CaCl$_2$.

The alkaline- and SDS-PAGE patterns at 75°C were very similar to those at 80°C (Figures 3.32 and 3.31), but the band intensity was greater at 75°C than at 80°C. The

extent of aggregation was less at 75°C (Figure 3.32) than at 80°C (Figure 3.31), which was less than at 85°C (Figure 3.27).

3.3.4.2 RP-HPLC

The combined effect of the heating temperature and the added calcium on the heat-induced aggregation of the whey proteins in CWPC solutions was also investigated by RP-HPLC. The results for α-lactalbumin and β-lactoglobulin individually are shown in Figure 3.33.
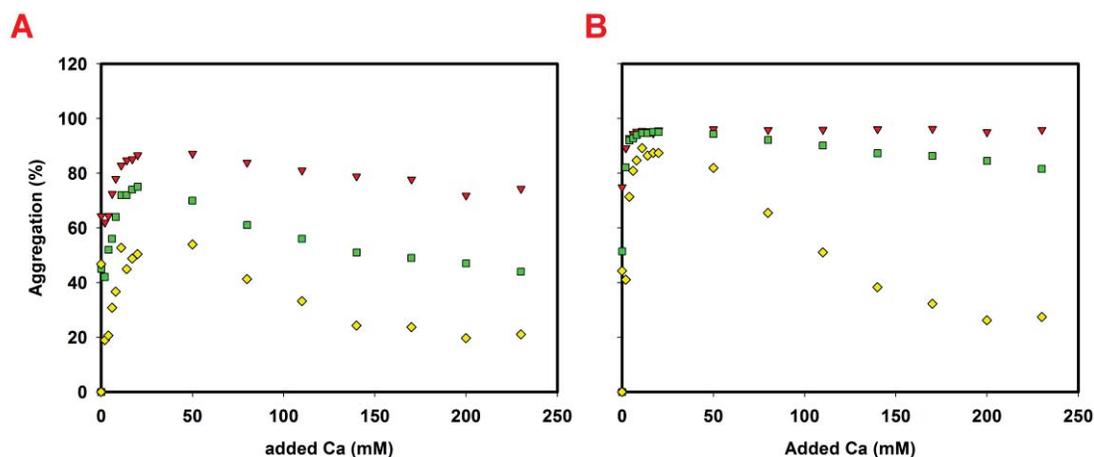
Figure 3.33: Aggregation of α-lactalbumin (**A**) and β-lactoglobulin (**B**) in heated (5 min) CWPC solutions (4%, w/w protein) quantified by RP-HPLC; ▼ 85°C, ■ 80°C, and ◇ 75°C.

The extent of aggregation at all three temperatures and all levels of added calcium was greater for β-lactoglobulin than for α-lactalbumin. However for both proteins, the extent of aggregation was greater at 85°C than at 80°C, itself greater than at 75°C. In the case of α-lactalbumin (Figure 3.33**A**), at the three temperatures, the extent of aggregation increased dramatically with increasing levels of added calcium, reaching its maximum in the sample with 20 mM added calcium, and then decreasing slowly with further increase in the levels of added calcium. At 75°C, in the samples with 110 to 230 mM, the extent of aggregation reached a constant value, ~ 20%. In the case of β-lactoglobulin (Figure 3.33**B**), at 85°C, the extent of aggregation increased dramatically with increasing levels of added calcium. It reached its maximum of 96% in the sample with 8 mM added calcium and then plateaued at that value with further increase in the level of added calcium. At 80 and 75°C, the extent of aggregation increased quickly with increasing levels of added calcium up to 11 mM, reaching maxima of 95 and 87%

aggregation respectively. Then, the extent of aggregation decreased with further increase in the added calcium concentrations. However, at 80°C the decrease in the extent of aggregation was much slower than at 75°C, reaching 82% compared with 17% at 75°C.

Figure 3.34 shows the extent of aggregation of the total whey proteins when CWPC solutions were heated at three different temperatures, 75, 80 and 85°C for 5 min in the presence of different levels of added calcium.
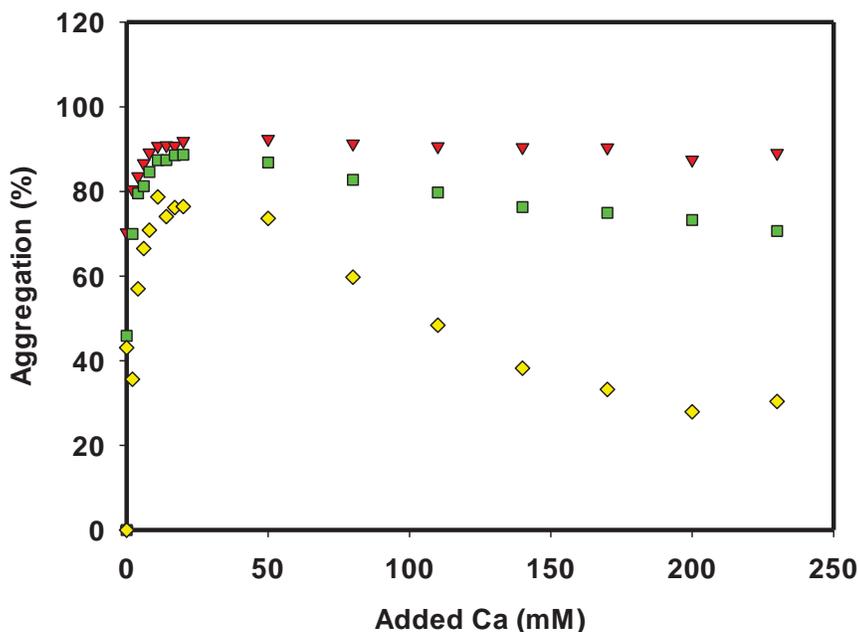
Figure 3.34: Aggregation of total whey proteins in heated (5 min) CWPC solutions (4%, w/w protein) quantified by RP-HPLC; ▼ 85°C, ■ 80°C, and ◇ 75°C.

As expected, the extent of aggregation of the total whey proteins was similar to that of β-lactoglobulin alone at all three temperatures and all added calcium concentrations. However, at 85°C, the extent of aggregation did not plateau at the maximum value (92% was reached in the heated sample with 20 mM added calcium) but slightly decreased with further increase in the levels of added calcium to 89% aggregation.

Figure 3.35 shows the chromatograms obtained for CWPC solutions heated at 85, 80 and 75°C for 5 min without added calcium, and with 20 and 230 mM of added calcium, the control being the unheated solution without added calcium.
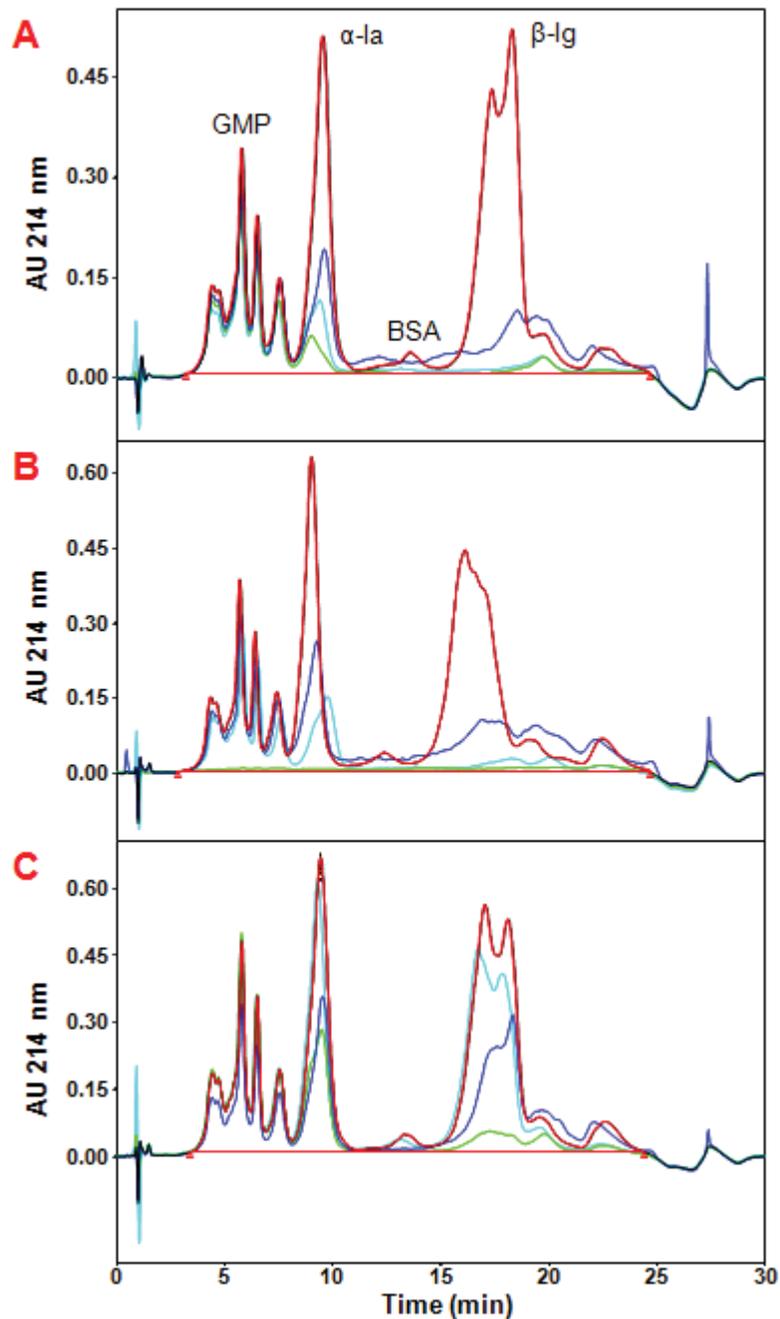
Figure 3.35: Chromatograms of heated (5 min) CWPC solutions (4%, w/w protein) at 85°C (**A**), 80°C (**B**) and 75°C (**C**); — unheated, no added $CaCl_2$; — heated, no added $CaCl_2$; — heated with 20 mM added $CaCl_2$; — heated with 230 mM added $CaCl_2$.

Similarly to the WPI and AWPC solutions, at 85°C (Figure 3.35**A**), the chromatogram of the heated solution without addition of calcium showed that the remaining soluble β-lactoglobulin molecules in solution underwent a lot of denaturation; the shape of the absorbance peak was dramatically different to that of the unheated sample without added calcium. With addition of calcium, for both levels, the β-lactoglobulin

absorbance peak was almost non-existent; only a very small amount of soluble β-lactoglobulin molecules remained in solution. In the case of α-lactalbumin, the shape of the absorbance peak for all heated solutions remained the same as that of the unheated solution, but was much smaller. It seemed that the soluble α-lactalbumin molecules were still in their native state after heat treatment. However, it is interesting to note that the α-lactalbumin absorbance peak of the heated solution with 230 mM of added calcium was bigger than that of the heated solution with 20 mM of added calcium but smaller than that of the heated solution without added calcium.

At lower heating temperature, 80 and 75ºC (Figures 3.35**B** and **C**), the effect of the concentration of added calcium on the heat-induced denaturation and aggregation of the whey proteins was more noticeable as the effect of the heating temperature was less dramatic, especially with respect to β-lactoglobulin. Similarly to the observation at 85ºC, α-lactalbumin and β-lactoglobulin molecules underwent some degree of denaturation and aggregation in the heated solutions without added calcium. In the heated solution with 20 mM of added calcium, the β-lactoglobulin absorbance peak mostly disappeared at 80ºC but was reasonably large at 75ºC. In the heated solution with 230 mM of added calcium, the β-lactoglobulin absorbance peak at 80°C almost disappeared, overlaying that of the heated solution with 20 mM added calcium. At 75ºC, there was a gradual decrease in the absorbance peak from the unheated solution without added calcium to the heated solution with 230 mM of added calcium, then to the heated solution without added calcium, and finally to the heated solution with 20 mM added calcium. For α-lactalbumin, in the heated solution with 20 mM of added calcium, the absorbance peak completely disappeared at 80°C. The absorbance peak of the heated solution without 230 mM added calcium did not overlay that of the heated solution without added calcium. At 75°C, the α-lactalbumin absorbance peak of the heated solution with 20 mM added calcium almost overlaid that of the heated solution without added calcium, and that of the heated solution with 230 mM almost overlaid that of the unheated solution without added calcium.

3.3.4.3 DSC

PAGE and RP-HPLC results showed that there was an effect of added calcium on the heat-induced aggregation of the whey proteins in the CWPC solutions. As reported in the previous sections (3.3.2.3 and 3.3.3.3), DSC experiments showed that added

calcium affected the heat-induced denaturation of the whey proteins in WPI and AWPC solutions. Therefore the effect of added calcium on the heat-induced denaturation of the whey proteins in CWPC solutions was investigated by DSC. Results for the onset and peak temperatures are shown in Figure 3.36.

The onset and peak temperatures decreased with increasing levels of added calcium up to 20 mM and the increased with further increase in the added calcium concentration to reach onset and peak temperatures higher than those of the control sample (without addition of calcium). The peak temperature of the sample with 20 mM added calcium was about 7ºC less than that of the control sample.
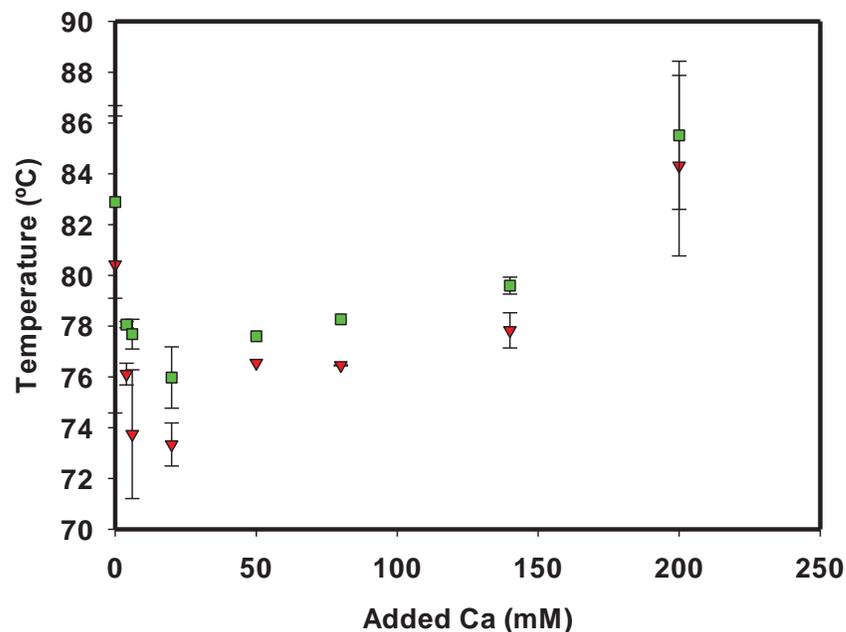
Figure 3.36: Differential scanning calorimetry temperature spectrum of CWPC solutions (4%, w/w protein); ▼ onset temperature; ■ peak temperature.

Regarding enthalpy of unfolding, the values were smaller and the error bars were bigger for CWPC solutions than for WPI solutions. The results were then more difficult to interpret and analyse, and therefore they are not shown and discussed in this section.

3.3.4.4 Conclusions

The same experiments were run with CWPC as with the WPI and AWPC solutions, at same protein concentration, 4% (w/w), under the same experimental conditions. PAGE, RP-HPLC and DSC were used to investigate the effect of added calcium on the heat-

induced denaturation and aggregation of the whey proteins in AWPC. Again, CD and 2D NMR experiments were not done with CWPC solutions because of the poor information obtained in the case of the WPI solutions.

Similarly to WPI and AWPC, PAGE results showed that the loss of alkaline-monomeric and SDS-monomeric α-lactalbumin and BSA increased with increasing added calcium concentration up to a certain level varying between 20 and 80 mM depending on the protein and then decreased with further increase in added calcium levels. However, in contrast to WPI and AWPC solutions, the loss of alkaline-monomeric and SDS-monomeric β-lactoglobulin increased dramatically with only a small addition of calcium (~ 4 mM), and then remained at that level with levels of added calcium increasing up to 230 mM. As for WPI and AWPC, from the results of the experiments without and with adjustment of the pH with NaOH or HCl (Figures 3.27, 3.28 and 3.29), and those of the experiments with added calcium chloride and added sodium chloride (Figures 3.27 and 3.30), it was concluded that the effect observed was due only to the addition of calcium and not to the variation in pH and ionic strength. Regarding the results of the experiments at different heating temperatures (Figures 3.37, 3.31 and 3.32), over the range of added calcium levels, the loss of alkaline-monomeric and SDS-monomeric α-lactalbumin and bovine serum albumin in CWPC decreased when heating temperature decreased from 85 to 75ºC. In the case of alkaline-monomeric and SDS-monomeric β-lactoglobulin, the trends observed at 80 and 75°C were different from that observed at 85°C for β-lactoglobulin. At 80 and 75°C, the loss of alkaline-monomeric and SDS-monomeric β-lactoglobulin increased with increasing added calcium levels up to 50 and 20 mM, respectively, then decreased with further increase in added calcium levels.

As for WPI and AWPC, the quantification obtained by means of RP-HPLC experiments supported the PAGE results, and the shape of the peaks on the RP-HPLC chromatograms gave some information as to whether α-lactalbumin and β-lactoglobulin molecules remaining in solutions were in their denatured or native state. Similarly to WPI and AWPC solutions, remaining α-lactalbumin molecules appeared to be in their native state, as the peak kept the same shape under heat treatment with and without added calcium at all three heating temperatures studied (Figure 3.35). In contrast, β-lactoglobulin molecules appeared to be in their denatured state at 80 and 85°C for all

levels of added calcium, and in the solution with 20 mM of added calcium when heated at 75°C (Figure 3.35**C**). This was different from the WPI and AWPC solutions where even though much protein was aggregated at 20 mM added calcium, the remaining molecules in solution appeared to be in their native state (Figures 3.11**C** and 3.25**C**).

Further observations of the effect of added calcium on the heat-induced denaturation of the whey proteins were carried out using DSC. As observed for WPI and AWPC, these experiments demonstrated that the denaturation temperature varied with the level of added calcium (Figure 3.36). The denaturation temperature reached a minimum at which the maximum extent of aggregation was observed by PAGE and RP-HPLC (Figures 3.27 and 3.35), ~ 20 mM of added calcium. In contrast to WPI and AWPC solutions, at higher levels of added calcium, the denaturation temperature increased to a higher value than the denaturation temperature of the CWPC solution without added calcium (Figure 3.36).

When studying the effect of added calcium on the heat-induced denaturation and aggregation of whey proteins in different systems, some differences between AWPC and WPI were observed. These differences and their potential impact on mechanisms are discussed in the following section.

3.3.5 Comparison of the three whey protein systems: WPI, AWPC and CWPC

Overall, the effect of added calcium chloride on the heat-induced denaturation and aggregation of the whey proteins seemed to be similar in WPI, AWPC and CWPC solutions. It would be easy to assume that these three whey protein systems would have similar properties and functionality under the experimental conditions studied. However, it is important to determine the differences, even if small, in the behaviour of the whey proteins from different protein systems as these differences might impact the final products after processing. In the following discussion, the relatively small differences between the three protein systems will be highlighted by comparing directly the results from the same types of experiments.

3.3.5.1 PAGE

The PAGE patterns showing the effect of added calcium on the heat-induced aggregation of whey proteins in WPI, AWPC and CWPC followed similar trends. The

extent of aggregation increased with increasing levels of added calcium up to a critical added calcium concentration, and then decreased with further increase in levels of added calcium. The critical added calcium concentration was the added calcium concentration at which maximal aggregation occurred. This critical concentration was between 20 and 80 mM added calcium for the three whey protein systems studied. From the quantification of the PAGE band intensities, the relative amounts of native proteins, proteins aggregated by disulphide bonds and proteins aggregated by hydrophobic interactions could be established as shown in Figure 3.37 (Havea, Singh, Creamer, & Campanella, 1998). The different types of protein-protein interactions during aggregation might give some understanding of the types of aggregation occurring when WPI, AWPC and CWPC solutions were heated in the presence of added calcium.
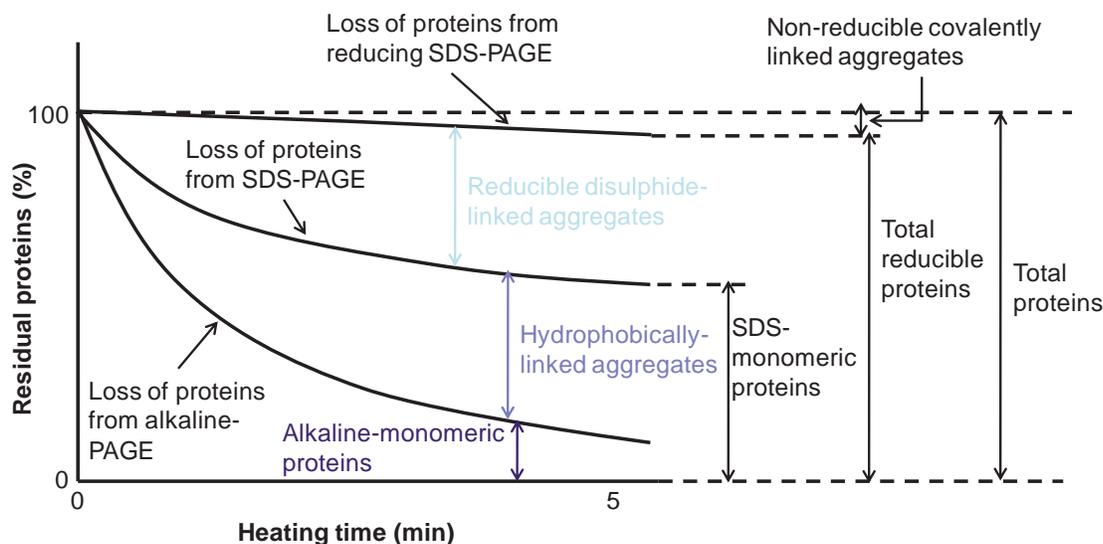
Figure 3.37: Schematic representation of the relationship between the relative amounts of different forms of proteins (e.g. `alkaline-monomeric', `SDS-monomeric', `total reducible') in heated whey protein concentrate solutions; reproduced from Havea, Singh, Creamer, and Campanella (1998).

Comparison at 85⁰C

Comparison at 85ºC

Figure 3.38 shows the different types of β-lactoglobulin aggregates formed in WPI (**A**), AWPC (**B**) and CWPC (**C**) heated at 85°C for 5 min. For the three systems, heat treatment had a great effect on the aggregation of β-lactoglobulin; > 80% of β-lactoglobulin molecules aggregated. Interestingly, less native-like β-lactoglobulin molecules remained in CWPC than in WPI, with the residual native β-lactoglobulin proportion being intermediate between the proportions in WPI and AWPC. Similar amounts of disulphide-linked aggregates were observed in WPI and AWPC solutions

without added calcium, but a higher amount of disulphide-linked aggregates was observed in CWPC. Thus, a lower amount of hydrophobically-linked aggregates were present in CWPC than in AWPC, which was itself slightly larger than in WPI.
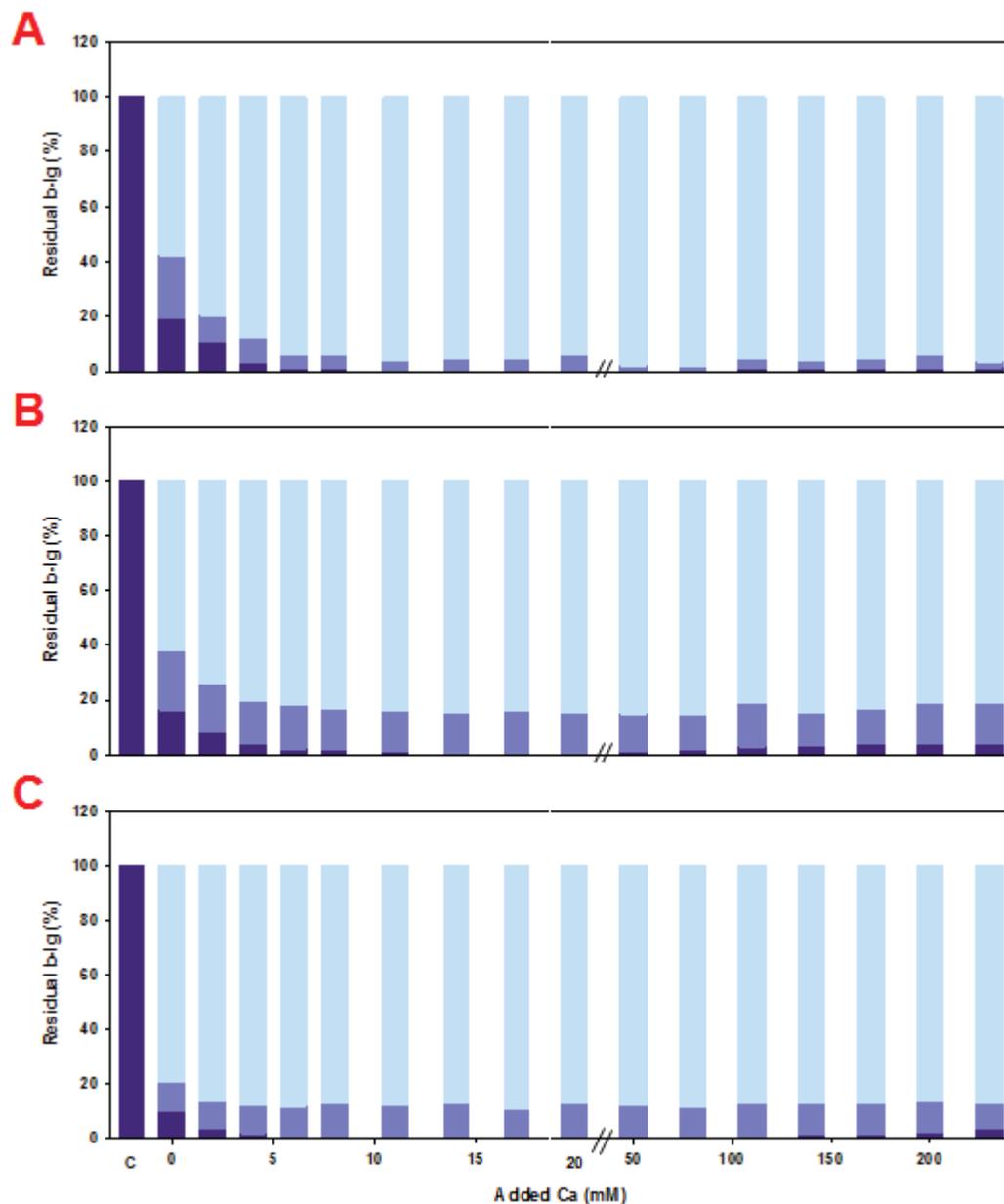
Figure 3.38: β-lactoglobulin aggregate types in heated (85°C, 5 min) whey protein solutions (4%, w/w protein): (**A**) WPI, (**B**) AWPC, and (**C**) CWPC, quantified by PAGE; ▉ alkaline-monomeric proteins (native-like), ▉ hydrophobically-linked aggregates, ▢ disulphide-linked aggregates, **C** control sample (unheated without added $CaCl_2$).

As seen previously, addition of calcium enhanced the extent of aggregation of β-lactoglobulin. In the case of WPI that appeared as a large increase in disulphide-linked

aggregates at the expense of hydrophobically-linked aggregates and residual native-like molecules. In contrast, for AWPC and CWPC, the proportions of hydrophobically-linked aggregates appeared to remain similar over the range of added calcium levels, and the disappearance of native-like β-lactoglobulin resulted in a slight increase in disulphide-linked aggregates. However, the proportions of hydrophobically-linked aggregates in AWPC were slightly but consistently higher than in CWPC.

Comparison at 80⁰C

Figure 3.39 shows the different types of β-lactoglobulin aggregates formed in WPI (**A**), AWPC (**B**) and CWPC (**C**) heated at 80°C for 5 min. In solutions without addition of calcium, the effect of heat treatment at 80°C on the aggregation of β-lactoglobulin was lower than at 85°C but still more than half of the initial amount of β-lactoglobulin aggregated. More aggregation occurred in CWPC than in AWPC and WPI. However, similar proportions of hydrophobically-linked aggregates were observed for AWPC and CWPC, and a larger amount for WPI. This resulted in a greater amount of disulphide-linked aggregates in CWPC than in AWPC, which was itself larger than in WPI.

The addition of calcium enhanced the extent of aggregation and, because of the lower temperature, it was possible to detect the differences in the effect of added calcium caused by changes in its concentration. The extent of aggregation increased with increase in added calcium levels to a maximum and then decreased with further increase in added calcium levels. Over the range of added calcium concentrations, the extent of aggregation was less for AWPC than for WPI and CWPC. At low and high added calcium levels, there appeared to be more aggregation in CWPC than in WPI, but at intermediate levels (10 < x < 50 mM), it was the opposite. For WPI, the increase in aggregated molecules due to the addition of calcium resulted in larger amounts of disulphide-linked aggregates at the expense of native-like proteins and hydrophobically-linked aggregates. For CWPC, less hydrophobically-linked aggregates were formed at high levels of added calcium (> 50 mM). Therefore, at lower added calcium levels (< 50 mM), the increase in aggregated molecules on calcium addition was through disulphide-bonding. CWPC and WPI were more similar in behaviour than AWPC and WPI.
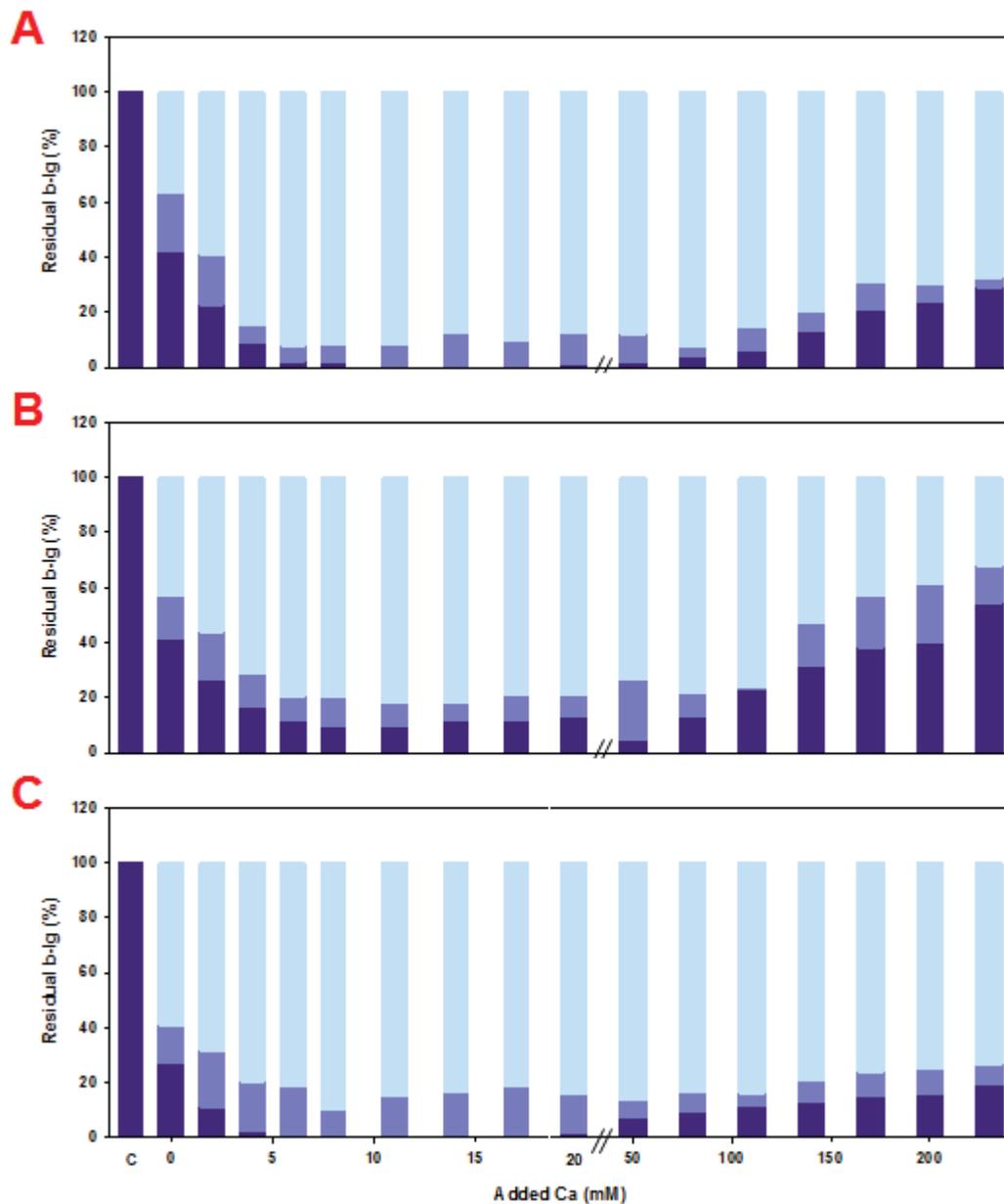
Figure 3.39: β-lactoglobulin aggregate types in heated (80°C, 5 min) whey protein solutions (4%, w/w protein): (**A**) WPI, (**B**) AWPC, and (**C**) CWPC, quantified by PAGE; ■ alkaline-monomeric proteins (native-like), ■ hydrophobically-linked aggregates, ■ disulphide-linked aggregates, **C** control sample (unheated without added $CaCl_2$).

Comparison at 75°C

Figure 3.40 shows the different types of β-lactoglobulin aggregates formed in WPI (**A**), AWPC (**B**) and CWPC (**C**) heated at 75°C for 5 min. The effect of heat treatment on the aggregation of β-lactoglobulin was less at 75°C than at 80°C and thus at 85°C, which allowed a better understanding of the effects of added calcium as these depended on

calcium concentrations. Again, at all levels of added calcium there was less aggregation in AWPC solutions than in WPI and CWPC solutions.
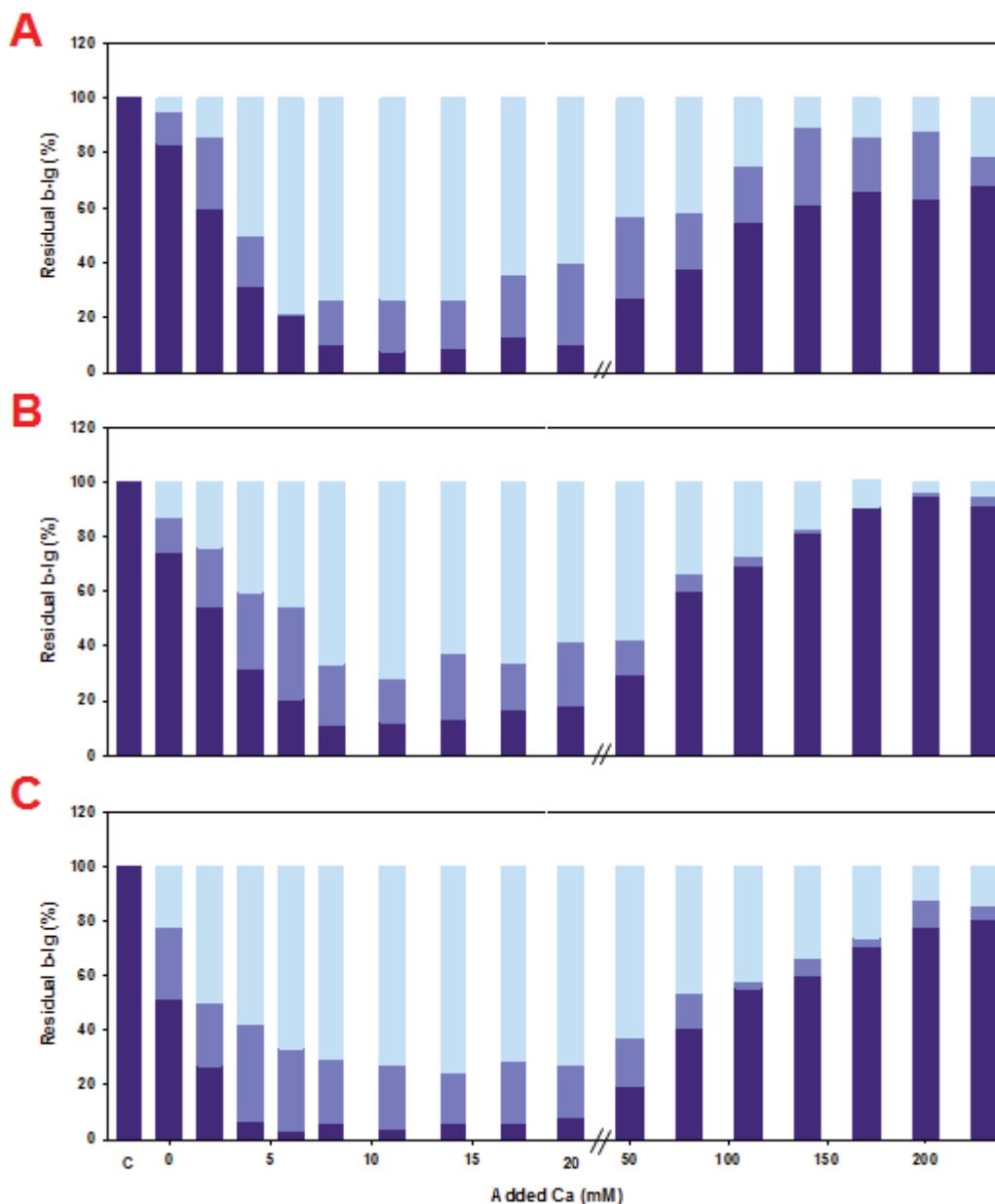
Figure 3.40: β-lactoglobulin aggregate types in heated (75°C, 5 min) whey protein solutions (4%, w/w protein): (**A**) WPI, (**B**) AWPC, and (**C**) CWPC, quantified by PAGE; ■ alkaline-monomeric proteins (native-like), ■ hydrophobically-linked aggregates, ■ disulphide-linked aggregates, **C** control sample (unheated without added $CaCl_2$).

In the CWPC solution without added calcium, about half of the initial amount of β-lactoglobulin molecules aggregated on heating, and about half of the aggregates were

disulphide-linked and another half hydrophobically-linked. In the case of AWPC solution without added calcium, similarly to CWPC solution without added calcium, half of the aggregates were disulphide-linked and the other half hydrophobically-linked, but only ~ 25% of β-lactoglobulin molecules had aggregated. For WPI solution without added calcium, less than 20% of β-lactoglobulin molecules aggregated, mainly via hydrophobic interactions.

As seen previously, the addition of calcium enhanced the extent of aggregation, which increased with increasing levels of added calcium to a maximum and then decreased with further increase in added calcium concentration. For both AWPC and CWPC, addition of calcium up to ~ 20 – 50 mM resulted in an increase in the proportion comprising hydrophobically- and disulphide-linked aggregates at the expense of native-like β-lactoglobulin molecules. At added calcium concentrations > 50 mM, much fewer β-lactoglobulin molecules aggregated, mainly via disulphide bonds. In contrast, for WPI, as added calcium levels increased up to ~ 15 mM, the proportions of hydrophobically-linked aggregates remained similar to that for WPI solution with no added calcium; thus the increase in aggregation occurred via disulphide bonds. At levels of added calcium higher than 15 mM, the extent of aggregation decreased with increasing added calcium levels, and the amount of hydrophobically-linked aggregates too variable to conclude on a trend. At high levels of added calcium (> 100 mM) the smaller extent of aggregation that occurred was mainly via hydrophobic interactions, except for the solution with 230 mM added calcium.

 Figures showing aggregate types for α-lactalbumin and bovine serum albumin can be found in Appendix 1.

3.3.5.2 RP-HPLC

RP-HPLC appeared to be a good method for quantifying the extent of aggregation of whey proteins, with limited possibility of experimental errors. Therefore, the effects of added calcium on the extent of aggregation of whey proteins in WPI, AWPC and CWPC are compared in the following at each of the heating temperatures studied previously, 85, 80 and 75°C.

Comparison at 85ºC

Figure 3.41 shows the effect of added calcium on the heat-induced aggregation of (**A**) α-lactalbumin and (**B**) β-lactoglobulin in WPI, AWPC and CWPC solutions when heated at 85ºC for 5 min.
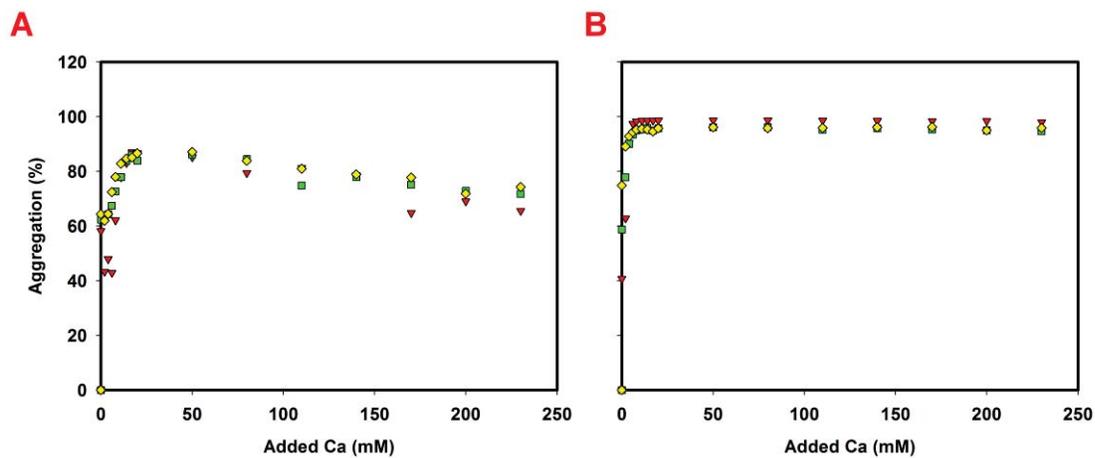
Figure 3.41: Aggregation of α-lactalbumin (**A**) and β-lactoglobulin (**B**) in heated (85ºC, 5 min) whey protein solutions (4%, w/w protein) quantified by RP-HPLC: comparison of the three systems; ▼ WPI, ▪ AWPC, ◆ CWPC.

