STUDIES ON HEAT-INDUCED INTERACTIONS AND GELATION OF WHEY PROTEIN

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TO 'ESETA, my mother

"By wisdom the Lord laid the earth's foundation, by understanding he set the heavens in place; by his knowledge the deeps were divided, and the clouds let drop the dew."

Proverbs 3:19-20 (The Holy Bible)

ABSTRACT

The purpose of this study was to gain greater understanding of the interactions of whey proteins during heat-induced gelation of whey protein concentrate (WPC) solutions. Attention was focused on gaining better knowledge of the relationship between composition of WPC and its ability to form heat-induced gels, and to explore the mechanisms of protein aggregation and gelation in WPC solutions.

Interactions of whey proteins (β-lactalbumin, α-lactalbumin and BSA) were studied in three types of commercial WPC (rennet, cheese and acid) solutions, as well as in pure protein model systems, using one-dimensional (1D-) and two-dimensional (2D-) polyacrylamide gel electrophoresis (PAGE), size exclusion chromatography, light scattering and ultracentrifugation. The formation and structure of aggregates and gels were determined by oscillatory rheometry, confocal scanning laser microscopy (CSLM) and transmission electron microscopy (TEM) techniques.

Examination of heated (75 °C) rennet WPC solutions at a range of concentrations (10-120 g/kg, pH 6.8) revealed that the extent of protein aggregation and the formation of the intermediate molecular weight products were concentration-dependent. The rates of loss of β-lactoglobulin, α-lactalbumin and BSA during heating increased as the WPC concentration was increased from 10 to 120 g/kg. 2D-PAGE showed that some disulphide-linked β-lactoglobulin dimers were present in heated 10 g/kg solution, but very little was present in heated 120 g/kg solution. SDS was able to dissociate monomeric protein from high molecular weight aggregates in heated 120 g/kg WPC solution but not in 10 g/kg WPC solution. This suggested that in addition to disulphide-linked aggregates, hydrophobic aggregates involving β-lactoglobulin, α-lactalbumin and BSA were formed in heated WPC solutions at high protein concentrations.

Examination of the heated acid WPC and cheese WPC solutions (120 g/kg), using 1D-PAGE and size exclusion chromatography, revealed that the loss of β-lactoglobulin, α-lactalbumin and BSA from the cheese WPC solution was faster than the loss of the same proteins from the acid WPC solutions. It was also found that a considerable proportion of aggregates formed in heated cheese WPC solution was linked by hydrophobic association, whereas the aggregates formed in heated acid WPC solutions were linked predominantly by disulphide bonds. TEM and CLSM showed that

the aggregates formed in cheese WPC solution were relatively large and "particulated," whereas the aggregates formed in acid WPC solution were small and "fine stranded." The gels formed from the heated cheese WPC solutions had low gel strength and high syneresis, whereas the gels obtained from the acid WPC had high gel strength and good water holding capacity.

Results of the dialysis experiments revealed that the differences between the properties of the acid WPC and the cheese WPC gels could be explained largely by their different mineral compositions. Relatively higher concentrations of divalent cations, Ca and Mg, in the cheese WPC was considered to be responsible for high rates of loss of native-like proteins, and the formation of large, hydrophobically-associated and "particulated" aggregates. High concentrations of monovalent cation in the acid WPC solutions probably resulted in slower loss of native-like proteins and formation of small and "fine-stranded" aggregates.

Attempt was made to characterise the nature of "insoluble" material in the unheated acid and cheese WPC solutions. Although, both the acid and the cheese WPC solutions contained considerable amounts of "insoluble" material, the amounts in the cheese WPC were greater. This material contained disproportionately higher levels of aggregated BSA and the minor whey proteins; in the cheese WPC it also contained considerable amounts of aggregated β-lactoglobulin and α-lactalbumin as well as phospholipids. The "insoluble" material in acid WPC, had higher casein content. The presence of this material did not appear to affect the gelation characteristics of the cheese WPCs, but had a positive effect on acid WPC gelation.

Studies on model systems of pure proteins showed that β-lactoglobulin, α-lactalbumin and BSA interacted to form homogeneous aggregates of each other as well as heterogeneous aggregates. 2D-PAGE clearly showed that when a mixture of these proteins was heated, initially BSA formed aggregates with itself and β-lactoglobulin and α-lactalbumin formed co-aggregates at a later stage of heating. Based on these results, the structure of WPC gel was suggested to be a heterogeneous network formed largely by co-polymers of β-lactoglobulin and α-lactalbumin embedded with "clusters" or "strands" of BSA aggregates.

Based on the results of this study, recommendations are made on how this information can be used in the development of new or improved whey products.

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CHAPTER 1
INTRODUCTION

Whey proteins represent a major source of highly functional proteins for the food industry. In recent years, considerable effort has been focused on understanding and improving the functionality of whey protein products to enhance their utilization by the food industry. However, commercial preparations of whey proteins may vary markedly in their functional properties which reflect differences in composition, processing treatments and extent of protein denaturation. There are a number of different processing methods used to manufacture whey products. In most cases, the whey is concentrated by ultrafiltration and evaporation prior to spray drying into powder. The manufacture of acid WPC involves ultrafiltration of acid casein whey at pH 4.3 - 4.5, whereas the manufacture of cheese WPC involves ultrafiltration of cheese or rennet whey at pH 6.3 - 6.5; this results in WPCs with different mineral content and profile. Furthermore, there are other materials such as GMP, bacterial debris etc. present in cheese WPC. There is no clear knowledge of the relationship between either the manufacturing processes or the WPC composition and WPC functionality.

The most important functional property of WPC is the ability to form heat-induced gels. Whey proteins, when heated above a critical temperature, undergo conformational changes followed by subsequent protein-protein interactions resulting in a precipitation or gelation, depending on protein, concentration, heating rate and environment conditions (pH, salt concentrations and type of salt). The mechanisms responsible for gel formation by whey proteins are not fully understood because of the complicated interactions among different proteins of whey and the interactions between protein and non-protein components. Although much information exists concerning factors affecting gel formation and properties of whey protein gels, there are limited data on the kinetics of gel formation and gelling properties of the individual proteins.

The important initial step in heat-induced gelation of whey proteins is the denaturation process which exposes the functional groups. Under appropriate conditions, the denatured molecules aggregate to form a three-dimensional gel network. Sulphydryl-disulphide interchange reactions and hydrophobic interactions are important

in cross-linking the protein molecules into a gel network. For the formation of a highly ordered gel, it is essential that the aggregation step proceeds at a slower rate than the denaturation step. Thus it is important to control the denaturation and aggregation process in order to obtain structures with desired textural properties. Although, denaturation of whey proteins and the factors that influence denaturation have been the subjects of a large number of investigations, most studies have been concerned with denaturation *per se* in dilute solutions and the undesirable aggregation that occurs under certain conditions rather than with denaturation as part of the overall desirable gelation process. Limited data is available in the literature on denaturation and aggregation of whey proteins at relatively high concentrations and in the ionic environments that are likely to exist in food systems where whey proteins are used as gelling agents.

One approach to developing a better understanding of the gelation of whey proteins is to take a model solutions of individual proteins in well defined systems, and determine effects of single and multiple factors on the rates of denaturation, aggregation and gelation processes. From this approach, the contribution of each protein and non-protein component to the gelation of the whole whey protein systems can be determined.

Extensive studies on the denaturation, aggregation and gelation of β-lactoglobulin have been reported (e.g. de Wit & Klarenbeek, 1981; Paulsson *et al.*, 1986; Xiong, 1992), although less information is available on the behaviour of α-lactalbumin, BSA and immunoglobulins. Some interactions between individual whey proteins have been identified; for example addition of BSA or α-lactalbumin to β-lactoglobulin has been shown to enhance gel strength. However, the mechanisms of interactions between these proteins have not been fully elucidated. It is known that "soluble" aggregates of polymerised whey proteins are formed during the early stages of heat-induced gelation and that subsequent polymerization results in the formation of a rigid gel network. For instance, McSwiney (1994*a,b*) showed that about 80% of the protein was polymerised involving disulphide bonds and hydrophobic interactions before an appreciable increase in gel strength was observed. It was concluded that during the earlier stages of heating, β-lactoglobulin aggregated to form relatively large molecular weight species and gelation resulted from interaction between these species.

Relatively little information is available on the sizes and composition of these aggregates, especially in the mixed whey protein systems.

Recent studies (Gezimati *et al.*, 1996*a,b*), have shown that when a mixture of BSA and β-lactoglobulin was heated under some gelling conditions, there was no obvious interactions between these proteins, whereas when a mixture of β-lactoglobulin and α-lactalbumin was heated under the same conditions, these proteins interacted to form heterogeneous aggregates which formed the structure of the gel network. It is not known whether these interactions take place in a heated WPC systems.

The purpose of this study was to gain a greater knowledge of the relationship between the composition of WPC and its ability to gel, and to explore the range of the interactions of proteins that occur during heat treatment of WPC solutions under gelling conditions. It also sought to gain information that could relate the molecular properties of individual whey proteins and the macroscopic properties of heat-induced WPC gels. An attempt was made to define the types of interactions that led to the formation of different types of aggregates, and how the various types of aggregates formed relate to the properties of the heat-induced WPC gels.

CHAPTER 2
LITERATURE REVIEW

2.1. Introduction

Whey is a by-product of cheese or casein manufacture. Whey can be defined as being "the liquid remaining after removal of casein from milk". Casein can be separated from milk in a number of different ways, resulting in the production of different types of whey. There are generally two types of whey: sweet and acid whey. Sweet whey (consists of two categories: rennet and cheese whey) is produced after casein is separated from milk by addition of rennet. Acid whey is produced after removal of casein from milk by direct addition of mineral acids or by *in situ* production of lactic acid by added starter bacteria. Figure 2.1 shows the production processes for some of the milk proteins and their associated whey represented in a simplified form (Sienkiewicz & Riedel, 1990). For decades, the dairy industry considered whey a waste product and it was usually disposed of or used as animal feed. More recently, whey has been recognised as a major source of nutritional and functional ingredients (de Wit, 1990). Consequently, a number of whey protein products are now manufactured and used as food ingredients in a wide range of applications (Sienkiewicz & Riedel, 1990).

The most important functional property of the whey proteins is their ability to form heat-induced gels. The industrial uses of the whey products, however, are limited mainly because of inconsistency of product quality and composition, and lack of understanding of how the proteins behave in food systems. Better understanding of the nature of whey proteins in industrial whey products and how they behave under different heating conditions can lead to better control of industrial processing of the whey protein products, and development of new processes or products with predictable or tailor-made functional properties. This review discusses the development in the area of whey protein chemistry and functionality with specific emphasis on the interactions of whey proteins during heat-induced gelation. It covers the publications up to 1995, the time when this project was started. More specific and current (1996-1998) literature is cited in the results and discussion section of relevant chapters.