At 85ºC, the extents of aggregation of α-lactalbumin and β-lactoglobulin (Figure 3.40) were similar for the three whey protein systems (WPI, AWPC, and CWPC). The extent of aggregation increased with increasing the levels of added calcium up to a maximum and then decreased with further increase in the added calcium concentrations. However, the extent of aggregation of α-lactalbumin in the samples with added calcium concentrations ranging from 50 to 230 mM was less in WPI solutions than AWPC and CWPC solutions (Figure 3.41**A**). In the case of β-lactoglobulin, the extent of aggregation in WPI solutions was slightly higher than in AWPC and CWPC solutions (Figure 3.41**B**), the extents of aggregation of β-lactoglobulin in AWPC and CWPC were similar. Interestingly, Figure 3.42, showing the effect of added calcium on the heat-induced aggregation of total whey proteins in WPI, AWPC and CWPC solutions when heated at 85ºC for 5 min, is very similar to Figure 3.41**B**, except that the extent of aggregation of total whey proteins was slightly less in CWPC solutions than in WPI and AWPC solutions, these last two systems being similar. However, the extent of aggregation of the total proteins in WPI, AWPC and CWPC was not significantly different at 85°C.
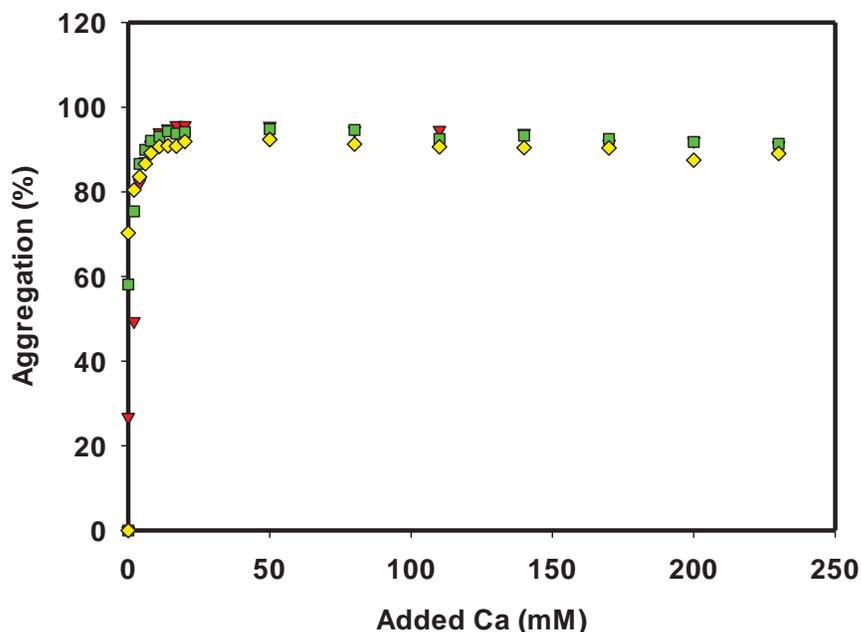
Figure 3.42: Aggregation of total whey proteins in heated (85ºC, 5 min) whey protein solutions (4%, w/w protein) quantified by RP-HPLC: comparison of the three systems; ▼ WPI, ▪ AWPC, ◇ CWPC.

Comparison at 80ºC

As seen previously, a greater extent of aggregation of whey proteins occurred at 85ºC (Figures 3.1, 3.17 and 3.27) than at 80ºC (Figures 3.5, 3.27 and 3.31). Figure 3.43 shows the effect of added calcium on the heat-induced aggregation of (**A**) α-lactalbumin and (**B**) β-lactoglobulin in WPI, AWPC and CWPC solutions when heated at 80ºC for 5 min.

The extent of aggregation increased with increasing added calcium concentration to a maximum and then decreased with further increase in added calcium levels. Both α-lactalbumin and β-lactoglobulin aggregated more in WPI solutions, than in CWPC solutions, where the aggregation was greater than in AWPC solutions (Figure 3.42). Interestingly, for both proteins, the rate of decrease in the extent of aggregation (after the maximum) was less in the CWPC solutions than in the WPI and AWPC solutions, leading to final similar values for CWPC and WPI samples with 230 mM added calcium, whereas the maximum extent of aggregation value was higher in WPI solutions than CWPC solutions.
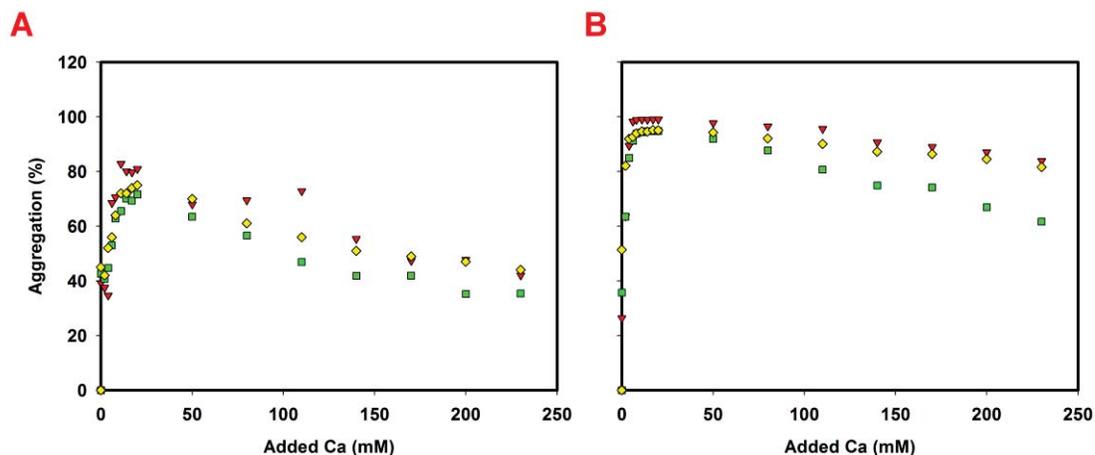
Figure 3.43: Aggregation of α-lactalbumin (**A**) and β-lactoglobulin (**B**) in heated (80ºC, 5 min) whey protein solutions (4%, w/w protein) with added CaCl$_2$ quantified by RP-HPLC: comparison of the three systems; ▼ WPI, ▪ AWPC, ◆ CWPC.

The effect of added calcium on the heat-induced aggregation of total whey proteins in WPI, AWPC and CWPC solutions when heated at 80ºC (5 min) is shown in Figure 3.44.
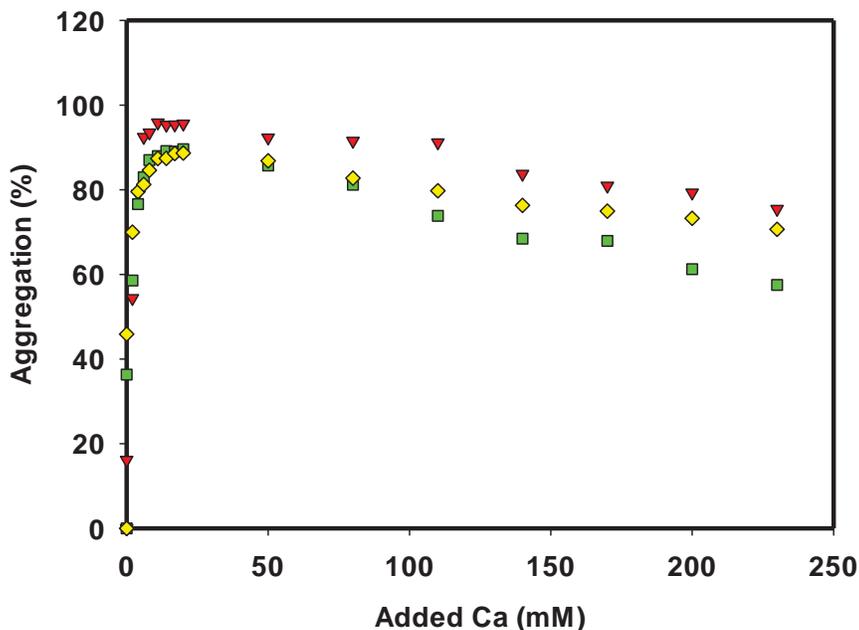
Figure 3.44: Aggregation of total whey proteins in heated (80ºC, 5 min) whey protein solutions (4%, w/w protein) quantified by reversed-phase HPLC: comparison of the three systems; ▼ WPI, ▪ AWPC, ◆ CWPC.

The extent of aggregation curve for the total whey proteins (Figure 3.44) was similar to that for β-lactoglobulin only, but the values were lower than those for β-lactoglobulin

(Figure 3.43**B**). As well, as for α-lactalbumin and β-lactoglobulin, the rate of decrease in the extent of aggregation with increasing added calcium concentrations was lower in the CWPC solutions than in the WPI and AWPC solutions.

Comparison at 75ºC

Figure 3.45 shows the effect of added calcium on the heat-induced aggregation of (**A**) α-lactalbumin and (**B**) β-lactoglobulin in WPI, AWPC and CWPC solutions when heated at 75ºC for 5 min.
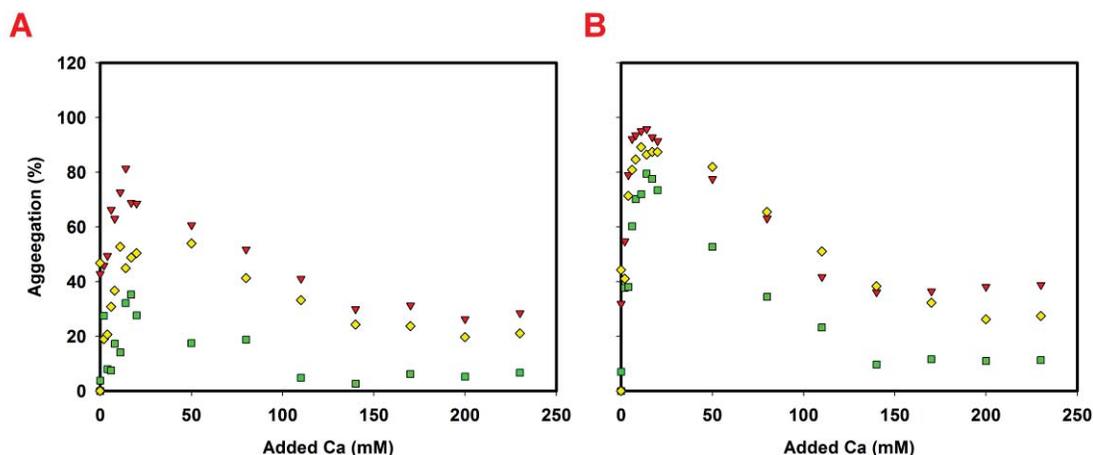
Figure 3.45: Aggregation of α-lactalbumin (**A**) and β-lactoglobulin (**B**) in heated (75ºC, 5 min) whey protein solutions (4%, w/w protein) quantified by RP-HPLC: comparison of the three systems; ▼ WPI, ■ AWPC, ◆ CWPC.

As seen previously, at 75ºC the extent of aggregation of α-lactalbumin bottomed out to a constant value for the samples of the three whey protein systems with 110 to 230 mM of added calcium. However, the extent of aggregation was slightly more in the WPI solutions than in the CWPC solutions, which was itself more than in the AWPC solutions. In the case of β-lactoglobulin, the extent of aggregation bottomed out to a constant value, ~ 10% for the AWPC samples with 110 to 230 mM added calcium and ~ 40% for the WPI samples with 140 to 230 mM added calcium. For the CWPC solutions, the extent of aggregation continued to decrease steadily, from ~ 80% to ~ 20%, with increasing levels of added calcium. It is important to note that the extent of aggregation was similar for the WPI and CWPC samples with 50 to 140 mM added calcium, especially for α-lactalbumin.

Figure 3.46 shows the effect of added calcium on the heat-induced aggregation of total whey proteins in WPI, AWPC and CWPC solutions when heated at 75ºC for 5 min. It was very similar to that observed for β-lactoglobulin (Figure 3.45**B**) but the values were slightly lower than for β-lactoglobulin.
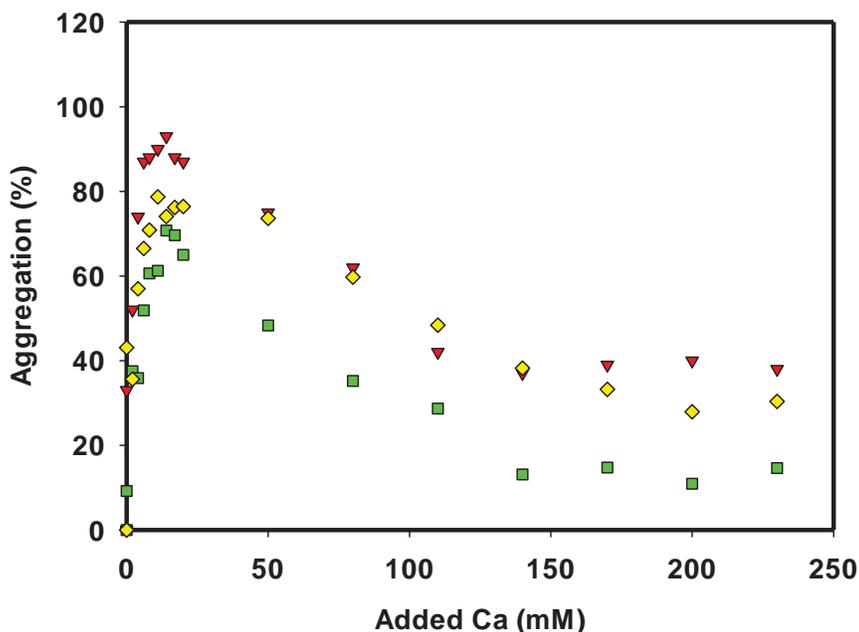
Figure 3.46: Aggregation of total whey proteins in heated (75ºC, 5 min) whey protein solutions (4%, w/w protein) quantified by RP-HPLC: comparison of the three systems; ▼ WPI, ■ AWPC, ◇ CWPC.

3.3.5.3 DSC

As reported earlier in this chapter, for the three whey protein systems, the denaturation temperature decreased with increasing added calcium levels up to the same critical concentration of 20 mM, and then increased with further increase in the added calcium level. However, the variation in the denaturation temperature varied slightly depending on the different whey protein systems. Figure 3.47 shows the effect of added calcium on the temperature of denaturation of the whey proteins in WPI, AWPC and CWPC solutions.
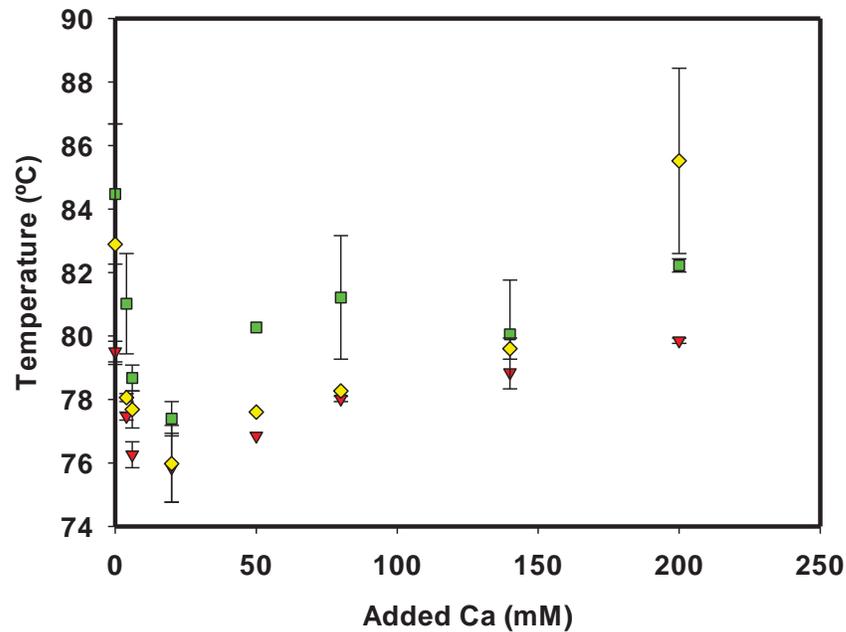
Figure 3.47: Differential scanning calorimetry temperature spectrum of whey protein solutions (4%, w/w protein): comparison of the peak temperature of the three systems; ▼ WPI, ■ AWPC, ◇ CWPC.

For the three systems, the minimum temperature was reached in the samples with 20 mM added calcium; however, the denaturation temperature of the AWPC samples was higher than those of the WPI and CWPC samples for all samples except that with 200 mM added calcium. The denaturation temperature of the AWPC sample with 140 mM added calcium was similar to those of the WPI and CWPC samples at that same added calcium concentration. The denaturation temperatures of the WPI samples were similar but slightly lower than those of the CWPC solutions for all samples except those without added calcium and with 200 mM added calcium. It was interesting to notice that for the solutions with 200 mM added calcium, the WPI solution had a similar denaturation temperature to that of the WPI control solution, whereas the AWPC and CWPC solutions had denaturation temperatures different from those of the their respective control solutions. For the CWPC, the denaturation temperature of the control solution was lower than that of the 200 mM added calcium solution, whereas for the AWPC the opposite was the case, the denaturation temperature of the control solution being higher than that of the 200 mM added calcium solution. Even though the trend of the effect of added calcium on the denaturation temperature was the same for the three systems studied, sensitivities of the systems to the addition of calcium were different.

That is also true for the decrease in the denaturation temperature. With 20 mM added calcium, the denaturation temperature was lowered by 4°C for WPI solution but by 6°C for AWPC solution and by 7°C for CWPC solution. The denaturation temperature reflects the heat stability of the whey protein conformations: the lower it is, the more sensitive the proteins are to heat-induced denaturation (Xiong, 1992). This suggested that, for the solutions with 20 mM added calcium, the whey proteins in the CWPC solution were more unstable than those in the AWPC solution, themselves more unstable than those in the WPI solution.

3.4 Overall conclusions

In the individual whey protein system studied, the RP-HPLC results showed that temperature had an effect on the extent of aggregation. As well described in the literature, the extent of aggregation was greater at higher temperature. In addition, it is widely agreed in the literature that the divalent cations (Ca^{2+} and Mg^{2+}) promote heat-induced whey protein aggregation in three different ways: (1) electrostatic shielding, (2) ion-specific hydrophobic interactions, and (3) cross-linking of adjacent protein anionic groups to form protein-calcium (or magnesium)-protein bridges (Ju & Kilara, 1998b; Wang & Damodaran, 1991).

Depending on the temperature at which the three different systems were compared, differences in the effect of added calcium on the extent of aggregation of the whey proteins were observed. At 85°C, these differences were very small; the extent of aggregation was almost the same in the whey protein systems (Figure 3.41). The effect of temperature overwhelmed the effect of the differences in composition of the three systems. However at 80 and 75°C, differences were more noticeable (Figures 3.43 and 3.45). With the high levels (50 to 230 mM) of added calcium compared with the levels of divalent cations initially present in the three whey protein systems (0.86 mM in WPI, 3.32 mM in AWPC and 5.63 mM in CWPC), one would expect to observe similar effects on the heat-induced aggregation of the whey proteins in the three systems. However, overall at 80 and 75°C (Figures 3.43 and 3.45), the extent of aggregation was much less in the AWPC solutions than in the CWPC solutions, which was itself less than in the WPI solutions. Havea et al. (2002) showed that the differences in the aggregation behaviours of AWPC and CWPC were due to the differences in mineral composition, the monovalent cations (K^+ and Na^+) limiting the extent of divalent cation

(Ca^{2+} and Mg^{2+}) bridging between protein molecules. AWPC contains more monovalent cations than CWPC, ~ 20 and 13 mM respectively, and CWPC contains slightly more monovalent cations that WPI, 13 and 11 mM respectively (Table 3.1). These differences in monovalent cation concentrations and the conclusions of Havea et al. (2002) correlated well with the differences in extent of aggregation observed in WPI, CWPC and AWPC solutions at 75°C for all added calcium concentrations (Figure 3.45) and at 80°C for solutions with 50 to 230 mM added calcium (Figure 3.43). At 80°C for the solutions with levels of added calcium up to 20 mM (Figure 3.43), the extent of aggregation was similar in AWPC and CWPC solutions but higher in WPI solutions; the effect of temperature probably overwhelmed the effect of the differences in initial mineral compositions in the case of AWPC and CWPC. However, the higher extent of aggregation of the WPI solutions could be explained by the lower initial concentration of divalent cations. The whey proteins would then have had more carboxylates available to interact with the soluble added calcium which would have promoted aggregation by forming protein-calcium-protein bridges (Li, Hardin, & Foegeding, 1994; Simons, Kosters, Visschers, & de Jongh, 2002).

The DSC results are in agreement with the RP-HPLC results. The denaturation temperatures of the AWPC solutions with added calcium levels ranging from 0 to 140 mM were higher than those of the CWPC and WPI solutions (Figure 3.46); the whey protein conformations are less destabilised in the AWPC solutions than in the CWPC and WPI solutions. Comparing the CWPC and WPI solutions, the denaturation temperatures for the WPI solutions were slightly lower than those for the CWPC solutions; the whey protein conformations were slightly more destabilised in WPI than in CWPC. However, the solutions with 200 mM added calcium behaved differently to the trend (Figure 3.46). The whey protein conformations in the CWPC solution were the least destabilised, even less destabilised by heat than in the control CWPC solution. Does this mean that high levels of added calcium have a protective effect? For the WPI solution, the destabilisation of the whey protein conformations was similar to that of the control WPI solution, but for the AWPC solution it was less than that of the control AWPC solution. It would have been interesting to investigate the effect of added calcium above this point (200 mM added calcium to 4%, w/w protein whey protein solutions) and understand what mechanism would then be involved and what differences there would be between the three different systems.

When the WPI, AWPC and CWPC results from the same types of experiments were compared directly, some differences in the heat-induced behaviour of the whey proteins were noticeable. However, the effect of added calcium on whey protein denaturation and aggregation in those systems appeared to be similar overall. At this stage of the study, these three whey protein systems were expected to behave in similar ways when used as raw materials in the manufacture of denatured whey protein products.

Nevertheless, such assumptions needed to be verified. Whey proteins have been used mainly as gelling or texturising agents in various food products, e.g. processed meat, yoghurt, baked goods, etc. Investigating the effect of calcium on the gelling properties of the whey proteins in WPI, AWPC and CWPC, and then comparing the results, appeared logically to be the next step of this study. This will be discussed in Chapter 4.

CHAPTER 4   Effect of Added Calcium on the Heat-induced Gelation of Whey Proteins in Three Different Commercial Systems: Study and Comparison

4.1 Introduction

Whey protein products have been used as functional ingredients in many food applications, mainly as gelling agents (Kinsella & Whitehead, 1989). The effect of minerals on heat-induced whey protein aggregation has been studied extensively over the past two decades. The mineral content of commercial whey protein products such as whey protein concentrates has been shown to play a major role in the heat-induced aggregation of whey protein solutions prepared from CWPC and AWPC (Havea, Singh, & Creamer, 2002; Schmidt, Illingworth, Ahmed, & Richter, 1978). Havea et al. (2002) suggested that the monovalent cations and the divalent cations influenced protein aggregation via different mechanisms. The high calcium content of CWPC promoted relatively rapid losses of native proteins and the formation of large aggregates (> 0.22 μm) during heating. The rapid formation of large protein aggregates was suggested to be due to the formation of calcium bridges between negatively charged residues, as well as hydrophobic interactions. In contrast, the low calcium content of AWPC limited the formation of calcium bridges and led to the formation of small, predominantly disulphide-linked, aggregates.

The role of calcium in the thermal denaturation and aggregation of whey protein has been reported to involve three phenomena: calcium bridging (Bryant & McClements, 1998; Hongsprabhas, Barbut, & Marangoni, 1999; Simons, Kosters, Visschers, & de Jongh, 2002), electrostatic shielding of negative charges on the protein (Hongsprabhas & Barbut, 1997d), and ion-induced conformational changes that could lead to hydrophobic interactions at elevated temperatures (Kinsella, Whitehead, Brady, & Bringe, 1989; Wang & Damodaran, 1991). The effect of calcium on heat-induced changes in whey proteins has been the subject of numerous publications in recent years (Hongsprabhas, Barbut, & Marangoni, 1999; O'Kennedy & Mounsey, 2009; Simons, Kosters, Visschers, & de Jongh, 2002; Xiong, Dawson, & Wan, 1993). These studies were carried out predominantly on model systems with well-defined protein compositions, usually pure β-lactoglobulin at a low concentration of 1 or 2% (w/w), and

defined mineral buffer systems. It is usually difficult to meaningfully extrapolate the findings from these studies to commercial whey protein products in which many factors, such as mixtures of different protein components with different physico-chemical properties, and heterogeneity of the mineral environment, are at play.

Although the above studies, among many others, provide some information about the impact of various minerals on the aggregation and/or gelation of whey proteins, the controlling mechanisms of the effects of different minerals on the denaturation, aggregation and gelation of whey proteins in more complex systems (e.g. WPC) are not fully understood. The objective of this study was to investigate the effect of added calcium on the heat-induced denaturation, aggregation and gelation of whey protein solutions prepared from commercial whey protein products with different mineral contents. These are referred to in the text as WPI, AWPC and CWPC. The gelation part of the study is treated in this chapter (Chapter 4) while the denaturation and aggregation part of the study are detailed in the previous chapter, Chapter 3.

It should be noted that the current study was orientated to industry, where there is much interest in the functional properties of overall protein systems. In this context, the actual impact of treatments such as the addition of calcium chloride to protein systems at macro levels is of significant technological importance. These products may contain denatured whey proteins, but the effect of added calcium on the total protein system is of significant interest. It should also be noted that gelation properties of the heated whey protein systems are the main issue when it comes to the manufacture of denatured whey protein products. Understanding the denaturation and aggregation behaviours may provide some useful insights into mechanisms, but unless there is good understanding of the gelation behaviour, it would be difficult to predict how a whey protein system would behave in commercial processes for manufacturing whey protein products.

4.2 Materials and Methods

4.2.1 Composition of whey protein powders
See Chapter 3, section 3.2.1.

4.2.2 Preparation of the whey protein solutions

Whey protein solutions were prepared by reconstituting appropriate quantities of whey protein powders (WPI, AWPC or CWPC) in milli-Q water so that the protein concentration was superior to final protein concentration needed. Appropriate volumes of a 2 M calcium chloride ($CaCl_2$) solution were added to give a range of different final added calcium concentration solutions (see section 3.2.2). Then, each solution was topped up with milli-Q water so that the final protein concentration was 4.8% (w/w) for the viscoelastic properties and 12% (w/w) for the compression test.

For the ζ-potential measurements, whey protein solutions were reconstituted in milli-Q water from whey protein powders (WPI, AWPC or CWPC) at a protein concentration of 1% (w/w). The protein concentration was 1% (w/w) instead of 4% (w/w) because of the limitation of the technique. When the whey protein powders are made, there is always a small amount of denatured proteins present in the product. This amount of "pre-denatured" proteins was neglected for the other analysis techniques as the proteins were anyway denatured during the experiments. But in the case of the ζ-potential measurements, the proteins were not denatured before measurement and having denatured proteins potentially aggregated, even in a small amount, would have affected the results. Thus, the samples needed to be filtered before measurement and at 4% (w/w) protein concentration the solutions were too concentrated to be filtered properly. The samples were then diluted to 1% (w/w) protein concentration. No minerals were added to these protein solutions, but pH was adjusted with 1 M HCl solution to give four different samples of each protein type: reconstitution pH = 6.7 for WPI and AWPC and 6.8 for CWPC, pH = 6, 5.5 and 5.

4.2.3 Heat treatment of the whey protein solutions

The solutions analysed by dynamic rheometrical measurements and compression testing were not heat treated before analysis. The heat treatment was applied during dynamic rheometrical measurements and to form gels for the compression testing (see sections 4.2.4 and 4.2.5).

The samples used for ζ-potential measurement were not heat treated at all.

4.2.4 Viscoelastic properties

Whey protein solutions (4.8%, w/w protein) with various levels of added calcium chloride (0, 4, 10, 20, 80, 140 and 200 mM) were prepared from each of the whey protein powders. Dynamic rheometrical measurements were made using a stress-controlled Paar Physica rheometer model UDS200 fitted with its cup and bob geometry (bob diameter: 26.63 mm). A 19 mL sample of each test solution was placed in the cup. The top of the solution was about 1 mm above the top of the bob. A layer of paraffin oil was placed on the surface of the solution to avoid evaporation during heating. The changes in the viscoelastic properties of the solution were monitored during heating using the rheometer in oscillatory mode at a frequency of 0.1 Hz and a shear strain amplitude of 0.1. The solution was heated under shear from 20 to 85°C at a rate of 1°C min^{-1}, held at 85°C for 30 min, then cooled to 20°C at a rate of 1°C min^{-1} and held at 20°C for 10 min. The storage modulus G' of the solution was measured every minute during this heating and cooling cycle (Havea, Watkinson, & Kuhn-Sherlock, 2009). The control for each whey protein product examined was the solution with no added salt. Every solution was tested in duplicate at least.

WPI contain more β-lactoglobulin than AWPC and CWPC, therefore WPI would be expected to form stiffer gels. For some viscoelastic properties experiments, 9 and 15 g L^{-1} of pure β-lactoglobulin (provided by the Fonterra Co-operative Group Ltd) was added to AWPC and CWPC solutions (4.8%, w/w protein prior to β-lactoglobulin addition), respectively, in order to match the β-lactoglobulin level in WPI and be able to compare better the gelling properties of WPI, AWPC and CWPC. The amount of pure β-lactoglobulin added to AWPC and CWPC solutions was calculated from the concentrations of each individual protein in 4.8% (w/w protein) WPI, AWPC and CWPC solutions obtained by RP-HPLC (Table 4.1).

Table 4.1: Individual whey protein concentrations in 4.8% (w/w protein) WPI, AWPC and CWPC solutions analysed by RP-HPLC prior to β-lactoglobulin addition.

	α-La (g L^{-1})	β-Lg (g L^{-1})	BSA (g L^{-1})
CWPC	5.577	13.215	0.479
AWPC	5.616	19.106	0.303
WPI	5.755	25.505	0.910

4.2.5 Compression test

Whey protein solutions (12%, w/w protein) with various levels of added calcium chloride (0, 12, 30, 60, 240, 420 and 600 mM) were prepared from each of the WP powders. The WP solutions were then placed in 400 cm long, 30 mm diameter, medium-walled polycellulosic plastic tubes the ends of which were then closed off using rubber bands to make stiff "sausages". These sausages were then placed in a thermostatically controlled water bath (85 ± 0.5°C) for 30 min. It took approximately 48 s to heat the centre of each tube to 84.7°C. After heating, the tubes were removed from the water bath and immediately placed under cold running water (~ 22.5°C) for 30 min before being stored at 4°C overnight. The resulting gels were analysed using a range of techniques.

Each gel was cut to give 6 cylindrical slices (30 x 25 mm) using a wire cutter and a template. Each slice was wrapped in plastic film to prevent moisture loss, placed in a sealed container, and then kept at 5ºC for 2 hrs to equilibrate before testing. In each test, the sample was placed between the upper 85 mm diameter Teflon plate and the lower 95 mm x 105 mm Teflon plate of a Texture Analyser TA-HD (Stable Micro Systems, Godalming, Surrey, UK) equipped with a 500 N load cell and controlled by Texture Expert Exceed version 2.64 software. The surfaces of the plates were lubricated with paraffin oil to minimize friction. The sample was then compressed to 90% of its original height at a rate of 0.83 mm s^{-1}. The Texture Analyser was in a thermostatically controlled (13ºC) room and compression took place in the instrument's thermostatically controlled chamber at 5ºC. Force-distance data were automatically recorded during each test (Havea, Watkinson, & Kuhn-Sherlock, 2009).

The testing conditions, and the calculation of fracture stress and fracture strain were based on the technical protocols for cheese testing and analysis developed by the International Dairy Federation (International Standards Organization and International Dairy Federation, 2006). Fracture stress was measured as the first peak stress as a function of strain. Fracture strain (Hencky strain) was the strain corresponding to the fracture stress (Figure 4.1).
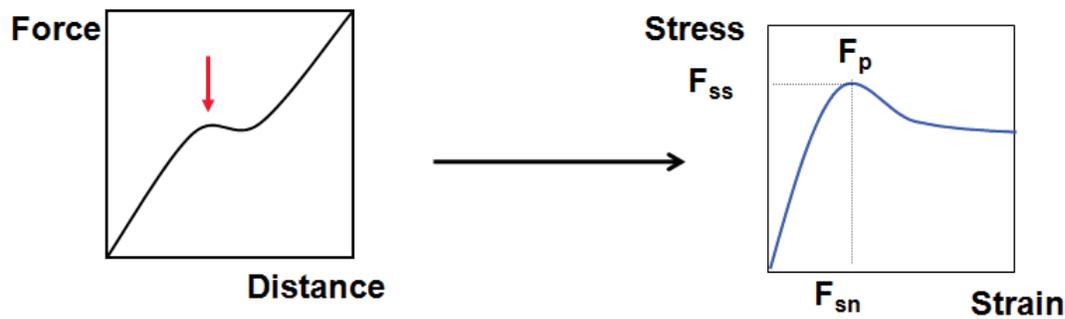
Figure 4.1: Determination of the fracture point in the compression test analysis; F_p = fracture point; F_{ss} = fracture stress; F_{sn} = fracture strain.

The results were plotted on a texture map to provide information on gel textures, details of the texture characteristics are shown on Figure 4.2.
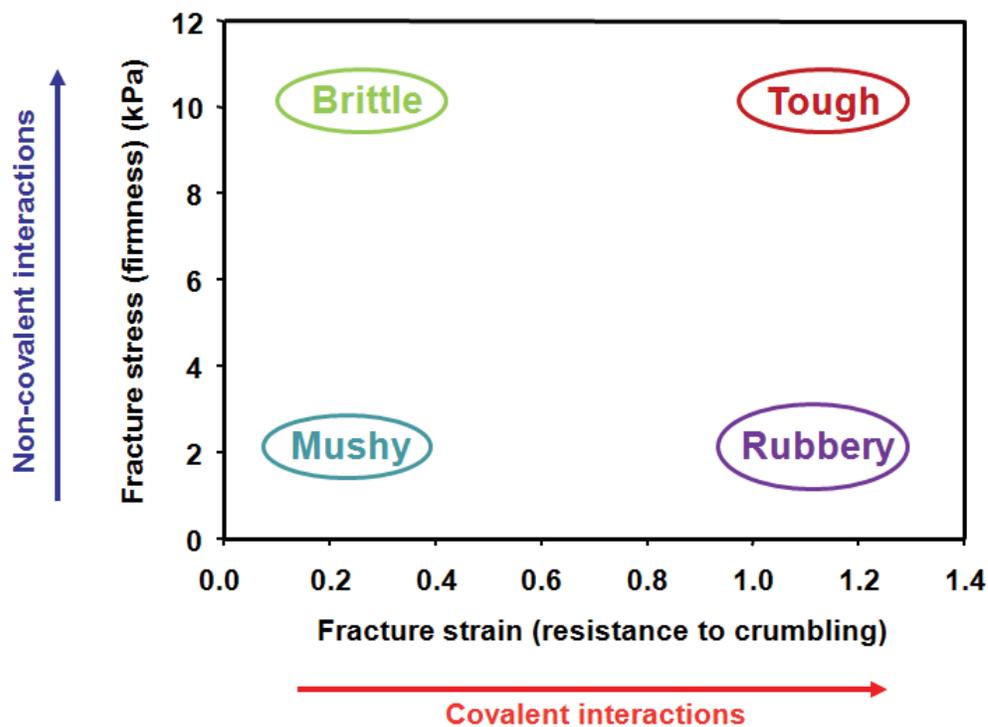
Figure 4.2: Map of texture characteristics and protein-protein interactions; from Havea, Watkinson, and Kuhn-Sherlock (2009).

4.2.6 Transmission Electron Microscopy (TEM)

Whey protein gel "sausages" were prepared as described in section 4.2.5.

Fixation

The sample is cut into ~ 1 mm^3 cubes and put into a bijoux bottle containing 6.25% gluteraldehyde in 0.2 M imidazole buffer. This was stored at 5°C for 2 days. The sample was rinsed twice with 0.2 M imidazole buffer over two hours. The buffer was removed

and the sample placed in 1% (v/v) osmium tetroxide in 0.2 M Na cacodylate overnight. The sample was rinsed twice with distilled water.

Dehydration

The dehydration process was carried out at 5°C in 25% (v/v) acetone (15 min) then in 50%, 70% and 90% (v/v) acetone (for 30 min each) followed by 100% (v/v) acetone (3 changes over 90 min).

Embedding

The acetone was then replaced with 1:1 100% Acetone: Procure 812 embedding resin, and the sample put on rollers for 12 h. Then this acetone mixture was replaced with 100% Procure 812 for 12 - 24 h. Then a cube of the sample was placed into a BEEM embedding capsule and cured at 60°C for 48 h.

Sectioning

The embedded samples were sectioned to a thickness of 90 nm using a Leica Ultracut R microtome. These sections were mounted on 3 mm copper/rhodium grids and stained using uranyl acetate and lead citrate before examination in a Philips Transmission Electron Microscope (TEM) (Philips, 201C, The Netherlands) at an accelerating voltage of 60 kV.

4.2.7 Electrophoretic mobility and ζ-potential determination

The electrophoretic mobilities and hence the calculated ζ-potentials of whey proteins were determined using a Malvern Zetasizer Nano instrument, and the associated Malvern Multi-8 64 channel correlator and clear disposable electrophoresis cell which incorporated a folded capillary (Malvern Instruments Ltd, Malvern, Worcs., UK). The temperature of the electrophoresis cell was maintained at 25°C by a water jacket which was temperature controlled by the Peltier system associated with the electrophoresis cell. An applied voltage of 150 V was used in all experiments.

Samples were filtered with 0.45 μm MiniSart Sartorius filter before analysis. Samples were done in triplicates.

4.2.8 Statistical analysis

Data collected from duplicate experiments were used to calculate the standard errors in the quantification of proteins using the rheological measurements and the compression tests using SigmaPlot (2002 for Windows version 8.02; SPSS Inc., Chicago, IL).

4.3 Results and Discussion

4.3.1 Composition of whey protein powders
See Chapter 3, section 3.3.1.

4.3.2 Whey protein isolate (WPI)
The investigation of the effect of added calcium on the heat-induced denaturation and aggregation of WPI solutions in Chapter 3 gave some interesting results. To get a better understanding of the effect of added calcium, this investigation of the heat-induced gelation of WPI solutions was carried out. To be able to link the results from both parts of the study, the experimental conditions had been kept similar as much as possible. Therefore, the viscoelastic properties were investigated at the same protein and added calcium concentrations as in Chapter 3 (apart from the slight increase in protein concentration to 4.8%, w/w). In the case of the fracture properties, the WPI solutions had to be at high enough concentration to form gels that could be sliced with as little damage as possible. Thus, the fracture properties were investigated at higher (three folds) protein and added calcium concentrations than in Chapter 3 experiments, keeping the same calcium/protein ratio (mM/%, w/w).

4.3.2.1 Viscoelastic properties
The effect of added calcium chloride on the gel stiffness (storage modulus, G') of the WPI solutions (4.8%, w/w protein, pH 6.7) during heating is shown in Figure 4.3.
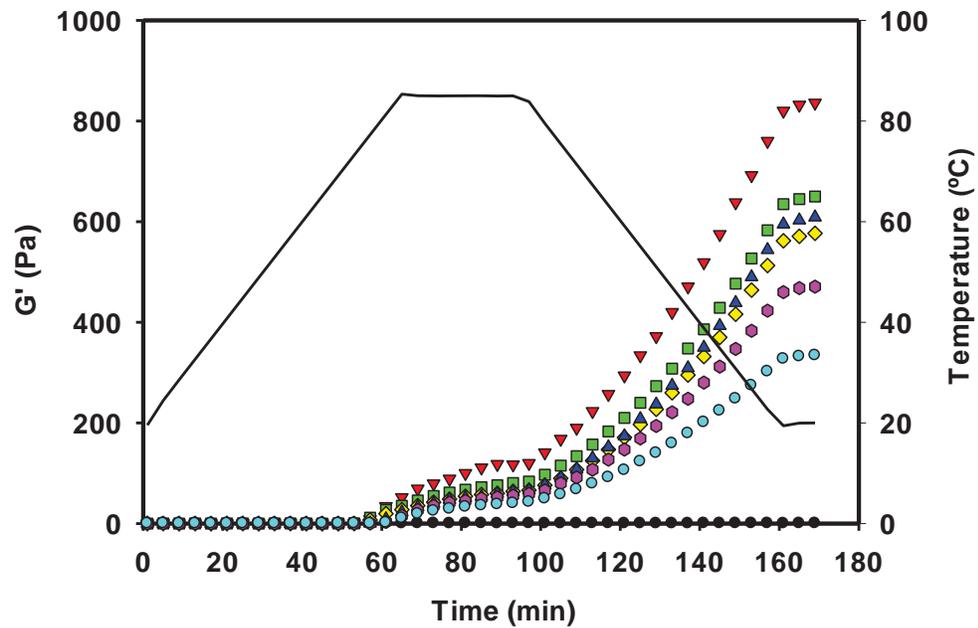
Figure 4.3: Effect of added CaCl$_2$ on thermally-induced changes in the storage modulus (G') of the WPI solutions (4.8%, w/w protein), heated from 20 to 85°C at 1°C min^{-1}, held at 85°C for 30 min, cooled to 20°C at -1°C min^{-1} and held at 20°C for 10 min. G' was measured every minute; ● no added CaCl$_2$; ▼ 4 mM added CaCl$_2$; ■ 10 mM added CaCl$_2$; ◇ 20 mM added CaCl$_2$; ▲ 80 mM added CaCl$_2$; ⬡ 140 mM added CaCl$_2$; ◯ 200 mM added CaCl$_2$; ▬ temperature.

Without the addition of calcium to the WPI solution, no measurable changes in the values of G' were observed, i.e. no gel networks were formed. The gel formed with 4 mM of added calcium was the strongest (highest G'$_{final}$), 846 ± 9.0 Pa (Figure 4.3). As the level of added calcium increased further, G'$_{final}$ decreased finally to 359 ± 25 Pa (for the gel formed with 200 mM of added calcium), indicating increasingly weaker gels.

The observation that weaker gels were formed from solutions with higher levels of added calcium, and that the extent of aggregation was lower, was probably attributable to the effect of calcium on protein denaturation and aggregation (see Chapter 3).

4.3.2.2 Fracture properties

The effect of added calcium on the fracture stress and the fracture strain of WPI gels is shown in Figure 4.4. In contrast to the control solution (4.8%, w/w protein) for the dynamic rheometrical experiments, the control solution (12%, w/w protein) for the

compression test formed a gel under heat treatment without the addition of calcium. The fracture stress of the WPI control gel was ~ 4 kPa and the fracture strain ~ 0.72.
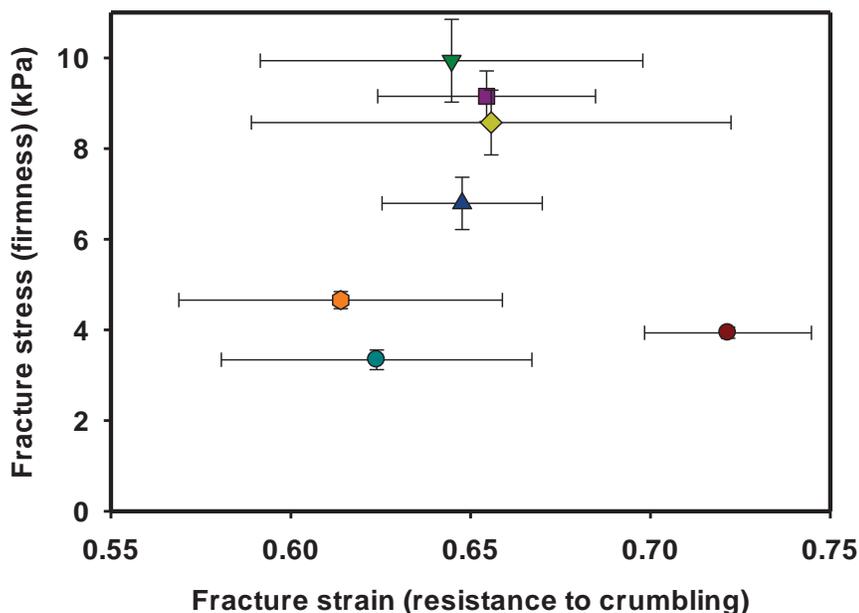
Figure 4.4: Effect of added $CaCl_2$ on the fracture stress and fracture strain of WPI gels: 12% (w/w) protein, heated at 85ºC for 30 min, $0 \leq$ added $CaCl_2 \leq 600$ mM. Fracture stress and fracture strain were measured by a 90% compression test on the gel; ● no added $CaCl_2$; ▼ 12 mM added $CaCl_2$; ■ 30 mM added $CaCl_2$; ◆ 60 mM added $CaCl_2$; ▲ 240 mM added $CaCl_2$; ● 420 mM added $CaCl_2$; ● 600 mM added $CaCl_2$. Error bars: $\pm$ standard error, $n = 2$.

The fracture stress of the WPI gels appeared to follow similar trends to those observed for G' in the small deformation oscillatory tests (Figure 4.3). The gels without added calcium had a fracture stress of ~ 4 kPa. The gels with 12 mM added calcium had more than double the initial fracture stress (~ 10 kPa). The fracture stress then decreased with further increases in the level of added calcium, to ~ 3.5 kPa for the WPI gels containing 600 mM added calcium. In contrast, the fracture strain decreased slightly when calcium was added to the WPI solution, from ~ 0.72 for the control solution to ~ 0.65 for the 4 mM added calcium solution. Further increases in added calcium level did not affect the fracture strain much; the fracture strain varied between ~ 0.62 and ~ 0.66.

4.3.2.3   TEM

Figure 4.5 shows the effect of added calcium on the structure of the WPI gels.
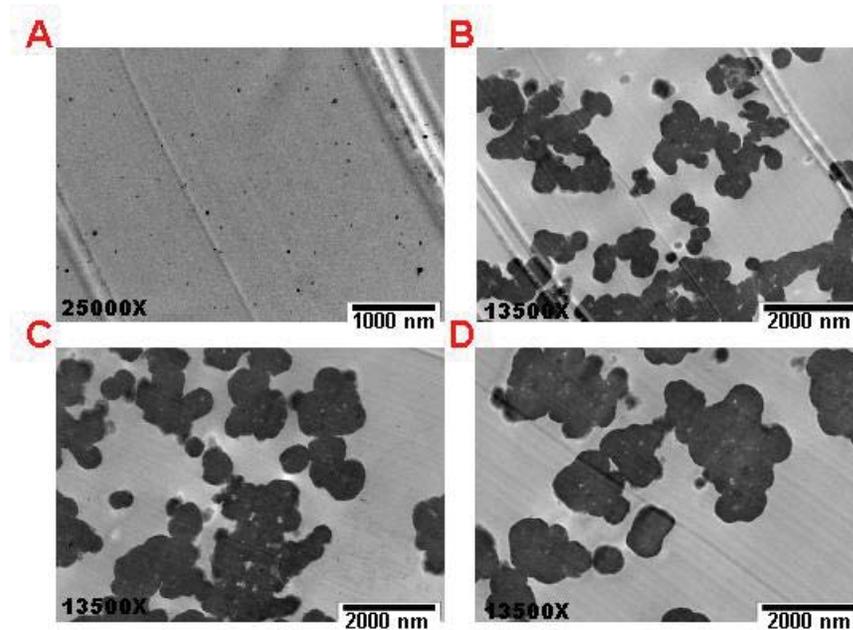
Figure 4.5: Effect of added CaCl$_2$ on the structure of WPI gels. TEM micrographs of 12% (w/w) protein solutions heated at 85ºC for 30 min; **A** 0 mM added CaCl$_2$; **B** 60 mM added CaCl$_2$; **C** 240 mM added CaCl$_2$; **D** 600 mM added CaCl$_2$.

The TEM micrographs show that the control WPI gel (Figure 4.5**A**) had a very fine homogeneous structure (difficult to see even at 25,000 times magnification). With 60 mM of added calcium, round aggregates of ~ 500 nm were formed (Figure 4.5**B**). With 240 and 600 mM added calcium (Figures 4.5**C** and **D**), even bigger aggregates, > 2,000 nm, were formed. The structure of the WPI gels changed from fine-stranded to particulate with addition of calcium. With further increase of added calcium levels, the WPI gels become coarser.

4.3.2.4   Conclusions

The results from the viscoelasticity measurements and from the fracture properties measurements were in agreement. The stiffest gels formed at the lowest added calcium level, and then the gel stiffness decreased with further increase in added calcium level. These results were in relatively good correspondence with the findings discussed in Chapter 3, where the maximal extent of aggregation was found at an optimal added calcium concentration, and above this optimum concentration, the extent of aggregation decreased with further increase in added calcium level. This confirmed that the observed effect of added calcium on the heat-induced gelation of WPI was related to the effect of added calcium on heat-induced protein aggregation. The TEM micrographs showed that the addition of calcium to WPI solutions completely changed the

microstructure of the gels formed. This suggested that the addition of calcium influenced the aggregation mechanism, probably by modifying the balance between attractive and repulsive forces leading to aggregation of the proteins.

4.3.3 Acid whey protein concentrate (AWPC)

4.3.3.1 Viscoelastic properties

The effect of added calcium on the heat-induced gelation of whey proteins in AWPC was studied using the same experimental techniques as those used for WPI. As AWPC contain less β-lactoglobulin than WPI (Table 3.1), a lower gel stiffness for AWPC gels was expected. Figure 4.6 shows the effect of added calcium on thermally-induced changes in the storage modulus (G') of the AWPC solutions (4.8%, w/w protein).
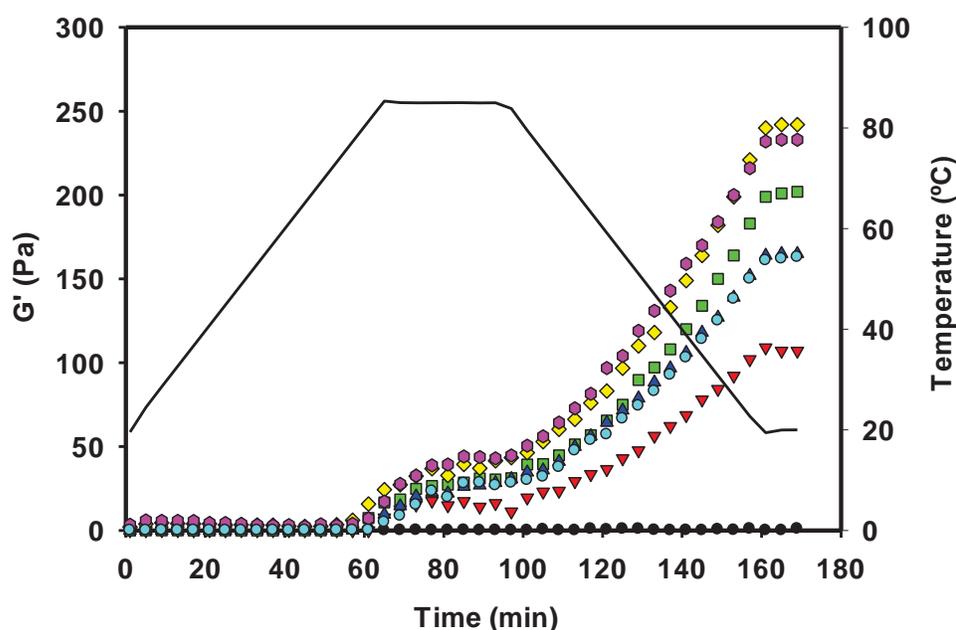
Figure 4.6: Effect of added $CaCl_2$ on thermally-induced changes in the storage modulus (G') of the AWPC solutions (4.8%, w/w protein), heated from 20 to 85°C at 1°C min^{-1}, held at 85°C for 30 min, cooled to 20°C at -1°C min^{-1} and held at 20°C for 10 min. G' was measured every minute; ● no added $CaCl_2$; ▼ 4 mM added $CaCl_2$; ■ 10 mM added $CaCl_2$; ◆ 20 mM added $CaCl_2$; ▲ 80 mM added $CaCl_2$; ⬢ 140 mM added $CaCl_2$; ⦿ 200 mM added $CaCl_2$; — temperature.

Similarly to the WPI solutions, the solution without addition of calcium did not form a gel under heat treatment. The final storage modulus G'$_{final}$ increased with increasing added calcium up to 20 mM, at which the gel was the strongest, 225 $\pm$ 18 Pa (Figure

4.6). Further increases in the level of added calcium resulted in decreases in storage modulus G'$_{final}$ to 152 ± 11 Pa. The final storage modulus G'$_{final}$ values of the gels formed with 80 and 200 mM added calcium were not significantly different. It is interesting to note that the weakest AWPC gel was formed with 4 mM added calcium whereas the stiffest WPI gel was formed with 4 mM added calcium.
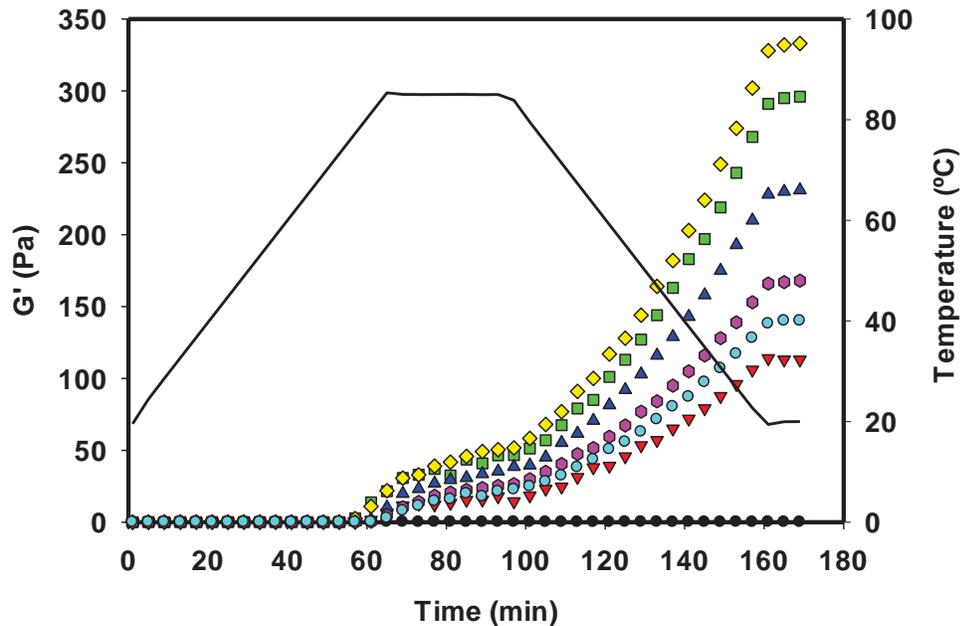
Figure 4.7: Effect of added CaCl$_2$ on thermally-induced changes in the storage modulus (G') of the AWPC solutions (4.8%, w/w protein) with β-lg level topped up to β-lg level in WPI, heated from 20 to 85ºC at 1ºC min^{-1}, held at 85ºC for 30 min, cooled to 20ºC at -1ºC min^{-1} and held at 20ºC for 10 min. G' was measured every minute; ● no added CaCl$_2$; ▼ 4 mM added CaCl$_2$; ■ 10 mM added CaCl$_2$; ◆ 20 mM added CaCl$_2$; ▲ 80 mM added CaCl$_2$; ⬠ 140 mM added CaCl$_2$; ◉ 200 mM added CaCl$_2$; — temperature.

As expected, at all levels of added calcium, the AWPC gels were less stiff than the WPI gels owing to the lower amount of β-lactoglobulin in AWPC compared with WPI. However, the AWPC gels were dramatically less stiff than the WPI gels. To understand if this was due only to the less β-lactoglobulin content of AWPC or its intrinsic properties as a complex system, pure β-lactoglobulin was added to AWPC solutions to match the β-lactoglobulin level in WPI. The total protein concentration was then > 4.8% (w/w). Figure 4.7 shows the effect of added calcium on the storage modulus, G', when AWPC solutions were heated after the addition of β-lactoglobulin.

As observed without added β-lactoglobulin, the addition of calcium was needed for structure development to occur as evidenced by an increase in storage modulus G'. The AWPC solutions with 4 mM and with 20 mM of added calcium formed the least stiff and the stiffest gels, respectively, whether β-lactoglobulin was added to AWPC solutions or not. However, at the other levels of added calcium, the gel stiffness ranking changed on addition of pure β-lactoglobulin. The most significant changes were for the AWPC gels made with 140 mM and 200 mM of added calcium. Without addition of β-lactoglobulin, the 140 mM gel was almost as stiff as the 20 mM gel (Figure 4.6) whereas with addition of β-lactoglobulin, it was only half as stiff as the 20 mM gel (Figure 4.7). The 200 mM gel stiffness was not significantly different from that of the 80 mM gel when there was no addition of β-lactoglobulin (Figure 4.6), but was ~ 100 Pa less than that of the 80 mM gel with addition of β-lactoglobulin (Figure 4.7).

AWPC gels with β-lactoglobulin addition (Figure 4.7) were stiffer than AWPC gels without addition of β-lactoglobulin (Figure 4.6), but their gel stiffness was still markedly lower than that of the WPI gels (Figure 4.3). The stiffest AWPC gel with β-lactoglobulin addition (332 ± 3.0 Pa) (Figure 4.7) was almost as stiff as the least stiff WPI gel (359 ± 25 Pa) (Figure 4.3 and Table 4.2). This suggested that the differences in gel stiffness between the two systems were not due to the difference in protein composition.

4.3.3.2 Fracture properties

As the difference in protein composition was not the cause of the difference in gelling behaviour, and as the present study did not have the primary aim of investigating the gelling properties of model systems, the level of β-lactoglobulin in AWPC was not increased to match the level of β-lactoglobulin in WPI for the fracture properties experiments.

The effect of added calcium on the fracture stress and fracture strain of the AWPC gels is shown in Figure 4.8.
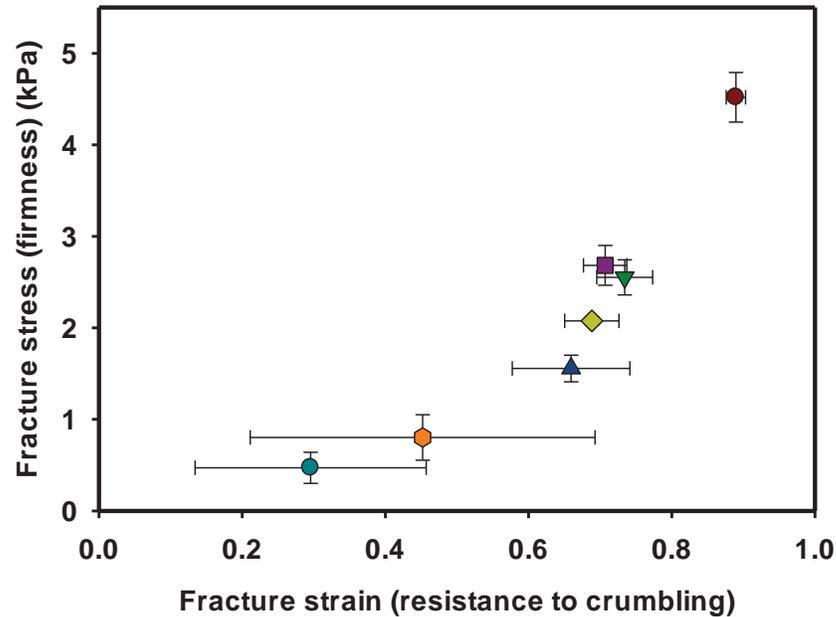
Figure 4.8: Effect of added $CaCl_2$ on the fracture stress and fracture strain of AWPC gels: 12% (w/w) protein, heated at 85ºC for 30 min, 0 < added $CaCl_2$ < 600 mM. Fracture stress and fracture strain were measured by a 90% compression test on the gel; ● no added $CaCl_2$; ▼ 12 mM added $CaCl_2$; ■ 30 mM added $CaCl_2$; ◆ 60 mM added $CaCl_2$; ▲ 240 mM added $CaCl_2$; ● 420 mM added $CaCl_2$; ● 600 mM added $CaCl_2$. Error bars: ± standard error, $n = 2$.

The highest fracture stress (~ 4.8 kPa) and fracture strain (~ 0.9) were observed in the control gels with no added calcium (Figure 4.8). Both the fracture stress and the fracture strain decreased with increasing levels of added calcium to ~ 0.5 kPa and ~ 0.3, respectively. The fracture stresses and fracture strains of the gels with 12 and 30 mM were not significantly different.

4.3.3.3   TEM
Figure 4.9 shows the effect of added calcium on the structure of the AWPC gels.
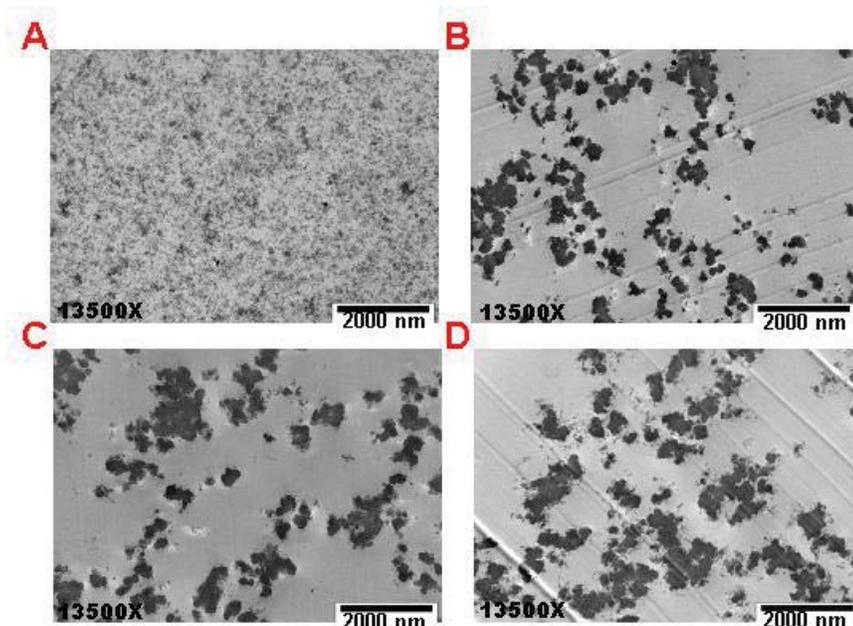
Figure 4.9: Effect of added CaCl$_2$ on the structure of AWPC gels. TEM micrographs of 12% (w/w) protein solutions heated at 85ºC for 30 min; **A** 0 mM added CaCl$_2$; **B** 60 mM added CaCl$_2$; **C** 240 mM added CaCl$_2$; **D** 600 mM added CaCl$_2$.

The control AWPC gel had a relatively fine structure (Figure 4.8**A**), similar to that reported previously (Havea, Singh, & Creamer, 2002). With 60 mM added calcium, relatively larger protein aggregates (100 – 200 nm) were formed (Figure 4.8**B**). The aggregate size did not seem to increase much with further increasing levels of added calcium (Figures 4.8**C** and **D**). The addition of calcium markedly changed the microstructure of the AWPC gels, from a fine network of small homogeneous aggregates to a loose particulate structure. At higher added calcium levels, the aggregates showed a higher degree of "hairiness".

4.3.3.4   Conclusions

As for WPI, to be able to link the results on the effect of added calcium on the heat-induced denaturation and aggregation of AWPC solutions with those on the heat-induced gelation of these solutions, the experimental conditions had been kept as similar as possible. This explains the protein and calcium concentrations chosen for the small and large deformation experiments.

In contrast with WPI, the correspondence between the viscoelastic properties and the fracture properties results of AWPC was not as good as for WPI. The first difference between the two sets of results was that the addition of calcium was needed to observe

development of structure in the small deformation experiments, whereas in the large deformation experiments, the AWPC gels with no added calcium showed the highest hardness. The second difference observed was that in the small deformation experiments, the gel stiffness increased with increase in added calcium levels up to 20 mM, and then decreased with further increase in added calcium levels. In the large deformation experiments, both fracture stress and fracture strain decreased with increasing added calcium levels. From these experiments, it was difficult to determine if these differences in AWPC behaviour under heat treatment were due to the difference in protein concentration, the higher amount of minerals initially present in AWPC, or the heating conditions. On the other hand, the small deformation experiments gave results in good agreement with the results from Chapter 3. At 4 – 5% (w/w) protein concentration, there was an optimal concentration of added calcium at which the greatest extent of aggregation occurred, leading to the stiffest gels. The TEM micrographs showed that adding calcium to AWPC considerably increased the size of the aggregates compared with those found in the control gels without added calcium, making the gels coarser.

As expected, AWPC solutions formed weaker gels than WPI solutions. However, the AWPC gels were dramatically weaker than the WPI gels under the same experimental conditions. The first thought to explain this difference was that AWPC contained less β-lactoglobulin that WPI. To be able to compare the AWPC results with WPI results, pure β-lactoglobulin was added to AWPC solutions to match the β-lactoglobulin level in WPI. Viscoelastic properties measurements were then made again. The AWPC gels were stiffer with β-lactoglobulin addition than without, but they were still markedly less stiff than the WPI gels.

4.3.4 Cheese whey protein concentrate (CWPC)

4.3.4.1 Viscoelastic properties

The same experimental techniques used with WPI were used to study the effect of added calcium on the heat-induced gelation CWPC, with 4.8% (w/w protein) solutions. Similarly to AWPC, CWPC contain less β-lactoglobulin than WPI but slightly more than AWPC (Table 3.1), the gel stiffness of CWPC gels was expected to be lower than that of WPI gels and to be similar if not slightly higher than that of AWPC gels. Figure

4.10 shows the effect of added calcium on thermally-induced changes in the storage modulus (G') of the CWPC solutions (4.8%, w/w protein).
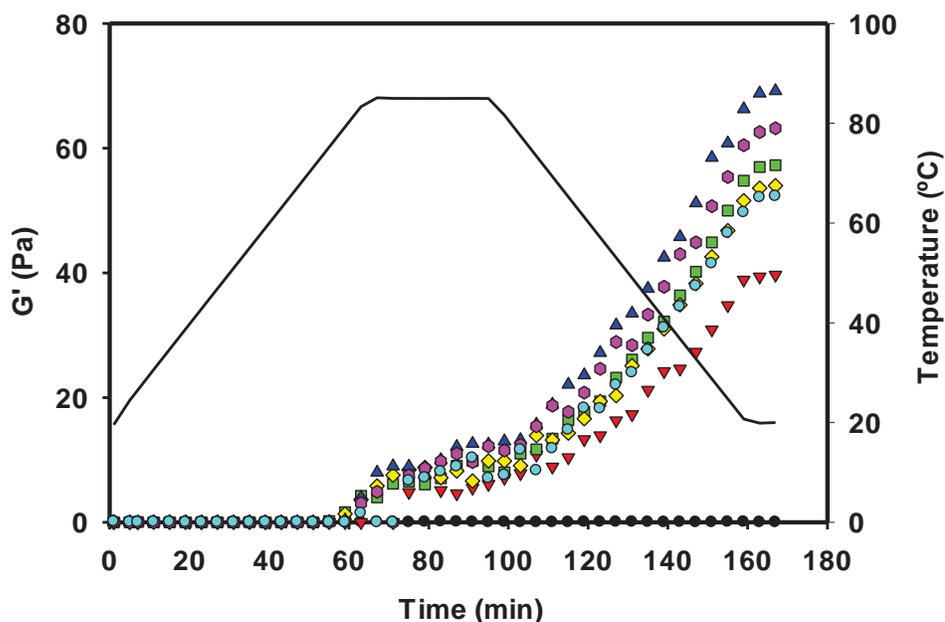
Figure 4.10: Effect of added $CaCl_2$ on thermally-induced changes in the storage modulus (G') of the CWPC solutions (4.8%, w/w protein), heated from 20 to 85ºC at 1ºC min^{-1}, held at 85ºC for 30 min, cooled to 20ºC at -1ºC min^{-1} and held at 20ºC for 10 min. G' was measured every minute; ● no added $CaCl_2$; ▼ 4 mM added $CaCl_2$; ■ 10 mM added $CaCl_2$; ◆ 20 mM added $CaCl_2$; ▲ 80 mM added $CaCl_2$; ● 140 mM added $CaCl_2$; ● 200 mM added $CaCl_2$; — temperature.

Similarly to WPI and AWPC solutions, without addition of calcium the CWPC solution did not form a gel under heat treatment. The final storage modulus G'$_{final}$ increased with increasing added calcium up to 80 mM, at which the gel was the stiffest, 73 ± 4.0 Pa (Figure 4.10). Further increases in the level of added calcium resulted in decreases in G'$_{final}$ to 55 ± 2.0 Pa. The final storage modulus G'$_{final}$ values of the gels formed with 10, 20 and 200 mM added calcium were not significantly different. It is interesting to note that the less stiff CWPC gel was formed with 4 mM added calcium whereas the stiffest WPI gel was formed with 4 mM added calcium.

As expected, at all levels of added calcium, the CWPC gels were dramatically less stiff than the WPI gels owing to the lower amount of β-lactoglobulin in CWPC than in WPI.

Interestingly, the CWPC gels were also significantly less stiff than the AWPC gels. The stiffest CWPC gel (73 ± 4.0 Pa) was ~ 50 Pa less stiff than the least stiff AWPC gel (121 ± 13 Pa) (Table 4.2). To understand if these observations were due only to the differences in protein composition between the three protein systems or if they were due to their intrinsic properties as complex systems, pure β-lactoglobulin was added to CWPC solutions to match the β-lactoglobulin level in WPI. The total protein concentration was then > 4.8% (w/w). Figure 4.11 shows the effect of added calcium on the storage modulus, G', when CWPC solutions were heated after the addition of β-lactoglobulin.
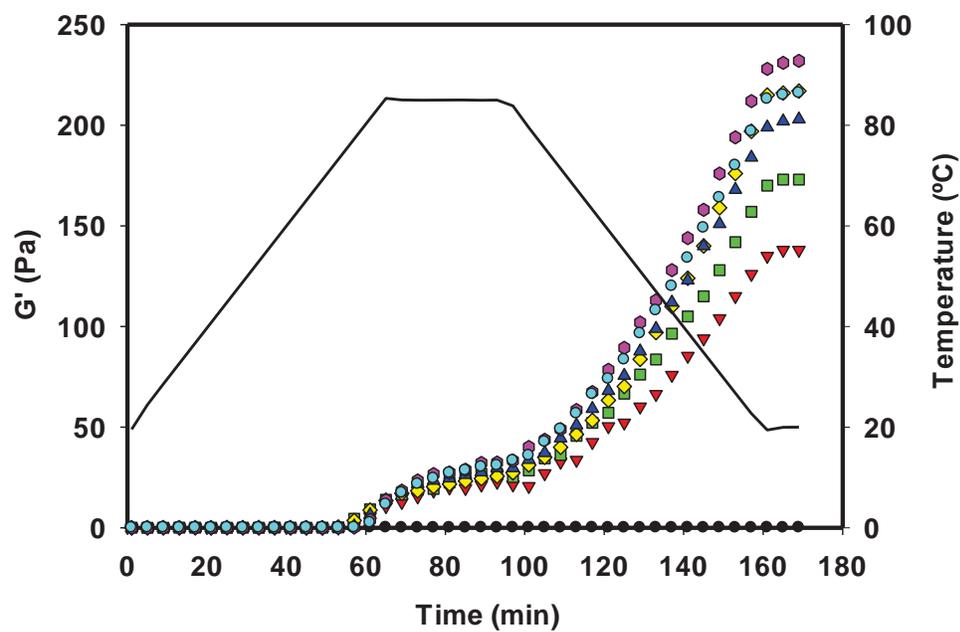
Figure 4.11: Effect of added $CaCl_2$ on thermally-induced changes in the storage modulus (G') of the CWPC solutions (4.8%, w/w protein) with β-lg level topped up to β-lg level in WPI, heated from 20 to 85ºC at 1ºC min^{-1}, held at 85ºC for 30 min, cooled to 20ºC at -1ºC min^{-1} and held at 20ºC for 10 min. G' was measured every minute; ● no added $CaCl_2$; ▼ 4 mM added $CaCl_2$; ■ 10 mM added $CaCl_2$; ◆ 20 mM added $CaCl_2$; ▲ 80 mM added $CaCl_2$; ⬟ 140 mM added $CaCl_2$; ◒ 200 mM added $CaCl_2$; — temperature.

Even with addition of β-lactoglobulin to CWPC solutions, without addition of calcium no gel formed. With the addition of β-lactoglobulin, the CWPC solutions with 4 mM still formed the least stiff gel, but the stiffest gel was formed with 140 mM of added calcium in contrast to 80 mM of added calcium without addition of β-lactoglobulin.

With the addition of β-lactoglobulin, the final storage moduli, G'_{final}, values of the gels formed with 20, 80 and 200 mM added calcium were not significantly different than those observed when there was no added β-lactoglobulin.

CWPC gels with β-lactoglobulin addition (Figure 4.11) were stiffer than CWPC gels without addition of β-lactoglobulin (Figure 4.10), but their gel stiffness was still markedly lower than that of the WPI gels (Figure 4.3). The stiffest CWPC gel with β-lactoglobulin addition (221 ± 13 Pa) was almost as stiff as the least stiff WPI gel (359 ± 25 Pa) (Table 4.2). This suggested that the differences in gel stiffness between the two systems were not due to the difference in protein composition. It is also interesting to note that the CWPC gels with addition of β-lactoglobulin had similar stiffness to the AWPC gels without addition of β-lactoglobulin, over the range of added calcium levels used (Table 4.2).

4.3.4.2 Fracture properties

For the same reasons as for AWPC, the level of β-lactoglobulin in CWPC was not increased to match the level of β-lactoglobulin in WPI to study the effect of added calcium on the fracture properties of CWPC. The effect of added calcium on the fracture stress and fracture strain of the CWPC gels is shown in Figure 4.12.
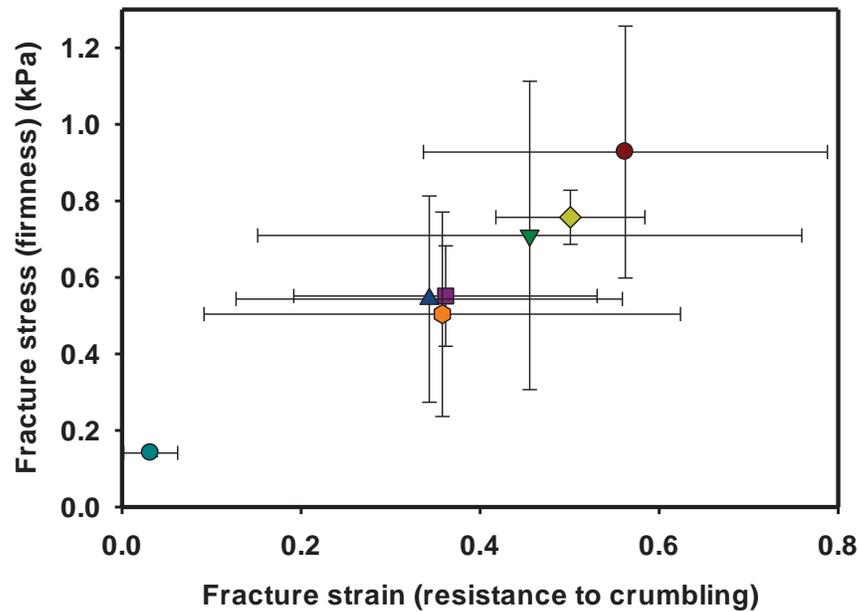
Figure 4.12: Effect of added $CaCl_2$ on the fracture stress and fracture strain of CWPC gels: 12% (w/w) protein, heated at 85ºC for 30 min, 0 < added $CaCl_2$ < 600 mM. Fracture stress and fracture strain were measured by a 90% compression test on the gel; ● no added $CaCl_2$; ▼ 12 mM added $CaCl_2$; ■ 30 mM added $CaCl_2$; ◆ 60 mM added $CaCl_2$; ▲ 240 mM added $CaCl_2$; ◓ 420 mM added $CaCl_2$; ● 600 mM added $CaCl_2$. Error bars: ± standard error, $n = 2$.

The compression test results (Figure 4.12) showed that the addition of calcium resulted in negligible changes in fracture stress but that there appeared to be some decrease in fracture strain. It is difficult to determine any effect of the added calcium on the CWPC gels. However, it is worth pointing out that fracture stress and fracture strain values of the CWPC gel with 600 mM added calcium were significantly lower than those of the other CWPC gels.

4.3.4.3 TEM
The effect of added calcium on the structure of the CWPC gels is shown in Figure 4.13.
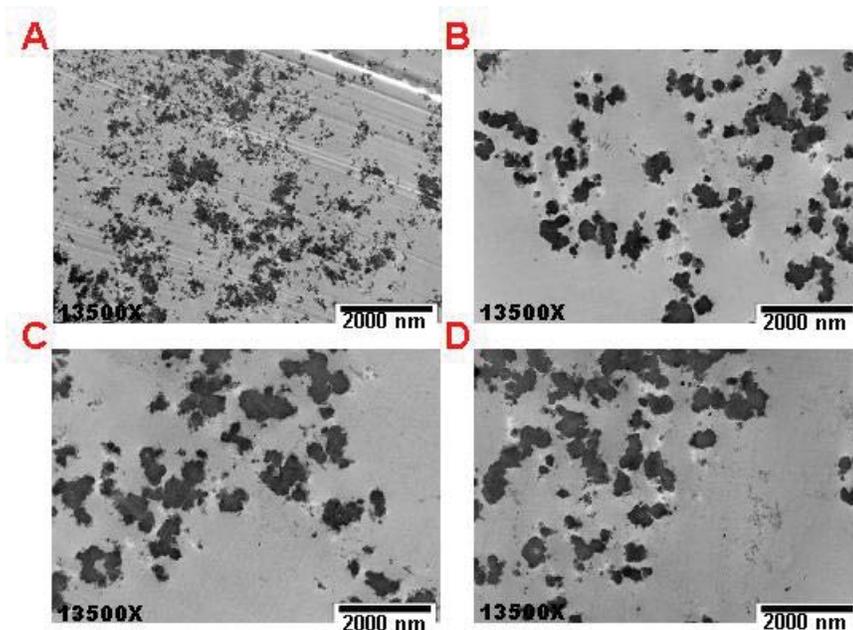
Figure 4.13: Effect of added $CaCl_2$ on the structure of CWPC gels. TEM micrographs of 12% (w/w) protein solutions heated at 85ºC for 30 min; **A** 0 mM added $CaCl_2$; **B** 60 mM added $CaCl_2$; **C** 240 mM added $CaCl_2$; **D** 600 mM added $CaCl_2$.

The control CWPC gel had a relatively coarse aggregate structure (~ 50 nm, Figure 4.13**A**). With 60 mM added calcium, larger protein aggregates (~ 300 nm) were formed (Figure 4.13**B**). This structure did not seem to change much with the addition of greater amounts of calcium (Figures 4.13**C** and **D**). Similarly to WPI and AWPC, addition of calcium changed the microstructure of the CWPC gels, from hairy, irregularly shaped aggregates to particulate aggregates.

4.3.4.4 Conclusions

As for WPI and AWPC, to be able to link the observations of the effect of added calcium on heat-induced denaturation and aggregation to the observations on heat-induced gelation of CWPC solutions, the experimental conditions had been kept as similar as possible. This explains the protein and calcium concentrations chosen for the small and large deformation experiments.

Similarly to AWPC, the viscoelastic properties data and fracture properties data for CWPC were somewhat difficult to reconcile. As for AWPC, the addition of calcium was needed before the development of structure was observed in the small deformation experiments, but, in the large deformation experiments, the CWPC gels without addition of calcium showed the highest hardness. It should be noted that the error bars

for the fracture stress and fracture strain values for CWPC gels were overlapping (except for the gels with 600 mM added calcium), making any interpretation difficult (Figure 4.12). The viscoelastic properties data showed the same trend as observed for WPI and AWPC, and were in good agreement with the results from Chapter 3. The gel stiffness increased with increase in added calcium levels up to a maximum at 80 mM, and then decreased with further increase in added calcium levels. The TEM micrographs showed that adding calcium to CWPC increased the size of the aggregates compared with those seen in the control gels without added calcium, making the gels coarser.

Similarly to AWPC, CWPC solutions were expected to form weaker gels than WPI solutions. The CWPC gels were in fact dramatically weaker than the WPI gels and also weaker than the AWPC gels, under the same experimental conditions. Again, pure β-lactoglobulin was added to CWPC solutions to match the β-lactoglobulin level in WPI. Viscoelastic properties measurements were then made again. It appeared that the CWPC gels were stiffer with β-lactoglobulin addition than without, but they were still dramatically less stiff than the WPI gels, and less stiff than AWPC gels with added β-lactoglobulin. It should be noted that the CWPC gels with β-lactoglobulin addition were of similar hardness to the AWPC gels without β-lactoglobulin addition.