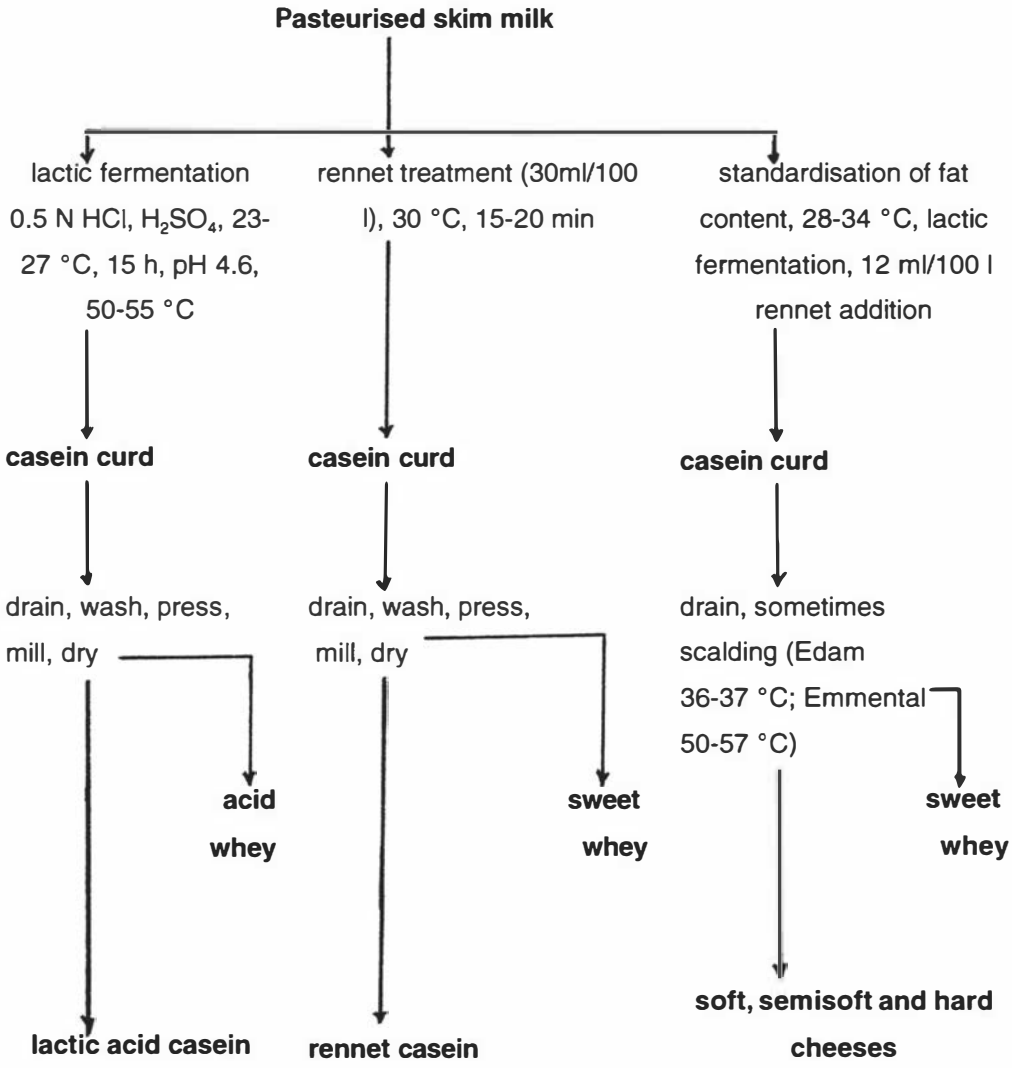


Figure 2.1. Manufacture of different cheeses and casein products and their wheys (adapted from Sienkiewicz & Riedel, 1990).

2.2. Whey composition

Whey composition varies depending on the milk source, the processing methods used and the cheese or casein type (Mulvihill & Donovan, 1987). Table 2.1 compares the composition of sweet and acid whey. Sweet whey has a higher pH, and total solids, protein, and lactose concentrations but lower concentrations of calcium and potassium than acid whey. Detailed information on the factors influencing whey composition can be found in the literature (e.g. Marshall, 1982; Olling & van Luin, 1988; Sienkiewicz & Riedel, 1990; Pearce, 1992).

Table 2.1. Typical composition of sweet (rennet and cheese) and acid (lactic acid and mineral acid casein) whey (adapted from Mulvihill & Donovan, 1987)

Component	Composition (g/l)			
	Sweet whey		Acid whey	
	Rennet casein	Cheddar cheese	Lactic acid casein	Mineral acid casein
Total solids	66	67	64	63
Total protein (N × 6.38)	6.6	6.5	6.2	6.1
Lactose	52.3	52.4	44.3	46.9
Minerals (ash)	5.0	5.2	7.5	7.9
Calcium	0.5	0.4	1.6	1.4
Potassium	1.0	0.5	2.0	2.0
Sodium	0.5	0.5	0.5	0.5
pH	6.4	5.9	4.6	4.7

2.3. Whey products

The components of whey can be isolated by different processes for production of various products. The most important are the whey protein products, but, in recent years, manufacture of lactose and mineral products from whey has gained commercial significance worldwide. Commercial production of lactose is done by either crystallisation or precipitation methods. Lactose is manufactured from either whey or de-proteinated milk serum from both sweet and acid whey (Hobman, 1984). The commercial manufacture of lactose has been reviewed by Zadow (1984), Bos (1987) and Harper (1992). Production of ethyl alcohol by fermentation of the lactose is widely used in the dairy industry. Detailed information on ethanol production using lactose can be found in several reviews, including Dale (1984), Whalen *et al.* (1987) and Zakrezecoski & Zmarlicki (1988). Commercial production of minerals (especially calcium) from whey has been exploited by the New Zealand dairy industry. Calcium phosphate can be precipitated, separated from whey, dried and sold as mineral powder. Calcium phosphate can be used to increase the levels of calcium in milk and other dairy products.

The protein products include various whey powders, whey protein concentrates (WPCs) and whey protein isolates (WPIs), and fractionated proteins, such as α-lactalbumin and β-lactoglobulin (Mulvihill, 1992). The most important commercial whey protein products are WPC (up to ~ 82% protein) and WPI (~ 95% protein). These two products are used widely as gelling agents in various food products. This review focuses mainly on WPC.

2.3.1. Whey protein concentrate (WPC) manufacture

Sweet or acid whey derived from cheese or casein production can be used for the manufacture of WPC. The manufacture of high quality WPC with consistent composition and functional properties requires whey of high quality. During the processing of cheese or casein, it is necessary to keep the numbers of psychrotropic and thermoduric microorganisms minimal. Large numbers of psychrotropic microorganisms can cause proteolytic activities which have been shown to influence the functional properties of WPC (Phillips *et al.*, 1987*a*). The presence of excessive

numbers of thermoduric microorganisms can result in acid production during the manufacture of WPC. A simplified schematic flow diagram of the manufacture of both sweet and acid WPC is given in Fig. 2.2. The processing steps shown in Fig. 2.2 can be grouped into three broad categories: whey pretreatment, ultrafiltration (UF) and diafiltration (DF), and concentration and drying.

Whey pretreatment

There is a range of whey pretreatment processes that may be carried out to improve flux and reduce fouling during UF, or to manipulate the chemical composition or functional properties of WPC. Details of the actual processes used are generally considered proprietary by the manufacturers. However, the following processes are commonly used.

Clarification. Whey contains a variety of soluble and insoluble components that have the potential to cause fouling of the membranes and reduce flux during UF. The accumulation of microorganisms, fine casein particles, lipoprotein material, mineral precipitates and denatured whey proteins has been shown to contribute to concentration polarisation and fouling (Lee & Merson, 1976). Fouling during the UF of acid whey is mainly caused by proteins whereas for sweet whey it is predominantly caused by calcium phosphate (Hiddink *et al.*, 1981). More information on fouling can also be found in Merin *et al.* (1983). Centrifugal clarification to remove particulate matter such as fat, casein fines and microorganisms is widely practised commercially (Hobman, 1992).

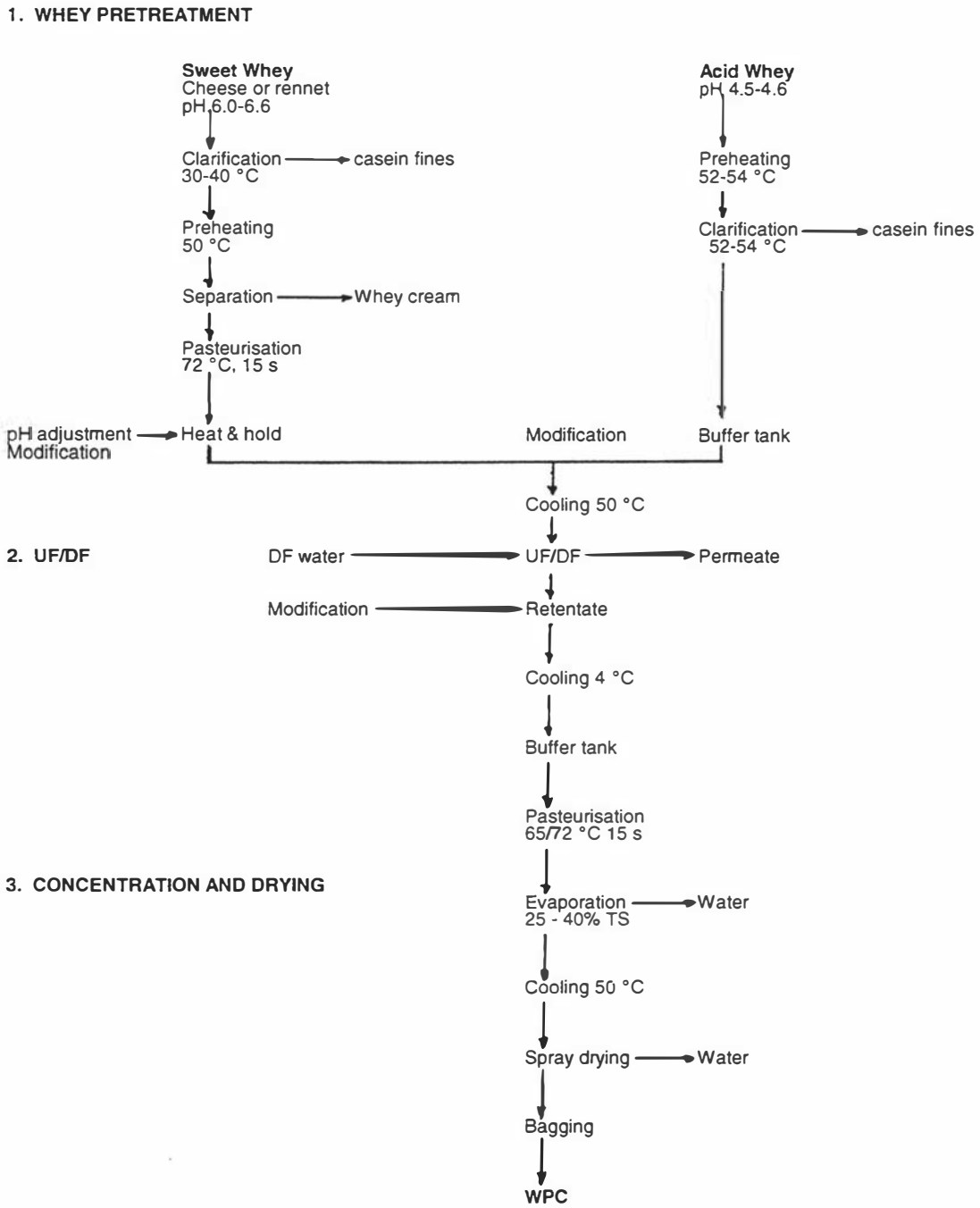


Figure 2.2. Simplified schematic flow diagram for the production of WPC (adapted from Nielsen, 1988).

Preheating and pasteurisation. Preheating whey to at least the temperature of UF and holding prior to UF has been shown (Hiddink *et al.*, 1981) to be particularly effective in reducing flux decline. It has been suggested that preheating tends to reduce the precipitation of calcium phosphate during UF. The clarified sweet whey, particularly in the case of cheese whey, is commonly pasteurised at 72 °C for 15 s and may be stored at low temperature (e.g. < 6 °C) prior to further processing. In comparison, acid whey is generally not pasteurised because such a heat treatment at the natural pH of the whey (~ pH 4.6) can result in protein denaturation. Furthermore, because acid whey is stored at ~ 54 °C, it is sufficiently inhibitory to the growth of microorganisms to avoid the need for pasteurisation (Hobman, 1992).

Preconcentration. Whey can be preconcentrated using two or three stage evaporators prior to UF. The advantages of this for WPC production include reduction of whey transportation costs, storage volumes and energy consumption, the possibility of improved fat separation, increased total solids and a reduction in the water to be removed during evaporation and drying (Nielsen, 1988).

Ultrafiltration (UF) and diafiltration (DF)

UF is a pressure-driven filtration process in which porous membranes are used to separate the components of a solid-liquid mixture on the basis of size and shape and, in some instances, charge (Sienkiewicz & Riedel, 1990). The pore dimensions are typically in the range from 1 to 100 nm nominal diameter, although commercially available UF membranes usually have an effective diameter of between 2 and 10 nm (Beaton & Steadly, 1982). In practice, any given membrane will have a distribution of pore sizes. Thus, the molecular weight cut-off of a membrane may be sharp or diffuse.