4.3.5 Comparison of the three whey protein systems studied

4.3.5.1 Rheological properties

Viscoelastic characteristics of the three whey protein systems studied are summarised in Table 4.2. Figure 4.14 shows, specifically, the effect of 4 mM of added calcium on thermally-induced changes in the storage modulus (G') of these systems. When the whey protein systems (WPI, AWPC and CWPC) with no added calcium were heated, no measurable changes in the values of G' were observed, i.e. no gel networks were formed (Table 4.2). When 4 mM calcium was added, all the whey protein systems formed gel networks (Figure 4.14).

When AWPC and CWPC systems were topped up with pure β-lactoglobulin to match the β-lactoglobulin level of the WPI system, the gels formed with 4 mM of added calcium were still weaker than the corresponding WPI gel (Figure 4.15). Interestingly, AWPC and CWPC gels had about the same stiffness when the β-lactoglobulin level was

topped up to that of WPI, whereas without this modification, the stiffness of the AWPC gel with 4 mM added calcium was greater than the stiffness of the CWPC gel with 4 mM added calcium.
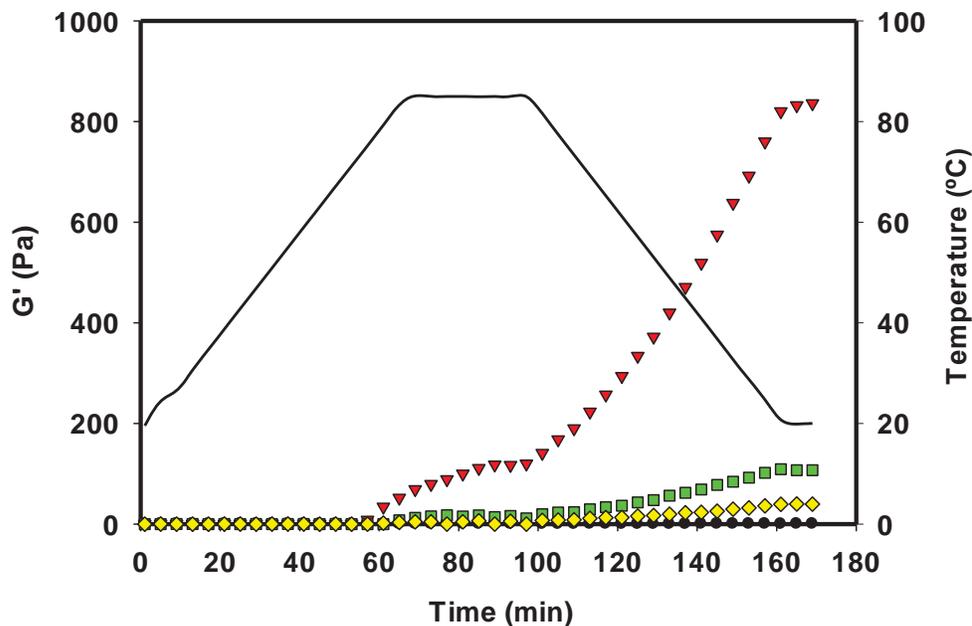
Figure 4.14: Effect of 4 mM added CaCl$_2$ on thermally-induced changes in the storage modulus (G') of the whey protein solutions, 4.8% (w/w) protein, heated from 20 to 85ºC at 1ºC min^{-1}, held at 85ºC for 30 min, cooled to 20ºC at -1ºC min^{-1} and held at 20ºC for 10 min. G' was measured every minute; ● control (WPI, no added CaCl$_2$), ▼ WPI, ■ AWPC, ◆ CWPC; — temperature.

The effect of added calcium on the gel point, defined as either the heating time or the temperature at which a measurable value of G' was first observed, followed similar trends in all three whey protein systems. The lowest gel points were found in heated protein solutions with 10 mM or 20 mM added calcium. Beyond these levels, the gel point increased with increases in added calcium. Comparing WPI with AWPC and CWPC (without the addition of β-lactoglobulin in both cases), at any level of added calcium the gel point for WPI was lower than that for either AWPC or CWPC. Interestingly, when β-lactoglobulin was added to AWPC and CWPC, at any level of added calcium the gel point for AWPC was higher than that for either WPI or CWPC (Table 4.2). The stiffest WPI gel (846 ± 8.0 Pa) was more than twice as stiff as the stiffest AWPC gel with added β-lactoglobulin (332 ± 3.0 Pa), and almost four times

stiffer than the stiffest CWPC gel with added β-lactoglobulin (221 ± 13 Pa). The G'$_{final}$ values were high at low levels of added calcium ($\leq$ 20 mM), but then decreased with increasing levels of added calcium.
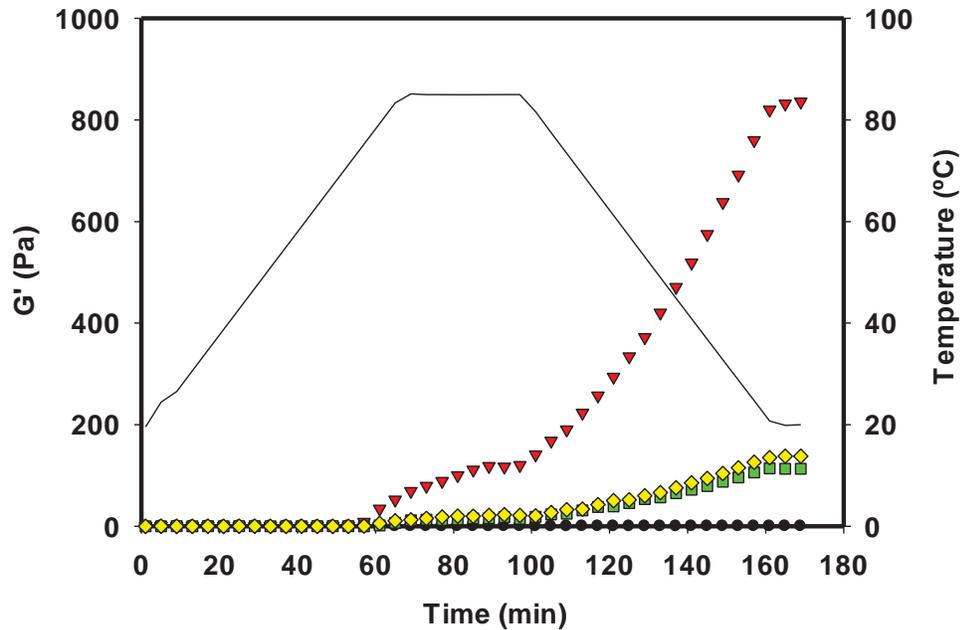
Figure 4.15: Effect of 4 mM added CaCl$_2$ on thermally-induced changes in the storage modulus (G') of the whey protein solutions, 4.8% (w/w) protein, heated from 20 to 85ºC at 1ºC min^{-1}, held at 85ºC for 30 min, cooled to 20ºC at -1ºC min^{-1} and held at 20ºC for 10 min. G' was measured every minute; ● control (WPI, no added CaCl$_2$, ▼ WPI, ■ AWPC with β-lg level topped up to β-lg level in WPI, ◇ CWPC with β-lg level topped up to β-lg level in WPI; — temperature.

Table 4.2: Rheological characteristics of heated whey protein solutions (4.8%, w/w protein) ($\pm$ standard error, $n = 2$).

Specific characteristics	Added CaCl$_2$ (mM)						
	0	4	10	20	80	140	200
WPI							
Gel point[a] (°C)	No gel	76 ± 1	74 ± 1	75 ± 1	78 ± 1	80 ± 1	81 ± 1
Gel point[b] (min)	No gel	56 ± 1	54 ± 1	55 ± 1	58 ± 1	60 ± 1	61 ± 1
G'$_{85}$[c] (Pa)	No gel	53 ± 1	39 ± 3	32 ± 5	27 ± 4	18 ± 2	11 ± 1
G'$_{final}$[c] (Pa)	No gel	846 ± 8.0	683 ± 32	627 ± 49	610 ± 1.0	496 ± 25	359 ± 25
AWPC							
Gel point[a] (°C)	No gel	82 ± 2	77 ± 1	79 ± 1	81 ± 1	83 ± 1	84 ± 1
Gel point[b] (min)	No gel	62 ± 2	57 ± 1	59 ± 1	61 ± 1	63 ± 1	64 ± 1
G'$_{85}$[c] (Pa)	No gel	8.0 ± 1	15 ± 2	18 ± 6	9.0 ± 1	11 ± 6	5.0 ± 1
G'$_{final}$[c] (Pa)	No gel	121 ± 13	197 ± 5.0	225 ± 18	195 ± 38	156 ± 9.0	152 ± 11
AWPC with β-lg level topped up to β-lg level in WPI							
Gel point[a] (°C)	No gel	82 ± 1	76 ± 1	76 ± 1	81 ± 1	83 ± 1	84 ± 1
Gel point[b] (min)	No gel	62 ± 1	56 ± 1	56 ± 1	61 ± 1	63 ± 1	64 ± 1
G'$_{85}$[c] (Pa)	No gel	7.0 ± 1	21 ± 1	22 ± 1	11 ± 1	5.0 ± 1	3.0 ± 1
G'$_{final}$[c] (Pa)	No gel	98 ± 15	295 ± 2.0	332 ± 3.0	221 ± 11	173 ± 5.0	143 ± 4.0
CWPC							
Gel point[a] (°C)	No gel	82 ± 1	78 ± 1	78 ± 1	81 ± 1	81 ± 1	83 ± 1
Gel point[b] (min)	No gel	62 ± 1	58 ± 1	58 ± 1	61 ± 1	61 ± 1	63 ± 1
G'$_{85}$[c] (Pa)	No gel	3.0 ± 1	5.0 ± 1	6.0 ± 1	5.0 ± 1	4.0 ± 1	3.0 ± 1
G'$_{final}$[c] (Pa)	No gel	35 ± 5.0	53 ± 4.0	60 ± 6.0	73 ± 4.0	64 ± 1.0	55 ± 2.0
CWPC with β-lg level topped up to β-lg level in WPI							
Gel point[a] (°C)	No gel	78 ± 1	76 ± 1	75 ± 1	78 ± 1	79 ± 1	80 ± 1
Gel point[b] (min)	No gel	58 ± 1	56 ± 1	55 ± 1	58 ± 1	59 ± 1	60 ± 1
G'$_{85}$[c] (Pa)	No gel	12 ± 2	14 ± 1	15 ± 2	14 ± 1	13 ± 1	11 ± 1
G'$_{final}$[c] (Pa)	No gel	139 ± 2.0	167 ± 6.0	210 ± 9.0	205 ± 2.0	221 ± 13	208 ± 9.0

[a]Temperatures at which a measurable increases in G' were first observed. [b]Times at which a measurable increases in G' were first observed. [c]G'$_{85}$ and G'$_{final}$ are the values of G' when 85°C was reached and at the end of the heating cycle, respectively.

Figure 4.16 shows the effect of total calcium (original calcium plus added calcium) on the G'$_{final}$ of 4.8% (w/w protein) whey protein solutions prepared from the three whey protein powders. The protein solutions (4.8%, w/w) made from these whey protein products required higher protein concentrations of around 8 to 10% (w/w) to form heat-induced gels. The total calcium concentrations in the original whey protein solutions (4.8%, w/w protein) were 2.1, 8.4 and 11.2 mM for WPI, AWPC and CWPC. It is clear that at 4.8% (w/w protein) added calcium induced gel formation in all of the whey protein solutions regardless of the original calcium concentration. Although the CWPC solutions had the highest calcium content (11.2 mM), they still needed added calcium to induce gel formation during heating. In contrast, although the WPI solution had a low initial calcium content (2.1 mM), a stiff gel was formed when 4 mM calcium was added (giving an overall residual calcium content of 6.1 mM, which was lower than the initial calcium content of the CWPC solutions). It is also clear that the initial calcium content had an effect on G'$_{final}$; the lower the initial content, the higher was the G'$_{final}$ at 4 mM added calcium (Figures 4.14 and 4.16).
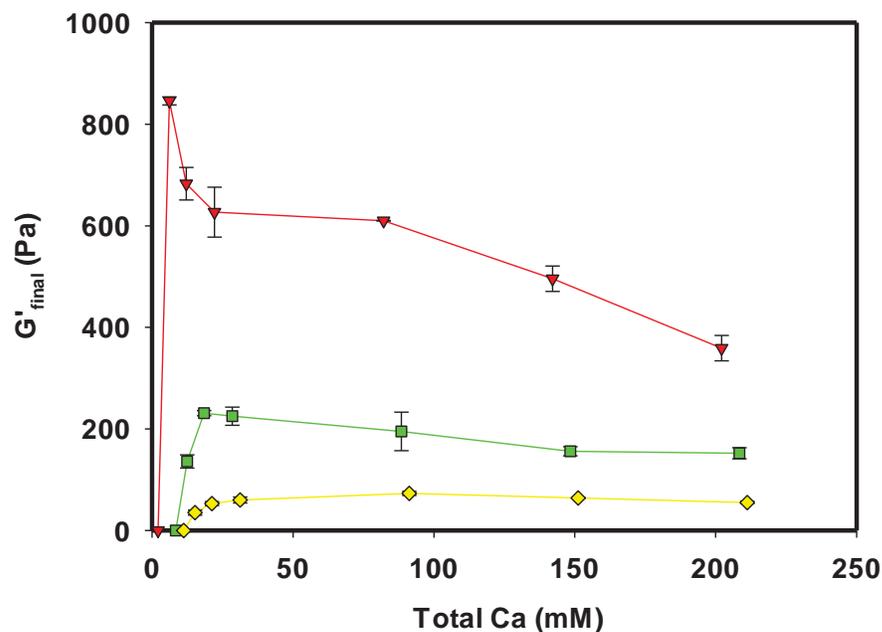
Figure 4.16: Effect of the total Ca on the G'$_{final}$ of the heat-induced gels (4.8%, w/w protein) prepared from ▼ WPI, ■ AWPC, and ◇ CWPC. The whey protein solutions were heated from 20ºC to 85ºC at 1ºC min^{-1}, held for 30 min, cooled from 85ºC to 20ºC at -1ºC min^{-1}, and then held for 10 min. Error bars: ± standard error, $n = 2$.

Without modification of the AWPC and CWPC systems (by the addition of β-lactoglobulin) the AWPC gels were stiffer than the CWPC gels over the range of added calcium concentration (Figure 4.16). In contrast, when AWPC and CWPC systems were topped up with pure β-lactoglobulin to match the β-lactoglobulin levels in WPI systems, at low levels of added calcium (< 100 mM), the AWPC gels were stiffer than the CWPC gels whereas at higher added calcium levels (>100 mM), the CWPC gels were stiffer than the AWPC gels (Figure 4.17).
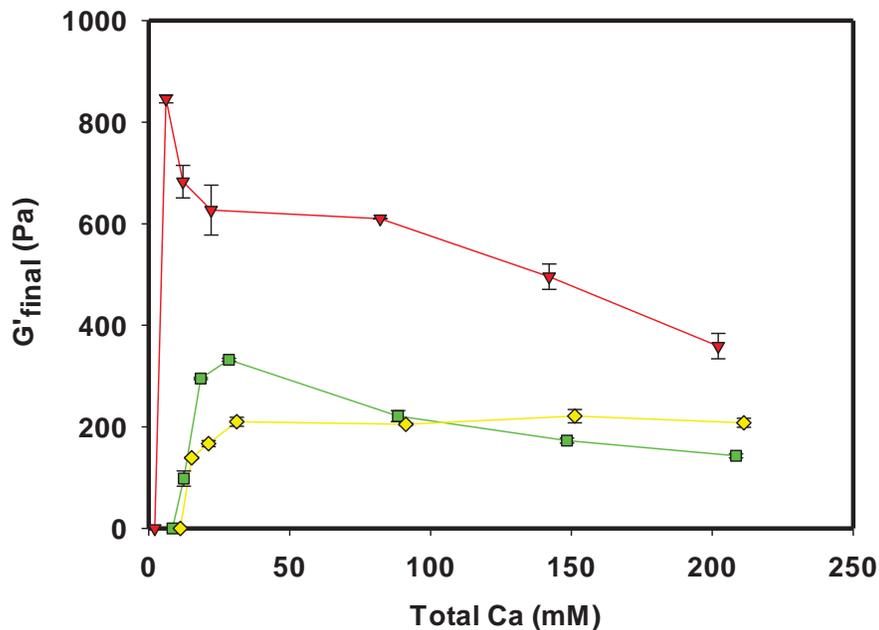
Figure 4.17: Effect of the total Ca on the G'$_{final}$ of the heat-induced gels (4.8%, w/w protein) prepared from ▼ WPI, ■ AWPC with β-lg level topped up to β-lg level in WPI, and ◆ CWPC with β-lg level topped up to β-lg level in WPI. The whey protein solutions were heated from 20ºC to 85ºC at 1ºC min^{-1}, held for 30 min, cooled from 85ºC to 20ºC at -1ºC min^{-1}, and then held for 10 min. Error bars: ± standard error, n = 2.

It appears that the calcium initially present in the whey protein solutions was probably bound to the proteins. As such, it would have been ineffective in inducing aggregation of the whey proteins during heating. Simons et al. (2002) suggested that calcium needs to be bound specifically to carboxylate groups with a threshold affinity (for binding as such) to trigger β-lactoglobulin aggregation. Protein aggregation itself is then driven by exposure of, and interaction between, denatured parts of the protein molecules without hindrance from electrostatic repulsion between the protein molecules. Different final gel

firmness (G') values for the three protein systems were observed at a given level of added calcium (Figure 4.14). It is unclear whether these differences could partly be attributable to the different degrees of calcium binding by the proteins in the systems. When the initial system contains less calcium (e.g. WPI), more sites could be available for calcium binding, hence the effect would be more pronounced. The opposite might be true when the calcium content is high (e.g. CWPC). The ζ-potential results at pH 6.7 (Table 4.2) suggested that the available carboxylate groups were limited in the AWPC and CWPC systems, compared with the WPI system, so that the binding of added calcium might have been significant in the WPI system, resulting in more interactions and much stiffer gels (Figure 4.16) and the formation of much larger protein aggregates (see Figure 4.21) than those formed in either the AWPC system or the CWPC system. The differences between AWPC and CWPC are probably attributable to the different effects of high sodium (AWPC) and high calcium (CWPC) in these products (Havea, Singh, & Creamer, 2002).

Table 4.3: ζ-potentials (mV) of the unheated whey protein solutions, 1% (w/w) protein (values are significantly different within the confidence interval at 95%, $P < 0.05$).

pH	WPI	AWPC	CWPC
6.7	-28	-24	-24
6	-21	-18	-18
5.5	-14	-14	-15

To compare the three whey protein systems in terms of their qualitative response to calcium, a shift plot of the effect of total calcium on the G'$_{final}$ of 4.8% (w/w protein) whey protein solutions is shown in Figure 4.17. The ratio G'/G'$_{max}$ was calculated by dividing the value of the G'$_{final}$ at each calcium level by the maximum G'$_{final}$ obtained in the range of total calcium level concerned. The ratio G'/G'$_{max}$ then ranged from 0 and 1.
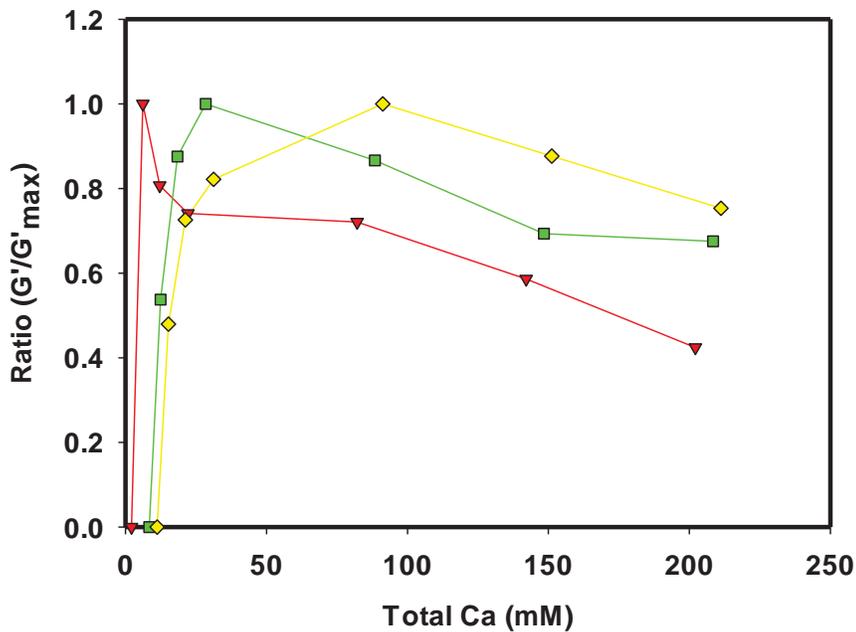
Figure 4.18: Effect of the total Ca on G'/G'_{max} of the heat-induced gels (4.8%, w/w protein) prepared from ▼ WPI, ▪ AWPC, and ◆ CWPC. The whey protein solutions were heated from 20ºC to 85ºC at 1ºC min^{-1}, held for 30 min, cooled from 85ºC to 20ºC at -1ºC min^{-1}, and then held for 10 min.

The WPI, AWPC and CWPC shift plot curves in Figure 4.18 were very different below 50 mM of total calcium. The main difference between them was that the stiffest gel was obtained at different levels of total calcium, ~ 6 mM total calcium for WPI, ~ 28 mM total calcium for AWPC and ~ 91 mM total calcium for CWPC. Therefore the peak of the curve is shifted to the right for AWPC and further to the right for CWPC. However, above 50 mM of total calcium, the three plots were relatively similar in terms of the downwards sloping part of the curve. The slope appeared to be similar and, with a transposition to higher ratio values, the WPI plot would overlay the AWPC plot. With further transposition to higher values, both the WPI and AWPC plots would overlay the CWPC plot. That is, at above 50 mM of total calcium, WPI, AWPC and CWPC were qualitatively relatively similar in terms of their responses to calcium.
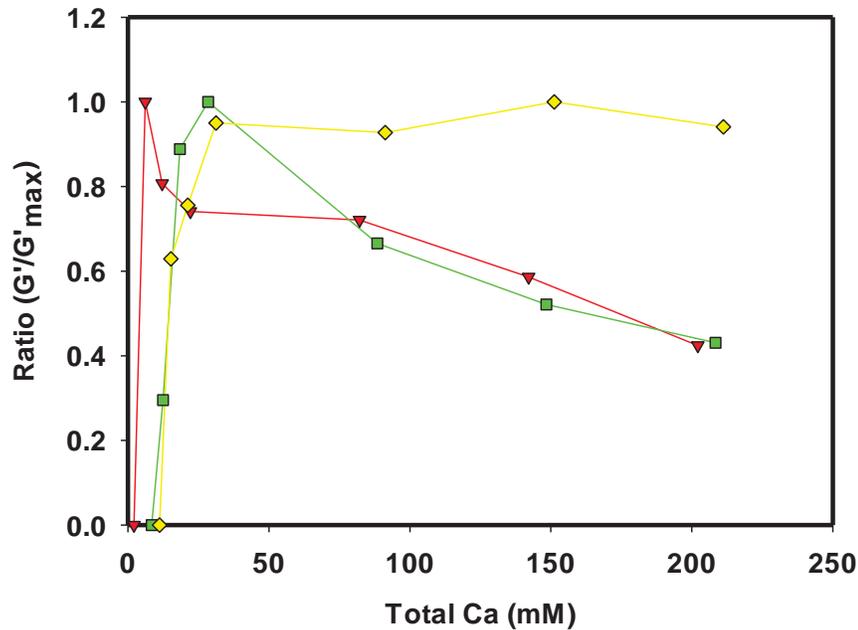
Figure 4.19: Effect of the total Ca on G'/G'$_{max}$ of the heat-induced gels (4.8%, w/w protein) prepared from ▼ WPI, ■ AWPC with β-lg level top up to WPI level, and ◆ CWPC with β-lg level top up to WPI level. The whey protein solutions were heated from 20ºC to 85ºC at 1ºC min^{-1}, held for 30 min, cooled from 85ºC to 20ºC at -1ºC min^{-1}, and then held for 10 min.

It is interesting to note that WPI and AWPC plots in Figure 4.19 were very different below 50 mM of total calcium but very similar above 50 mM of total calcium; that is, at above 50 mM of total calcium, WPI and AWPC were qualitatively similar in terms of their responses to calcium. However, below 50 mM of total calcium, the main difference between them was that the stiffest gel was obtained at different levels of total calcium, ~ 6 mM total calcium for WPI and ~ 28 mM total calcium for AWPC. Therefore the peak of the curve was shifted to the right for AWPC.

CWPC was very different from WPI and AWPC (Figure 4.19). For both WPI and AWPC, the stiffest gel was obtained with an optimal level of total calcium, and further increase in the total calcium level led to a decrease in gel stiffness. On the contrary, for CWPC it seemed that a total calcium threshold needed to be reached to obtain the stiffest gel and beyond this threshold, stiffness remained largely constant at its maximum level.

4.3.5.2 Fracture properties

High protein concentration is desirable when manufacturing a whey protein product, either functional or not functional, as the feed material would need to be heated at high protein concentration in order to make the commercial process economically feasible. To understand the effect of added calcium on the textural properties of whey protein gels at such high protein concentrations, heat-induced whey protein gels (12%, w/w protein) were prepared from the whey protein products. These gels were then analysed using large deformation compression tests and transmission electron microscopy (TEM). Figure 4.20 shows the effect of added calcium on the fracture stress and fracture strain of gels made from the three whey protein systems studied.
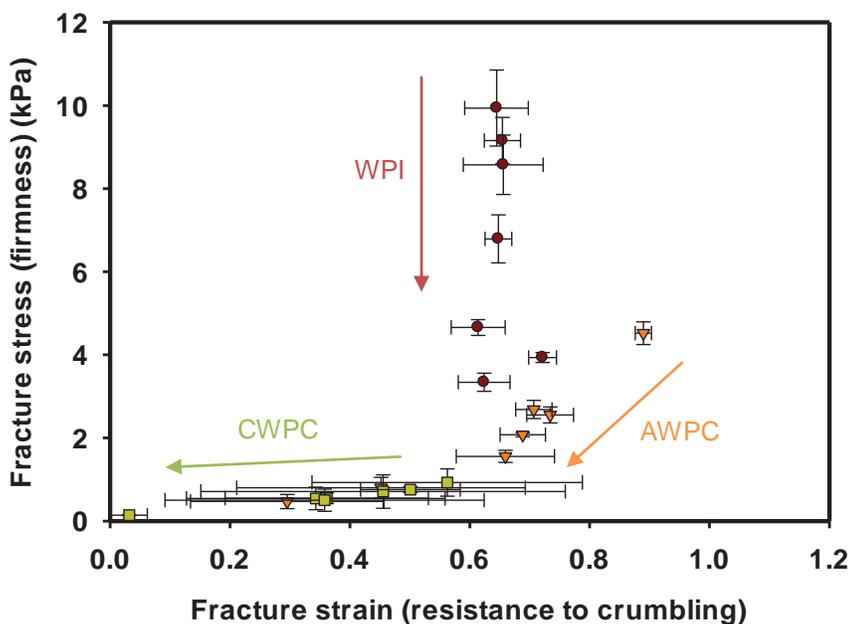
Figure 4.20: Effect of added $CaCl_2$ on the fracture stress and fracture strain of the whey protein gels: 12% (w/w) protein, heated at 85ºC for 30 min, $0 <$ added $CaCl_2 < 600$ mM. Fracture stress and fracture strain were measured using a 90% compression test on the gel; • WPI, ▽ AWPC, or ▫ CWPC. Error bars: ± standard error, $n = 2$.

The large deformation test results (Figure 4.20) revealed further information about the functional properties of the three whey protein systems. In contrast to the heated 4.8% (w/w protein) whey protein solutions (Figure 4.14 and 4.16), the 12% (w/w protein) whey protein solutions without added calcium formed gel networks upon heating.

When the gels were cut for compression testing, the control WPI gels were observed to be strong and clear, and could be described as being rubbery (Havea, Carr, & Creamer, 2004; Havea, Watkinson, & Kuhn-Sherlock, 2009). When calcium was added, the gels became very opaque and displayed considerable syneresis. Syneresis appeared to increase with increase in the level of added calcium. The gels with high levels of added calcium (e.g., 240 mM) were similar to sponges; water could readily be squeezed out of the gels. Mulvihill and Kinsella (1988) reported similar behaviour in β-lactoglobulin gels (17%, w/v, pH 8) heated at 90ºC for 30 min in the presence of high (> 4,000 mM) concentrations of sodium chloride. In contrast, the control CWPC gels (with no added calcium) were slightly more opaque than the control WPI gels. They were relatively strong and rubbery. The gels became slightly more opaque and less rubbery with increase in the level of added calcium up to ~ 240 mM. With further increases in the level of added calcium, the gels became "soft" and "mushy" (Havea, Carr, & Creamer, 2004). AWPC and CWPC gels released relatively small amounts of serum.

Serum release from the protein gels during the compression test is reported here only as an obvious difference between the WPI gels and the AWPC and CWPC gels. A proper analysis of this phenomenon following the method of van den Berg, van Vliet, van der Linden, van Boekel, and van de Velde (2007a) would have been interesting. However, this analysis was not performed when the experiments were carried out and will be the subject of future experiments. For the purposes of this study, the measured strains and stresses for the protein gels are reported here as the observed fracture stress and observed fracture strain uncorrected for serum release (Figure 4.20).

Zirbel and Kinsella (1988) reported maximal hardness (force (N) at 70% compression) at 20 mM added calcium in gels formed from 20% (w/w) protein WPI solutions. The difference between their and our results could probably be attributed to the different protein concentrations and the different methods of preparing the whey protein solutions. Kuhn and Foegeding (1991a, 1991b) suggested that a minimal amount of calcium was required for optimal gelation and that the initial calcium concentration played a beneficial role in promoting gelation unless this concentration was too high. Mulvihill and Kinsella (1988) and Schmidt et al. (1979) reported that gel strength increased to a maximum and then decreased with increasing calcium concentration. This could have been due to an excessive extent of intra-chain calcium bridging, with the protein matrix

collapsing into large, dense, non-continuous aggregates surrounded by an aqueous medium.

Recent work on the impact of different types of protein interactions on the functional properties of whey protein gels (Havea, Carr, & Creamer, 2004; Havea, Watkinson, & Kuhn-Sherlock, 2009) suggested that the intermolecular disulphide linkages between the denatured protein molecules were responsible for the rubberiness (fracture strain) of heat-induced whey protein gels (Figure 4.20). The degree of non-covalent association determines the fracture stress of whey protein gels. It appears that the addition of 4 mM calcium to the WPI systems resulted in a considerable degree of non-covalent intermolecular interaction, resulting in a high fracture stress (Figure 4.4). However, further additions of calcium resulted in decreasing fracture stresses with no apparent changes in fracture strain. This suggests that a specific phenomenon, possibly calcium bridging, was probably responsible for the intermolecular interactions. This specific interaction did not affect the degree of disulphide linkages, and hence there was no change in fracture strain (Figure 4.4). Addition of calcium to AWPC solutions (Figure 4.7) resulted in a decrease in both fracture stress and fracture strain. This indicated that the addition of calcium resulted in non-covalent interactions occurring at the expense of the formation of disulphide bonds, in comparison with WPI. It should be noted that the WPI solutions contained significantly more β-lactoglobulin, which is more susceptible to the formation of non-covalent associations when heated in the presence of added calcium (Chapter 3, Figure 3.1). Increasing the level of added calcium resulted in β-lactoglobulin forming a greater and greater proportion of non-covalent interactions, which dominated the gelling behaviour of the WPI gels (Figure 4.4).

4.3.5.3 Transmission electronic microscopy (TEM)

The effects of added calcium on the microstructures of the three types of whey protein gel (12%, w/w protein) studied is shown in Figure 4.21.
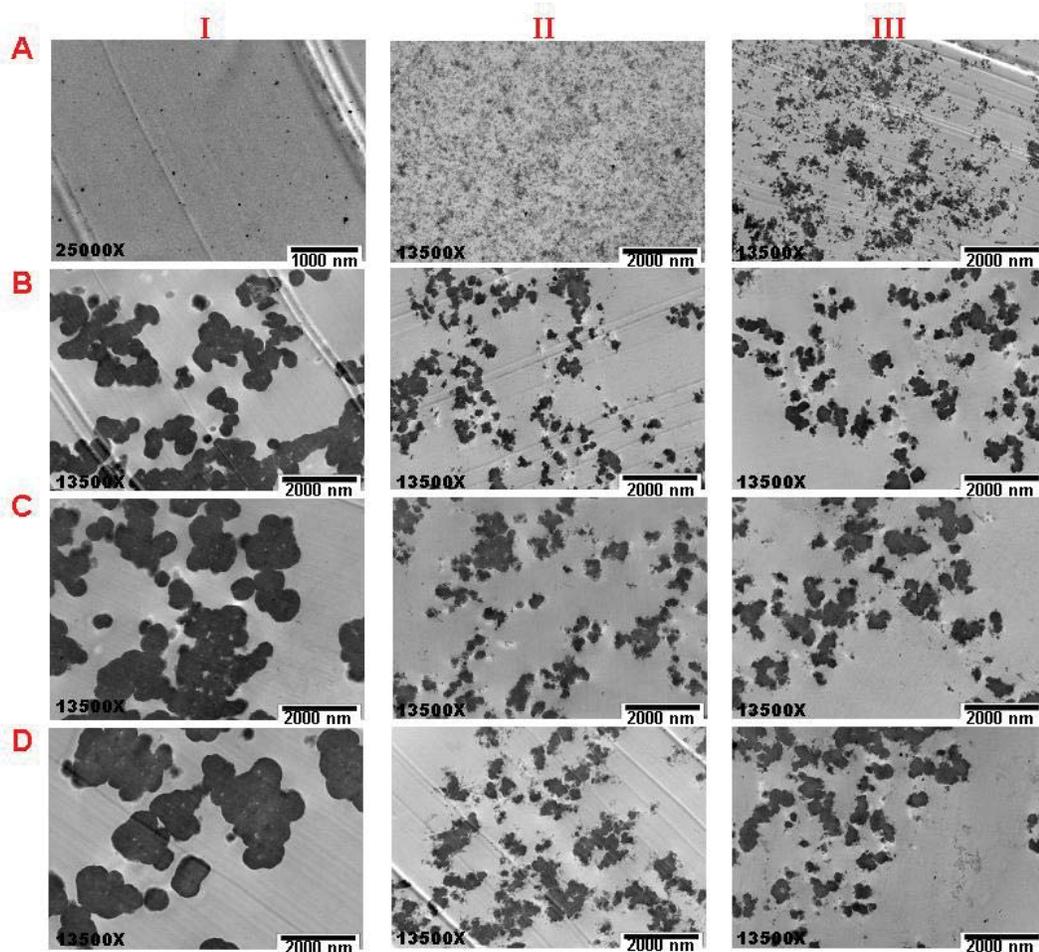
Figure 4.21: Effect of added CaCl$_2$ on the structure of whey protein gels. TEM micrographs of 12% (w/w) protein solutions heated at 85ºC for 30 min; **I** WPI; **II** AWPC; **III** CWPC. **A** 0 mM added CaCl$_2$; **B** 60 mM added CaCl$_2$; **C** 240 mM added CaCl$_2$; **D** 600 mM added CaCl$_2$.

Verheul and Roefs (1998a) described gelation as a two-phase process: the formation of the primary spatial structure and then an increase in the amount and/or stiffness of the bonds in the gel, which does not change the spatial structure. It appears that calcium promoted the formation of coarser gels (Havea, Singh, & Creamer, 2002; Ju & Kilara, 1998b; Morr & Josephson, 1968; Parris, Anema, Singh, & Creamer, 1993) in terms of primary spatial structure and that, as the calcium concentration increased, the amount and/or stiffness of the bonds in the gel diminished, leading to weaker gels. Added calcium appeared to have less effect on the microstructure of the AWPC and CWPC gels (Figures 4.21**II** and **III**) than on that of the WPI gels. The structures of the control gels (Figures 4.21**AII** and **AIII**) were relatively coarse. When calcium was added, larger aggregates were formed, although the aggregates were not as large as those

formed in the WPI gels with added calcium. The large coarse structure of the WPI gels probably explains the observed lack of water-holding capacity.

Mulvihill and Kinsella (1988) observed a maximum gel strength (17% β-lactoglobulin, pH 8, 90ºC, 30 min) at 10 mM calcium with microstructures described as comprising evenly dispersed aggregates that appeared to be linked by stronger well-defined, aggregate-type strands. At higher levels of calcium chloride (25 – 100 mM) the dispersed matrix collapsed into very large, densely packed protein aggregates in large areas of free aqueous space. These results were consistent with those of the current study.

4.4 Overall conclusions

The effects of added calcium on the heat-induced denaturation, aggregation and gelation of whey protein solutions prepared from commercial whey protein products (WPI, AWPC and CWPC) are complex, and were maximal at certain levels. It appeared that addition of calcium interfered with the types of interactions leading to the formation of protein aggregates during heating. It was considered that addition of calcium resulted in the formation of more non-covalent associations. Such associations dominated the properties of the heat-induced WPI gels. Addition of calcium to AWPC solutions resulted in the formation of non-covalent associations at the expense of disulphide linkages during heating. Addition of calcium to CWPC solutions had little apparent effect probably due to a limited number of sites available for calcium binding.

Understanding the effect of added calcium on the functionality of whey proteins is useful information for food companies, as is understanding the effect of added calcium on the behaviour of whey proteins during processing. Studying the kinetic parameters of the effect of added calcium on the heat-induced aggregation of whey proteins in a complex system appeared logically to be the next step of this work. Because of the growing commercial interest in adding more whey proteins to food products, the initial focus was on investigating the effect of added calcium on the kinetics of whey protein aggregation with varying protein levels (5 to 35%, w/w ts) and a fixed added calcium level (20 mM). This is discussed in Chapter 5.

CHAPTER 5 Kinetics of Whey Protein Heat-induced Denaturation and Aggregation at Different Total Solids Concentrations in the Absence or Presence of Added Calcium

5.1 Introduction

The nature, extent and rate of heat-denaturation/aggregation of whey proteins are influenced by the pH, ionic strength, protein concentration, heating temperature, heating time, etc. (Harwalkar, 1986). Kinetics of whey protein heat-induced denaturation and aggregation have been of much interest as, for industrial purposes, kinetic parameters are important for establishing the right processes for manufacturing whey protein products or milk products. Because of the complexity of milk or whey protein systems, changing one processing parameter might lead to different milk or whey products. Thus, it is difficult to extrapolate the kinetic data from a general experiment to all processes. Therefore, many studies are available in the literature, using different milk-based media: skim milk (Dannenberg & Kessler, 1988; Hillier & Lyster, 1979; Kessler & Beyer, 1991; Lyster, 1970; Oldfield, Singh, & Taylor, 2005b; Wehbi, Perez, Sanchez, Pocovi, Barbana, & Calvo, 2005), whole milk (Anema & McKenna, 1996; Ye, Singh, Oldfield, & Anema, 2004), simulated milk ultrafiltrate (O'Kennedy & Mounsey, 2009), whey protein concentrates or isolates (Harwalkar, 1986; Hinrichs & Rademacher, 2004; Mounsey & O'Kennedy, 2007; Spiegel, 1999; Spiegel & Huss, 2002), or proteins in water or buffer (Chen, Chen, Nguang, & Anema, 1998; de Wit & Swinkels, 1980; Galani & Apenten, 1999b; Gough & Jenness, 1962; Harwalkar, 1980b; Hoffmann & van Mil, 1997; Larson & Jenness, 1952; Park & Lund, 1984; Relkin & Launay, 1990; Roefs & de Kruif, 1994; Sava, van der Plancken, Claeys, & Hendrickx, 2005; W. H. Sawyer, Norton, Nichol, & McKenzie, 1971; Tanford, Bunville, & Nozaki, 1959; Tolkach & Kulozik, 2005, 2007; Verheul, Roefs, & de Kruif, 1998) at different protein concentrations and salt concentrations, ionic strength, pH, etc.

However, even though the values of the kinetic parameters were different depending on the experimental conditions, there is a general agreement on the mechanism of denaturation/aggregation of β-lactoglobulin (Roefs & de Kruif, 1994) and of whey proteins, which is largely dominated by β-lactoglobulin behaviour under heat-treatment.

The mechanism has 2 steps: the first is the unfolding or denaturation of the proteins exposing the reactive free thiols and hydrophobic residues and the second one is the aggregation or polymerisation of these activated proteins through disulphide exchange reactions and non-covalent interactions (hydrophobic, electrostatic, van der Waals). Depending on the heating temperature, either one step or the other will be the rate-limiting reaction. At low heating temperature, up to 80 - 90ºC, the unfolding of the proteins is rate-limiting. At high heating temperature, the aggregation is rate-limiting. The temperature at which the change occurs is called the transition temperature.

From all of the different studies cited above, it appears that the order of reaction for α-lactalbumin in any system has been found to be always 1. But for β-lactoglobulin, the order of reaction varies between 1 and 2. Similarly, the transition temperature has always been found to be 80ºC for α-lactalbumin and to vary between 80 and 90ºC for β-lactoglobulin.