When a pressure gradient is applied across the membrane, the liquid is forced to flow through the pores to the low pressure side, transporting with it any components that are smaller than the size of the membrane pores. By this process, a concentrate of high molecular weight (retentate) and a permeate fraction of low molecular weight are obtained (Kessler, 1988). The permeate obtained from UF of whey consists of water, lactose, minerals, vitamins and amino

acids whereas the retentate contains higher molecular weight material (protein, fat) and water, and small proportions of low molecular weight matter. Whey proteins of high purity can be produced in a multi-phase process by additional DF. DF is a process by which the retentate is diluted with water and further ultrafiltered. This allows further removal of low molecular weight material from the retentate.

UF membranes are available in many forms but tubular membranes are most popular in modern UF applications because of their simplicity and versatility. The membranes are available with a wide range of porosity and separability in a molecular mass range from 500 to 200,000 (Sienkiewicz & Riedel, 1990). Several criteria must be satisfied to ensure the technical and economic viability of a particular UF application (Beaton & Steadly, 1982).

(a) Membranes that have appropriate selectivity characteristics to achieve the desired separation must be available.

(b) A flux of sufficient magnitude must be achievable under appropriate operating conditions to ensure that the capital and operating costs are acceptable.

(c) Flux must be capable of being maintained or restored by use of cleaning procedures after sufficiently long periods of operation to ensure that the operating costs are acceptable.

(d) The operating life of membranes must be sufficient to ensure that the operating costs (e.g. associated with membrane replacement) are acceptable.

(e) The equipment must be of appropriate design and capable of operation under conditions that are pertinent to the specific application, e.g. materials of construction, cleaning in place (CIP) and sanitisation.

It is important that the parameters of the UF plant for processing whey are appropriately designed (Madsen, 1985). A critical factor for the design is the shear stress at the surface of the membrane. The chosen shear stress must be of a sufficiently high value to avoid the formation of gel layer at the membrane surface and enable attainment and maintenance of high flux. This should not result in excessive energy consumption, mechanical forces within membranes (which may cause damage), or shear damage to protein. The number of stages to be used and the membrane area per stage can also be optimised. Other factors influencing the design and configuration include

the type, composition and quality of the whey, time and temperature of operation, throughput and the composition of the WPC.

UF of whey is commonly carried out at ~ 50 °C. This temperature is generally considered optimum where acceptable fluxes are achieved and the problems with microorganisms and thermal denaturation of proteins are avoided. Operation at lower temperatures (e.g. 10 °C) is currently used by some WPC manufacturers in New Zealand. The major disadvantages of such low temperatures are the lower fluxes achieved and more susceptibility to microbiological contamination (Nielsen, 1988).

DF is employed to produce WPC with protein contents > 65%. The quality of the water used for DF is of particular importance to avoid membrane fouling. Purified water (demineralised, evaporator condensate or reverse osmosis permeate) is commonly used. The number of DF stages and the volume of water used are dependent on the design and operation of the UF plant and the WPC specification. Typically, the flow rate of DF water to each stage ranges from 40 to 95% of the permeate flow rate from the stage (Hobman, 1992).

The retentate from the UF plant may need to be cooled (~ 4 °C) and stored until sufficient volume has been accumulated before drying commences (Hobman, 1992). Pasteurisation of the retentate using a heat treatment of ~ 72 °C for 15 s may also be necessary to reduce the number of bacteria. Nielsen (1988) reported that, for an 80% protein product, bacteria in the whey can be concentrated during UF by up to 130 times. Food grade chemicals may be added to the retentate (e.g. neutralisation of acid-whey-based retentate) to modify the physico-chemical properties of the powder.

Concentration and drying

The retentate may be concentrated before drying to minimise the cost of water removal and improve the physical properties of the powder. The use of an especially designed, high vacuum, low-boiling temperature (e.g. 50 °C) falling-film evaporator enables effective concentration of the retentate up to 40% total solids (Nielsen, 1988) while avoiding thermal denaturation of proteins. Recent improvements in the design of UF equipment enable retentates of high total solids to be produced and in some cases can eliminate the need for evaporation.

Drying of the retentate is usually performed using a spray drier fitted with nozzle atomisation. Typically, the inlet and outlet air temperatures used are 160-180 °C and less than 80 °C, respectively (Hobman, 1992).

2.4. Composition of whey protein concentrate

The composition of WPCs, although reflecting the source of the raw whey, can be influenced largely by the processing history. The degree of UF/DF applied during the processing of different WPC products is different among manufacturers, depending on the intended use of the product. There is a general drive to produce WPC with high protein content, as WPCs are marketed based on their protein content. Lactose and minerals vary considerably between various WPCs.

Commercially available WPCs contain from 35 to 85% protein. If they are added to food on a dry basis, there will be large differences in functionality owing to the differences in protein contents. Most food formulations call for a certain protein content and thus WPCs are generally utilised on a constant protein basis. In this case, the differences due to protein content as such should be eliminated. As the protein content increases, the composition of other components in the WPC must also change, and these changes in composition might be expected to have an effect on functionality (Mangino, 1992*a, b*). The WPCs containing 35-55% protein are used largely for animal feed manufacture. WPC and WPI that contain > 70% protein are used extensively as functional and nutritional ingredients in medical, pharmaceutical and human food products such as infant formula, health food and drinks, high gel product applications and frozen foods (Morr & Ha, 1993). Table 2.2 shows the average composition of eight commercial WPCs (Morr & Foegeding, 1990; Morr & Ha, 1993).

Table 2.2. Chemical composition of commercial WPCs (%) (adapted from Morr & Foegeding, 1990)

	WPC*	
Component	**Range**	**Mean ± s.d.**
Moisture	4.14-6.01	5.31±0.66
Protein	72.0-76.6	73.8±1.64
Lactose	2.13-5.75	3.92±1.20
Total lipids	3.30-7.38	5.00±1.27
Phospholipids	0.80-1.54	1.28±0.23
Ash	2.52-6.04	4.28±1.29
Sodium	0.15-1.71	1.04±0.65
Potassium	0.07-0.46	0.25±0.17
Calcium	0.23-1.05	0.46±0.27
Magnesium	0.02-0.40	0.09±0.12
Phosphorus	0.20-1.30	0.44±0.35

* n = 8.

WPCs contain residual lipid despite attempts by producers to remove as much lipid as possible from the whey. Removal of residual lipid from whey has been shown to increase the UF flux and to improve WPC functionality (de Wit & de Boer, 1975). Glover (1985) showed that the lipid content of WPCs increased as the protein content increased. Residual lipid has long been recognised as being detrimental to the quality of WPCs with particular attention to the foaming and flavour of the product (Mangino, 1992). Sternberg *et al.* (1976) have reported that residual lipid also inhibits the gel-forming properties of WPC. The lipid found in WPC does not have the same composition as the bulk lipid of milk, but is greatly enriched in phospholipids and milkfat globule membrane material (Houlihan & Hirst, 1987). Maubois *et al.* (1987) reported that a special treatment to remove this residual lipid greatly enhanced the foaming characteristics of WPCs. Joseph & Mangino (1988*a, b*) demonstrated that milkfat globule membrane material was associated with the residual phospholipid

fraction of WPCs.

The concentration of lactose in WPC generally decreases as the concentration of protein increases. The lactose content of commercially available WPCs ranges from 0.1 to 46% (de Wit *et al.*, 1986). Lactose is a reducing sugar and can react with proteins via non-enzymic browning producing less nutritious and lower functional products. Lactose can increase the heat stability of proteins and lactose concentration has been shown to be related to the solubility of whey proteins following heat treatment (Zadow, 1986*a, b*).

The mineral content of whey is altered as the whey is concentrated to form WPCs. The method of processing can have a large effect on the mineral content. Peltonen-Shalaby & Mangino (1986) reported that the ash content of commercially available WPCs, produced by UF, electrodialysis and metaphosphate complex formation, ranged from 0.98 to 12.18%. Calcium and phosphorus contents ranged from 0.014 to 21.80% and 0.26 to 3.53% respectively.

WPCs have been shown to function better in a number of applications where either minerals have been removed or their content has been modified (Mangino, 1992*b*). Such applications include uses in ice cream, infant formulae, bakery products and dietetic foods. The mineral that has received most attention regarding its effect on functionality is calcium. Schmidt (1981) showed that the strength of WPC gels was dependent on the calcium content of the WPC. The addition of calcium increased the gel strength until it reached a maximum; further increase in calcium caused a decrease in gel strength. At low concentrations, calcium increased the gel strength by aiding the formation of the crosslinks that are necessary for proper gel formation (Mangino, 1984). At higher concentrations of calcium, protein precipitation occurred at a faster rate than the crosslink formation and the gel was weakened. Commercially available WPCs, both acid (Liao & Mangino, 1987) and sweet (Kohnhorst & Mangino, 1985), contain calcium at a level where it weakens the gel strength and, thus, the gel strength generally increases with a reduction of calcium (Mangino, 1992*a, b*).

2.5. Whey proteins

Whey proteins are globular to ellipsoid in structure, relatively soluble and heat labile, with the exception of the proteose peptones (PP). The most important whey proteins are β-lactoglobulin, α-lactalbumin, bovine serum albumin (BSA) and immunoglobulins. The other proteins include lactoperoxidases, lysozyme, lactoferrin (Lf), lactollin and many others. These proteins have different properties as a result of differences in amino acid composition and spatial arrangement. The structure and properties of various whey proteins have been reviewed by Swaisgood (1982), Eigel *et al.* (1984), Mulvihill & Donovan (1987) and Whitney (1988) (Table 2.3).

Table 2.3. Characteristics of whey proteins (adapted from Mulvihill & Donovan, 1987; Walstra & Jenness, 1984)

Whey protein	Concentration in milk (g/kg)	MW (Da)	Isoelectric point (pH)	Disulphide bonds
β-Lactoglobulin	3.3	18,363	5.13	2
α-Lactalbumin	1.2	14,147	4.2-4.5	4
BSA	0.4	66,267	4.7-4.9	17
Immunoglobulin	0.7	$(1.5\text{-}10)\times10^5$	5.5-8.3	21
PP & other minor whey proteins	0.8	$(4.1\text{-}40.8)\times10^3$	-	0

2.5.1. β-Lactoglobulin

β-Lactoglobulin was among the first proteins to be crystallised and is well characterised (Creamer *et al.*, 1983; Hambling *et al.*, 1992). Seven generic variants have been identified, β-lactoglobulin A-F, (Eigel *et al.*, 1984) with A and B dominating and occurring at almost equal frequency (Hambling *et al.*, 1992). The β-lactoglobulin monomer comprises 162 amino acids with one free thiol group (Cys^{121}) and two disulphide bridges (Cys^{106}-Cys^{119} and Cys^{66}-Cys^{160}) (Papiz et al., 1986). The A and B variants differ at positions 64 and 118, where Asp and Val in β-lactoglobulin A are

replaced by Gly and Ala in β-lactoglobulin B.

Depending on pH, ionic strength and temperature, β-lactoglobulin may exist as a monomer, a dimer or an octamer (McKenzie & Sawyer, 1967). At room temperature and physiological pH, β-lactoglobulin exists mainly as a dimer but dissociates into monomers at elevated temperatures. The dimer is stable between pH 5.5 and pH 7.5, but dissociates due to strong electrostatic repulsions below pH 3.5. Between pH 3.5 and pH 5.2, the dimers form octomers. Above pH 7.0, β-lactoglobulin undergoes reversible conformational changes, and above pH 8.0, it is unstable and forms aggregates of denatured proteins (Lyster, 1972).

β-Lactoglobulin belongs to the lipocalin family of proteins, which all contain a β-barrel consisting of eight antiparallel β-strands (see Fig. 2.3). Each successive β-strand is adjacent to the preceding one and they fold into a calyx. The ninth β-strand is at the dimer interphase and forms an anti-parallel β-sheet interaction with the associated monomer. The free thiol group, Cys^{121}, and the disulphide bridge, Cys^{106}-Cys^{119}, are buried whereas the other disulphide bridge, Cys^{66}-Cys^{160}, is positioned on the outer surface in a mobile region of the molecule. β-Lactoglobulin, like most lipocalin proteins, can bind small hydrophobic molecules within the calyx (Townend *et al.*, 1967; Green *et al.*, 1979; Papiz *et al.*, 1986).

The biological function of β-lactoglobulin is still unknown. Being stable under acidic conditions and gastric proteolysis has suggested that it is a carrier of retinol from maternal milk to neonate via specific receptors in the intestine (Papiz *et al.*, 1986) and possibly facilitates vitamin A esterification (Ong, 1985). Other studies have indicated that retinol and fatty acids are bound to β-lactoglobulin in milk (Garrick, 1986; Puyol *et al.*, 1991), and that their uptake in the intestinal tract is enhanced by this association (Said *et al.*, 1989).

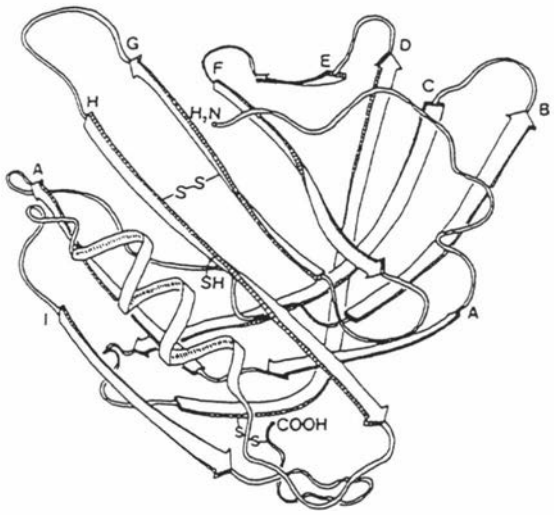


Figure 2.3. Tertiary structure of β-lactoglobulin. The arrows indicate strands of β-sheets, marked A-I (Papiz *et al.*, 1986)

2.5.2. α-Lactalbumin

α-Lactalbumin is the second most abundant, 20%, of the whey proteins in bovine milk. It is a small compact globular protein, consisting of 123 amino acid residues with a stable conformation between pH 5.4 and pH 9.0. It is stabilised by four disulphide bonds (Eigel *et al.*, 1984; Mckenzie & White, 1991; Brew & Grobler, 1992). α-Lactalbumin possesses an essential amino acid profile, rich in lysine, leucine, threonine, tryptophan and cystine. There are three known generic variants, A, B and C (Eigel *et al.*, 1984). The secondary structure at physiological pH consists of 26% α-helix, 14% β-structure and 60% unordered structure (Robbins & Holmes, 1970). α-Lactalbumin has also been shown to exhibit high homology to type-c lysozyme (Mckenzie & White, 1991). At pH values below its isoelectric point (pH 4.2), α-lactalbumin forms dimers and trimers, the association being fast and reversible and greater at 10 °C than at 25 °C. Above pH 9.0 and below pH 4.0, α-lactalbumin undergoes conformational changes without causing irreversible aggregation (Lyster, 1972; Shukla, 1973). Upon heating, reversible conformational changes in α-lactalbumin can be detected at pHs near neutrality, but no precipitation occurs (Baer *et al.*, 1976). Heating at more severe conditions (e.g. pH 6.8, 100 °C for 30 min) causes the

formation of disulphide bonds between α-lactalbumin molecules (Chaplin & Lyster, 1986).

α-Lactalbumin is a calcium-binding metalloprotein, which is also capable of binding zinc and probably other metals. Removal of the calcium reduces the heat stability of the protein. The biological activity of α-lactalbumin is to promote the binding of glucose to galactosyltransferase, which enhances the transfer of galactose from uridine diphosphogalactose to glucose, an essential step in the formation of lactose at physiological concentrations of glucose (McKenzie, 1971; Walstra & Jenness, 1984; Fox, 1989; Brew & Grobler, 1992).

2.5.3. Bovine serum albumin (BSA)

BSA is identical to the serum albumin found in the blood stream, and represents ~ 5% of the total whey proteins. The protein is synthesised in the liver and gains entry to the milk through the secretory cells. BSA has the longest single polypeptide chain of all the whey proteins, consisting of 582 amino acid residues, and has a molecular weight of 66,000 Da (Eigel *et al.*, 1984). It has 17 disulphide bonds, which stabilise its tertiary structure, and one free thiol group at position 34. It is a monomer and displays some micro-heterogeneity . The secondary structure consists of 55% α-helix, 16% β-sheet and 29% unordered structure (Reed *et al.*, 1975). The precise three-dimensional crystal structure of BSA is not known and the current view of the structure is that the molecule exists in three major domains, each consisting of two large double loops and a small double loop with the overall shape as a 3:1 ellipsoid (Brown, 1977). The precise biological function of BSA is not known, but it has been suggested that it functions as a carrier of small molecules, such as fatty acids (Walstra & Jenness, 1984).

2.5.4. Immunoglobulin (Ig)

Ig is a fraction of a complex heterogeneous mixture of large glycoproteins which possess antibody activity. These proteins represent ~ 10% of the whey proteins. Four distinct classes of Ig occur in bovine milk: IgM, IgA, IgE and IgG (Eigel *et al.*, 1984); the last is subdivided into IgG1 and IgG2. Two heavy (50-70 kDa) and two light (22.4

kDa) polypeptide chains, linked by disulphide bridges, form the basic subunit in each class. The molecular weight of Ig varies between ~ 150 and 900 kDa and the amino acid sequence is variable. IgG1, with an isoelectric point of 6.3-7.0 (Eigel *et al.*, 1984), is the main Ig class in bovine milk, and represents ~ 80% of the total Ig (Fox, 1989). These proteins are easily denatured by heat (Lyster, 1972).

2.5.5. Proteose peptones (PP)

PP are not considered to be "real" whey proteins as they are fragments of β-casein. In a broad sense, they are chemically defined as those milk proteins remaining soluble at pH 4.6 following heating at 95-100 °C for 30 min, but insoluble in 8-12% trichloroacetic acid (Mulvihill & Donovan, 1987). They range in molecular weight from 4,000 to 22,000 Da, depending on the variant. They are named after their electrophoretic mobility: PP-3, PP-5, PP-8-fast, PP-8-slow (Walstra & Jenness, 1984; Mulvihill & Donovan, 1987). No disulphide or sulphydryl residues have been identified. Some of the PP are the only proteins in whey having distinct soap-like characteristics. This is due mainly to the distribution of hydrophobic and hydrophilic sections (Dybing & Smith, 1991).

2.5.6. Minor whey proteins

β_2-Microglobulin is a single polypeptide chain consisting of approximately 100 amino acid residues (molecular weight of 11,800 Da). Transferrin is a common blood plasma protein. Lactoferrin and transferrin are both iron binding proteins that exist as large single chain polypeptides of 600-700 residues. Lactoferrin is an inhibitor of bacteria because it deprives them of iron, but the antibacterial effect is not significant because of its very low concentration (Walstra & Jenness, 1984).

2.6. Molecular forces governing protein structure and protein interactions

Molecular forces that hold the protein structure together and are involved in protein interactions include covalent disuphide linkages and non-covalent, electrostatic, van der Waals; hydrogen and hydrophobic forces (Howell, 1992). Figure 2.4 illustrates these forces.

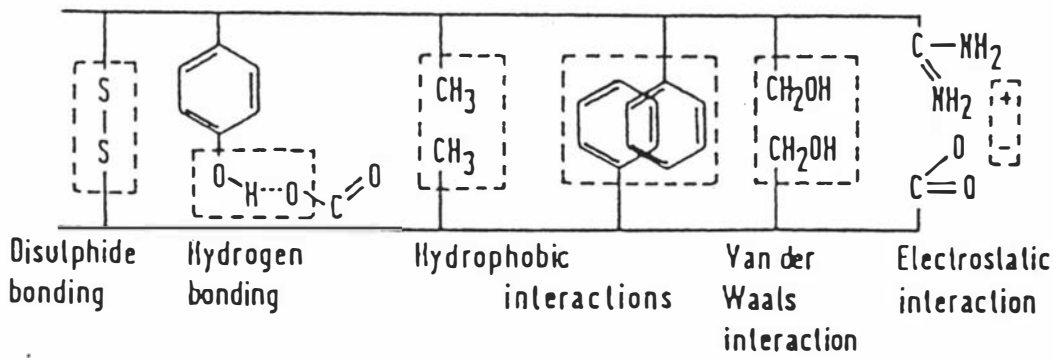


Figure 2.4. Molecular forces that govern the protein molecular structure and interactions (Howell, 1992).

The primary covalent bond within the protein molecules is the peptide bonds that hold the primary structure together. The peptide bond has a heat of formation of ~ 100 kcal/mol (Whitaker, 1977*a, b*). The peptide bond is not broken during denaturation but is readily hydrolysed by strong acid, alkali or proteolytic enzymes. More importantly, covalent disulphide bonds are involved in the tertiary structure and are therefore affected by denaturation during heat treatment. The disulphide bond has a heat of formation of ~ 50 kcal/mol. Disulphide bonds are formed between two Cys residues; the sites depend on the conformation of the polypeptide chain (Shimada & Cheftel, 1989). The number of disulphide bonds within a specific protein is determined by genetic origin. Disulphide bonds between two protein molecules (e.g. during heat-induced aggregation) are formed via thiol group (-SH) oxidation into S-S bonds and/or SH-induced S-S interchange reactions (Beveridge *et al.*, 1984; Dunkerley & Zadow, 1984; To *et al.*, 1985; Yasuda *et al.*, 1986; Shimada & Cheftel, 1988).

Electrostatic interactions occur between two charged molecules. Protein molecules are zwitterions which carry a net positive or net negative charge depending on their isoelectric point and the environmental pH. Electrostatic interactions are repulsive for like charges and attractive for opposite sign charges. They occur over large distances and vary inversely with distance. Electrostatic bonds have a heat of formation of ~ 10-20 kcal/mol (Whitaker, 1977*a*).

Van der Waals' forces are weak attractive interactive forces between polar and non-polar molecules. They occur over short distances when the positively charged nuclei and negatively charged electrons of two approaching molecules affect the electron cloud distribution of each molecule. Dipoles are induced and reversed and the resulting oscillating dipoles reduce the total energy of the interacting molecules, thereby creating an attractive force (Greighton, 1983).

The hydrogen bond is a particular type of electrostatic force between polar molecules. The H atom is shared between an acid (proton donor) group, e.g. -OH or -NH, and a base (proton acceptor), e.g. -C=O. It is not clear exactly how the bond is formed; it may be considered as an intermediate stage in the transfer of a proton from an acid to a base. The energy of formation of the hydrogen bond depends on the geometric angle with which the bond is formed but is reported to be in the range 1-6 kcal/mol (Whitaker, 1977*a,b*; Schultz & Schirmer, 1979; Greighton, 1983).

The hydrophobic interaction occurs between the non-polar regions of protein molecules when in an aqueous environment. When the protein molecules are surrounded by water, the polar regions interact via hydrogen bonding and electrostatic interactions with water whereas the non-polar residues are strongly tucked and hidden within the tertiary structure of the molecules. During the denaturation process, the hydrophobic residues are exposed resulting in strong interactions between non-polar residues of denatured molecules and form hydrophobically-associated aggregates. The hydrophobic interaction has an energy of formation of ~ 1- 4 kcal/mol (Howell, 1992).

As a result of these forces being created between the amino acid side chains, the primary structure will fold in an ordered fashion, forming the secondary and tertiary structures that give rise to a unique compact native structure which possesses the lowest feasible free energy. The most abundant regular secondary structures found in proteins are the α-helix and β-pleated sheet. When the protein structure is disrupted (e.g. during heating), these molecular forces cause different intra- and/or intermolecular interactions that give the lowest feasible free energy. Depending on the environmental conditions, this gives rise to the formation of protein aggregates or a gel network.

Denaturation can be defined as any major alteration in the original native or tertiary structure of the protein without hydrolysis of the primary covalent bonds, i.e.

the changes are restricted mainly to those occurring in the secondary structures (de Wit, 1981; de Wit & Klarenbeek, 1984; Mulvihill & Donovan, 1987). Each protein has a unique structure and therefore displays different responses to changes in the environment. Depending on the protein and the environmental conditions, denaturation may be confined to a segment of the protein or may involve the complete protein molecule (Brendts, 1967).