The kinetics of heat-induced whey protein denaturation and aggregation have been well studied in different media but most of the time at low protein concentrations. Fewer studies were done at high β-lactoglobulin concentration. Park and Lund (1984) studied β-lactoglobulin thermal denaturation at a concentration of 10% (w/v), and Relkin and Launay (1990) within a concentration range up to 24% (w/w). In the present study, kinetic parameters were established at high protein concentrations, up to 35% (w/w) total solids, and the effect of 20 mM added calcium on the kinetic parameters was investigated.

From the previous results, WPI and CWPC whey proteins appeared to behave differently. The added calcium had a dramatic effect on the aggregation and gelation of whey proteins in WPI and a very small effect in CWPC. For both systems, if they were used for the kinetic study, accurate interpretation of the results would have then been difficult or limited. On the contrary, the effect of added calcium on the aggregation and gelation of whey proteins in AWPC was greater than in CWPC but not as dramatic as in WPI, enabling an accurate interpretation of the results. Thus, AWPC appeared logically to be the system to use for the kinetic study.

5.2 Materials and Methods

5.2.1 Composition of acid whey protein concentrate and preparation of the solutions

AWPC powder was provided by the Fonterra Co-operative Group Ltd, New Zealand and its composition is shown in Table 5.1.

Table 5.1: Compositions of the acid whey protein product used in this study

Composition		AWPC
Protein	%, w/w	80.4
Fat	%, w/w	5.43
Ash	%, w/w	3.81
Lactose	%, w/w	4.08
Moisture	%, w/w	4.96
Minerals	mmol kg^{-1} protein	
	Ca	74.375
	K	471.15
	Mg	7.6667
	Na	14.043

Different amounts of AWPC powder were dissolved in Milli Q water to give total solids (ts) concentrations of 5, 10, 15, 25 and 35% (w/w). These total solids concentrations correspond to 4, 8, 12, 20 and 28% (w/w) protein concentrations. A second set of AWPC solutions was made at these concentrations, with each solution containing 20 mM of calcium chloride. All samples (5 to 35%, w/w, with 0 and 20 mM added calcium chloride) heated for 20 and 80 s were done in triplicates to be able to calculate the statistical significance of the results.

5.2.2 Heat treatment of AWPC solutions

1 mL samples were heated in sealed Pasteur pipettes at 70, 75, 80, 85, 90, 95 and 100ºC in a water bath for 10, 20, 30, 40, 50, 60, 80, 100 and 120 s. As soon as tubes were taken from the water bath, they were put in an ice water bath for an hour to cool down and stop the reaction. The control was the unheated sample.

It is important to note that the pH was left as it was after heat treatment and was not adjusted to pH 4.6. Thus denatured proteins were not precipitated as described in the

literature (de Wit & Swinkels, 1980). However, O'Kennedy and Mounsey (2009) showed that levels of denaturation and aggregation were similar with or without pH adjustment.

5.2.3 Reverse-Phase High-Performance Liquid Chromatography (RP-HPLC)

The proteins measured by RP-HPLC as soluble were the native and denatured states or soluble aggregates. So the kinetic parameters determined by these experiments are related to the formation of insoluble aggregates of whey proteins under heat treatment. After the heat treatment, samples were diluted to 1% (w/w) protein concentration and centrifuged at 16,000 g for 3 minutes to remove any insoluble materials. The supernatants were then analysed by RP-HPLC.

The RP-HPLC system consisted of a Waters 2690 Alliance Separation Module (Waters, Milford, MA, USA) interfaced with a Waters 486 MS tunable absorbance detector and a Waters Millenium 32 data acquisition and manipulation system. A 1 mL Resource RPC column (Pharmacia Biotech) was operated at room temperature (RT) and at flow-rate of 1 mL min^{-1}. The column was equilibrated in 80% solvent A (0.1%, v/v, TFA in milli-Q water) and after sample injection a 1 min isocratic period was applied followed by a series of linear gradients to 100% solvent B (0.09%, v/v, TFA, 90%, v/v, MeCN in Milli-Q water) as described by Elgar et al. (2000). Detection was by absorbance at 214 nm and total run time was 30 min.

Acetonitrile (MeCN; far UV grade) and trifluoroacetic acid (TFA; HPLC grade) were from BDH (Poole, UK). Q Sepharose Fast Flow and Sephadex G-75 were from Pharmacia Biotech (Uppsala, Sweden). All other buffers and reagents were analytical grade or better. Aqueous buffers (eluents) were filtered through 0.45 μm cellulose acetate membranes (Millipore, Bedford, MA, USA) and degassed prior to use.
Queued samples were refrigerated at 5ºC.

5.2.4 Concentration normalisation

To measure the effect of the initial total solids concentration on the aggregation of β-lactoglobulin and α-lactalbumin, we needed to compare the results from the different experiments. Therefore, β-lactoglobulin and α-lactalbumin concentrations are given as a

percentage of their initial concentrations, normalised at 100% in the unheated solutions (controls) for each total solids concentration.

The results were given for β-lactoglobulin and α-lactalbumin together as an overview of the effect of total solids and the effect of added calcium on the loss of soluble proteins during heating. The results were significant for P < 0.001. β-Lactoglobulin and α-lactalbumin are not present in equal proportions in milk. Therefore, they are not present in equal proportions in the AWPC used for this study; the ratio (determined by RP-HPLC) was β-lactoglobulin/α-lactalbumin 70:30 in the control solution. Thus, the combined α-lactalbumin plus β-lactoglobulin concentration after a given heating time was calculated by summing the percentage remaining of each protein, the percentages being weighted according to these proportions.

5.3 Results and Discussion

An overall picture of the loss of soluble whey proteins (α-lactalbumin plus β-lactoglobulin) as determined by total solids concentration, heating temperature and heating time is presented in Section 5.3.1. Then, the calculated kinetic parameters for heat-induced changes in β-lactoglobulin and α-lactalbumin, in the absence of and in the presence of added calcium, are presented and discussed for each protein individually in Sections 5.3.2 and 5.3.3. In conclusion (Section 5.4), the kinetics of β-lactoglobulin and α-lactalbumin heat-induced denaturation/aggregation and the effect of calcium on the kinetics are compared.

5.3.1 AWPC: effect of initial total solids concentration, heating temperature and heating time on the loss of soluble whey proteins during heating

5.3.1.1   Without calcium chloride added to the whey protein solutions

Figure 5.1 shows the combined effect of heating temperature, heating time and initial total solids concentration on the loss of soluble (native molecules, denatured molecules and soluble aggregates) proteins (α-lactalbumin plus β-lactoglobulin), measured by RP-HPLC. The other whey proteins were also measured by RP-HPLC but they were not of interest in this study. This method of analysing the data, contour plots, allows an overview of the effects of heating temperature, heating time and total solids concentration all at once. The effects of each parameter will then be discussed more in detail.
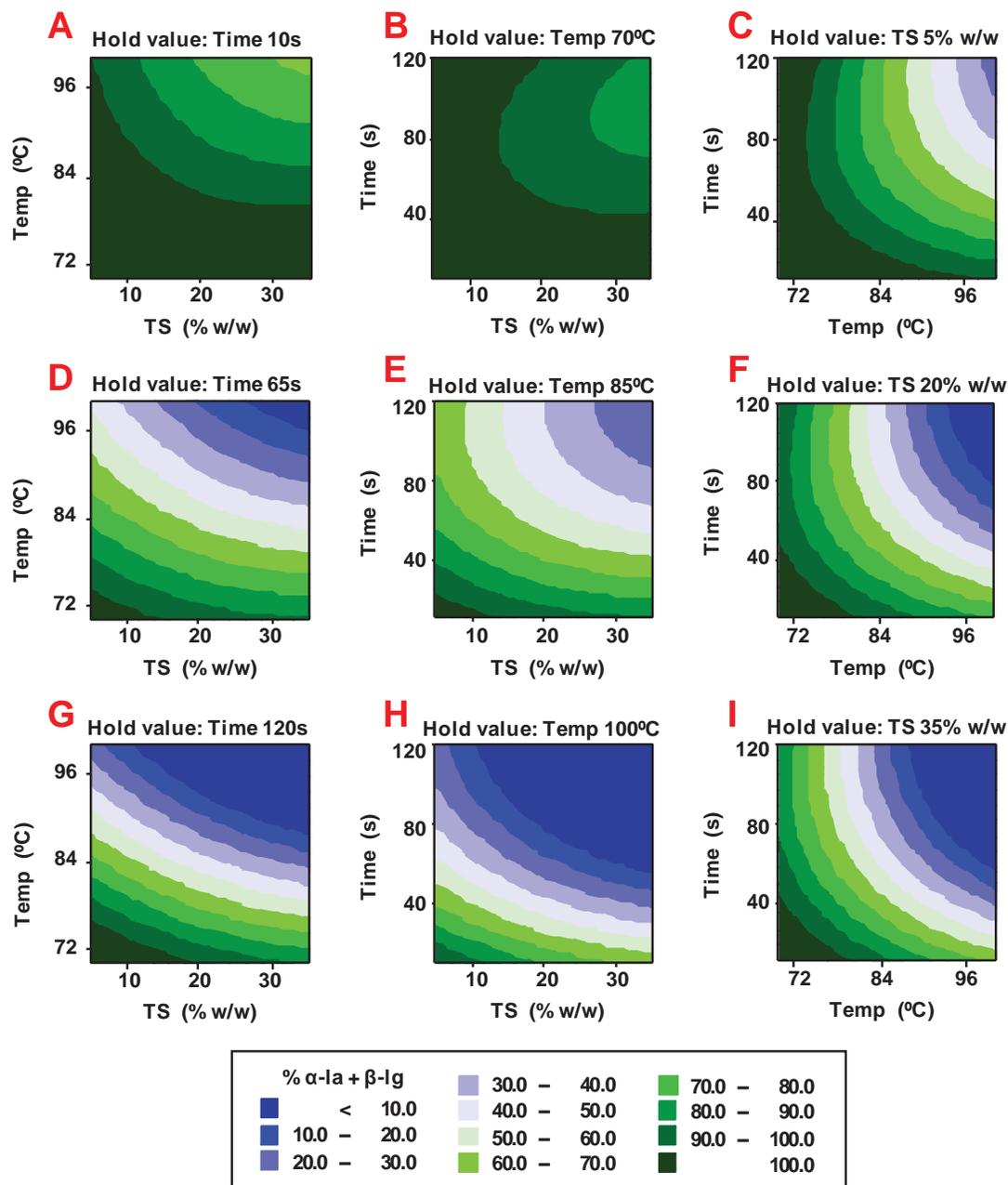
Figure 5.1: Effect of temperature, time and total initial protein concentration on the concentration of soluble β-lactoglobulin plus α-lactalbumin during heating without addition of $CaCl_2$. Graphs in the left hand column show the effect of heating temperature (**Temp**) vs. total solids concentrations (**TS**) at low (**A**), medium (**D**) and high (**G**) heating time (**Time**) hold values. The middle graphs show the effect of heating time (**Time**) vs. total solids concentrations (**TS**) at low (**B**), medium (**E**) and high (**H**) heating temperature (**Temp**) hold values. Graphs in the right hand column show the effect of heating time (**Time**) vs. temperature (**Temp**) at low (**C**), medium (**F**) and high (**I**) total solids concentration (**TS**) hold values.

The combination of high heating temperature, high heating time and high total solids concentration was necessary to see a large extent of insoluble aggregate formation (bottom graphs). It seemed that heating temperature had a greater effect than heating time and total solids concentration. At high total solids concentration, even after 120 s of heating, there was not much aggregation at low heating temperature (**I**). In contrast, at high heating temperature, significant insoluble aggregate formation occurred at all heating times and all total solids (**H**). Looking at the top graphs, at low heating temperature (**B**) there was little aggregation even after 120 s of heating at high total solids concentration. On the contrary, at low total solids concentration (**C**), there was a small blue area indicating that most of the proteins aggregated when heated for a long time at high temperature.

In the literature (Anema & McKenna, 1996; de Wit, 2009; Hillier, Lyster, & Cheeseman, 1979), the denaturation and aggregation kinetics of whey proteins are described as temperature dependent. As stated above, in the low temperature range, the denaturation step or unfolding is the rate-limiting reaction whereas in the high temperature range, the aggregation step is the rate-limiting reaction. This influence of the heating temperature on the denaturation/aggregation reaction is obvious in Figure 5.1; whey protein solutions needed to be heated above a certain temperature to observe any aggregation at a given heating time and total solids. Higher protein concentration and longer heating time increased the extent of insoluble aggregate formation. At a given temperature - time combination, the extent of insoluble aggregate formation increased with total solids (**C**, **F**, **I**).

5.3.1.2   With 20 mM calcium chloride added to the whey protein solutions

Previously, in Chapter 4, it has been demonstrated that 20 mM of calcium added to a 4% (w/w) protein solution (or 5%, w/w ts solution in the case of AWPC) significantly promoted the aggregation of whey proteins during heating at 85ºC for 5 minutes, especially of β-lactoglobulin. It was therefore interesting to study the effect of added calcium on the aggregation kinetics of the whey proteins. And so, adding 20 mM of calcium to the solutions appeared to be the best option to see the greatest changes on 5% (w/w) ts solution (4%, w/w protein solution). As before, the effect of 20 mM of added calcium on the different total solids whey protein solutions was investigated.

Figure 5.2 shows the combined effect of heating temperature, heating time and initial total solids concentration on the loss of soluble α-lactalbumin and β-lactoglobulin with 20 mM of calcium added to the solutions. As previously, the contour plots give an overview of the effects of heating temperature, heating time and total solids concentration when added calcium is present. The comparison of Figures 5.1 and 5.2 shows the effect of calcium on the thermal denaturation/aggregation of the two main whey proteins, α-lactalbumin and β-lactoglobulin.

The graphs in Figure 5.2 are very different to the graphs in Figure 5.1. It seems that the addition of calcium reversed the concentration effect on the heat-induced denaturation/aggregation of whey proteins. This was not that obvious in the right hand graphs (**C**, **F**, **I**), where the graphs were very similar for the three levels of total solids concentrations, with a slightly bigger darkest blue area (less than 10% soluble proteins left in solution) at low total solids concentration (**C**). The left hand graphs show clearly that at low (**A**) and medium (**D**) heating time, there was more aggregation at low total solids concentrations than at high total solids concentrations. At high heating time (**G**), the aggregation extent is relatively even in the total solids concentrations range, with more aggregation at higher heating temperature. But looking at the middle column of graphs, whether the heating temperature was low (**B**), medium (**E**) or high (**H**), there was more aggregation at low total solids than at high total solids concentrations.

The addition of 20 mM of calcium chloride to the whey protein solutions increased significantly the extents of aggregation of whey proteins at low total solids concentrations whereas at high total solids concentrations, it had no such effect. It seemed that addition of 20 mM of calcium chloride speeded up aggregation at low total solids but slightly slowed down the aggregation of whey proteins at high total solids concentrations. Sherwin and Foegeding (1997) showed that the ratio of added calcium (mM)/protein (%, w/w) was an important parameter in the effect of added calcium on whey protein aggregation. In the 35% (w/w) ts solution, there was 7 times more protein than in the 5% (w/w) ts, for the same amount of available calcium, 20 mM. At low total solids concentrations, the amount of calcium free to bind with the proteins was enough to improve the aggregation extent. But at high total solids concentrations the addition of calcium did not seem to influence the whey protein aggregation behaviour.
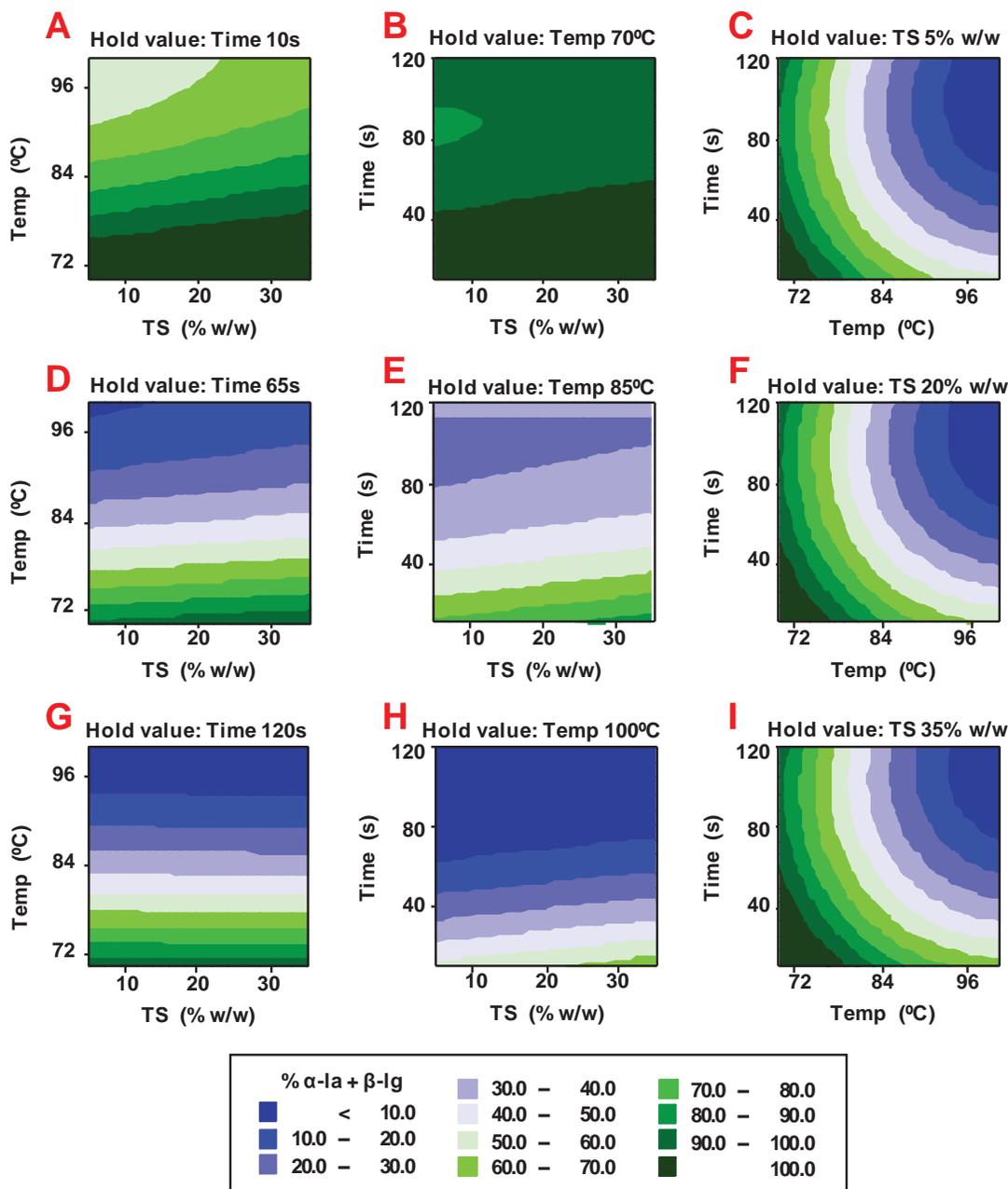
Figure 5.2: Effect of temperature, time and total initial protein concentration on the loss of soluble β-lactoglobulin plus α-lactalbumin during heating with 20 mM added $CaCl_2$. Graphs in the left hand column show the effect of heating temperature (**Temp**) vs. total solids concentrations (**TS**) at low (**A**), medium (**D**) and high (**G**) heating time (**Time**) hold values. The middle graphs show the effect of heating time (**Time**) vs. total solids concentrations (**TS**) at low (**B**), medium (**E**) and high (**H**) heating temperature (**Temp**) hold values. Graphs in the right hand column show the effect of heating time (**Time**) vs. temperature (**Temp**) at low (**C**), medium (**F**) and high (**I**) total solids concentration (**TS**) hold values.

5.3.1.3 Conclusions

From this overview of the results, it can be concluded that the thermal aggregation of α-lactalbumin and β-lactoglobulin is temperature dependent. When the heating temperature increased, the aggregation extents increased (Tolkach & Kulozik, 2007). De Wit (2009) reviewed the thermal behaviour of β-lactoglobulin, and reported that under 70ºC β-lactoglobulin undergoes reversible conformational changes and that the unfolding of the molecules occurs between 70 and 80ºC. This temperature is pH dependent (Relkin, 1996), being 70ºC at pH 7 and 80ºC at pH 3. Irreversible conformational changes in β-lactoglobulin structure take place as well between 70 and 80ºC which lead to the aggregation of the molecules. Increasing the heating time (Anema & McKenna, 1996; Dannenberg & Kessler, 1988) and the initial total solids concentration (Verheul, Roefs, & de Kruif, 1998) increased the extents of these reactions resulting in a larger loss of soluble proteins in solution. Relkin and Launay (1996) suggested that increasing the concentration promoted the thermal denaturation of β-lactoglobulin.

The addition of 20 mM of calcium seemed to increase significantly the aggregation extents at low initial total solids concentrations, 5 and 10 % (w/w). O'Kennedy and Mounsey (2009) observed that heating a 1% (w/w) β-lactoglobulin solution in the presence of 5 mM added calcium chloride significantly increased the level of denaturation/aggregation compared with heating in water or 100 mM added sodium chloride. The ratio β-lactoglobulin/calcium chloride in that study was similar to the ratio obtained with the 10% (w/w) total solids solution in the present study.

At high total solids concentrations, the aggregation extents with added calcium chloride were very similar to those without addition of calcium chloride, or even slower. Sherwin and Foegeding (1997) demonstrated that the calcium-associated thermal aggregation of β-lactoglobulin and WPI was affected by the calcium chloride to protein stoichiometry. Table 5.2 gives the calcium to protein ratios for each AWPC solution used to determine the effect of 20 mM added calcium chloride on the kinetics of aggregation.

Table 5.2: Calcium to protein ratios in the kinetics experiments.

Total solids	%, w/w	5	10	15	25	35
Protein concentration	%, w/w	4	8	12	20	28
Calcium from AWPC	mM	0.73	1.47	2.20	3.67	5.14
Total calcium in solution	mM	20.73	21.47	22.20	23.67	25.14
Calcium/protein ratio	mM/%, w/w	5.18	2.68	1.85	1.18	0.90

According to Sherwin and Foegeding study (1997), a maximum rate existed between 3.33 and 23.3 calcium/protein (mM/%, w/w). The ratio obtained for the 5% (w/w) ts solution was within this range, but not the ratios for 10 and 15% (w/w) ts solutions (though these values were still close to the lower limit of the range). However, an increase in the aggregation extents for these latter solutions was also observed. The ratio range given by Sherwin and Foegeding (1997) was established with pure β-lactoglobulin solutions or WPI solutions. This might explain why in this present study with AWPC solutions, the ratios observed for 10 and 15% (w/w) ts were not within the range even though the extents of aggregation increased by the addition of calcium.

Results showing the combined effect of heating temperature, heating time and total solids concentration, and of added calcium, on the aggregation of whey proteins in AWPC will now be presented for β-lactoglobulin and then α-lactalbumin individually.

5.3.2 β-Lactoglobulin: effect of initial total solids concentration, heating temperature and heating time on the loss of soluble β-lactoglobulin during heating

5.3.2.1 Loss of soluble proteins

Without added calcium

Figure 5.3 shows the loss of soluble β-lactoglobulin, measured by HPLC, against time while heated at different temperatures, 70 to 100ºC, at different total solids concentrations, 5 to 35% (w/w).

The loss of soluble β-lactoglobulin increased with heating time and temperature, but at different rates depending on the initial total solids concentration. At higher total solids concentration, β-lactoglobulin aggregated faster; 6% of soluble β-lactoglobulin was left after 120 s of heating at 100ºC for the 35% (w/w) ts solution as against 44% for the 5%

(w/w) ts solution, i.e. the extent of denaturation increased with total solids concentration. This is in agreement with the results of Relkin and Launay (1990), who looked at the concentration effects on the kinetics of β-lactoglobulin heat denaturation by differential scanning calorimetry (DSC). For that study, they used samples of β-lactoglobulin dispersed in distilled water at pH 3.2 with concentrations from 3.5 to 24%, heated at 82.5ºC for different periods of time. They found that increasing the concentration resulted in increased denaturation. This is not in agreement with earlier studies on cheese whey (Guy, Vettel, & Pallansch, 1967; Hillier, Lyster, & Cheeseman, 1979; Nielsen, Coulter, Morr, & Rosenau, 1973) where the authors described maximum protection against denaturation at an intermediate total solids concentration (20%, w/v). McKenna and O'sullivan (1971) reported results similar to those of Guy et al.(1967), Nielsen et al. (1973) and Hillier et al. (1979) in concentrated skim milks heated at 75 and 80ºC.

An interesting observation was that for all total solids concentrations, the heating temperatures could be organised in two groups. The first one was 70 and 75ºC, where for 5 and 10% (w/w) ts solutions, no significant aggregation was observed and, for 15, 25 and 35% (w/w) ts solutions, there was relatively little aggregation. Slightly more aggregation was observed when heated at 75 than at 70ºC but a steady value was not reached. At these two heating temperatures, the loss of soluble β-lactoglobulin was almost linear with increasing heating time. The second group was 80 to 100ºC, where aggregation occurred relatively quickly and reached a steady value at around 80 s of heating for 25% and 35% (w/w) ts. A recent review on the thermal behaviour of bovine β-lactoglobulin (de Wit, 2009) reported the unfolding of β-lactoglobulin at between 70 and 80ºC, as well as its irreversible conformational changes and its aggregation. The predominance of one phenomena or another one depended on many factors like salt concentration and ionic strength, protein concentration, heating rate, etc. De Wit (2009) reported the aggregation of β-lactoglobulin at between 80 and 95ºC.
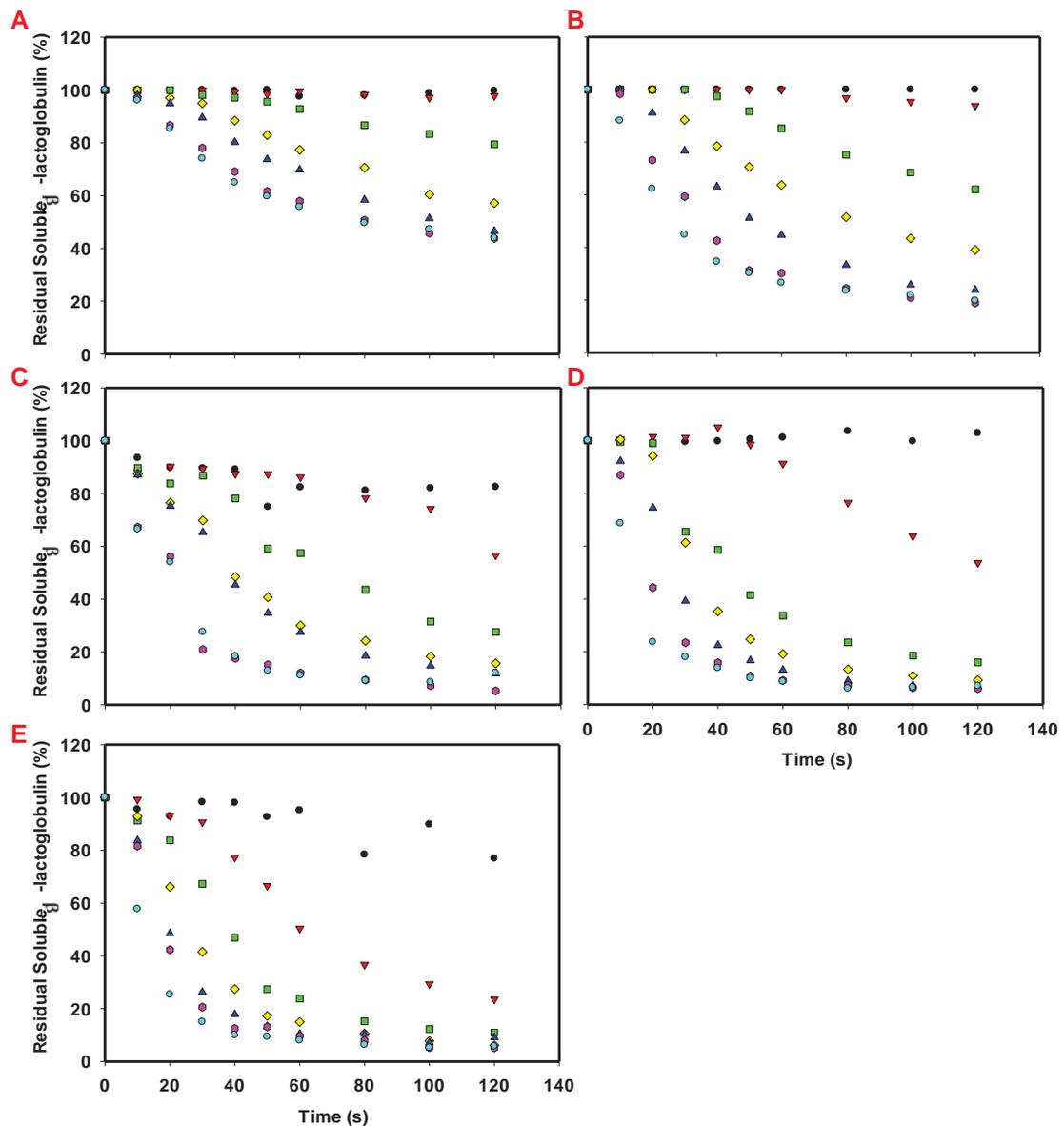
Figure 5.3: Loss of soluble β-lactoglobulin during heating at ● 70ºC, ▼ 75ºC, ■ 80ºC, ◇ 85ºC, ▲ 90ºC, ⬠ 95ºC and ○ 100ºC; at different total solids concentrations: **A** 5% (w/w) ts, **B** 10% (w/w) ts, **C** 15% (w/w) ts, **D** 25% (w/w) ts and **E** 35% (w/w) ts and without addition of $CaCl_2$.

With added calcium

Figure 5.4 shows the loss of soluble β-lactoglobulin (native, denatured molecules and soluble aggregates), measured by RP-HPLC, against time while being heated at different temperatures, 70 to 100ºC, and for different total solids concentrations, 5 to 35% (w/w) ts, each solution containing 20 mM of added calcium. As expected, for the 5% (w/w) ts solution, aggregation occurred much faster with increasing heating time and temperature than without added calcium, except at 70ºC where little aggregation

occurred. With 20 mM of added calcium, only 5% of soluble β-lactoglobulin was left after heating at 100ºC for 120 s as against 44% of soluble β-lactoglobulin left without addition of calcium under the same heating conditions (Figure 5.3). Similarly, for the 10% (w/w) ts solution, aggregation was faster than without added calcium, 5% of soluble β-lactoglobulin being left with 20 mM added calcium as against 20% of soluble β-lactoglobulin being left without added calcium, for the heating conditions of 100ºC for 120 s. The aggregation was also slightly faster than without added calcium for the 15% (w/w) ts solution, 7% of soluble β-lactoglobulin being left with 20 mM added calcium as against 12% of soluble β-lactoglobulin being left without addition of calcium, for the heating conditions of 100ºC for 120 s. In the case of 25 and 35% (w/w) ts solutions, the effect of added calcium on the aggregation of β-lactoglobulin was different, and almost opposite to the effect of added calcium on 5, 10 and 15% (w/w) ts solutions. Indeed, with the addition of 20 mM of calcium to both 25 and 35% (w/w) ts solutions, the aggregation of β-lactoglobulin was slightly slower at the lower heating temperatures, 70 to 85ºC. For the 25 and 35% (w/w) ts solutions, with 20 mM added calcium 32 and 18% (respectively) of soluble β-lactoglobulin were left after heating at 80ºC for 120 s as against 16 and 11% (respectively) of soluble β-lactoglobulin being left, at the same heating conditions, without the addition of calcium (Figure 5.3). Above 85ºC, the amounts of soluble β-lactoglobulin left were similar with or without addition of calcium. The maximum aggregation observed (corresponding to around 6% of β-lactoglobulin remaining soluble) was about the same whether or not calcium had been added.
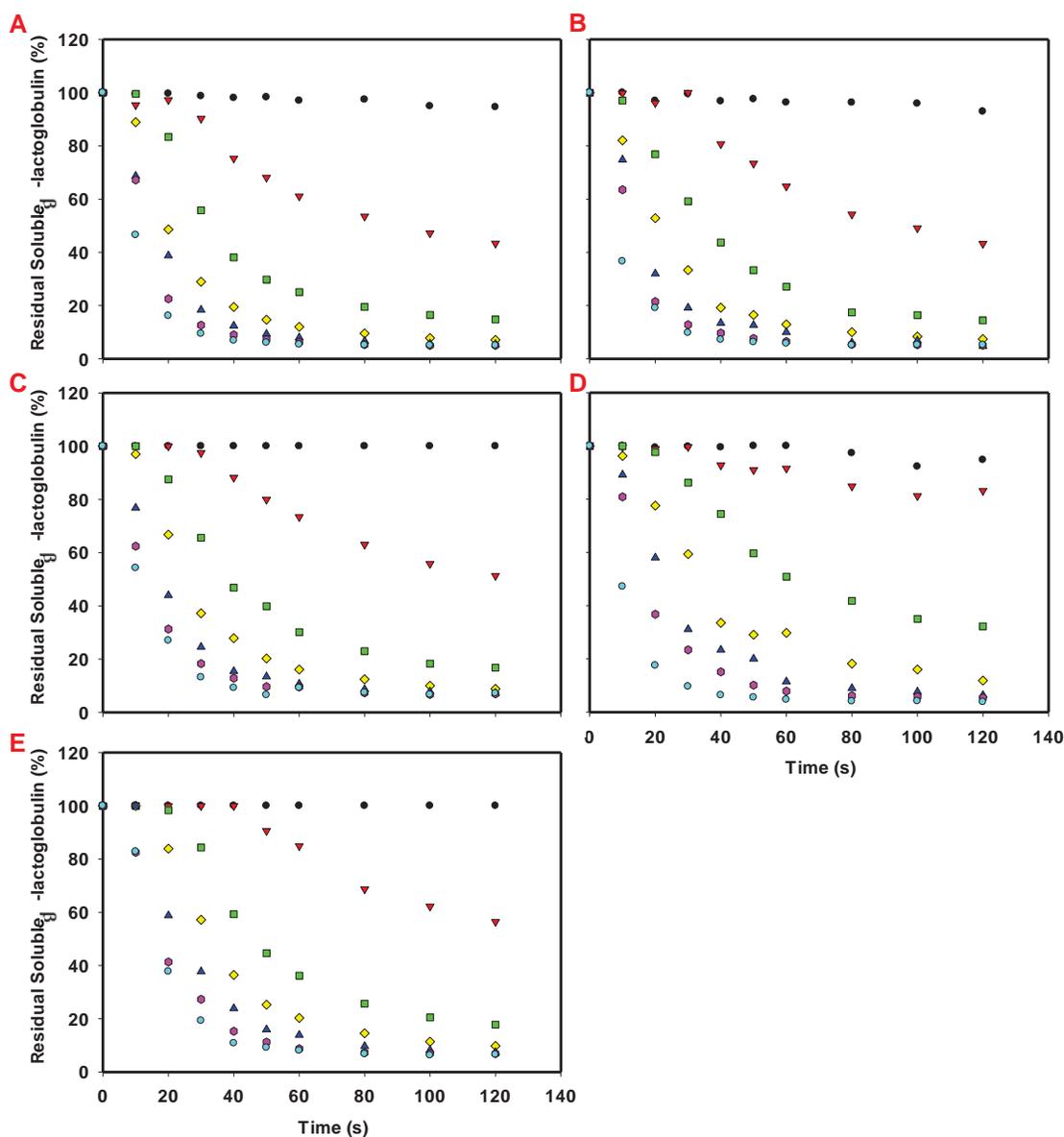
Figure 5.4: Loss of soluble β-lactoglobulin during heating at ● 70ºC, ▼ 75ºC, ■ 80ºC, ◇ 85ºC, ▲ 90ºC, ⬠ 95ºC and ◯ 100ºC; at different total solids concentrations: **A** 5% (w/w) ts, **B** 10% (w/w) ts, **C** 15% (w/w) ts, **D** 25% (w/w) ts and **E** 35% (w/w) ts and with 20 mM added $CaCl_2$.

Even though changes were observed in the loss of soluble β-lactoglobulin, the two groups of heating temperatures noticed in Figure 5.4 remained the same. The aggregation was still slow at 70 and 75ºC without reaching maximum aggregation, whereas at 80ºC and above, the loss of soluble β-lactoglobulin was faster and tended to bottom out to a constant value. And an exponential type model could still be applied on the losses of soluble β-lactoglobulin over time during heating.

From the study of the loss of soluble β-lactoglobulin during heating, it appeared that, without added calcium, β-lactoglobulin aggregation was most sensitive to heating temperature, then to total solids concentration and finally to heating time. But when 20 mM of calcium was added to the solutions, the behaviour of β-lactoglobulin under heating was remarkably different at low total solids concentrations. To quantify these observations, it was important to look at the kinetic parameters (rate constants, activation energies, orders of reaction).

5.3.2.2 Aggregation kinetics

The order of thermal aggregation of β-lactoglobulin was determined by integration of the general rate equation

$$-dC/dt = k_n C_t^n \tag{5.1}$$

(n = order of reaction, k_n = rate constant and C_t = concentration of soluble protein at time t) which yields an equation that describes the extent of reaction at any one moment as a function of the instantaneous protein concentration

for $n \neq 1$

$$(C_t/C_0)^{(1-n)} = 1 + (n-1)k_n C_0^{(n-1)} t \tag{5.2}$$

and for $n = 1$

$$\ln(C_t/C_0) = -k_1 t \tag{5.3}$$

Equations 5.2 and 5.3 were used to generate plots of $\ln(C_t/C_0)$ versus t (for assumed n = 1) and $(C_t/C_0)^{(1-n)}$ versus t (for assumed n = 1, 1.2, 1.5, 1.7 and 2). These choices for the value of n were driven by the literature (Chime, Konrad, Kleinschmidt, & Gorzki, 2009; Gough & Jenness, 1962; Harwalkar, 1980b; Hillier & Lyster, 1979; Iametti, DeGregori, Vecchio, & Bonomi, 1996; Kessler & Beyer, 1991; Lyster, 1970; Park & Lund, 1984; Relkin & Launay, 1990; W. H. Sawyer, Norton, Nichol, & McKenzie, 1971; Verheul, Roefs, & de Kruif, 1998) where the order of β-lactoglobulin denaturation/aggregation reaction is described to vary between 1 and 2. The order of reaction n is then deduced as that pertaining to the most linear plot, i.e. the plot for which the R^2 value (correlation coefficient) is maximal. The slopes of the straight lines give the values of the observed rate constants $k = (n-1)k_n C_0^{(n-1)}$ from which the rate constant k_n is calculated for any (constant) temperature of heating.

Without added calcium

First order reaction was eliminated at each total solids concentration as linear relationships were never obtained. Linear relationships were obtained for the other reaction order values (n = 1.2, 1.5, 1.7 and 2). However, the order of reaction was not consistent and varied over the range of total solids concentrations (5 to 35%, w/w ts). To be able to compare and explain the results, kinetic parameters had to be determined for the same order of reaction at all total solids concentrations. Overall, the best linear regression fits at the different total solids concentrations was obtained for the value n = 1.7. Figure 5.5 shows these fits for 5% (w/w) ts. The common order of reaction then chosen was n = 1.7.
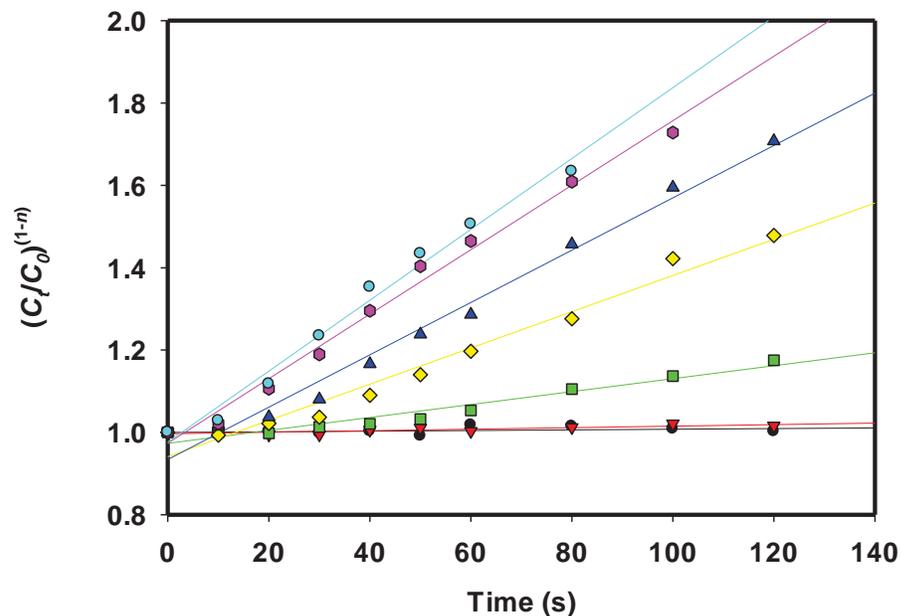
Figure 5.5: Aggregation of β-lactoglobulin in heated AWPC solutions, 5% (w/w) ts, without addition of $CaCl_2$; at ● 70ºC, ▼ 75ºC, ■ 80ºC, ◇ 85ºC, ▲ 90ºC, ● 95ºC and ○ 100ºC; n = 1.7.