During denaturation, the forces stabilising the native protein structure are disrupted, particularly the hydrogen bonds and van der Waals' interactions, the protein structure collapses and a new unidentified random coil structure is obtained. In some cases, it may involve the disruption of disulphide bonds leading to complete unfolding of the polypeptide chain. Denaturation exposes the hydrophobic amino acid residues buried deep within the protein structure causing an increase in the reactivity of such groups. There is increased reactivity of Cys residues because they can undergo oxidation to disulphide (S-S) or cysteic acid ($-S_3OH$) groups; disulphide interchange may occur leading to the formation of intermolecular disulphide bonds (de Wit, 1984; Mulvihill & Donovan, 1987; Kella & Kinsella, 1988).

Denaturation is often reversible but in most cases it is irreversible because of the interplay of many factors, and subsequent reactions occurring after denaturation make it impossible for the proteins to return to their native state. The unfolded protein molecules may associate to form aggregates of irreversibly denatured molecules and such associations may finally lead to precipitation, coagulation and/or gelation. Oxidation of thiols and disulphides mainly results in irreversible aggregation (Donovan & Mulvihill, 1987*a, b*; de Wit, 1989).

Physical and chemical agents, such as heat, salts, pressure, organic solutes and solvents (ethanol, mercaptoethanol), extremes of pH (acids and alkali), chaotropic agents, urea, guanidinium chloride and sodium dodecyl sulphate (SDS) can induce denaturation (Donovan & Mulvihill, 1987*b*).

Manifestations of denaturation include loss of solubility and/or biological activity, changes in binding characteristics, alterations in spectral properties, a more negative optical rotation, a decrease in α-helix content, exposure of thiol groups and enhanced susceptibility to proteolytic digestion (de Wit & Swinkels, 1980; Park &

Lund, 1984; Relkin & Launay, 1990).

Denaturation of proteins may be assessed through measurements of loss of solubility, reactivity of the thiol groups, electrophoretic analysis, loss of antigenic activity and use of differential scanning calorimetry (DSC) (de Wit & Klarenbeek, 1981; Bernal & Jelen, 1985). These methods are based on different physical or chemical properties of the protein and so it is difficult to compare the denaturation results obtained.

2.7. Heat-induced denaturation of proteins

An aqueous environment is essential for maintaining the native structure of a protein. Heating globular proteins in an aqueous environment increases the thermal motion of the numerous structural elements in the polypeptide chain. These movements are all related to the energy supplied in the form of heat, leading to the rupture of intermolecular bonds, particularly the hydrogen bonds, van der Waals interactions and disulphide bonds.

Heat-induced unfolding of globular proteins is accompanied by an endothermal heat effect. Calorimetry can effectively be used to monitor the extent of heat denaturation as it is sensitive to the changes in enthalpy associated with denaturation (de Wit, 1981, 1984). The main contributors to the heat effect are the rupture of intramolecular hydrogen bonds and van der Waals interactions, the formation of bonds between water and the exposure of certain amino acid residues on the protein. At high protein concentrations, aggregation of the unfolded protein molecules may proceed immediately. In contrast to unfolding, aggregation is generally an exothermic process.

Sulphydryl and sulphydryl-disulphide interchange reactions and molecular collision frequency, due to increased kinetic energy, are enhanced at higher temperatures. The rate of protein denaturation increases 20-30 times for every 10 °C rise in temperature.

2.7.1. Heat-induced denaturation of whey proteins

Because of the heterogeneity of the whey protein system and the individual proteins exhibiting different responses towards heat, the thermal denaturation of total whey

protein reflects the collective response of the component proteins (de Wit & Klarenbeek, 1984). The thermal behaviour of the purified individual proteins cannot be directly compared with their behaviour in heterogeneous systems, such as in milk and whey systems. For example, the resistance of whey proteins to thermal denaturation in individual protein systems (8-10% concentration, 0.7 M phosphate buffer, pH 6.0) follows the order: β-lactoglobulin > Ig > BSA > α-lactalbumin (de Wit & Klarenbeek, 1984). However, the resistance of the same proteins to thermal denaturation in milk (Larson & Rolleri, 1955; Dannenberg & Kessler, 1988*a,b*; Singh & Creamer, 1991*a*) and rennet whey (Donovan & Mulvihill, 1987*a,b*) follows the order: α-lactalbumin > β-lactoglobulin > BSA > Ig. The major whey proteins have different thermal transition temperatures between 60 and 100 °C (Table 2.4).

Table 2.4. Thermal denaturation temperatures and enthalpies of whey proteins. Adapted from de Wit (1984) and Kinsella & Whitehead (1989).

Protein	Td (°C)	Ttr (°C)	ΔH (kJ/mol)
β-Lactoglobulin	78	83	311
α-Lactalbumin	62	68	253
BSA	64	70	803
Ig	72	89	500

Td is the initial denaturation temperature; Ttr is the temperature at the DSC peak maximum; ΔH is the enthalpy of denaturation.

There is no clear agreement on which of the two main genetic variants of β-lactoglobulin is the most heat labile. Dannenberg & Kessler (1988) showed that, in skim milk heated in the range 70-150 °C, β-lactoglobulin B is less stable than β-lactoglobulin A. This is in agreement with the studies of Gough & Jenness (1962) and Pernell-Clunies *et al.* (1988) in skim milk and whole milk, respectively. In buffered solutions, β-lactoglobulin B is also less stable (Sawyer, 1968; McKenzie *et al.*, 1971). This is not the case in cheese whey, where β-lactoglobulin A is less stable than

β-lactoglobulin B at temperatures above 90 °C (Hillier *et al.*, 1979). Using DSC, Imafidon *et al.* (1991*a,b*) observed that 10% solutions (simulated milk ultrafiltrate (SMUF)) of β-lactoglobulin A were more heat sensitive than those of β-lactoglobulin B. The protein concentration could be important in determining the relative stability of the variants, as McSwiney *et al.* (1994*a, b*) reported a 10% solution of β-lactoglobulin B to be more heat stable than β-lactoglobulin A (in 20 mM imidazole buffer, 0.1 M NaCl, pH 7.0, heated at 75-85 °C). Varunsatian *et al.* (1983) found that, in a 1% solution of WPC, β-lactoglobulin A was more heat sensitive than β-lactoglobulin B. The differences in the reported heat stabilities of the β-lactoglobulin variants could be attributed to a wide range of heating media and the techniques used.

α-Lactalbumin has been noted as the most heat stable of the whey proteins (Larson & Rolleri, 1955; de Wit, 1981; Lyster, 1970) mainly because of its ability to revert to its native state following heat treatment (Wont *et al.*, 1988). Ruegg *et al.* (1977) observed renaturation levels of 90% in systems containing only α-lactalbumin. Work using DSC by several workers (Bernal & Jelen, 1984; de Wit & Klarenbeek, 1984; Dannenberg & Kessler, 1988*b*) showed that α-lactalbumin has the lowest denaturation temperature but requires a large amount of heat for unfolding. DSC studies also show that the denaturation of α-lactalbumin is irreversible in complex whey systems and when heated in the presence of calcium chelators, such as EDTA (Bernal & Jelen, 1984).

BSA has an irreversible thermal transition with the free thiol group being responsible. The rate of denaturation of BSA has been reported to follow either first- or second-order kinetics because of the complexity of the reactions (Donovan & Mulvihill, 1987*a,b*). Fatty acids stabilise BSA against heat denaturation (de Wit, 1989).

2.7.2. Kinetics of whey protein denaturation

Studies on denaturation kinetics show a wide variation in both the reaction orders and the kinetic constants. A broad range of media and a variety of analytical techniques, including solubility at pH 4.6, quantitative electrophoresis, DSC and HPLC, have been used in different studies. The denaturation kinetics of β-lactoglobulin and α-lactalbumin have been extensively researched, whereas Ig and BSA have received only minor

attention. The denaturation kinetics of β-lactoglobulin have been studied extensively in skim milk (Lyster, 1970; Hillier & Lyster, 1979; Manji & Kakuda, 1986; Dannenberg & Kessler, 1988*a*; Dalgleish, 1990), cheese whey (Hillier & Lyster, 1979), SMUF (Park & Lund, 1984), buffered solutions (Gough & Jenness, 1962; Harwalkar, 1980*a,b*) and distilled water (de Wit & Swinkels, 1980; Relkin & Launay, 1990). Just as the denaturation of proteins is affected by many factors, the reaction kinetics are also affected by the same factors.

There is no clear agreement on the reaction order for β-lactoglobulin denaturation in skim milk. Reaction orders of 1.5 (Dannenberg & Kessler, 1988), pseudo first (Dalgleish, 1990) and second have been obtained (Hillier & Lyster, 1979; Manji & Kukuda, 1986). In other media, reaction orders also vary. For example, the reaction order of β-lactoglobulin dissolved in SMUF is 2 (Park & Lund, 1984). In distilled water, pseudo first-order (Harwalkar, 1980*b*), first-order (de Wit & Swinkels, 1980) and second-order (Relkin & Launay, 1990) have all been found. There is greater agreement on the denaturation of α-lactalbumin, which has been found to follow first-order kinetics in skim milk (Hillier & Lyster, 1979; Manji & Kukuda, 1986; Dannenberg & Kessler, 1988) and cheese whey (Baer *et al.*, 1976). Of the other whey proteins, the denaturation of Ig follows a second-order reaction in whole milk (Resmini *et al.*, 1989), but the denaturation of BSA could not be described by either simple first or second-order kinetics (Hillier & Lyster, 1979).

Protein concentration is important in determining the reaction order. In distilled water (Harwalkar, 1980) and phosphate buffer (Gough & Jenness, 1962; de Wit & Swinkels, 1980) at concentrations below 1%, the order is 1 for the denaturation of β-lactoglobulin. At 3.5% β-lactoglobulin, the denaturation in distilled water follows a second-order reaction but further increases up to 24% have no effect on the order (Relkin & Launay, 1990). Park & Lund (1984) also found a reaction order of 2 for 10% β-lactoglobulin in phosphate buffer. β-Lactoglobulin denaturation can be described as second-order in the pH range from 6.0 to 9.0, and as third-order below pH 5.0 (Park & Lund, 1984). Dannenberg & Kessler (1988*b*) found that the ratio of whey protein to casein affects the reaction order. Adjusting the casein/whey protein ratio from 0/100 (whey protein solution) to 83/17 (skim milk) decreases the order from 2.0 to 1.5.

From the varied reaction orders of β-lactoglobulin, it is apparent that the mechanism of denaturation is complex. The reaction order and the denaturation mechanism in distilled water and phosphate buffer systems may be significantly different from those in milk. Harwalkar (1980*a,b*) and Sawyer *et al.* (1971) have suggested that denaturation in distilled water and phosphate media is pseudo first-order involving two consecutive first-order reactions. From the evidence of Arrhenius plots, it seems more plausible to treat denaturation as two consecutive reactions (Hillier & Lyster, 1979). It should be noted that the kinetic equations are only a 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.

2.8. Factors affecting the denaturation of whey proteins

2.8.1. Effect of temperature

Heat-induced denaturation of whey proteins as analysed by reverse-phase HPLC indicates that denaturation proceeds in two stages (Parris *et al.,* 1991). Between 60 and 70 °C, denaturation of whey proteins increases slowly but progresses rapidly between 80 and 90 °C. Reversible changes in the whey protein structure occur mainly at temperatures up to 60 °C as these reactions are governed mainly by hydrophobic association which is enhanced as the temperature increases up to 60 °C (Scheraga *et al.,* 1962; de Wit & Klarenbeek, 1984) and above this temperature the changes become irreversible. No denaturation effects are observed for β-lactoglobulin between 50 and 65 °C. Secondary transition temperatures are observed above 100 °C for β-lactoglobulin and BSA (de Wit & Klarenbeek, 1981); these were attributed to the unfolding of residual structures that are partially stabilised at 80 °C.

2.8.2. Effect of pH

Individual proteins exhibit different responses to pH, probably due to different content and distributions of polar residues (Donovan & Mulvihill, 1987*a*). β-Lactoglobulin has been shown to be least stable, whereas BSA is most stable at pH 6.7. α-Lactalbumin and BSA were shown to be unfolded at pH 3.0 prior to heat treatment (de Wit &

Klarenbeek, 1984). Donovan & Mulvihill (1987*b*) showed that the denaturation of α-lactalbumin was relatively independent of pH in the range 4.5-7.0. However, Hillier *et al.* (1979) reported that α-lactalbumin heated at pH 4.0 had a slower rate of denaturation than at pH 6.0. Denaturation of β-lactoglobulin decreases with a decrease in pH from 7.0 to 4.5 (Donovan & Mulvihill, 1987*b*). The relatively high net negative charge of β-lactoglobulin at neutral pH results in strong intramolecular repulsive forces, thus facilitating opening up and denaturation of the β-lactoglobulin molecule.