The values of the slopes (the observed rate constants k) and the rate constant $k_{1.7}$ corresponding to the different total solids concentrations are given in Table 5.3.

Table 5.3: Observed rate constants $k = 0.7 \times k_{1.7} C_0^{0.7}$ and rate constants $k_{1.7}$ for β-lactoglobulin in heated AWPC solutions without addition of $CaCl_2$, at each heating temperature and each total solids concentration.

Temperature (ºC)		70	75	80	85	90	95	100
$0.7 \times$ $k_{1.7} C_0^{0.7}$ $(s^{-1} \times 10^{-3})$	5% (w/w) ts	0.07 $R^2 = 0.88$	0.10 $R^2 = 0.86$	1.20 $R^2 = 0.88$	3.60 $R^2 = 0.93$	5.50 $R^2 = 0.96$	7.40 $R^2 = 0.99$	8.20 $R^2 = 0.98$
	10% (w/w) ts	0.40 $R^2 = 0.66$	1.00 $R^2 = 0.90$	3.60 $R^2 = 0.93$	8.40 $R^2 = 0.96$	15.70 $R^2 = 0.96$	22.40 $R^2 = 0.94$	26.90 $R^2 = 0.97$
	15% (w/w) ts	1.70 $R^2 = 0.59$	2.90 $R^2 = 0.81$	10.70 $R^2 = 0.91$	21.20 $R^2 = 0.96$	26.80 $R^2 = 0.95$	55.40 $R^2 = 0.98$	52.50 $R^2 = 0.95$
	25% (w/w) ts	0.20 $R^2 = 0.92$	3.10 $R^2 = 0.72$	20.50 $R^2 = 0.94$	35.20 $R^2 = 0.95$	49.10 $R^2 = 0.96$	64.10 $R^2 = 0.97$	76.70 $R^2 = 0.99$
	35% (w/w) ts	1.60 $R^2 = 0.81$	12.30 $R^2 = 0.88$	30.50 $R^2 = 0.94$	47.80 $R^2 = 0.97$	47.80 $R^2 = 0.87$	63.30 $R^2 = 0.97$	76.50 $R^2 = 0.96$
$k_{1.7}$ $(s^{-1} mg^{-1} g \times 10^{-3})$	5% (w/w) ts	0.01	0.02	0.20	0.60	0.91	1.23	1.36
	10% (w/w) ts	0.04	0.10	0.37	0.87	1.63	2.33	2.80
	15% (w/w) ts	0.10	0.18	0.66	1.31	1.66	3.42	3.24
	25% (w/w) ts	0.01	0.21	1.40	2.41	3.36	4.38	5.24
	35% (w/w) ts	0.10	0.81	2.00	3.14	3.14	4.15	5.02

The rate constants $k_{1.7}$ increased with heating temperature and with total solids concentrations, which is in agreement with the literature (Anema, Lee, & Klostermeyer, 2006; Anema & McKenna, 1996; Dannenberg & Kessler, 1988; Gough & Jenness, 1962; Oldfield, Singh, & Taylor, 2005b; Spiegel, 1999). It is interesting to note that at 35% (w/w) ts, the rate constants at 90, 95 and 100ºC are similar to those for 25% (w/w) ts. Some R^2 values (correlation coefficient) were low, $R^2 < 0.80$, probably due to experimental errors.

Figure 5.6 illustrates the concentration dependence of the rate constants $k_{1.7}$. The effect of concentration on the rate constants was more important at low temperature and low total solids concentration. There was a sharp increase in the rate constants from 5 to 15 % (w/w) total solids at 70 and 75ºC. This increase was slower at 80 and 85ºC. At 90, 95 and 100ºC, the rate constants increased only slightly with total solids concentration. At higher total solids concentration, 15 to 35% (w/w), the rate constants increased slightly and seemed to reach a plateau value, except for 35% (w/w) at 75ºC.
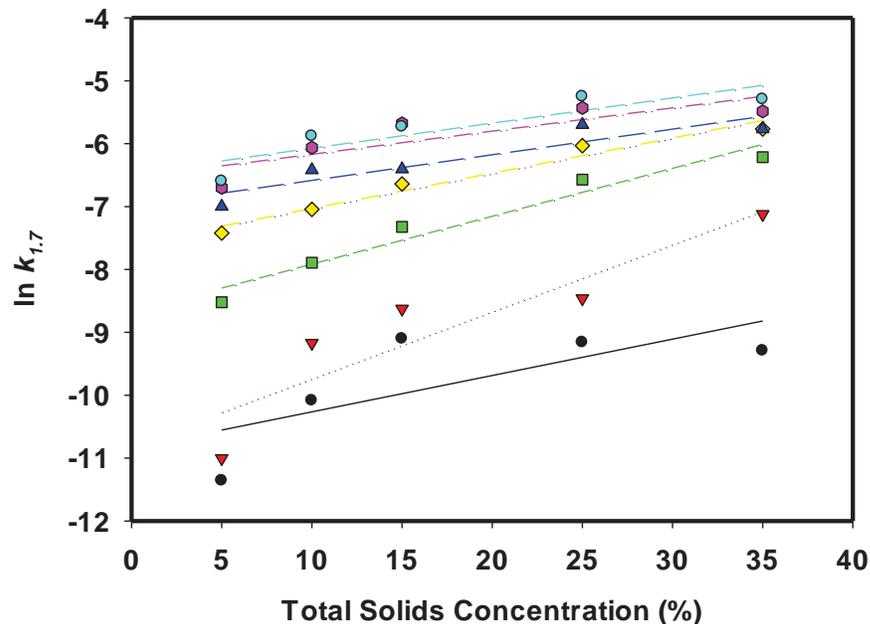
Figure 5.6: Effect of total solids concentration on the rate constants $k_{1.7}$, without addition of CaCl$_2$, at ● 70ºC, ▼ 75ºC, ■ 80ºC, ◆ 85ºC, ▲ 90ºC, ⬤ 95ºC and ⬤ 100ºC.

With added calcium
Similarly to the case of no addition of calcium chloride, no linear relationships were obtained for a reaction order of unity. But linear relationships were obtained for order of reaction values between 1 and 2 (n = 1.2, 1.5, 1.7 and 2). Best fit were obtained at different order of reaction values over the range of total solids concentrations studied. For a given temperature and ts, the reaction order n was different for *with* and for *without* calcium. To be able to compare the results and explain them, an order of reaction of n = 1.7 was again chosen (Figure 5.7).
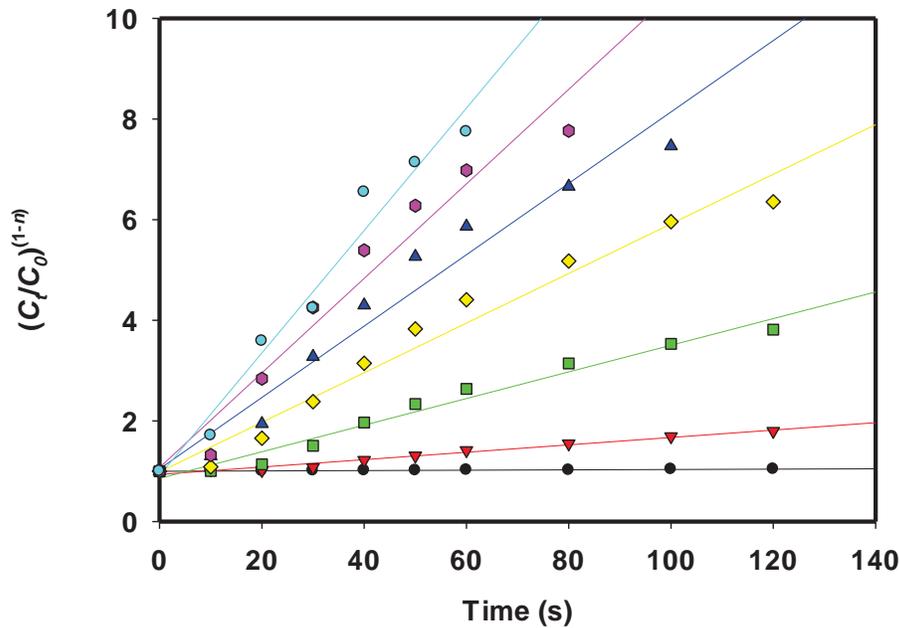
Figure 5.7: Aggregation of β-lactoglobulin in heated AWPC solutions, 5% (w/w) ts, with 20 mM added CaCl$_2$, at ● 70ºC, ▼ 75ºC, ■ 80ºC, ◆ 85ºC, ▲ 90ºC, ⬣ 95ºC and ⭕ 100ºC; n = 1.7.

The observed rate constants k_{Ca} and the rate constants $k_{1.7Ca}$ corresponding to the different total solids concentrations are given in Table 5.4.

Adding 20 mM of calcium chloride to the whey protein solutions accelerated the aggregation reactions at 5, 10 and 15% (w/w) total solids concentrations (Figures 5.5 and 5.7). The increase in the rate constant $k_{1.7Ca}$ was greater for 5% (w/w) ts than for 10% (w/w) ts, and this was greater than for 15% (w/w) ts. For 25 and 35% (w/w) ts, adding 20 mM of calcium chloride slowed down the aggregation reactions: the rate constants $k_{1.7Ca}$ were lower than the rate constants $k_{1.7}$, except for 25% (w/w) ts at 100ºC. It was difficult to determine if this change in the rate constants $k_{1.7Ca}$ was actual or just an artefact in the results. To be able to explain the behaviour of the rate constant at that point, further experiments at temperatures higher than 100ºC would be needed. Some R^2 values (correlation coefficient) were low, R^2 < 0.80, probably due to experimental errors.

Table 5.4: Observed rate constants $k_{Ca} = 0.7 \times k_{1.7Ca}C_0^{0.7}$ and rate constants $k_{1.7Ca}$ for β-lactoglobulin in heated AWPC solutions with 20 mM added CaCl$_2$ at each heating temperature and each total solids concentration.

Temperature (ºC)		70	75	80	85	90	95	100
$0.7 \times$ $k_{1.7Ca}C_0^{0.7}$ $(s^{-1} \times 10^{-3})$	5% (w/w) ts	0.30 $R^2 = 0.93$	6.50 $R^2 = 0.96$	24.60 $R^2 = 0.97$	49.10 $R^2 = 0.97$	71.60 $R^2 = 0.96$	95.30 $R^2 = 0.96$	119.70 $R^2 = 97$
	10% (w/w) ts	0.40 $R^2 = 0.79$	10.00 $R^2 = 0.91$	24.70 $R^2 = 0.96$	46.60 $R^2 = 0.97$	64.00 $R^2 = 0.96$	92.80 $R^2 = 0.97$	117.60 $R^2 = 0.97$
	15% (w/w) ts	0.30 $R^2 = 0.94$	4.50 $R^2 = 0.89$	22.60 $R^2 = 0.91$	39.20 $R^2 = 0.98$	55.60 $R^2 = 0.96$	71.00 $R^2 = 0.97$	99.30 $R^2 = 0.94$
	25% (w/w) ts	0.30 $R^2 = 0.95$	2.50 $R^2 = 0.62$	9.80 $R^2 = 0.94$	26.80 $R^2 = 0.96$	54.90 $R^2 = 0.93$	74.80 $R^2 = 0.97$	131.40 $R^2 = 0.98$
	35% (w/w) ts	0.10 $R^2 = 0.77$	3.20 $R^2 = 0.73$	18.40 $R^2 = 0.92$	33.80 $R^2 = 0.97$	46.40 $R^2 = 0.97$	62.30 $R^2 = 0.94$	74.50 $R^2 = 0.94$
$k_{1.7Ca}$ $(s^{-1}mg^{-1}g \times 10^{-3})$	5% (w/w) ts	0.05	1.10	4.16	8.31	12.12	16.13	20.26
	10% (w/w) ts	0.04	1.01	2.50	4.71	6.47	9.38	11.88
	15% (w/w) ts	0.02	0.35	1.77	3.07	4.35	5.56	7.78
	25% (w/w) ts	0.02	0.14	0.53	1.46	2.99	4.07	7.16
	35% (w/w) ts	0.004	0.14	0.82	1.50	2.06	2.76	3.30

Figure 5.8 shows the effect of total solids concentration on the rate constants $k_{1.7Ca}$ when 20 mM of calcium was added. In the presence of calcium, the trend was opposite to that described for no added calcium: the rate constant decreased slightly with increasing total solids concentration.
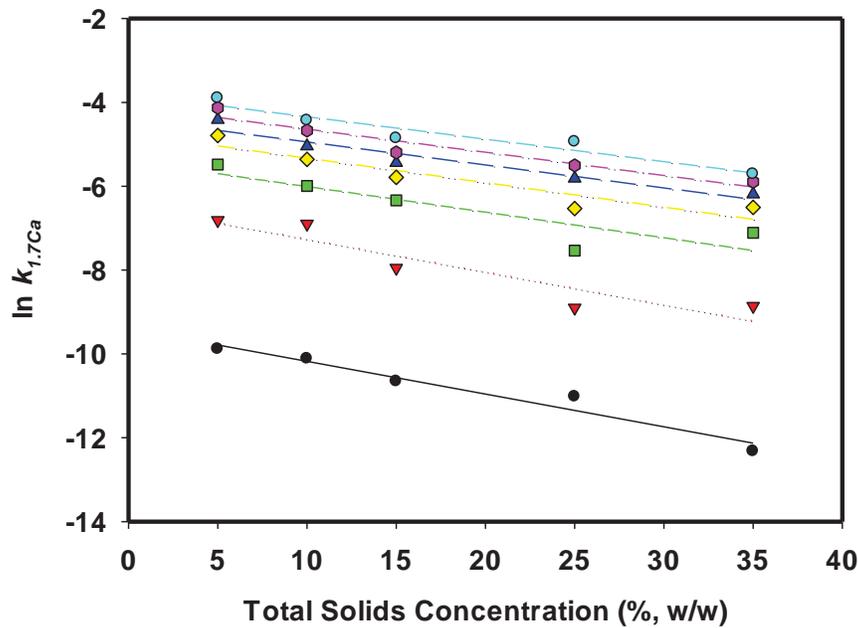
Figure 5.8: Effect of total solids concentration on the rate constants $k_{1.7Ca}$, with 20 mM

added CaCl$_2$, at ● 70ºC, ▼ 75ºC, ■ 80ºC, ◆ 85ºC, ▲ 90ºC, ● 95ºC and ● 100ºC.

5.3.2.3 Temperature dependence and activation energy

The relationship between the rate constants k_n and the temperature of the reaction is

given by the Arrhenius equation:

$$k_n = k_0 exp(-E_a/RT) \qquad\qquad (5.4)$$

(E_a = activation energy, R = universal gas constant, k_0 = frequency factor, and T =

absolute temperature).

The logarithms of the constants ($\ln k_n$) obtained from the slopes of the previous linear

regressions and corrected for protein concentration differences were plotted against the

reciprocal of absolute temperature for all total solids concentrations for without and

with addition of calcium chloride. For each total solids concentration without or with

addition of calcium, the relationship ($\ln k_n$ against $1/T$) was linear within certain

temperature ranges. However, a marked change in temperature dependence occurred at

a temperature called the transition temperature T_t. At temperatures below the transition

temperature T_t, the slope of the denaturation line was steep and at higher temperatures it

was less steep. The only explanation for this effect of temperature on the slope is that

there were two reactions taking place at the same time and that each predominated in a

certain temperature range (Kessler & Beyer, 1991). The linearity in the two temperature

ranges allowed the activation energies E_a to be calculated by linear regressions and the transition temperature T_t to be determined from the intersection of the best-fit lines for the two temperature ranges.

Without added calcium

Figure 5.9 shows the Arrhenius plot for the aggregation of β-lactoglobulin without addition of calcium chloride, in 5% (w/w) ts solution as an example; similar plots were obtained at all total solids concentrations.
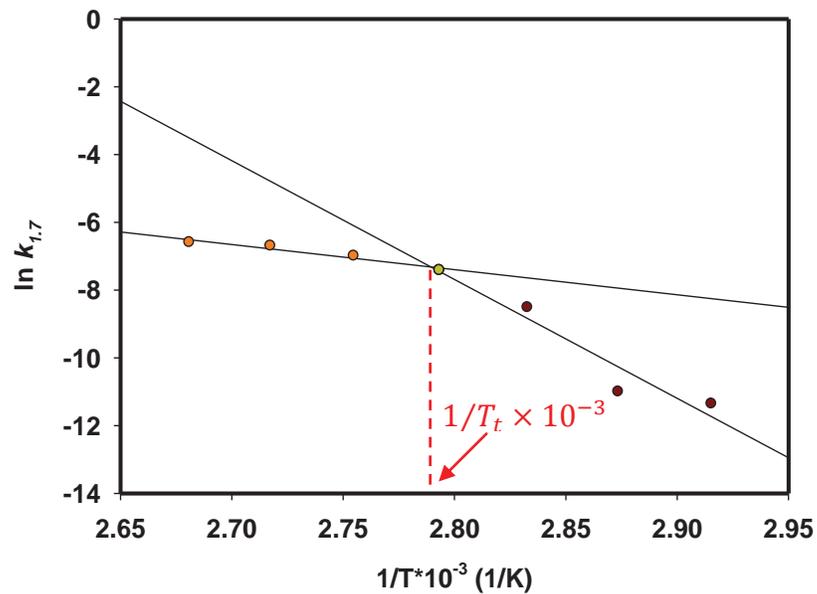
Figure 5.9: Arrhenius plot for the aggregation of the β-lactoglobulin in heated AWPC solutions, 5% (w/w) ts, without addition of $CaCl_2$, $n = 1.7$; ● data points used for the denaturation step regression ($T < T_t$), ● data points used for the aggregation step regression ($T > T_t$), ● data point used in both regressions.

The transition temperature T_t and activation energies E_a varied over the range of total solids concentrations (Table 5.5).

Table 5.5: Transition temperatures T_t and activation energies E_a for β-lactoglobulin aggregation in heated AWPC solutions without addition of $CaCl_2$ as a function of total solids concentration.

Total solids concentration (w/w)		5%	10%	15%	25%	35%
T_t (°C)		85	82	83	80	80
E_a (kJ mol^{-1})	$T < T_t$	292 $R^2 = 0.93$	559 $R^2 = 0.99$	179 $R^2 = 0.93$	537 $R^2 = 0.98$	311 $R^2 = 0.95$
	$T > T_t$	62 $R^2 = 0.94$	90 $R^2 = 0.93$	91 $R^2 = 0.93$	58 $R^2 = 0.99$	46 $R^2 = 0.93$

The transition temperature T_t slightly decreased with increasing total solids concentration, from 85 to 80°C. This is partly in agreement with the literature, where two different transition temperatures are reported: 85°C (Anema & McKenna, 1996; Spiegel, 1999) and 90°C (Anema, Lee, & Klostermeyer, 2006; Dannenberg & Kessler, 1988; Kessler & Beyer, 1991; Tolkach & Kulozik, 2005, 2007).

The activation energy E_a for the temperature range $T < T_t$ did not exhibit a definite trend as total solids content varied; its value fluctuated within the range 179 to 560 kJ mol^{-1}. However, values were still within the range described in the literature as corresponding to the activation energy for protein denaturation, the unfolding of the protein molecules leading to a change in their configuration (Anema, Lee, & Klostermeyer, 2006; Anema & McKenna, 1996; Dannenberg & Kessler, 1988; Kessler & Beyer, 1991; Oldfield, Singh, & Taylor, 2005b; Relkin & Launay, 1990; Spiegel, 1999; Tolkach & Kulozik, 2005, 2007). In contrast, the value obtained for the activation energy E_a for the temperature range $T > T_t$ increased with increasing concentration up to 15% (w/w) ts ($E_a = 91$ kJ mol^{-1}) and then decreased to $E_a = 46$ kJ mol^{-1}. These values for the activation energy E_a are described in the literature as being usual for chemical reactions where bonds are formed, in this case the aggregation step.

With added calcium

Figure 5.10 shows the Arrhenius plot for β-lactoglobulin with the addition of 20 mM of calcium chloride, in 5% (w/w) total solids solution as an example; similar plots were obtained at all total solids concentrations.
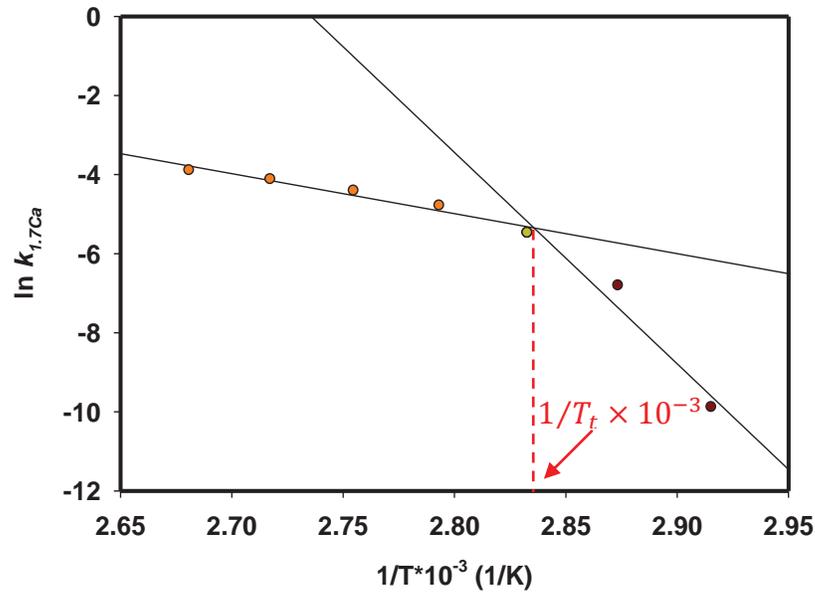
Figure 5.10: Arrhenius plot for the aggregation of the β-lactoglobulin in heated AWPC solutions, 5% (w/w) ts, with 20 mM added $CaCl_2$, $n = 1.7$; ● data points used for the denaturation regression ($T < T_t$), ● data points used for the aggregation regression ($T > T_t$), ● data point used in both regressions.

Adding calcium chloride greatly affected the overall aggregation of β-lactoglobulin. This was obvious when comparing the transition temperatures T_t and activation energies E_a (Table 5.6) with those obtained previously for no addition of calcium chloride (Table 5.5).

Table 5.6: Transition temperature T_{tCa} and activation energies E_{aCa} for β-lactoglobulin aggregation in heated AWPC solutions with 20 mM added $CaCl_2$ at each total solids concentration.

Total solids concentration (w/w)		5%	10%	15%	25%	35%
T_{tCa} (ºC)		80	80	80	81	81
E_{aCa} (kJ mol^{-1})	$T < T_t$	444 $R^2 = 0.95$	416 $R^2 = 0.91$	477 $R^2 = 0.97$	392 $R^2 = 0.98$	363 $R^2 = 0.99$
	$T > T_t$	66 $R^2 = 0.99$	70 $R^2 = 0.99$	67 $R^2 = 0.99$	113 $R^2 = 0.98$	59 $R^2 = 0.99$

With 20 mM of added calcium chloride, the transition temperature T_{tCa} and activation energy E_{aCa} varied less within the range of total solids concentrations studied than they did without addition of calcium chloride. The transition temperature was 80 - 81ºC, equivalent to the transition temperature T_t obtained at 25 and 35% (w/w) ts when no calcium chloride was added. The activation energy E_{aCa} for the aggregation (T > T_t) step varied between 60 and 70 kJ mol^{-1}, except at 25% (w/w) ts where E_{aCa} = 113 kJ mol^{-1}. For the denaturation step (T < T_t), the activation energy E_{aCa} variability was slightly higher but values were still within the range described in the literature, E_{aCa} = 418± 56 kJ mol^{-1}.

5.3.2.4 Conclusions

The kinetic parameters of the denaturation/aggregation of β-lactoglobulin obtained in this study (see sections 5.3.2.2 and 5.3.2.3) were not dramatically different from the ones found in the literature. There were slight differences owing to the different experimental conditions (de Wit, 2009; Mulvihill & Donovan, 1987), but the present overall kinetic study was in agreement with the literature. The order of denaturation/aggregation was found to be *n* = 1.7 for β-lactoglobulin, which is in agreement with the literature, where it has been reported as varying between 1 and 2 (Chime, Konrad, Kleinschmidt, & Gorzki, 2009; Gough & Jenness, 1962; Harwalkar, 1980b; Hillier & Lyster, 1979; Iametti, DeGregori, Vecchio, & Bonomi, 1996; Kessler & Beyer, 1991; Lyster, 1970; Park & Lund, 1984; Relkin & Launay, 1990; W. H. Sawyer, Norton, Nichol, & McKenzie, 1971; Verheul, Roefs, & de Kruif, 1998). The Arrhenius plot showed a marked change in the temperature dependence of the rate constant $k_{1.7}$, giving two temperature ranges separated by the transition temperature T_t. In this study, the transition temperature T_t varied between 80 and 85ºC, decreasing with increasing total solids concentration. This was not in complete agreement with the literature, where two different transition temperatures T_t have been reported, 85ºC (Anema & McKenna, 1996; Spiegel, 1999) and 90ºC (Anema, Lee, & Klostermeyer, 2006; Dannenberg & Kessler, 1988; Kessler & Beyer, 1991; Tolkach & Kulozik, 2005, 2007). The linearity in the two temperature ranges observed in the Arrhenius plots allowed the calculation of the activation energy E_a for each range.

As expected, the total solids concentration had a great effect on the rate constants $k_{1.7}$ for the aggregation of β-lactoglobulin, the protein molecules aggregating much faster

with increasing total solids concentration. However, the aggregation rate seemed to reach a limit, as at temperatures equal to or higher than 90°C, the rate constants $k_{1.7}$ were similar for 25 and 35% (w/w) solutions. Further experiments with total solid concentrations above 35% (w/w) would be needed to confirm or refute this hypothesis. The longer the heating time, the greater is the extent of change in the concentration of the proteins left in solution. The greater extent of change in concentration and the heating temperature affected the rate constants $k_{1.7}$, synergistically with the total solids concentration; the rate constant $k_{1.7}$ increased with heating temperature. To obtain the fastest and greatest extent of aggregation of β-lactoglobulin molecules, high total solids whey protein solutions had to be heated at high temperature for a long enough time.

According to Spiegel and Huss (2002) the reaction order for the β-lactoglobulin denaturation in WPC is independent of pH in the pH range 3.5 – 6.7 but is strongly dependent on the presence of calcium. In this study, adding 20 mM of calcium chloride to the whey protein solutions affected significantly the kinetic parameters for β-lactoglobulin denaturation/aggregation. However, this effect of added calcium varied depending on the total solids concentration of the protein solutions. As the same amount of calcium chloride (20 mM) was added to the different protein solutions but the total solids concentration of these solutions varied from 5 to 35% (w/w), the calcium (mM)/protein (%, w/w) ratio decreased as total solids increased. This is why the effect of added calcium on the denaturation/aggregation kinetics of β-lactoglobulin also decreased as total solids increased. Indeed, for the 25 and 35% (w/w) ts solutions, the rate constants $k_{1.7}$ and $k_{1.7Ca}$ were similar at heating temperatures equal to or higher than 90°C. For these experimental conditions, high heating temperature and high total solids concentration, there was no effect of added calcium on the rate constant. The ratio calcium (mM)/protein (%, w/w) was then around 1. In addition, when the total solids concentration increased in the presence of added calcium, the rate constant $k_{1.7Ca}$ decreased, a trend opposite to the one observed without added calcium. It appeared that adding 20 mM of calcium slowed down the aggregation of β-lactoglobulin at higher total solids concentrations, where the ratio calcium (mM)/protein (%, w/w) decreased. By extrapolation, this is in agreement with the literature. Indeed, Spiegel and Huss (2002) found that calcium removal from a WPC solution, giving 0.05% calcium for 10% (w/w) protein, had a heat stabilizing effect at a low lactose content. Similarly, Faka *et al.* (2009) showed that the heat-stability of low-heat skim milk powder reconstituted

at 25% (w/w) ts can be improved by reducing the free calcium concentration of the original milk to 1.14 mM or lower prior to evaporation and spray drying.

5.3.3 α-Lactalbumin: effect of initial total solids concentration, heating temperature and time on the loss of soluble α-lactalbumin during heating

5.3.3.1 Loss of soluble proteins

Without added calcium

Figure 5.11 shows the loss of soluble (native, denatured molecules and soluble aggregates) α-lactalbumin, measured by RP-HPLC, while being heated at different temperatures, 70 to 100°C, for different total solids (ts) concentrations, 5 to 35% (w/w).

Similarly to the loss of soluble β-lactoglobulin, the loss of soluble α-lactalbumin increased with heating temperature and time, but was generally slower than the loss of soluble β-lactoglobulin. For 5% (w/w) ts solution, 54% of soluble α-lactalbumin remained after heating at 100°C for 120 s as against 44% of soluble β-lactoglobulin. For 35% (w/w) ts solution with the same heating conditions, 6% of soluble α-lactalbumin remained, the same as for β-lactoglobulin, but no bottoming out was observed for α-lactalbumin. Another major difference compared with the loss of soluble β-lactoglobulin was that the two groups of curves (at low and high temperatures, respectively) were not observed. The loss of soluble α-lactalbumin was almost linear with heating time for each heating temperature at all total solids concentrations. The rate of aggregation increased with increasing heating temperature.
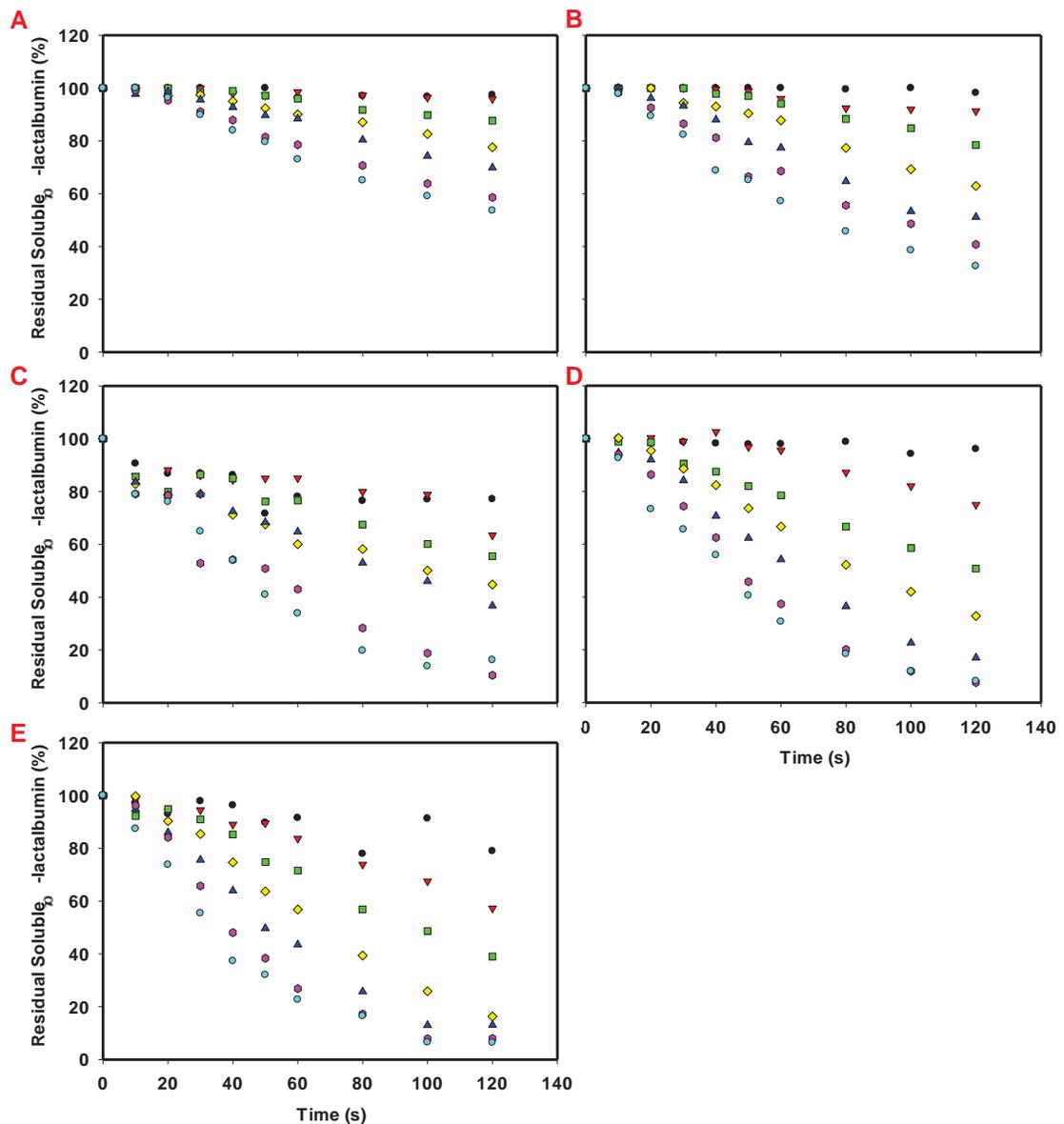
Figure 5.11: Loss of soluble α-lactalbumin during heating at ● 70ºC, ▼ 75ºC, ■ 80ºC, ◆ 85ºC, ▲ 90ºC, ● 95ºC and ○ 100ºC; at different total solids concentrations **A** 5% (w/w) ts, **B** 10% (w/w) ts, **C** 15% (w/w) ts, **D** 25% (w/w) ts and **E** 35% (w/w) ts and without addition of CaCl$_2$.

With added calcium

Figure 5.12 shows the loss of soluble α-lactalbumin, measured by HPLC, against time while heated at different temperatures, 70 to 100ºC, and for different total solids concentrations, each solution containing 20 mM of added calcium.
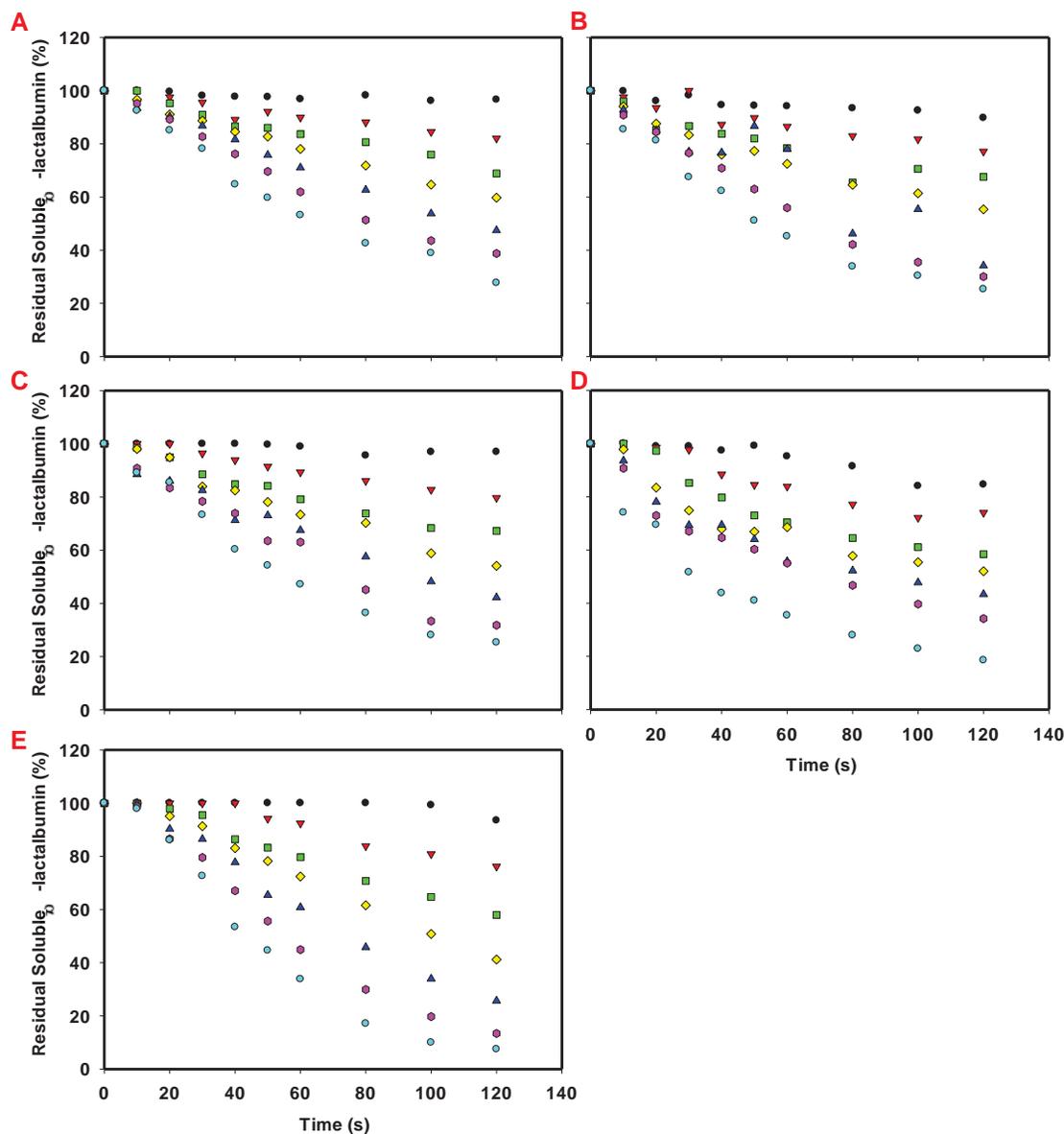
Figure 5.12: Loss of soluble α-lactalbumin during heating at ● 70ºC, ▼ 75ºC, ■ 80ºC, ◇ 85ºC, ▲ 90ºC, ⬢ 95ºC and ○ 100ºC; at different total solids concentrations **A** 5% (w/w) ts, **B** 10% (w/w) ts, **C** 15% (w/w) ts, **D** 25% (w/w) ts and **E** 35% (w/w) ts and with 20 mM added $CaCl_2$.

The effect of added calcium on the loss of soluble α-lactalbumin was similar to the effect of added calcium on the loss of β-lactoglobulin. The addition of 20 mM of calcium enhanced the aggregation of α-lactalbumin at low total solids concentrations (5 and 10%, w/w ts) whereas it slightly slowed down the aggregation of α-lactalbumin at higher total solids concentrations (15, 25 and 35%, w/w ts). For the 5 and 10% (w/w) ts solutions, with 20 mM of added calcium, 28 and 25% (respectively) of soluble α-

lactalbumin remained after heating at 100ºC for 120 s as against 54 and 33% (respectively) of soluble α-lactalbumin left without addition of calcium, for the same heating conditions. In contrast, for the 15, 25 and 35% (w/w) ts solutions, with 20 mM of added calcium 25, 19 and 8% of soluble α-lactalbumin remained after heating at 100ºC for 120 s as against 16, 8 and 6% of soluble α-lactalbumin remaining without addition of calcium, for the same heating conditions.

As for β-lactoglobulin, the addition of calcium did not change the overall aggregation behaviour of α-lactalbumin. The loss of soluble α-lactalbumin was almost linear with increasing heating time for each heating temperature at all total solids concentrations. The rate of aggregation increased with increasing heating temperature.

5.3.3.2 Aggregation kinetics

The order of thermal aggregation of α-lactalbumin and the associated rate constants were determined in the same way as for β-lactoglobulin (see section 5.4.2).

Without added calcium

At all total solids concentrations, linear relationships were obtained when $\ln(C_t/C_0)$ was plotted against t (Figure 5.13), indicating that the aggregation of α-lactalbumin followed first-order reaction kinetics.
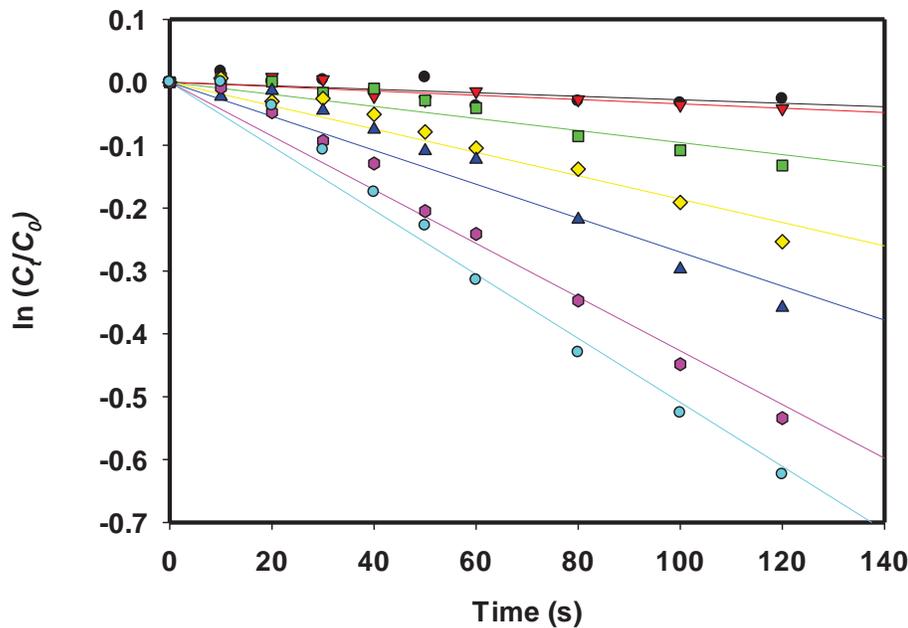
Figure 5.13: Aggregation of α-lactalbumin in heated AWPC solutions, 5% (w/w) ts, without addition of CaCl$_2$, at ● 70°C, ▼ 75°C, ■ 80°C, ◇ 85°C, ▲ 90°C, ⬟ 95°C and ◯ 100°C; $n = 1$.

The rate constants k_1 for α-lactalbumin are given in Table 5.7.

Table 5.7: Rate constants k_1 for α-lactalbumin in heated AWPC solutions without addition of CaCl$_2$ at each heating temperature and each total solids concentration.

Temperature (°C)		70	75	80	85	90	95	100
k_1 ($s^{-1} \times 10^{-3}$)	5% (w/w) ts	0.3 $R^2 = 0.52$	0.3 $R^2 = 0.76$	1.0 $R^2 = 0.87$	1.9 $R^2 = 0.95$	2.7 $R^2 = 0.94$	4.3 $R^2 = 0.98$	5.1 $R^2 = 0.98$
	10% (w/w) ts	0.9 $R^2 = 0.81$	1.8 $R^2 = 0.72$	2.6 $R^2 = 0.99$	4.3 $R^2 = 0.98$	6.4 $R^2 = 0.98$	8.6 $R^2 = 0.78$	10.4 $R^2 = 0.99$
	15% (w/w) ts	2.9 $R^2 = 0.52$	3.2 $R^2 = 0.69$	5.0 $R^2 = 0.90$	7.2 $R^2 = 0.93$	8.0 $R^2 = 0.97$	17.6 $R^2 = 0.95$	16.9 $R^2 = 0.98$
	25% (w/w) ts	0.5 $R^2 = 0.75$	1.7 $R^2 = 0.74$	5.0 $R^2 = 0.94$	8.1 $R^2 = 0.93$	13.1 $R^2 = 0.93$	19.6 $R^2 = 0.94$	19.5 $R^2 = 0.79$
	35% (w/w) ts	1.6 $R^2 = 0.76$	3.9 $R^2 = 0.92$	6.9 $R^2 = 0.93$	12.6 $R^2 = 0.91$	16.8 $R^2 = 0.94$	21.8 $R^2 = 0.96$	24.0 $R^2 = 0.98$

The rate constant k_1 increased with heating temperature and with total solids concentration, which is in agreement with the literature (Anema, Lee, & Klostermeyer, 2006; Anema & McKenna, 1996; Dannenberg & Kessler, 1988; Oldfield, Singh, & Taylor, 2005b; Wehbi, Perez, Sanchez, Pocovi, Barbana, & Calvo, 2005). Some R^2 values (correlation coefficient) were low, $R^2 < 0.80$, probably due to experimental errors.

Figure 5.14 shows the effect of total solids concentration on the rate constant k_1. As described for β-lactoglobulin without addition of calcium, there was a sharp increase in the rate constant at low total solids concentration, 5 to 15% (w/w), for all temperatures. The rate constant seemed to plateau at 15% (w/w); the anomalous values at 25% (w/w) at 70 and 75ºC might be explained by experimental error.
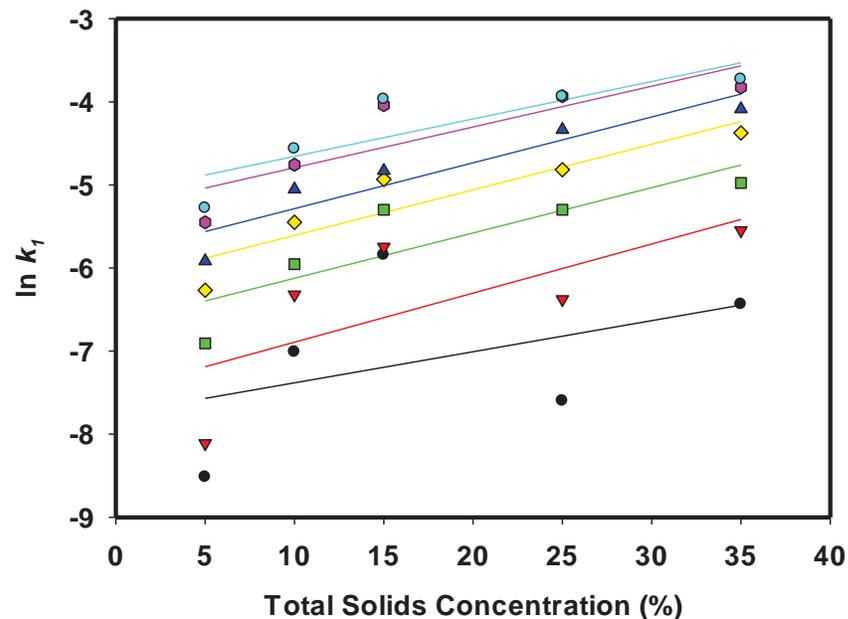
Figure 5.14: Effect of total solids concentration on the rate constants k_1, without addition of CaCl$_2$, at ● 70ºC, ▼ 75ºC, ■ 80ºC, ◇ 85ºC, ▲ 90ºC, ● 95ºC and ○ 100ºC.

With added calcium

Adding calcium to the solutions did not affect the order of the aggregation reaction of α-lactalbumin. Linear relationships were also obtained when $\ln(C_t/C_0)$ was plotted against t (Figure 5.15), indicating that the aggregation of α-lactalbumin followed first-order reaction kinetics.
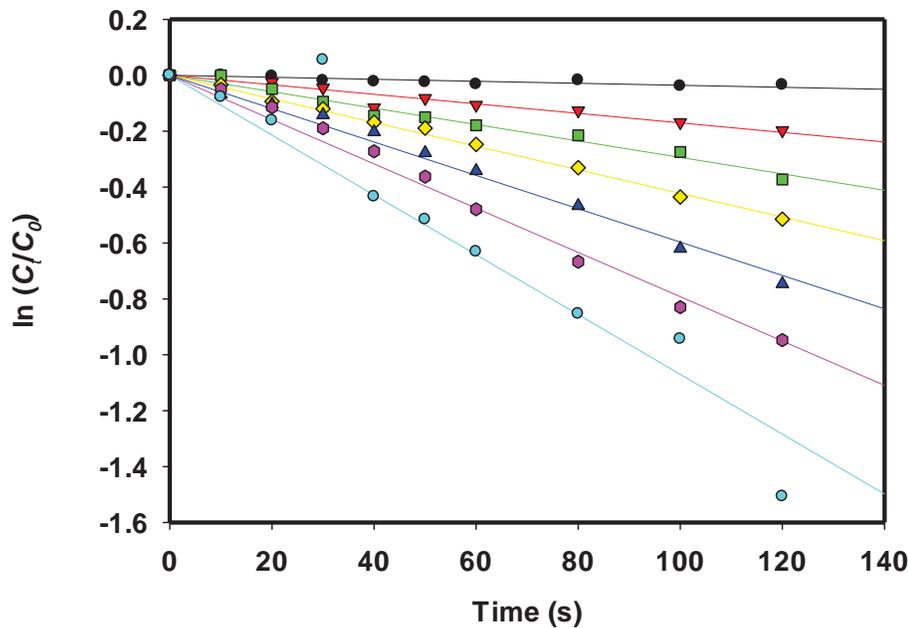
Figure 5.15: Aggregation of α-lactalbumin in heated AWPC solutions, 5% (w/w) ts, with 20 mM added CaCl$_2$, at ● 70°C, ▼ 75°C, ■ 80°C, ◇ 85°C, ▲ 90°C, ⬠ 95°C and ○ 100°C; $n = 1$.

The rate constants k_{1ca} for α-lactalbumin are given in Table 5.8.

Table 5.8: Rate constants k_{1ca} for α-lactalbumin in heated AWPC solutions with 20 mM added CaCl$_2$ at each heating temperature and each total solids concentration.

Temperature (°C)		70	75	80	85	90	95	100
k_{1ca} (s^{-1} × 10^{-3})	5% (w/w) ts	0.4 $R^2 = 070$	1.7 $R^2 = 0.91$	2.9 $R^2 = 0.98$	4.2 $R^2 = 0.99$	6.0 $R^2 = 0.99$	7.9 $R^2 = 0.99$	10.7 $R^2 = 0.91$
	10% (w/w) ts	0.9 $R^2 = 0.87$	2.2 $R^2 = 0.83$	3.9 $R^2 = 0.84$	5.2 $R^2 = 0.97$	7.4 $R^2 = 0.83$	10.0 $R^2 = 0.99$	12.2 $R^2 = 0.98$
	15% (w/w) ts	0.4 $R^2 = 0.92$	2.0 $R^2 = 0.98$	4.5 $R^2 = 0.82$	5.4 $R^2 = 0.64$	7.3 $R^2 = 0.98$	9.9 $R^2 = 0.97$	12.6 $R^2 = 0.97$
	25% (w/w) ts	1.3 $R^2 = 0.85$	4.1 $R^2 = 0.77$	5.0 $R^2 = 0.94$	6.3 $R^2 = 0.88$	9.2 $R^2 = 0.66$	9.5 $R^2 = 0.96$	15.6 $R^2 = 0.94$
	35% (w/w) ts	0.1 $R^2 = 0.90$	2.7 $R^2 = 0.91$	5.1 $R^2 = 0.99$	7.6 $R^2 = 0.89$	12.2 $R^2 = 0.82$	16.1 $R^2 = 0.97$	21.4 $R^2 = 0.96$

As for the rate constant k_1 (Table 5.7), the rate constant k_{1ca} increased with increasing heating temperature and total solids concentration. But comparing with the solutions without added calcium, α-lactalbumin aggregated more rapidly with the addition of 20 mM of calcium than without added calcium at low total solids concentrations (5 and 10%, w/w ts). However, at high total solids concentrations (15, 25 and 35%, w/w ts) α-lactalbumin generally aggregated more slowly with the addition of 20 mM of calcium than without added calcium. Some R^2 values (correlation coefficient) were low, $R^2 < 0.80$, probably due to experimental errors.

Figure 5.16 illustrates the effect of total solids concentration on the rate constant k_{1ca}. In contrast to the behaviour of β-lactoglobulin with 20 mM of added calcium, the values of the rate constants increased moderately with increasing total solids concentration. Again, experimental error would explain the scatter in the plots at 70 and 75ºC.
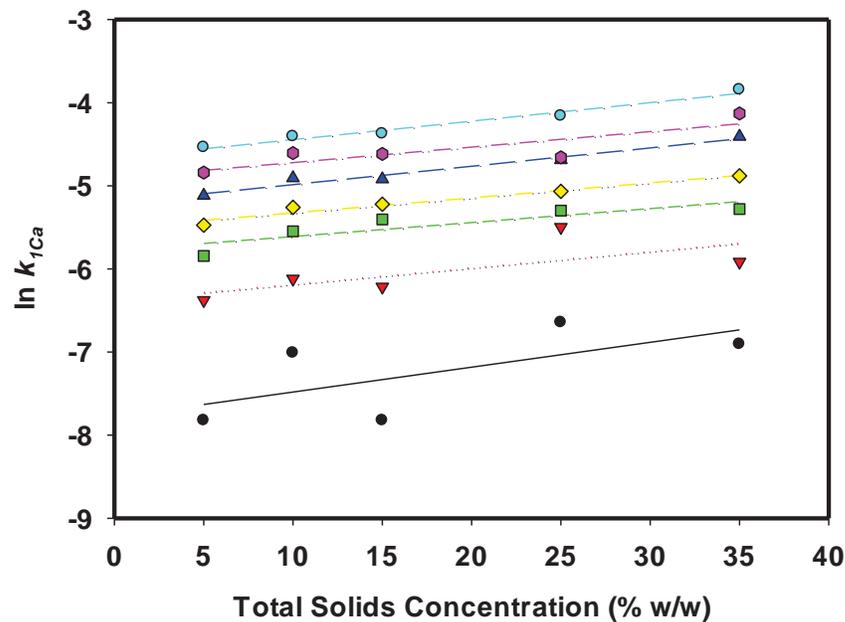
Figure 5.16: Effect of total solids concentration on the rate constants k_{1ca}, with 20 mM added CaCl$_2$, at ● 70ºC, ▼ 75ºC, ■ 80ºC, ◇ 85ºC, ▲ 90ºC, ● 95ºC and ○ 100ºC.

5.3.3.3 Temperature dependence and activation energy

The temperature dependence and the activation energy E_a were determined in the same way as was done in the case of β-lactoglobulin (see section 5.4.3).

Without added calcium

Figure 5.17 shows the Arrhenius plot for α-lactalbumin without addition of calcium chloride, in 5% (w/w) total solids solution, as an example. Similar graphs were obtained at 10, 25 and 35% (w/w) ts concentrations.
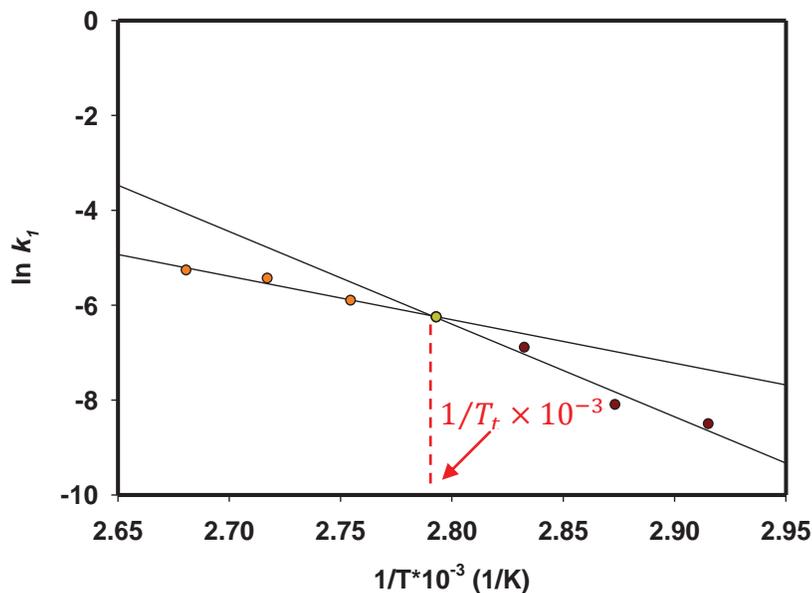
Figure 5.17: Arrhenius plot for the aggregation of the α-lactalbumin in heated AWPC solutions, 5% (w/w) ts, without addition of $CaCl_2$, $n = 1$; ● data points used for the denaturation regression $(T < T_t)$, ● data points used for the aggregation regression $(T > T_t)$, ● data point used in both regressions.

It is interesting to note that at 15% (w/w) ts, there was no marked change in the temperature dependence (Figure 5.18). Thus, there was no transition temperature T_t, and therefore only one activation energy E_a. This activation energy, $E_a = 70.52$ kJ mol^{-1}, corresponded to the aggregation reaction and is characteristic of a chemical reaction where bonds are formed (Table 5.9).
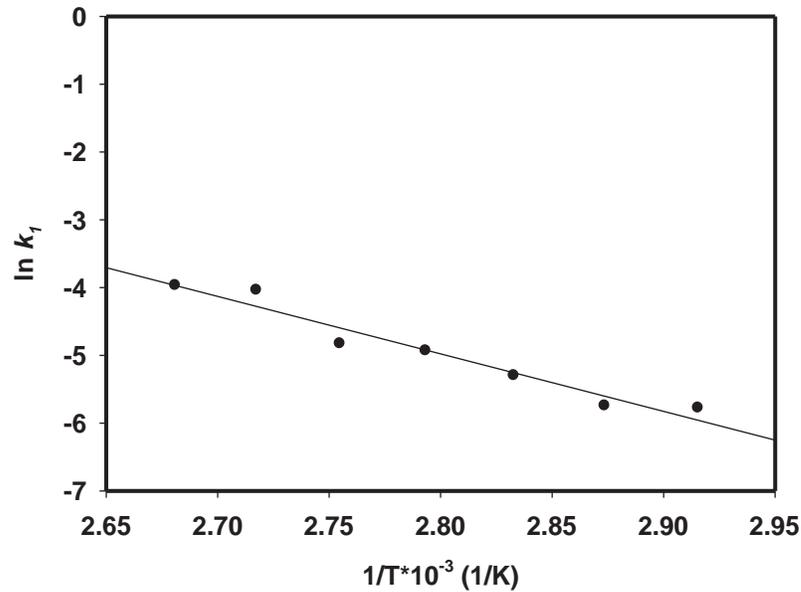
Figure 5.18: Arrhenius plot for the aggregation of the α-lactalbumin in heated AWPC solutions, 15% (w/w) ts, without addition of $CaCl_2$, $n = 1$.

Wehbi et al. (2005) studied the heat denaturation of α-lactalbumin in skim milk and observed, in the Arrhenius plot, a linear relationship in the temperature range studied, 78 to 94°C. They reported an activation energy $E_a = 88$ kJ mol^{-1} for the aggregation step.

The transition temperature T_t and activation energies E_a for α-lactalbumin at each total solids concentration are given in Table 5.9.

Table 5.9: Transition temperature T_t and activation energies E_a for α-lactalbumin in heated AWPC solutions without addition of $CaCl_2$ at each total solids concentration.

Total solids concentration (w/w)		5%	10%	15%	25%	35%
T_t (°C)		85	82	--	80	85
E_a (kJmol^{-1})	$T < T_t$	162 $R^2 = 0.97$	274 $R^2 = 0.94$	--	254 $R^2 = 0.99$	135 $R^2 = 0.99$
	$T > T_t$	76 $R^2 = 0.98$	79 $R^2 = 0.97$	71 $R^2 = 0.95$	79 $R^2 = 0.94$	49 $R^2 = 0.96$

The transition temperature T_t varied between 80 and 85°C without showing any specific trend within the total solids concentration range studied. This is not in agreement with

the literature where it has always been reported as 80ºC (Anema, Lee, & Klostermeyer, 2006; Anema & McKenna, 1996; Dannenberg & Kessler, 1988).

The activation energies, E_a, did not vary dramatically within the range of total solids concentrations studied, as in the case of β-lactoglobulin. For the temperatures below the transition temperature (T $< T_t$), the activation energy slightly increased and then decreased, E_a = 135 – 274 kJ mol^{-1}, which corresponded to the activation energy of the unfolding of the protein molecules, the denaturation step. And for the temperatures above the transition temperature (T $> T_t$), the activation energy E_a was relatively consistent, at between 70 and 80 kJ mol^{-1}, except at 35% (w/w) ts where it was lower, at E_a = 49 kJ mol^{-1}. These values were related to a chemical reaction where bonds are formed, i.e. the aggregation step. Overall, the values of the activation energy corresponded to values reported in the literature (Anema, Lee, & Klostermeyer, 2006; Anema & McKenna, 1996; Dannenberg & Kessler, 1988; Kessler & Beyer, 1991; Wehbi, Perez, Sanchez, Pocovi, Barbana, & Calvo, 2005).

With added calcium

Figure 5.19 shows the Arrhenius plot for α-lactalbumin with addition of 20 mM of calcium chloride, in 5% (w/w) total solids solution as an example. Similar graphs were obtained at all total solids concentrations.
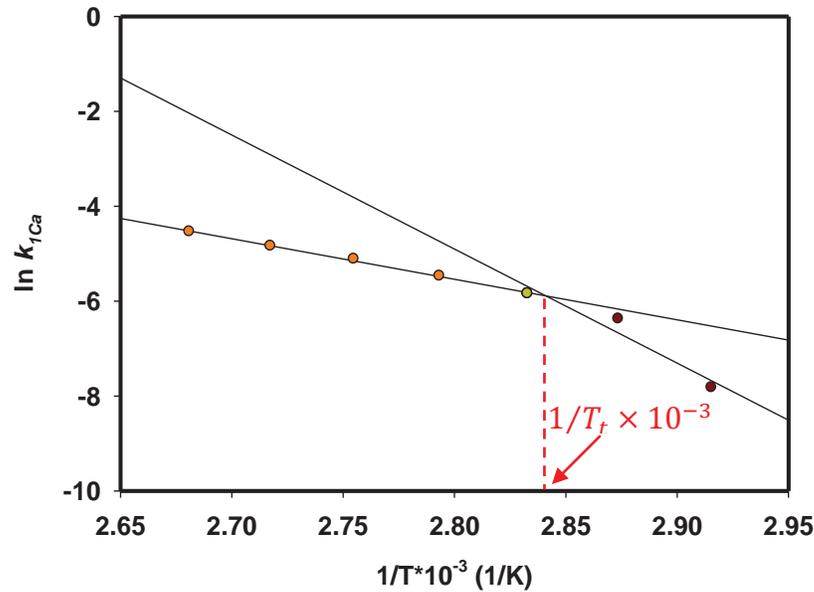
Figure 5.19: Arrhenius plot for the aggregation of the α-lactalbumin in heated AWPC solutions, 5% (w/w) ts, with 20 mM added $CaCl_2$, $n = 1$; ● data points used for the denaturation regression (T < T_t), ● data points used for the aggregation regression (T > T_t), ● data point used in both regressions.

Similarly to the case of β-lactoglobulin, adding calcium had a great effect on the overall aggregation of α-lactalbumin, giving a more consistent transition temperature T_t and more consistent activation energies E_a (Table 5.10).

Table 5.10: Transition temperature T_{tCa} and activation energies E_{aCa} for α-lactalbumin in heated AWPC solutions with 20 mM added $CaCl_2$ at each total solids concentration.

Total solids concentration (w/w)		5%	10%	15%	25%	35%
T_{tCa} (ºC)		80	80	79	79	79
E_{aCa} (kJmol^{-1})	T < T_t	200 $R^2 = 0.94$	148 $R^2 = 0.99$	269 $R^2 = 0.97$	153 $R^2 = 0.85$	447 $R^2 = 0.89$
	T > T_t	68 $R^2 = 0.99$	64 $R^2 = 0.99$	65 $R^2 = 0.99$	55 $R^2 = 0.96$	88 $R^2 = 0.99$

The transition temperature T_{tCa} was 79 − 80ºC within the total solids concentration range studied. The activation energy E_{aCa} for the range of temperatures below the transition temperature (T < T_t) varied slightly for total solids concentrations of up to

25% (w/w). E_{aCa} = 147 – 269 kJ mol^{-1} corresponding to the activation energy of the denaturation step. The activation energy E_{aCa} for the range of temperatures above the transition temperature (T > T_t) was between 55 and 69 kJ mol^{-1} for the same total solids range, corresponding to the activation energy of the aggregation step. There was an exception at 35% (w/w) ts where for both reaction steps, the activation energy was relatively high, E_{aCa} = 447 kJ mol^{-1} and E_{aCa} = 88 kJ mol^{-1} for denaturation and aggregation respectively. However, the activation energies E_{aCa} for both steps corresponded to the values reported in the literature (Anema, Lee, & Klostermeyer, 2006; Anema & McKenna, 1996; Dannenberg & Kessler, 1988; Kessler & Beyer, 1991; Wehbi, Perez, Sanchez, Pocovi, Barbana, & Calvo, 2005).

5.3.3.4   Conclusions

Overall, the kinetics of the heat-induced aggregation of α-lactalbumin observed in this study were in agreement with the literature. The reaction order was found to be n = 1 (Anema, Lee, & Klostermeyer, 2006; Anema & McKenna, 1996; Dannenberg & Kessler, 1988; Oldfield, Singh, & Taylor, 2005b). The rate constants increased with increasing heating temperature, also in agreement with the literature. From the Arrhenius plot displaying two linear ranges, the transition temperature T_t was determined. It varied between 80 and 85ºC; in the literature it is described as being 80ºC. The difference can be attributed to differences in experimental conditions. As well, the activation energy E_a for each of the linear ranges was calculated. And at T < T_t, the activation energy E_a value corresponded to that reported in the literature for the denaturation step (unfolding of the molecules). At T > T_t, the activation energy E_a corresponded to the aggregation step (formation of bonds) (Anema, Lee, & Klostermeyer, 2006; Anema & McKenna, 1996; Dannenberg & Kessler, 1988; Oldfield, Singh, & Taylor, 2005b; Wehbi, Perez, Sanchez, Pocovi, Barbana, & Calvo, 2005).

The rate constants were found to increase slightly with the total solids concentration. Anema et al. (2006) also observed the same dependence of the denaturation rate constants on protein concentration in skim milk. They concluded that α-lactalbumin aggregation had an apparent order of reaction n = 1; if it followed true first-order reaction kinetics, the rate constant should be independent of the total solids concentration. Oldfield et al. (2005b) found the order of reaction n of the heat-aggregation of α-lactalbumin to vary between 0.9 and 1.1 in skim milks.

The addition of 20 mM calcium did not have a great effect on the kinetic parameters. The aggregation of α-lactalbumin was still found to follow first order reaction kinetics. Compared with the case of β-lactoglobulin, the rate constants of α-lactalbumin denaturation/aggregation were slightly affected by the addition of calcium. They still slightly increased with increasing total solids concentration, and the scatter in the data was less than in the case of no addition of calcium. The increase was less sharp in the low total solids concentration range. Anema et al. (2006) found that the denaturation of α-lactalbumin in skim milk appeared to be unaffected by the total milk concentration, including calcium concentration.

5.4 Overall conclusions

From a first overview of the results, it appeared that β-lactoglobulin and α-lactalbumin behaved differently under heat treatment, which has already been described in the literature for low protein concentrations. However, at higher protein concentrations, both proteins kept much the same temperature dependence of reaction rate constant as at low protein concentrations. But denaturation/aggregation occurred more rapidly as more concentrated solutions enhanced the probability of "collision" between proteins or aggregates.

The addition of 20 mM of calcium influenced the aggregation behaviour of the whey proteins studied, α-lactalbumin and β-lactoglobulin. The added calcium did not seem to change the mechanism of aggregation as such but influenced the rate of aggregation, increasing it or decreasing it, depending on the total solids concentration of the solutions. This was probably due to the importance of the ratio of free calcium/ to protein as discussed earlier. Surprisingly, the addition of calcium enhanced the aggregation of α-lactalbumin at low total solids concentrations and slowed down it at high total solids concentrations. α-Lactalbumin is known as a calcium-binding protein, and at low total solids concentrations there would have been more free calcium available per protein to bind with and then protect α-lactalbumin from denaturation. Thus, the opposite effect of calcium on the aggregation of α-lactalbumin would have been expected: slower aggregation at low total solids concentrations and normal rate at higher total solids concentrations.

At 70 and 75ºC, some denaturation would have occurred but probably little aggregation. Thus, a proportion of the denatured proteins could have been soluble and thus would have been measured as being undenatured. This could have introduced errors, affecting the calculated rate constants.

CHAPTER 6   Overall Discussion and Recommendations for Future Work

Whey protein products have to be carefully chosen, depending on the commercial process and the functionality required in the final product. While whey proteins are still used for their functional (gelling) properties in many food applications, in the recent years they have also been valued for their high nutritional profile (Schaafsma, 2006). For example because of their uniquely high content of leucine, they are the best proteins for sports nutrition. These benefits have led to food manufacturers looking at ways of adding more whey proteins to their food products. However, the amount of whey proteins that can be added to food products is limited by the proteins' functionality, especially their ability to aggregate and form gels. For example, when producing beverages, if a high concentration of whey protein is added, there is a high risk of gelling during processing and also during shelf life. In baked goods and snack bars, a high whey protein content leads to the hardening of products during shelf life. Therefore, dairy industry is interested in developing new processes to make denatured whey proteins, in other words make whey proteins that do not form gels during food processing and over time, so that they can be added in greater amounts to food products for their nutritional properties alone. From the investigations presented in this thesis, addition of calcium appears to be a potential aid in making denatured whey protein products or at least in lowering the firmness of the gels formed during commercial processing, making whey proteins "processable" without blocking commercial plants. However, understanding the mechanism of protein-protein interactions in complex systems like commercial whey protein products under processing conditions is still crucial to helping to develop new whey protein products and food processes.

This study was carried out in a commercial environment with the aim of being able to apply the results to existing commercial processes, which means minimising as much as possible the changes to the processes. For example, the commercial processing of WPC involves UF/DF of whey at high total solids, followed by evaporation that increases the total solids even more, and then drying at the very high final total solids. This makes the process cost effective, and thus commercially feasible. The processing of denatured WPC needs to be similar: the whey proteins need to be denatured at high total solids,

and then dried. This requires considering where during the process the proteins should be heated and how to make sure that no gelation will occur during processing.

Another issue is the types of products produced. These depend on market demand. Currently in New Zealand (Fonterra Co-operative Group Ltd), three types of whey protein products are produced: CWPC, AWPC and WPI. AWPC and CWPC are mainly sold as gelling agents, but they have potential to be converted to non-functional (non-gelling) nutritional ingredients. The question is how these three products would behave if they were used as substrates in making denatured whey protein products in a commercial process. It is well known that under heat treatment whey proteins denature, aggregate and form gels; this is likely to happen during a commercial process for making denatured WPC but is not desired. Therefore, it is important to be able to manipulate the conditions to avoid the formation of a gel during processing. Addition of calcium appeared a potential aid in making denatured whey protein products and this study aimed to establish understanding of its effects.

The objectives of the work reported in this thesis were firstly to determine the effects of added calcium on the denaturation, aggregation and gelation of heated whey protein solutions prepared from three different commercial whey protein products (WPI, AWPC and CWPC). The second objective was to determine the effect of the added calcium to protein concentration ratio on the kinetics of heat-induced whey protein aggregation. The third was to make recommendations on the suitability of different whey protein systems for making denatured whey protein products, the usefulness of adding calcium as an aid in making such denatured whey protein products, and future study.

When the denaturation and aggregation behaviours of proteins in WPI, AWPC and CWPC were compared directly, the differences observed in the heat-induced behaviour of these whey protein systems were not dramatic but noticeable. At high temperature (e.g. 85°C), the effect of added calcium was overwhelmed by the effect of heat treatment. But at lower temperature (e.g. 75 – 80°C), the extent of aggregation increased with addition of calcium up to an optimal concentration (differing from one whey protein system to the other), and then decreased with further increase in added calcium concentration. Similarly, the denaturation temperature decreased with addition of

calcium up to the same critical concentration (differing from one whey protein system to the other), and then increased with further increase in added calcium levels. This variation of the denaturation temperature gave some information on how the added calcium was influencing the stability of the protein conformations. At this optimal added calcium concentration, the protein conformations were the most destabilised, whereas at higher added calcium levels, the protein conformations were stabilised. Therefore, a greater extent of aggregation occurred when the protein conformations were the most destabilised by the addition of calcium, as shown by the lower denaturation temperature. However, differences in the response to the effect of added calcium were observed between the three systems. The extent of aggregation was less in the AWPC system than in the CWPC system, itself slightly less than in the WPI system. In parallel, aggregation via disulphide bonding was less in the AWPC system than in the CWPC system, itself less than in the WPI system. And the denaturation temperature was higher for the AWPC system than for the CWPC and WPI systems, reflecting less destabilised whey protein conformations in the AWPC system, leading to less aggregation. These differences might be explained by the fact that the AWPC system contains more monovalent cations (Na^+ and K^+) than does the CWPC system, itself slightly more than does the WPI system. The monovalent cations appeared to limit the extent of protein-calcium-protein bridges by surrounding the protein molecules before the calcium could bind to them (Havea, Singh, & Creamer, 2002). However, at this stage of the study, it did not seem that these differences would have a major influence on the behaviour of the whey protein systems during processing; these three whey protein systems appeared to be similar as whey protein products and might have been expected to behave in similar ways when used as feed materials in the manufacture of denatured whey protein products.

Nevertheless, such assumptions needed to be verified by investigating the effect of added calcium on the gelling properties of the whey proteins in WPI, AWPC and CWPC, and then comparing the results to better understand the influence of the differences previously observed and described on the whey proteins denaturation and aggregation properties.

In contrast to its effect on heat-induced denaturation and aggregation, the effects of added calcium on the gelation properties of WPI, AWPC and CWPC solutions clearly

differed among the three systems. The stiffest gels for the three whey protein systems were obtained at different levels of added calcium. As well, addition of calcium to WPI solutions resulted in the formation of more non-covalent associations than covalent bonding, and these dominated the properties of the heat-induced WPI gels. There was a dramatic change in the texture of the WPI gels as the amount of added calcium was increased; they became much softer and more elastic, behaving like a "sponge". The addition of calcium to AWPC solutions resulted in the formation of non-covalent associations at the expense of disulphide linkages during heating. Again, there were great changes in the texture of the AWPC gels as added calcium was increased but not as dramatic as in the case of the WPI gels. The AWPC gels were softer and mushier. The addition of calcium to CWPC solutions had little apparent effect on the gels' texture. These differences between WPI, AWPC and CWPC were attributed to the different levels of calcium initially present in these whey protein systems. In a commercial environment for manufacturing denatured whey proteins, the effect of added calcium on the texture of the AWPC gels seemed to be of more interest than its effect on the WPI and CWPC gels. Indeed, addition of calcium affected the AWPC gel structure, which became softer and mushier, leaning towards more "processable" gels. In contrast, even though addition of calcium affected dramatically the texture of the WPI gels, those were still too hard to be "processable", with a high risk of blocking the manufacturing plant. Regarding the case of CWPC, with no addition of calcium the gels were already soft and the addition of calcium barely affected the texture of the CWPC gels, not giving any advantage of using calcium.

As important differences in the gelling properties of the three whey protein systems were observed, and as the denaturation and aggregation experiments carried out did not give enough information to explain these differences, investigation of the effect of calcium on the aggregation kinetics appeared to be necessary. From a first overview of the kinetics results, it appeared that β-lactoglobulin and α-lactalbumin behaved differently under heat treatment, a phenomenon that has already been described in the literature, though for low protein concentrations. In the present study, the addition of 20 mM of calcium influenced the aggregation behaviour of the whey proteins studied, α-lactalbumin and β-lactoglobulin. The added calcium did not seem to change the mechanism of aggregation as such but influenced the rate of aggregation, increasing it or decreasing it, depending on the total solids concentration of the solutions. This was

probably due to the importance of the ratio of free calcium to protein as discussed earlier. However, without addition of calcium, at higher protein concentrations, both proteins exhibited much the same temperature dependence of reaction rate constant as that at low protein concentrations. In the industrial context, these are very important findings as they provide information useful in determining processing conditions for higher total solids feed materials.

From the findings of the present study, a mechanism for the effect of added calcium on the heat-induced aggregation of whey proteins is proposed and shown in Figure 6.1. The results presented show that the extent of aggregation (measured by PAGE and HPLC) increases with addition of calcium up to a certain concentration (~ 20 mM for 4%, w/w protein), but then decreases with further increase in added calcium concentration. This was at first suspected of being an ionic strength effect but PAGE results showed that, at the same ionic strength, the effect of added calcium was greater than that of added sodium. These results suggest that the effect of added calcium is probably a combination of two effects: screening net charges and bridging protein molecules through specific calcium binding. At an optimal added calcium level, calcium ions screen the proteins' net charge by surrounding them (a screening effect), leading to maximal intermolecular interactions and subsequent aggregation, which are further enhanced by intermolecular calcium bridges (a bridging effect). At added calcium levels lower and higher than the optimal level, strong protein repulsion occurs limiting intermolecular interactions and subsequent aggregation. At added calcium levels lower than the optimal level ($\leq$ 20 mM for 4%, w/w protein), the proteins' net charge is not fully screened by the calcium ions; therefore the proteins are still slightly negatively charged resulting in repulsive forces between them. At low added calcium levels and up to the optimal added calcium level, the screening of the intermolecular repulsion by calcium ions as well as the specific calcium bridges control the rate and extent of aggregation. At added calcium levels higher than the optimal level ($\geq$ 20 mM, for 4 %, w/w protein), binding of calcium ions by protein molecules stabilises the secondary and tertiary structure of the protein, making them more resistant to heat-induced unfolding. In other words, more energy is required to destabilise the protein molecules and as a result the denaturation temperature increases (Schokker, Singh, Pinder, & Creamer, 2000). This idea is supported by the DSC measurements which showed that the denaturation temperature of whey proteins was higher, 80 to 85°C (depending on the

whey protein system) at high levels of added calcium (200 mM for 4%, w/w protein). Moreover, irrespective of added calcium concentration, at greater heating temperatures than the denaturation temperature, sufficient energy is present to cause protein heat-induced unfolding and consequent aggregation. Thus, the effect of heating temperature negates the stabilising effect of the high added calcium levels.

Not only is the effect of added calcium important in the heat-induced aggregation mechanism, but equally the denaturation and aggregation rate must be considered. The temperature dependence of the kinetic constants shows that high heating temperatures (e.g. $T \geq 85°C$) are higher than the transition temperature ($\sim 80°C$) and thus aggregation is the limiting step. This means that unfolding of the proteins happens rapidly and aggregation takes place slowly, relatively to unfolding. Therefore, protein molecules are mostly, if not completely, unfolded when intermolecular interactions occur. This leads to an overall fast aggregation mainly via disulphide-bonding, mostly due to the temperature effect rather than the calcium effect. In contrast, at temperatures lower than the transition temperature (but still higher than the whey protein denaturation temperature) (e.g. $\sim 75 - 80°C$), the temperature dependence of the kinetic constants shows that the denaturation step is rate-limiting. Therefore, the proteins unfold slowly, and aggregation occurs quickly, relatively to unfolding. The effect of calcium then dominates the temperature effect by allowing intermolecular interactions between partially unfolded protein molecules, the result of protein net charge screening and intermolecular calcium bridges. Aggregation, mainly via disulphide-bonding, can then take place even though the protein molecules are only partially unfolded.
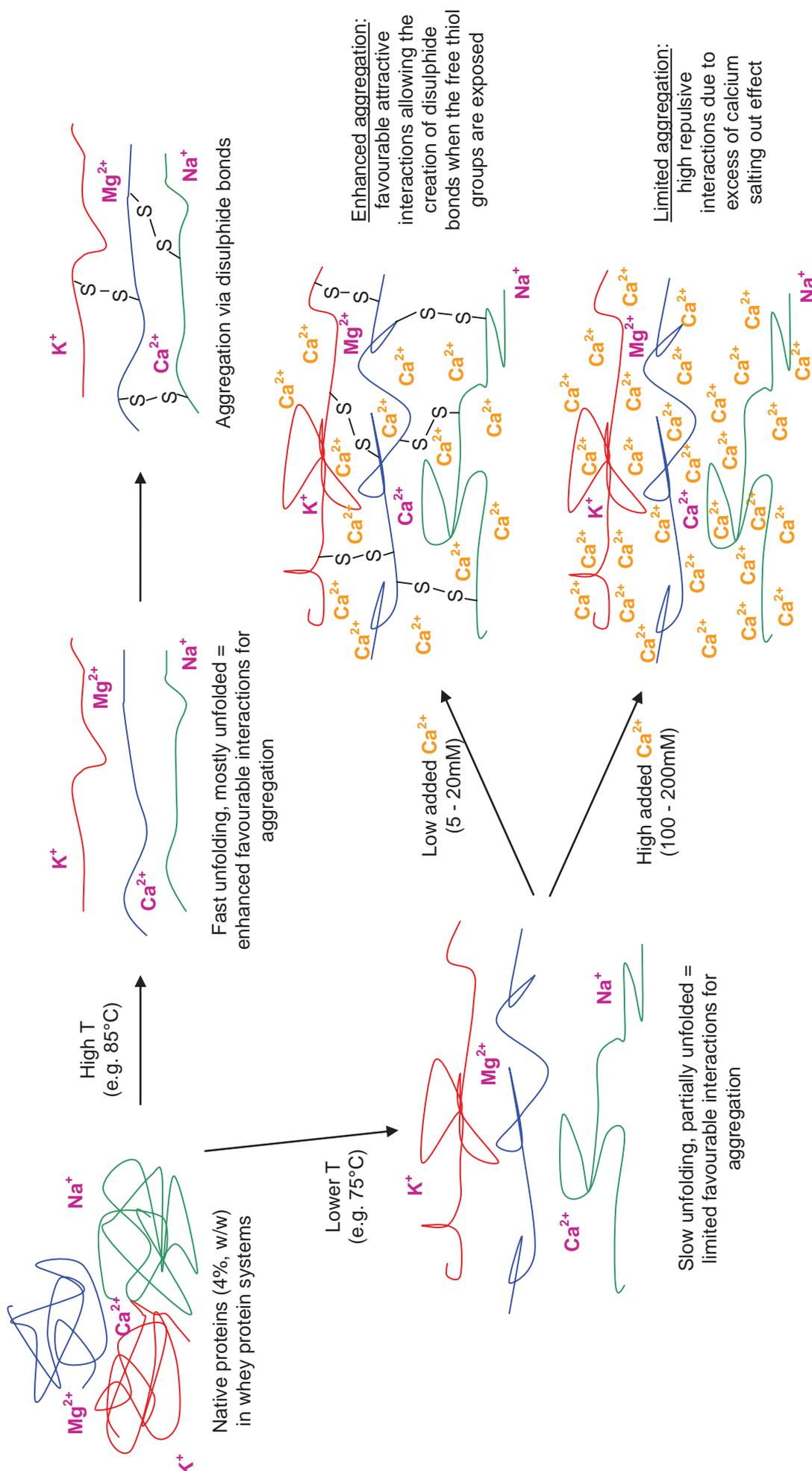
Figure 6.1: Diagram of the proposed mechanism for the effect of added calcium on the heat-induced aggregation of whey proteins; Ca^{2+} corresponds to added calcium and Ca^{2+}, Mg^{2+}, Na^+ and K^+ correspond to initial minerals.