Increasing the pH from 6.4 to 7.3 had negligible effects on the enthalpy of denaturation (ΔH) of β-lactoglobulin, whereas the denaturation temperature decreased from 79 to 74 °C, with the rate of denaturation increasing slightly (Relkin & Launay, 1990). Harwalkar (1979) reported that increasing the pH from 4.5 to 6.5 at zero ionic strength decreased the ΔH for β-lactoglobulin from 0.85 to 0.60 J/g protein and the denaturation temperature from 83 to 77 °C. Hegg (1980) demonstrated that the denaturation temperature of β-lactoglobulin increased from 78 to 83 °C between pH 2 and pH 4 and then progressively decreased with increasing pH, being approximately 78 °C at pH 6, 67 °C at pH 7 and 60 °C at pH 8. Increasing the pH from 6 to 8, caused a decrease in the enthalpy of denaturation from 0.83 to 0.23 J/g. The discrepancies in the published data reflect differences in protein preparations and the conditions under which tests were carried out (Hegg, 1980; de Wit, 1984; Bernal & Jelen, 1985; Kinsella & Whitehead, 1989).

2.8.3. *Effect of concentration*

Hillier *et al.* (1979) showed that increasing the concentration of cheese whey up to three-fold delayed the denaturation of β-lactoglobulin but hastened the denaturation of α-lactalbumin during heating at 80 °C. The effect of increasing levels of non-protein constituents (e.g. lactose) also delayed the thermal denaturation of both β-lactoglobulin and α-lactalbumin (Hillier & Lyster, 1979; Bernal & Jelen, 1985).

Kessler & Beyer (1991) showed that the reaction orders of the denaturation of whey proteins were constant over a wide range of concentration. The denaturation of β-lactoglobulin from WPI solutions ranging in concentration from 1 to 14 times the whey protein content of milk or cheese whey, during heating at 90 °C, could be

described as a second-order reaction. At lower initial whey protein concentrations (< 1 mg/l), the denaturation of β-lactoglobulin was found to be a higher-order reaction, suggesting that the mechanisms of the reactions following denaturation are influenced by the concentration of the proteins.

Qi *et al.* (1995) studied the denaturation of β-lactoglobulin using DSC at pH 6.75 and 8.05 in the concentration range 2-120 mg/ml. They reported that below 50 mg/ml the position of the maximum of the denaturation thermal peak becomes strongly dependent on concentration, passing through a minimum near 25 mg/ml and increasing towards the lowest concentrations at which measurements were practicable.

Earlier studies, in which milk protein was concentrated up to six-fold, showed that the level of denaturation of total whey protein increased with total whey protein concentration (Pierre *et al.*, 1977; McMahon & Yousif, 1993). Lyster (1970) found that doubling the concentration of β-lactoglobulin A in milk had no effect whereas doubling the concentration of β-lactoglobulin B gave an increased rate of denaturation of that protein. Increasing the concentration of β-lactoglobulin also gave a slight increase in the rate of denaturation of α-lactalbumin.

2.8.4. Effect of salts and ionic environments

The milk salts have a significant effect on the heat-induced denaturation and aggregation of whey proteins. The presence of salt seems to stabilise the protein quaternary structure against dissociation and denaturation. The effect of salt on the denaturation temperature of whey proteins is pronounced outside the isoelectric point. The effects of salts on protein structure involve two mechanisms (Kuhn & Foegeding, 1990; Xiong, 1992; Tang *et al.*, 1995):

(a) the electrostatic shielding effect, which is usually achieved at or below 0.2 ionic strength, is dependent only on the ionic strength of the medium.

(b) salts exert an ion-specific effect on hydrophobic interactions thus affecting the stability of the proteins at higher concentration through modification of the water structure which causes perturbations at the protein-water interface.

Varunsatian *et al.* (1983) showed that the effects of Ca, Mg and Na were to promote the denaturation and aggregation of whey proteins, but only on the alkaline

side of the isoelectric point (> pH 5.5). The effect of Ca was greater than that of Mg but both divalent cations affected protein denaturation and aggregation to a much greater extent than Na. It was suggested that Ca and Mg bind specifically to the heat denatured proteins to form aggregates. The effect of Na may be to mask some of the exposed ionic groups, thus altering the electrical double layer and facilitating protein-protein interactions (Xiong *et al.*, 1993). The DSC transition peaks of β-lactoglobulin were reported to be broader in $CaCl_2$ than in NaCl, suggesting that the binding of Ca to different structural domains of β-lactoglobulin differs considerably, whereas Na interacts with all parts of β-lactoglobulin similarly so that simultaneous unfolding occurs (Xiong *et al.*, 1993). Parris *et al.* (1993) showed that the extent of "soluble" aggregate formation (non-sedimentable protein material at 12,000 × *g* for 30 min at 4 °C) increased, with a concomitant decrease in large "insoluble" aggregates, as the Ca concentration of sweet whey was reduced. This indicated that Ca is involved in the formation of large "insoluble" aggregates.

Removal of Ca by EDTA decreased the denaturation temperature of α-lactalbumin by 20 °C, suggesting that binding of α-lactalbumin to Ca (metalloprotein) stabilises its tertiary structure (Shimada & Matsushita, 1981).

2.8.5. Effect of other constituents

Several studies have shown that the presence of other constituents, such as lactose, casein fines and fat, affects the denaturation of whey proteins. Lactose has a protective effect on the thermal denaturation of whey proteins (Hillier *et al.*, 1979; de Wit, 1981; de Wit & Klarenbeek, 1981). The denaturation temperature, measured by DSC, increases in the presence of lactose, with a further slight increase obtained when this sugar is replaced by glucose or galactose (Bernal & Jelen, 1985); it was suggested that lactose maintains or increases the hydration of the protein molecule, thus contributing to its stability. Pappas (1992) showed that addition of lactose to a β-lactoglobulin/casein mixture (dispersed in 30 mM sodium barbitol buffer, pH 7.0) reduced the calcium binding ability of proteins; hence the number of intermolecular electrostatic linkages that could be formed by calcium was reduced.

Pierre *et al.* (1977) showed that the increase in concentration of lactose, which occurs on evaporation but not during UF, reduces the rate of denaturation of total whey protein. In studies where both protein and non-protein material were concentrated by evaporation, the level of denaturation of total whey protein actually decreased with increasing total solids (McKenna & O'Sullivan, 1971).

The presence of casein enhances the denaturation of β-lactoglobulin (Kessler & Beyer, 1991). Fatty acid appears to stabilise the structure of BSA. Removal of naturally bound fatty acids from BSA, without altering the native structure, decreases the denaturation temperature by 7-12 °C in DSC studies (Bernal & Jelen, 1985).

2.9. Heat-induced gelation of whey proteins

The ability of whey proteins to form heat-induced gels and provide structural matrices for holding water, flavours, sugar and food ingredients is useful in food applications and new product development (Kinsella, 1976). Gel formation occurs when the denatured proteins aggregate in ordered, three-dimensional structures that trap the water in the system (Ferry, 1948). As the denaturation of the proteins is affected by many factors, the properties of whey protein gels are also affected by factors such as type of protein, protein concentration, temperature, pH, ionic strength and the presence of other ingredients such as lactose (Kinsella, 1976; Molder, 1985). The structure of protein gels can vary widely depending on these conditions, and has an impact on gel properties, such as rheological properties, sensory qualities and water-holding capacity. Generally, whey protein can form two types of heat-induced gel: fine-stranded gels, consisting of fine filaments, are formed when repulsion is large; particulated gels, consisting of large protein aggregates, are formed when the isoelectric point is approached (Clark *et al.*, 1981; Stading *et al.*, 1993). The kind of network formed is associated with changes in the balance between the attractive and repulsive forces between the aggregating particles (Clark & Ross-Murphy, 1987).

A wide range of techniques is used in studying gels. The gel point (which can be defined as the point on a temperature scale where the sol-gel transition in a polymer solution occurs) is usually determined using rheological measurement (Tang *et al.*, 1993, 1994). Rheological measurement is useful in gelation studies because it measures

various viscoelastic properties which are related to different gel properties including storage modulus (G′), loss modulus (G″) and phase angle (tan δ) (Tang *et al.*, 1993). Futhermore, this technique can measure the development of these properties during the heating of a protein system. Gel strength is usually measured by compression techniques, in which the force required to compress a well-defined size piece of gel is used as a measure of gel strength.

2.9.1. Mechanism of gel formation

Early investigators proposed a two-stage process for the gelation of globular proteins: the unfolding of globular protein molecules and the association of the unfolded (denatured) protein molecules to form a three-dimensional network (Ferry, 1948; Hermansson, 1979; Schmidt, 1981).

$$xP_N \rightarrow xP_D \qquad (1)$$

$$xP_D \rightarrow x(P_D)_x \qquad (2)$$

where x = number of protein molecules, P_N = native protein molecules, P_D = denatured protein molecules.

As mentioned earlier, heating a protein in solution weakens the bonds that maintain the secondary and tertiary structure, resulting in unfolding of the molecules and exposing reactive sites. Because reactive sites are exposed, the unfolded protein molecules interact by forming disulphide bonds and hydrophobic/ionic association takes place, immobilising large amounts of water, and a three-dimensional gel is formed.

Based on recent studies, a four-step sequence of events has been proposed in the heat-induced gelation of proteins (Schmidt, 1981; Aguilera, 1995):

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

Globular proteins taking a single conformation in their native state (N) are

transformed to the unfolded (U) or denatured state (D) under certain conditions (e.g. heating). Interactions of exposed hydrophobic regions are responsible for the formation of aggregates (A). These "soluble" aggregates are the basic building blocks leading to strand(s) or pro-gels, and ultimately gels (G).

Denaturation (unfolding) of native proteins

Denaturation of whey proteins usually occurs in the range 60-80 °C (Myers, 1990). The thermally induced gels from globular proteins have been shown to require a certain degree of unfolding of the protein as a first step (Clark & Ross-Murphy, 1987) but not total unfolding, which rarely occurs below 100 °C. As the unfolding proceeds, the amount of water bound to the protein increases (Mangino, 1992).

Recent studies using reverse phase HPLC suggest that unfolding of whey proteins by heat begins at 40 °C and proceeds slowly to achieve 10% denaturation at 62 °C. After renaturation to almost the initial state at 65 °C, the percentage denaturation follows a linear relationship with temperature and is 95% complete at 85 °C (Parris & Baginski, 1991). As opening of the molecular structure exposes a large number of reactive sites for intermolecular interactions, gelation of whey proteins is normally observed above 70 °C (Aguilera, 1995).

Molten globule state

Hirose (1993) recently postulated that the initial steps in gelation may involve a conformational intermediate state referred to as the "molten globule state". This molecular state can be viewed as not native but not fully denatured (unfolded). The protein molecules are partially unfolded, retaining the secondary structure, whereas the tertiary structure is slightly expanded with increased exposure of hydrophobic groups and enhancement in dynamic accessibility of the peptide N-H proton (Ptitsyn, 1992; Hirose, 1993). The exposed reactive sites have been shown to be responsible for the initial interactions that lead to aggregation in heated protein solutions (Doi, 1993; Hirose, 1993; Aguilera, 1995).

Aggregation of unfolded molecules

Aggregation of denatured or partially denatured globular proteins in solution has been recognised as an integral part of the heat-induced gelation process, although the molecular mechanisms responsible for the formation of aggregates during and after protein unfolding are not well known. Aggregation is considered to proceed through a sequence of unimolecular unfolding reactions and bimolecular association steps to yield higher-order polymeric structures (Aguilera, 1995). It is generally assumed that the driving forces for aggregations are non-specific interactions between the hydrophobic regions of unfolded polypeptide chains, but hydrogen bonding and ionic interactions are likely to participate as well (Clark & Ross-Murphy, 1987). Stabilisation of soluble aggregates may even proceed through disulphide crosslinking between polypeptide chains (Hillier *et al.*, 1980).

Strand formation of aggregates

The most outstanding microstructural feature of aggregate gels made from whey proteins is the presence of a homogeneous network of connected protein particles or aggregates forming a three-dimensional matrix and interstices filled by a liquid or aqueous solution (Aguilera, 1995). Tombs (1974) postulated two models for the formation of globular protein gels from aggregates: random clustering, usually referred to as "particulated" gels; string of beads, usually referred to as "fine stranded" gels. β-Lactoglobulin, BSA and whey protein products are known to form either type of gel, depending on pH and ionic strength (Stading & Hermansson, 1991). The network of particulated gels is composed of spherical particles linked together, forming the strands of the network (Stading *et al.*, 1993). β-Lactoglobulin gels formed at a fast heating rate (5-12 °C/min) showed strands of spherical particles of uniform size linked as a "string of beads" in a chain of single particles. Those formed at slower heating rates resulted in thicker strands of large particles, with a broad particle size distribution, fused together at many points (Stading *et al.*, 1993).

Several stabilising forces affecting gelation have been suggested at the molecular level: electrostatic forces, covalent bonds and hydrophobic interactions (Mangino, 1992*b*). Although it is not clear whether they stabilise the aggregates

themselves or the strand of aggregates, it appears that the primary overall stabilising forces are hydrophobic interactions (Clark *et al.*, 1981) and possibly ionic interactions (Konhorst & Mangino, 1985).

Association of strands and network formation

Protein gels are usually divided into physical gels and entangled networks. The former are strong gels, whereas the latter are formed by the topological entanglement of chains and are viscoelastic liquids without an equilibrium modulus (Ross-Murphy, 1992). Whey protein gels appear to be physical gels of infinite molecular weight (Aguilera, 1995).

2.9.2. Factors affecting the properties of whey protein gels

Gel properties are determined by the type of forces that hold the components together (Howell, 1978; Mangino, 1992*a*). If the network is too weak, the viscosity of the system will increase, but fluid flow will be possible and a gel will not form. If, on the other hand, the protein-protein interactions are too strong, the network will collapse and water will be expelled from the structure. A balance between the attractive forces necessary to form a network and the repulsive forces necessary to prevent its collapsing is required for gel formation (Mangino, 1992*a, b*).

Electrostatic interactions contribute to protein-protein association in most biological systems, and they can be altered by changes in pH (Xiong, 1992). The effect of pH on the physical appearance and rheological properties of WPC gels has been discussed in some detail by Stading & Hermannson (1991). The variations in gel properties with pH are attributed to variation in electrostatic interactions and disulphide bonding. At certain pH values, there is an optimum balance between protein-protein and protein-solvent interactions, resulting in maximum gel stiffness.

In a DSC study of the thermal aggregation of 10% WPI solutions (Xiong, 1992), it was observed that, at pH 5.5, major aggregation occurred after heating at ~ 71 °C. This was attributed to the interactions facilitated by the near isoelectric point pH values (4.8-5.3) of β-lactoglobulin and α-lactalbumin (Kinsella & Whitehead, 1989). It was suggested that, at pH 5.5, both proteins may substantially reduce their net

charges, thereby facilitating intermolecular interactions via hydrophobic and van der Waals' association. At pH 6.0, two transitions were observed, probably derived from α-lactalbumin and β-lactoglobulin. At pH 6.5 and above, the transitions were completely inhibited (Xiong, 1992). Hermansson (1979) observed no aggregation of a WPC suspension (9 mg/l) in distilled water at pH ≥ 8.0.

It appears that the mechanism of protein-protein interaction is different below and above pH 6.5. Zirbel & Kinsella (1988) reported that whey protein gels formed below pH 6.5 were opaque, whereas gels prepared above pH 6.5 were translucent. This difference has been inferred from the increased intermolecular disulphide bonds formed at increased pH (Hillier *et al.*, 1980). Between pH 6.0 and pH 10.0, whey protein gel strength was inversely related to the pH (Hillier *et al.*, 1980; Zirbel & Kinsella, 1988; Xiong & Kinsella, 1990; Xiong, 1992). Tang *et al.* (1993, 1994) reported that in moving from extreme pH values towards isoelectric points, heat-induced WPC gels encountered maximum G′ values. Englandsdal (1980) and Stading & Hermansson (1991) reported similar results. At these pHs, a balance between protein-protein interactions and the gel matrix structure was achieved. Excessive protein-protein interactions led to a geometric structure that was weak in some mechanical or rheological properties including gel stiffness, G′. However, weak protein-protein interactions also resulted in low gel stiffness. The maximum in G′ was thus governed by the balance between these two factors and would occur somewhere between weak and excessive protein-protein interactions. Therefore, between the pHs where the maxima occur, excessive protein-protein interactions lead to lower gel stiffness; outside this pH region, weak protein-protein interactions also lead to lower stiffness. At the pHs where the maxima occurred, an optimal balance between the extent of protein-protein interactions and the gel geometric structure led to a maximum in gel stiffness (Tang, 1993; Tang *et al.*, 1993., 1994).

It is well established that salts have a major effect on the properties of whey protein gels. Increasing levels of either NaCl or $CaCl_2$ increase gel strength, shear stress and other rheological properties until maximum values are reached, and then decrease with higher salt concentrations (Schmidt *et al.*, 1978, 1979; Mulvihill & Kinsella, 1988; Kuhn & Foegeding, 1991). Tang *et al.* (1994) also observed a similar effect of KCl on

the storage modulus, G′, of heated WPC solutions. A maximum in a gel property has generally been attributed to an optimal balance between protein-protein and protein-solvent interactions at a particular salt concentration. Divalent cations have a much greater effect on gel properties than monovalent cations (Tang *et al.*, 1993, 1994). Kuhn & Foegeding (1991*a,b*) demonstrated that a range of divalent cations (Ca, Mg, Ba) caused similar increases in shear stress and shear strain at failure of WPI gels and that the increase in shear stress at failure was much larger than that caused by a range of monovalent cations (Na, Li, K, Rb, Cs).

Xiong (1992) observed that a combination of 0.6 M NaCl and 50 mM sodium phosphate had a complex effect on protein-protein aggregation. At pH 6.0, WPI dispersed in 50 mM sodium phosphate showed a single and appreciable transition (77 °C), which did not recur when 0.6 M NaCl was also present. It was suggested that protein-protein aggregation at pH 6.0 was most sensitive to sodium phosphate in a low ionic strength environment and/or that at pH 6.0 NaCl had a dominant effect. It was also noted that at pH ≥ 7.0 protein aggregation in the presence of sodium phosphate was accelerated. This was attributed to phosphate being more ionisable at pH ≥ 7.0, resulting in increased charges, rendering a more favourable ionic environment for protein-protein association (Xiong, 1992; Tang *et al.*, 1995).

2.10. Heat-induced interactions of whey proteins

It is believed that a better understanding of the mechanisms of how the aggregates that precede gelation are formed can lead to better control of the production of WPCs with predictable gelling properties. To understand the heat-induced aggregation of the whey proteins in WPC systems, individual proteins have been studied separately by many workers. The heat-induced protein-protein interactions in model protein systems have been examined at low protein concentrations (for example, Griffin *et al.*, 1993; Hines & Foegeding, 1993), with some studies at higher concentrations (Matsuura & Manning, 1994; Hollar *et al.*, 1995; Qi *et al.*, 1995). The thiol-catalysed disulphide bond interchange reactions, which have been known for some time (McKenzie, 1971), are often invoked to explain many of the thermally induced whey protein interactions. McSwiney *et al.* (1994*a, b*) recently showed that, in addition to the disulphide bond

interchange reactions, stable non-covalently bonded aggregates appeared to be formed when 10% (w/v) solutions of β-lactoglobulin were heated.

2.10.1. Heat-induced interactions of β-lactoglobulin

Because β-lactoglobulin is the most abundant whey protein, it dominates the heat-induced behaviour of whey protein products, such as WPCs. Most of the research work, therefore, on the functionality of whey proteins has been carried out on β-lactoglobulin in model systems.

The heat-induced unfolding of β-lactoglobulin at low (< ~3) pH is essentially reversible (Tanford & De, 1961; Pace & Tanford, 1968; Gimel *et al.*, 1994) whereas, at high pH, irreversible reactions involving thiol-catalysed disulphide bond interchange lead to aggregation (Sawyer, 1968; McKenzie, 1971; McKenzie *et al.*, 1972; Ralston, 1973; Creamer, 1995). Electrophoresis results (Sawyer, 1968) showed that heating β-lactoglobulin A for 120 min at 75 and 97.5 °C led to different reaction products, confirming an earlier finding (Briggs & Hull, 1945). Griffin *et al.* (1993) found that, when β-lactoglobulin A was heated for 4 min at various temperatures, there appeared to be a maximum aggregate size when the solution was heated at ~ 85 °C, supporting the notion that there were two different reaction mechanisms with different temperature dependences. Considerable emphasis has been placed on the importance of Cys ^{121} in the aggregation reactions and blocking the thiol group gave a protein derivative that would not aggregate via disulphide interchange reactions (Sawyer, 1968; Ralston, 1973). These results were recently confirmed by Cairoli *et al.* (1994) and Iametti *et al.* (1995) who measured the concentrations of various polymer products by size-exclusion HPLC and SDS-PAGE.

As pointed out earlier, the early work on serum albumin gelation was interpreted (Ferry, 1948) in terms of a simple two-step reaction with native protein unfolding and the unfolded, or denatured, protein then aggregating to form macromolecular particles or gel strands. McKenzie (1971) concluded that β-lactoglobulin denaturation and aggregation at neutral pH involved several steps with a number of intermediate species and that the reactions included: dissociation of the native dimer to monomer, monomer conformational changes, disulphide interchange to form aggregates, oxidation of thiols

to disulphides, and non-covalent aggregations. A number of features of the heat-induced aggregation reaction at neutral pH have been examined further (Griffin *et al.*, 1993; Qi *et al.*, 1995) using light scattering techniques, calorimetry, circular dichroism and infrared spectroscopy. These authors explained their results in terms of a hypothesis similar to that of McKenzie (1971) and included dimer to monomer dissociation, unfolding of the monomeric protein to expose the single thiol group, and a thiol-catalysed disulphide interchange reaction.

Roefs & de Kruif (1994) suggested that a polymer-forming chain reaction is consistent with their light scattering results. They proposed a kinetic model for the temperature-induced denaturation and aggregation of β-lactoglobulin that recognised an initiation, a propagation and a termination step by analogy with polymer radical chemistry. However, there is evidence (McSwiney *et al.*, 1994*a*) that heat-induced aggregates formed in 5-10% heated β-lactoglobulin solutions contain appreciable quantities of β-lactoglobulin that dissociates to monomeric β-lactoglobulin in SDS solution. These and other results from several groups (Cairoli *et al.*, 1994; Huang *et al.*, 1994*a, b*; McSwiney *et al.*, 1994*b*) have led to suggestions that β-lactoglobulin probably forms intermediates bearing some similarity to the "molten globule" state (Hirose, 1995; Ptitsyn, 1995*a, b*).

2.10.2. Heat-induced interactions of α-lactalbumin

When α-lactalbumin is heated by itself under mild conditions (~ 80 °C, pH ~ 6.7), it does not form aggregates (Paulsson *et al.*, 1986; Matsudomi *et al.*, 1992; 1993; Calvo *et al.*, 1993; Calvo & Leaver, 1993; Hines & Foegeding, 1993). The lack of aggregate formation is largely explained by the lack of a free thiol group in the α-lactalbumin molecular structure (Eigel *et al.*, 1984). However, it has been shown by DSC studies (Rüegg *et al.*, 1977) that α-lactalbumin undergoes a reversible transition at 64 °C. When α-lactalbumin is heated under more severe conditions (100 °C, for 10-30 min), disulphide-linked polymers as well as modified monomers are formed (Chaplin & Lyster, 1986). The latter are probably in the "molten globule state" (Kuwajima, 1989; Hirose, 1993). Schnack & Klostermeyer (1980) showed that significant loss of disulphide bonds occurs when heating α-lactalbumin under the same conditions as those

used by Chaplin & Lyster (1986). It was suggested that, as the disulphide bond is lost, a group similar to a thiol is simultaneously formed, which is probably responsible for the intermolecular disulphide interactions of α-lactalbumin. There is no report in the literature on non-covalent interactions of α-lactalbumin.