As the three protein systems studied comprise the same proteins, similar aggregation mechanisms are expected. However, some differences were observed in the type of interactions leading to aggregation, and in gelation properties. These differences could be explained not by the effect of calcium on the aggregation and gelation mechanisms as such, but probably by the way the effect of added calcium is influenced by the initial mineral environment of the whey protein systems. During the manufacture of WPI and WPC, the minerals from the milk are in their ionic form, and would thus be expected to be in their ionic form when WPI and WPC are dispersed in water. However, from the results of the present study, the initial minerals do not seem to behave as if they were in their ionic form, as shown by the different gel properties obtained when ionic calcium was added. Unfortunately, the present study does not allow further understanding of the state of the original minerals of WPI and WPC owing to the limitations of the experimental techniques used. However, one skilled in the art, when thinking of the manufacturing processes for WPI and WPC, would ask the question "What happens between the proteins and minerals during spray drying?" It is known that whey proteins themselves are sensitive to spray drying conditions and their solubility is spray-drying-conditions-dependent (Anandharamakrishnan, Rielly, & Stapley, 2008). But WPI and WPC contain more than just whey proteins, and surely the other components would also be affected by the spray drying conditions. What does rapid water evaporation do to the ionic minerals? How does that affect the proteins? Several questions can be asked and thus can be the subject of future investigations.

Future work

The results discussed in this thesis are a good step forward to understanding how the effect of added calcium on the heat-induced behaviour of whey proteins varies with the whey protein product. However, a complete understanding is needed to be helpful for manufacturers when developing whey protein products. The results of this study were all produced in the laboratory, in static conditions which did not accurately reflect the real dynamic conditions of commercial plant. Further experiments carried out under shear will give a better understanding of the dynamic situation. These experiments would be designed to study the effect of shear on heat-induced denaturation and aggregation, on the types of interactions involved (non-covalent interactions versus disulphide bridges), and on gelation properties. In addition, investigating the effect of added calcium on whey protein systems heat-treated under shear will give a more

complete understanding of the use of calcium as an aid in manufacturing denatured whey proteins. It will also be important to study the effects of shear and added calcium at high protein concentration. A study of the effects of severe conditions of shear, flow rate, and other "extreme" processing conditions will be very useful for commercialisation of denatured whey proteins. Finally, further experiments will help to answer the question: will the effect of added calcium under shear and high protein be similar for the different whey protein systems studied?

As Sherwin and Foegeding (1997) demonstrated, the added calcium (mM) to protein (%) ratio contributed in large part to the effect of added calcium on the heat-induced aggregation of whey proteins. In the present work, the kinetics of the heat-induced aggregation of whey proteins was investigated at a fixed added calcium concentration but varying protein levels, in other words while varying added calcium (mM) to protein (%) ratio. Results showed that the rate of aggregation seemed to be added-calcium-to-protein-ratio-dependent. Further experiments with varying levels of added calcium and protein concentrations would deepen knowledge of this ratio-dependent kinetics.

New analytical techniques have been developed and studied to try and understand the mechanism of the denaturation, aggregation and gelation of whey proteins in complex systems. Wang et al. (2006) demonstrated that ultrasound can be used to quantify the degree of aggregation of α-lactalbumin. Ruis et al. (2008) used diffusing wave spectroscopy to study the influence of shear on the aggregation of sodium caseinate. Elshereef et al. (2006) demonstrated that fluorescence spectroscopy can be a useful tool for monitoring solubility and aggregation behaviour of β-lactoglobulin on heat treatment. Evaluating these analytical methods against the "classic" ones usually used, and showing their potential advantages in saving time analysing whey protein solutions, would be of great interest to food companies.

On a broader level, the effect of added calcium on the heat-induced denaturation, aggregation and gelation of whey proteins, which has been studied in this work, is an effect that is influenced by the different mineral compositions of the three different whey protein systems. However, commercial whey protein products contain not only whey proteins and minerals but also residual lipids and lactose. It is well known that whey proteins interact with lactose and lipids leading to modifications of whey protein

behaviour and functionality. Recently, El-Salam et al. (2009) reviewed the factors affecting the functional properties of whey protein products. These factors relate to the composition of the whey protein products, including the relative concentrations of whey protein fractions (dependent on milk composition and therefore on seasonality), minerals (pH, ionic strength, mono- and divalent cations), residual lipids, residual lactose (and degree of lactosylation). They also relate to the processing operations, including heat treatment, high hydrostatic pressure, ultrasound, and extrusion. Much knowledge is already available on the effects of compositional and processing factors, on an individual basis, in model and commercial complex systems. Many research teams have been more recently studying the combined effects of all of these factors in commercial complex systems and how they interact/compete/act synergistically. However, commercial whey protein products are used by the food industry in food applications with other food ingredients. And these other food ingredients potentially interact with whey proteins. The complexity is not only with the whey protein products as such but also with the food matrices they will be used in. Further studies combining all of these factors (whey protein product compositions, food processing conditions and food matrices) need to be carried out to be able properly to advise food companies about the selection of whey protein products for specific food applications.

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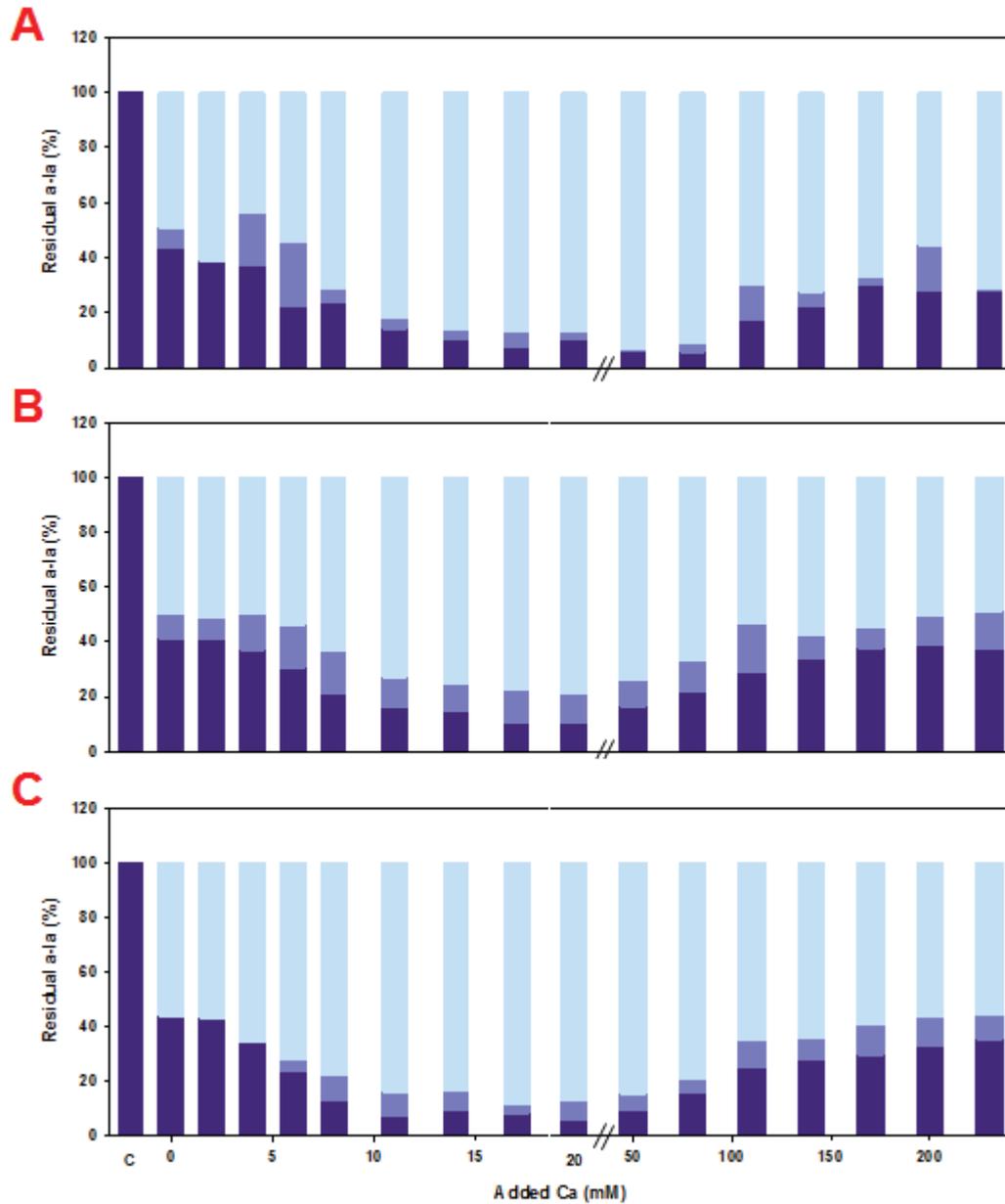
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APPENDIX 1 Protein aggregates in heated whey protein solutions with added calcium (Chapter 3)

- **α-Lactalbumin aggregates**

  o **Heated at 85°C**

α-Lactalbumin aggregates in heated (85°C, 5 min) whey protein solutions (4%, w/w protein): (**A**) WPI, (**B**) AWPC, and (**C**) CWPC, quantified by PAGE; ■ alkaline-monomeric proteins (native-like), ■ hydrophobically linked aggregates, ■ disulphide linked aggregates, **C** control sample (unheated without added $CaCl_2$).
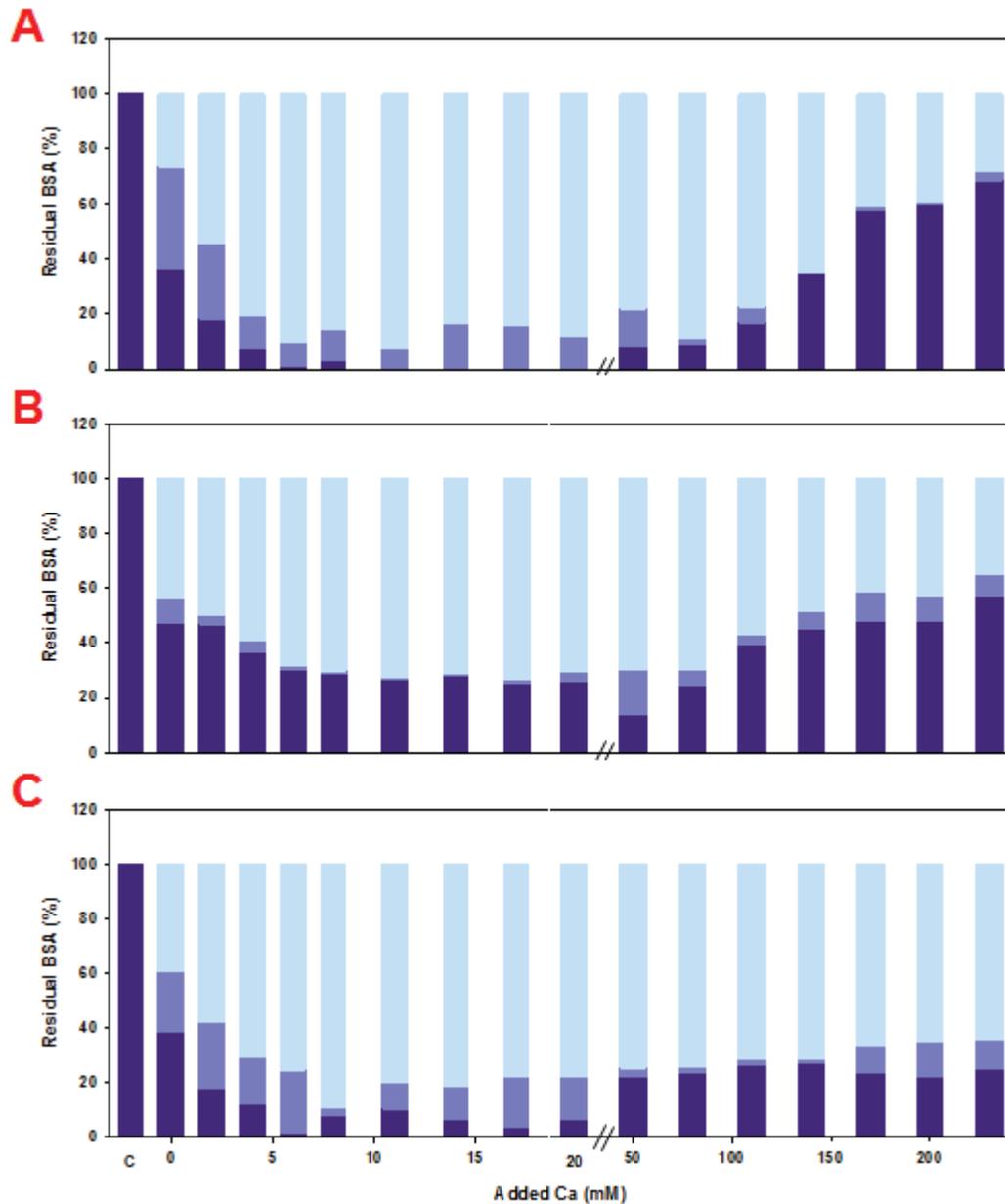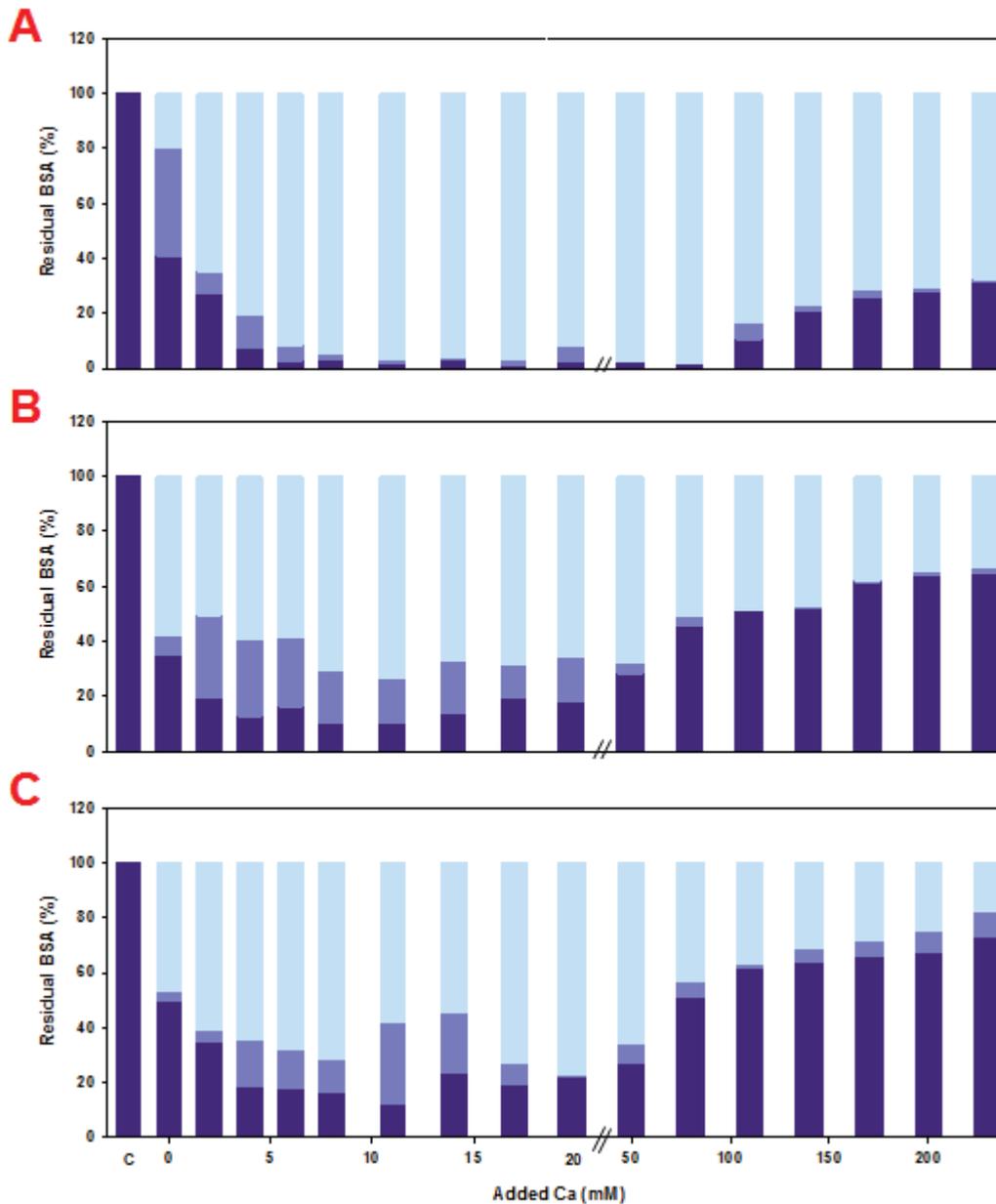
o   **Heated at 80°C**

α-Lactalbumin aggregates in heated (80°C, 5 min) whey protein solutions (4%, w/w protein): (**A**) WPI, (**B**) AWPC, and (**C**) CWPC, quantified by PAGE; ■ alkaline-monomeric proteins (native-like), ▮ hydrophobically linked aggregates, ▯ disulphide linked aggregates, **C** control sample (unheated without added CaCl₂).

○ **Heated at 75°C**

A

B

C

α-Lactalbumin aggregates in heated (75°C, 5 min) whey protein solutions (4%, w/w protein): (**A**) WPI, (**B**) AWPC, and (**C**) CWPC, quantified by PAGE; ▮ alkaline-monomeric proteins (native-like), ▮ hydrophobically linked aggregates, ▮ disulphide linked aggregates, **C** control sample (unheated without added CaCl$_2$).

- **Bovine serum albumin aggregates**
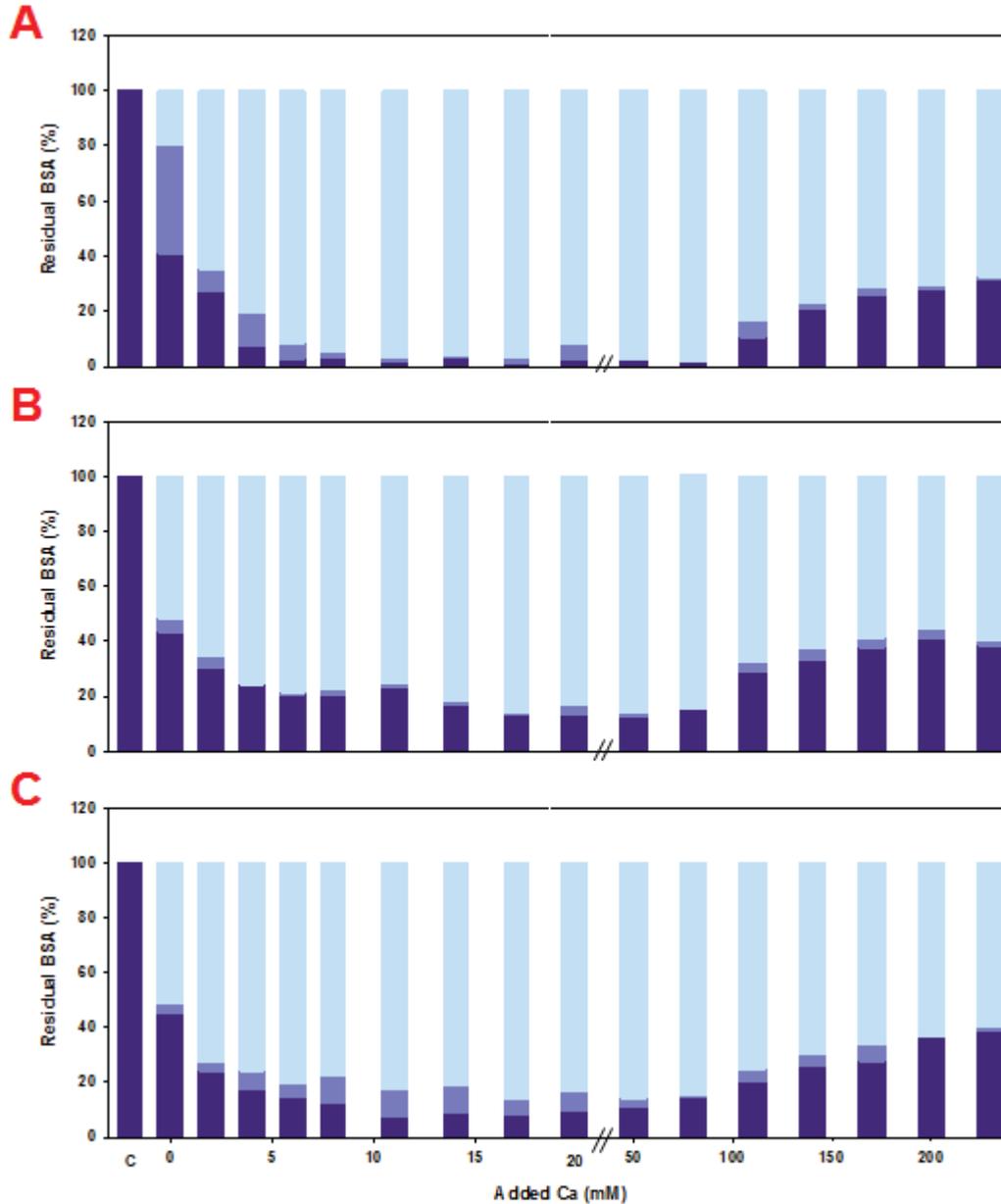
  o **Heated at 85°C**

Bovine serum albumin aggregates in heated (85°C, 5 min) whey protein solutions (4%, w/w protein): (**A**) WPI, (**B**) AWPC, and (**C**) CWPC, quantified by PAGE; ▮ alkaline-monomeric proteins (native-like), ▮ hydrophobically linked aggregates, ▯ disulphide linked aggregates, **C** control sample (unheated without added CaCl$_2$).

○   **Heated at 80°C**

Bovine serum albumin aggregates in heated (80°C, 5 min) whey protein solutions (4%, w/w protein): (**A**) WPI, (**B**) AWPC, and (**C**) CWPC, quantified by PAGE; ▇ alkaline-monomeric proteins (native-like), ▇ hydrophobically linked aggregates, ▇ disulphide linked aggregates, **C** control sample (unheated without added $CaCl_2$).

○   **Heated at 75°C**

Bovine serum albumin aggregates in heated (75°C, 5 min) whey protein solutions (4%, w/w protein): (**A**) WPI, (**B**) AWPC, and (**C**) CWPC, quantified by PAGE; ■ alkaline-monomeric proteins (native-like), ■ hydrophobically linked aggregates, ■ disulphide linked aggregates, **C** control sample (unheated without added $CaCl_2$).

APPENDIX 2 Rate constants values for β-lactoglobulin aggregation at different orders of reaction (Chapter 5)

- **Without added calcium**
  - $n = 1.2$

Temperature (ºC)		70	75	80	85	90	95	100
$0.2 \times k_{1.2}C_0^{0.2}$ $(s^{-1} \times 10^{-3})$	5% (w/w) ts	0.02 $R^2 = 0.88$	0.04 $R^2 = 0.86$	0.30 $R^2 = 0.88$	0.90 $R^2 = 0.94$	1.30 $R^2 = 0.97$	1.80 $R^2 = 0.98$	2.00 $R^2 = 0.98$
	10% (w/w) ts	0.10 $R^2 = 0.66$	0.30 $R^2 = 0.89$	0.90 $R^2 = 0.94$	1.90 $R^2 = 0.97$	3.10 $R^2 = 0.98$	4.20 $R^2 = 0.85$	5.30 $R^2 = 0.93$
	15% (w/w) ts	0.50 $R^2 = 0.56$	0.80 $R^2 = 0.84$	2.30 $R^2 = 0.95$	3.90 $R^2 = 0.98$	4.60 $R^2 = 0.98$	7.50 $R^2 = 0.91$	7.80 $R^2 = 0.88$
	25% (w/w) ts	0.05 $R^2 = 0.92$	0.80 $R^2 = 0.73$	3.80 $R^2 = 0.96$	5.50 $R^2 = 0.94$	6.90 $R^2 = 0.93$	8.80 $R^2 = 0.90$	10.70 $R^2 = 92$
	35% (w/w) ts	0.40 $R^2 = 0.82$	2.60 $R^2 = 0.92$	5.00 $R^2 = 0.95$	6.80 $R^2 = 0.97$	6.90 $R^2 = 0.78$	8.20 $R^2 = 0.86$	10.00 $R^2 = 0.79$
$k_{1.2}$ $(s^{-1}\%^{-1} \times 10^{-3})$	5% (w/w) ts	0.05	0.11	0.81	2.43	3.52	4.87	5.41
	10% (w/w) ts	0.24	0.71	2.13	4.49	7.33	9.93	12.53
	15% (w/w) ts	1.02	1.63	4.69	7.95	9.37	15.28	15.90
	25% (w/w) ts	0.10	1.68	7.97	11.54	14.47	18.46	22.45
	35% (w/w) ts	0.83	5.39	10.37	14.10	14.31	17.00	20.74

○ **$n = 1.5$**

Temperature (ºC)		70	75	80	85	90	95	100
$0.5 \times$ $k_{1.5}C_0^{0.5}$ $(s^{-1} \times 10^{-3})$	5% (w/w) ts	0.05 $R^2 = 0.88$	0.10 $R^2 = 0.86$	0.90 $R^2 = 0.88$	2.50 $R^2 = 0.93$	3.70 $R^2 = 0.96$	5.00 $R^2 = 0.99$	5.50 $R^2 = 0.98$
	10% (w/w) ts	0.30 $R^2 = 0.66$	0.087 $R^2 = 0.89$	2.50 $R^2 = 0.93$	5.50 $R^2 = 0.97$	9.70 $R^2 = 0.97$	13.40 $R^2 = 0.91$	16.50 $R^2 = 0.96$
	15% (w/w) ts	1.20 $R^2 = 0.58$	2.00 $R^2 = 0.82$	6.80 $R^2 = 0.93$	12.70 $R^2 = 0.97$	15.50 $R^2 = 0.97$	28.90 $R^2 = 07$	28.50 $R^2 = 0.93$
	25% (w/w) ts	0.10 $R^2 = 0.92$	2.10 $R^2 = 0.72$	12.20 $R^2 = 0.95$	19.60 $R^2 = 0.95$	26.00 $R^2 = 0.96$	33.70 $R^2 = 0.95$	40.50 $R^2 = 0.98$
	35% (w/w) ts	1.10 $R^2 = 0.81$	7.70 $R^2 = 0.90$	17.30 $R^2 = 0.95$	25.40 $R^2 = 0.98$	25.70 $R^2 = 0.84$	32.30 $R^2 = 0.94$	39.20 $R^2 = 0.91$
$k_{1.5}$ $(s^{-1}\%^{-1} \times 10^{-3})$	5% (w/w) ts	0.02	0.04	0.39	1.08	1.59	2.15	2.37
	10% (w/w) ts	0.09	0.02	.77	1.69	2.98	4.12	5.08
	15% (w/w) ts	0.25	0.42	1.44	2.69	3.29	6.13	6.05
	25% (w/w) ts	0.02	0.48	2.78	4.47	5.93	7.68	9.24
	35% (w/w) ts	0.24	1.71	3.83	5.63	5.69	7.15	8.68

○   *n* = 1.7

Temperature (ºC)		70	75	80	85	90	95	100
$0.7 \times$ $k_{1.7}C_0^{0.7}$ $(s^{-1} \times 10^{-3})$	5% (w/w) ts	0.07 $R^2 = 0.88$	0.10 $R^2 = 0.86$	1.20 $R^2 = 0.88$	3.60 $R^2 = 0.93$	5.50 $R^2 = 0.96$	7.40 $R^2 = 0.99$	8.20 $R^2 = 0.98$
	10% (w/w) ts	0.40 $R^2 = 0.66$	1.00 $R^2 = 0.90$	3.60 $R^2 = 0.93$	8.40 $R^2 = 0.96$	15.70 $R^2 = 0.96$	22.40 $R^2 = 0.94$	26.90 $R^2 = 0.97$
	15% (w/w) ts	1.70 $R^2 = 0.59$	2.90 $R^2 = 0.81$	10.70 $R^2 = 0.91$	21.20 $R^2 = 0.96$	26.80 $R^2 = 0.95$	55.40 $R^2 = 0.98$	52.50 $R^2 = 0.95$
	25% (w/w) ts	0.20 $R^2 = 0.92$	3.10 $R^2 = 0.72$	20.50 $R^2 = 0.94$	35.20 $R^2 = 0.95$	49.10 $R^2 = 0.96$	64.10 $R^2 = 0.97$	76.70 $R^2 = 0.99$
	35% (w/w) ts	1.60 $R^2 = 0.81$	12.30 $R^2 = 0.88$	30.50 $R^2 = 0.94$	47.80 $R^2 = 0.97$	47.80 $R^2 = 0.87$	63.30 $R^2 = 0.97$	76.50 $R^2 = 0.96$
$k_{1.7}$ $(s^{-1}\%^{-1} \times 10^{-3})$	5% (w/w) ts	0.01	0.02	0.20	0.60	0.91	1.23	1.36
	10% (w/w) ts	0.04	0.10	0.37	0.87	1.63	2.33	2.80
	15% (w/w) ts	0.10	0.18	0.66	1.31	1.66	3.42	3.24
	25% (w/w) ts	0.01	0.21	1.40	2.41	3.36	4.38	5.24
	35% (w/w) ts	0.10	0.81	2.00	3.14	3.14	4.15	5.02

○   *n = 2*

Temperature (ºC)		70	75	80	85	90	95	100
$k_2 C_0$ $(s^{-1} \times 10^{-3})$	5% (w/w) ts	0.10 $R^2 = 0.88$	0.20 $R^2 = 0.86$	1.80 $R^2 = 0.87$	5.60 $R^2 = 0.92$	8.70 $R^2 = 0.95$	11.80 $R^2 = 0.98$	12.80 $R^2 = 0.98$
	10% (w/w) ts	0.50 $R^2 = 0.67$	1.40 $R^2 = 0.90$	5.50 $R^2 = 0.92$	13.90 $R^2 = 0.95$	28.30 $R^2 = 0.94$	42.50 $R^2 = 0.96$	48.90 $R^2 = 0.98$
	15% (w/w) ts	2.50 $R^2 = 0.61$	4.40 $R^2 = 0.79$	18.40 $R^2 = 0.88$	40.60 $R^2 = 0.94$	54.10 $R^2 = 0.92$	132.50 $R^2 = 0.96$	116.60 $R^2 = 0.95$
	25% (w/w) ts	0.30 $R^2 = 0.92$	4.80 $R^2 = 0.70$	39.20 $R^2 = 0.91$	75.60 $R^2 = 0.93$	113.90 $R^2 = 0.95$	151.00 $R^2 = 0.96$	179.00 $R^2 = 0.98$
	35% (w/w) ts	2.00 $R^2 = 0.81$	21.80 $R^2 = 0.86$	63.30 $R^2 = 0.92$	111.20 $R^2 = 0.93$	108.20 $R^2 = 0.88$	156.40 $R^2 = 0.97$	187.60 $R^2 = 0.99$
k_2 $(s^{-1}\%^{-1} \times 10^{-3})$	5% (w/w) ts	0.005	0.01	0.08	0.26	0.40	0.55	0.59
	10% (w/w) ts	0.01	0.03	0.13	0.33	0.67	1.01	1.16
	15% (w/w) ts	0.03	0.05	0.21	0.46	0.61	1.49	1.31
	25% (w/w) ts	0.004	0.06	0.51	0.98	1.48	1.96	2.33
	35% (w/w) ts	0.02	0.27	0.78	1.36	1.33	1.92	2.30

- **With 20 mM of added calcium**

  - $n = 1.2$

Temperature (ºC)		70	75	80	85	90	95	100
$\mathbf{0.2 \times}$ $\boldsymbol{k_{1.2Ca}C_0^{0.2}}$ $(s^{-1} \times 10^{-3})$	**5% (w/w) ts**	0.05 $R^2 = 0.93$	1.60 $R^2 = 0.96$	4.40 $R^2 = 0.96$	7.00 $R^2 = 0.95$	9.50 $R^2 = 0.93$	12.30 $R^2 = 0.93$	15.30 $R^2 = 0.96$
	10% (w/w) ts	0.10 $R^2 = 0.78$	1.50 $R^2 = 0.91$	4.40 $R^2 = 0.96$	6.80 $R^2 = 0.90$	8.30 $R^2 = 0.84$	12.10 $R^2 = 0.86$	15.30 $R^2 = 0.88$
	15% (w/w) ts	0.09 $R^2 = 0.93$	1.10 $R^2 = 0.90$	4.10 $R^2 = 0.94$	6.10 $R^2 = 0.93$	8.10 $R^2 = 0.88$	10.30 $R^2 = 0.87$	13.70 $R^2 = 0.93$
	25% (w/w) ts	0.09 $R^2 = 0.95$	0.60 $R^2 = 0.66$	2.20 $R^2 = 0.95$	4.60 $R^2 = 0.96$	7.40 $R^2 = 0.90$	10.40 $R^2 = 0.97$	16.20 $R^2 = 0.93$
	35% (w/w) ts	0.03 $R^2 = 0.77$	0.80 $R^2 = 0.72$	3.50 $R^2 = 0.94$	5.40 $R^2 = 0.96$	6.70 $R^2 = 0.92$	8.70 $R^2 = 0.89$	10.50 $R^2 = 0.90$
$\boldsymbol{k_{1.2Ca}}$ $(s^{-1}\%^{-1} \times 10^{-3})$	**5% (w/w) ts**	0.14	4.35	11.96	19.03	25.82	33.44	41.59
	10% (w/w) ts	0.23	3.52	10.32	15.95	19.47	28.38	35.89
	15% (w/w) ts	0.20	2.40	8.94	13.30	17.67	22.46	29.88
	25% (w/w) ts	0.18	1.18	4.33	9.04	14.55	20.45	31.85
	35% (w/w) ts	0.06	1.48	6.49	10.01	12.42	16.13	19.46

○ **_n_ = 1.5**

Temperature (ºC)		70	75	80	85	90	95	100
$0.5 \times$ $k_{1.5Ca}C_0^{0.5}$ $(s^{-1} \times 10^{-3})$	5% (w/w) ts	0.20 $R^2 = 0.93$	4.30 $R^2 = 0.96$	14.50 $R^2 = 0.96$	26.20 $R^2 = 0.95$	27.00 $R^2 = 0.93$	48.70 $R^2 = 0.93$	60.90 $R^2 = 0.96$
	10% (w/w) ts	0.30 $R^2 = 0.78$	4.00 $R^2 = 0.91$	14.50 $R^2 = 0.96$	25.10 $R^2 = 0.90$	32.70 $R^2 = 0.84$	47.60 $R^2 = 0.86$	60.20 $R^2 = 0.88$
	15% (w/w) ts	0.20 $R^2 = 0.94$	3.00 $R^2 = 0.90$	13.30 $R^2 = 0.92$	21.60 $R^2 = 0.97$	30.00 $R^2 = 0.94$	38.10 $R^2 = 0.95$	52.10 $R^2 = 0.95$
	25% (w/w) ts	0.20 $R^2 = 0.95$	1.70 $R^2 = 0.64$	6.30 $R^2 = 0.94$	15.50 $R^2 = 0.97$	28.60 $R^2 = 0.93$	39.50 $R^2 = 0.98$	65.70 $R^2 = 0.97$
	35% (w/w) ts	0.08 $R^2 = 0.77$	2.20 $R^2 = 0.73$	11.10 $R^2 = 0.93$	19.00 $R^2 = 0.97$	24.90 $R^2 = 0.96$	32.90 $R^2 = 0.93$	39.50 $R^2 = 0.93$
$k_{1.5Ca}$ $(s^{-1}\%^{-1} \times 10^{-3})$	5% (w/w) ts	0.09	1.87	6.32	11.42	16.13	21.23	26.54
	10% (w/w) ts	0.09	1.21	4.37	7.57	9.86	14.35	18.15
	15% (w/w) ts	0.05	0.75	3.34	5.43	7.54	9.58	13.09
	25% (w/w) ts	0.04	0.33	1.22	3.01	5.55	7.66	12.74
	35% (w/w) ts	0.01	0.37	1.86	3.18	4.17	5.51	6.61

○   **n = 1.7**

Temperature (°C)		70	75	80	85	90	95	100
0.7 × $k_{1.7Ca}C_0^{0.7}$ (s^{-1} × 10^{-3})	**5% (w/w) ts**	0.30 $R^2 = 0.93$	6.50 $R^2 = 0.96$	24.60 $R^2 = 0.97$	49.10 $R^2 = 0.97$	71.60 $R^2 = 0.96$	95.30 $R^2 = 0.96$	119.70 $R^2 = 0.97$
	10% (w/w) ts	0.40 $R^2 = 0.78$	10.00 $R^2 = 0.91$	24.70 $R^2 = 0.96$	46.60 $R^2 = 0.97$	64.00 $R^2 = 0.96$	92.80 $R^2 = 0.97$	117.60 $R^2 = 0.97$
	15% (w/w) ts	0.30 $R^2 = 0.94$	4.50 $R^2 = 0.89$	22.60 $R^2 = 0.91$	39.20 $R^2 = 0.99$	55.60 $R^2 = 0.96$	71.00 $R^2 = 0.97$	99.30 $R^2 = 0.94$
	25% (w/w) ts	0.30 $R^2 = 0.95$	2.50 $R^2 = 0.62$	9.80 $R^2 = 0.94$	26.80 $R^2 = 0.96$	54.90 $R^2 = 0.93$	74.80 $R^2 = 0.97$	131.40 $R^2 = 0.98$
	35% (w/w) ts	0.10 $R^2 = 0.77$	3.20 $R^2 = 0.73$	18.40 $R^2 = 0.92$	33.80 $R^2 = 0.97$	46.40 $R^2 = 0.97$	62.30 $R^2 = 0.94$	74.50 $R^2 = 0.94$
$k_{1.7Ca}$ (s^{-1}%$^{-1}$ × 10^{-3})	**5% (w/w) ts**	0.05	1.10	4.16	8.31	12.12	16.13	20.26
	10% (w/w) ts	0.04	1.01	2.50	4.71	6.47	9.38	11.88
	15% (w/w) ts	0.02	0.35	1.77	3.07	4.35	5.56	7.78
	25% (w/w) ts	0.02	0.14	0.53	1.46	2.99	4.07	7.16
	35% (w/w) ts	0.004	0.14	0.82	1.50	2.06	2.76	3.30

- **$n = 2$**

Temperature (ºC)		70	75	80	85	90	95	100
$k_{2Ca}C_{0}$ $(s^{-1} \times 10^{-3})$	5% (w/w) ts	0.50 $R^2 = 0.93$	10.50 $R^2 = 0.95$	48.10 $R^2 = 0.96$	112.70 $R^2 = 0.98$	173.20 $R^2 = 0.97$	235.10 $R^2 = 0.97$	297.10 $R^2 = 0.96$
	10% (w/w) ts	0.60 $R^2 = 0.79$	9.70 $R^2 = 0.90$	48.60 $R^2 = 0.94$	105.50 $R^2 = 0.98$	158.50 $R^2 = 0.96$	226.90 $R^2 = 0.98$	290.00 $R^2 = 0.97$
	15% (w/w) ts	0.50 $R^2 = 0.94$	7.00 $R^2 = 0.89$	43.90 $R^2 = 0.87$	85.00 $R^2 = 0.98$	125.30 $R^2 = 0.97$	161.40 $R^2 = 0.97$	234.20 $R^2 = 0.92$
	25% (w/w) ts	0.40 $R^2 = 0.95$	3.80 $R^2 = 0.60$	16.50 $R^2 = 0.93$	53.70 $R^2 = 0.94$	131.40 $R^2 = 0.91$	175.40 $R^2 = 0.94$	335.60 $R^2 = 0.97$
	35% (w/w) ts	0.20 $R^2 = 0.77$	5.00 $R^2 = 0.73$	34.40 $R^2 = 0.90$	71.50 $R^2 = 0.95$	105.60 $R^2 = 0.96$	145.20 $R^2 = 0.94$	172.20 $R^2 = 0.94$
k_{2Ca} $(s^{-1}\%^{-1} \times 10^{-3})$	5% (w/w) ts	0.02	0.50	2.28	5.35	8.23	11.17	14.11
	10% (w/w) ts	0.01	0.22	1.10	2.40	3.60	5.16	6.59
	15% (w/w) ts	0.008	0.11	0.69	1.34	1.98	2.55	3.70
	25% (w/w) ts	0.004	0.04	0.16	0.50	1.24	1.65	3.15
	35% (w/w) ts	0.001	0.04	0.24	0.50	0.74	1.02	1.21

APPENDIX 3   Publications

The poster was presented at the 5th international symposium on food rheology and structure (ISFRS) 2009 in Zurich.