2.10.3. Heat-induced interactions of BSA

Thermal interactions of BSA have received little attention from food chemists. BSA is one of the most heat sensitive among the whey proteins, under a range of heating conditions at near neutral pHs (de Wit & Klarenbeek, 1984). A BSA concentration of 4% (w/v) was required for the formation of a self-supporting gel following heating at 80 °C for 30 min in 100 mM sodium phosphate buffer, pH 6.8 (Matsudomi *et al.*, 1993). Both β-lactoglobulin and α-lactalbumin cannot form self-supporting gels under these conditions of concentration and pH. This observation was supported by the results of Paulsson *et al.* (1986) and Gezimati (1995). A recent study (Gezimati, 1995) suggested that BSA undergoes thermal interactions in a similar fashion to that previously reported for β-lactoglobulin (McSwiney *et al.*, 1994*a, b*). That is, on the basis of native- and SDS-PAGE studies, BSA formed aggregates that were held together by hydrophobic interactions in addition to the well-documented sulphydryl-disulphide bond interchange reaction, except that BSA began to aggregate and gel at lower temperature (Gezimati, 1995). The similarity, however, should be expected on the basis that both BSA and β-lactoglobulin each contain a free thiol group, Cys^{34} for BSA (Carter & Ho, 1994) and Cys^{121} for β-lactoglobulin (Hambling *et al.*, 1992). It seems likely that the BSA aggregates observed by Gezimati (1995) were similar to those reported by Matsudomi *et al.* (1993) to occur in a solution of *N*-ethylmaleimide-treated BSA that had been heated.

2.10.4. Heat-induced interactions of β-lactoglobulin and α-lactalbumin

Besides self-aggregation, β-lactoglobulin can form heat-induced complexes with α-lactalbumin (Matsudomi *et al.*, 1992; Calvo *et al.*, 1993). The soluble complexes are formed mainly through thiol-disulphide interchange between β-lactoglobulin and α-lactalbumin (Matsudomi *et al.*, 1992; Calvo *et al.*, 1993). Denaturation

of α-lactalbumin is affected by the presence of β-lactoglobulin. The faster loss of α-lactalbumin when heated in the presence of β-lactoglobulin, compared with when it is heated alone, has been demonstrated using size exclusion chromatography (Elfagm & Wheelock, 1978; Hines & Foegeding, 1993; Calvo *et al.*, 1993), DSC (Paulsson & Dejmek, 1990) and gel filtration and HPLC (Matsudomi *et al.*, 1992). In contrast, loss of β-lactoglobulin was not affected by α-lactalbumin (Hines & Foegeding, 1993). Elfagm & Wheelock (1978) reported similar results at concentrations of 0.2% (w/v) β-lactoglobulin and 0.1% (w/v) α-lactalbumin. However, when there was a higher ratio of β-lactoglobulin to α-lactalbumin (3:1), α-lactalbumin had a marked effect in reducing the amount of aggregated β-lactoglobulin.

A few studies have shown that α-lactalbumin enhances β-lactoglobulin gel rigidity (Matsudomi *et al.*, 1992; 1993; Hines & Foegeding, 1993; Gezimati, 1995). These studies did not offer a clear explanation of the likely protein interactions that were involved in the enhancement of gel rigidity. Some of the suggestions (Gezimati, 1995) include the possibility that the aggregates formed by the interactions between β-lactoglobulin and α-lactalbumin are more extensively crosslinked by disulphide bonds than the aggregates formed by β-lactoglobulin-β-lactoglobulin interactions. In addition, there may be a greater number of disulphide bridges bonding the aggregates together in β-lactoglobulin and α-lactalbumin mixtures. It was also proposed that the gel network formed from β-lactoglobulin and α-lactalbumin consists of a single homogeneous network containing co-polymers of β-lactoglobulin and α-lactalbumin (Gezimati, 1995).

2.10.5. Heat-induced interactions of β-lactoglobulin and BSA

Hines & Foegeding (1993) found that the rate of loss of native β-lactoglobulin from solution at 80 °C was increased by the presence of BSA, indicating some synergistic effect. Matsudomi *et al.* (1994) found that, when mixtures of BSA and β-lactoglobulin were heated at 80 °C and examined at room temperature, the gels from the mixtures were stronger, again suggesting a synergistic effect. It would be expected that, when such a mixture is heated at lower temperatures (e.g. ≤ 70 °C), the number of β-lactoglobulin molecules that undergo the thermal transition would be less than the

number of BSA molecules; hence BSA will be the dominant species in the aggregates and the gel formed will be largely composed of BSA molecules. By contrast, when heating at high temperature (> 75 °C), each protein will form aggregates and the rates of aggregation of the two proteins will be more comparable. The interactions between the two proteins are, therefore, dependent on the rate of heating (Gezimati, 1995).

2.10.6. Heat-induced interactions of α-lactalbumin and BSA

The heat-induced interaction between α-lactalbumin and BSA was studied by Matsudomi *et al.* (1993). Their results showed that addition of ≥ 3% α-lactalbumin to 6% BSA caused a significant increase in gel hardness, following heating at 80 °C for 30 min in 100 mM sodium phosphate buffer, pH 6.8. BSA and α-lactalbumin interacted to form soluble aggregates through thiol-disulphide interchange reaction during gel formation. These heterogeneous aggregates had lower molecular weight than those formed by BSA alone. The enhancing effect of α-lactalbumin on the gel hardness of BSA was attributed to the formation of a finer, more uniform gel matrix. It was also reported that, during gel formation, BSA was involved in the formation of hydrophobically-associated aggregates which were dissociated under SDS-PAGE conditions, whereas there was no evidence of α-lactalbumin being involved (Matsudomi *et al.*, 1993).

2.10.7. Heat-induced interactions of β-lactoglobulin and α-lactalbumin and BSA

There are no published reports on thermal interactions in a mixture of β-lactoglobulin, α-lactalbumin and BSA. However, it would be expected that the interactions of these proteins in the different mixtures discussed above occur when the three proteins are heated together. Because of the different thermal transition temperatures of these proteins, it is likely that BSA would undergo aggregation to form the initial aggregates and that β-lactoglobulin and α-lactalbumin would form aggregates at some later stage. All kinds of homogeneous as well as heterogeneous aggregates would be expected to be formed between these proteins during heat treatment. It is not clear if there are specific preferential interactions among proteins; this area needs further investigation.

2.10.8. Heat-induced interactions of whey proteins in WPC solutions

The heat-induced interactions of whey proteins in WPC solutions have not been explored as yet. Most of the studies on WPCs deal with rheological properties of gels (e.g. Tang *et al.*, 1993, 1994). The purpose of model protein studies is to elucidate the likely mechanisms that may govern the interactions of whey proteins during heat treatment. However, it is difficult to directly relate these findings to those taking place in WPC systems. This is mainly because of the presence of many other proteins such as immunoglobulin, casein fines, lactose, fat and minerals, which have a significant effect on the interactions of these proteins. Further study is required to define the mechanisms of the interactions of these proteins during heat treatment in WPC systems.

2.11. Concluding remarks

Commercial WPCs are used in a wide range of applications in the food industry. The utilisation of WPCs, however, is limited because of significant variations in product quality and composition which also vary their functional properties. The variations of these products are due largely to variations in the composition of milk as well as the processing history. The utilisation of these products can be greatly enhanced if the variations in product quality and composition are eliminated. Extensive research has been carried out on the factors that influence the functional properties of WPCs. A considerable amount of information has been established on effects of factors such as pH, protein concentration, salts and ionic strength on thermal denaturation, aggregation and gelation of whey proteins. There is, however, very little understanding of how the various whey proteins interact during thermal treatment.

To understand the mechanisms of how the whey proteins interact during heat treatment, individual proteins have been studied extensively in single protein solutions or in different mixtures. Because of the wide range of solvent media and heating conditions under which these studies have been carried out, the results vary considerably. However, the results generally show that the interactions between the whey proteins are largely explained by the formation of disulphide bonds with some contributions by hydrophobic associations between denatured protein molecules. Understanding the mechanisms of how the whey proteins interact during thermal

treatments can lead to a better control of WPC manufacture and the production of WPC products with predictable functional properties.

2.12. The aim and objectives of the thesis

The aim of this work was to gain more insight into the heat-induced interactions of whey proteins in heated WPC solutions, in order to support utilisation of WPCs as food ingredients. This requires that the gap in knowledge between the molecular properties of individual proteins and the macroscopic properties of heat-induced WPC gels must be bridged. Most of the previous studies in this area have taken an approach where the interactions of the whey proteins are measured as changes in properties (e.g. gel strength) in response to some specific treatment (e.g. change in pH). This approach, however, does not provide much information about the mechanisms of how the whey proteins interact during heating. The current study was aimed at understanding the interaction of the whey proteins, at a molecular level, and then relating the findings to the macroscopic properties of the heated WPC systems.

The study was designed to meet the following objectives.

* To characterise the nature of the protein material in commercial WPC products.
* To understand the interactions of the major whey proteins β-lactoglobulin, α-lactalbumin and BSA during heat-induced gelation of WPC.
* To measure the degree of disulphide-linked and hydrophobically-associated aggregates formed during aggregation and gelation.
* To characterise the intermediate species that may be generated during heat-induced aggregation and gelation.
* To relate the aggregation behaviour of individual whey proteins to the gel properties of WPC systems.
* To report on the commercial implications of the findings.

CHAPTER 3
MATERIALS AND METHODS

3.1. Materials

Three commercial whey protein concentrate (WPC) powders were obtained from the New Zealand Dairy Board, Wellington, New Zealand. One was derived from cheese whey (ALACEN 392), one was derived from rennet whey (ALACEN 472) and one was derived from acid whey (ALACEN 342). β-Lactoglobulin, α-lactalbumin, bovine serum albumin (BSA) and the molecular weight standards were obtained from Sigma Chemical Co. (St. Louis, MO 63178, USA). The state of the proteins in these materials was assessed by native-PAGE and the most appropriate samples were selected for further examination. In all cases, there were small quantities of protein impurities which were usually other whey or blood proteins and polymers of β-lactoglobulin, α-lactalbumin or BSA. The chemicals used for preparation of electrophoresis buffers (obtained from Bio-Rad Laboratories, Hercules, CA 94547, USA) were of analytical grade. Water was purified using a MilliQ-system (Millipore Corp., Bedford, MA 01730, USA).

3.2. Composition analyses

The total protein content of the WPC powders was determined using the Kjeldahl method (AOAC, 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 phospholipid content was determined at the New Zealand Dairy Research Institute using the analytical method described by McDowell (1958). The moisture content was determined by oven-drying pre-weighed duplicate samples at 105 °C for 24 h, cooling in a desiccator for 2 h and then reweighing the samples. The GMP content was determined at the New Zealand Dairy Research Institute using cation-exchange chromatography as described by Léonil & Mollé (1991). 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).

The concentrations of β-lactoglobulin, α-lactalbumin and BSA were estimated using 1D-PAGE (Section 3.5.1). Solutions of pure proteins (β-lactoglobulin, α-lactalbumin or BSA) with various known concentrations were prepared, diluted with native or SDS (in the presence of 2-mercaptoethanol) sample buffer and then run on native or SDS gels. The variations of the band intensities with protein concentrations were used to produce standard curves for each of the whey proteins. These curves were then used to determine the concentration of each protein in the WPC powders.

3.3. Sample preparation

3.3.1. WPC solutions

WPC solutions (10, 30, 60 or 120 g/kg) were prepared by dissolving appropriate quantities of WPC powder in water followed by stirring for 2 h at room temperature (~ 20 °C) using a magnetic stirrer and adjusting the pH to 6.8 with 0.1 M NaOH or 0.1 M HCl.

3.3.2. Pure protein solutions

Pure protein solutions were prepared by dissolving appropriate amounts of protein powders in WPC permeate obtained from ultrafiltration of 100 g/kg WPC solutions. The WPC solutions were prepared by dissolving appropriate quantities of WPC powder in water, stirring for 2 h at room temperature using a magnetic stirrer, and adjusting the pH to pH 6.8 with 0.1 M NaOH or 0.1 M HCl. The solutions were then ultrafiltered using an ultrafiltration unit (KOH Membrane Systems, Inc. Wilmington, MA 01887, USA), equipped with a O-HF-131-VIV membrane; the permeate obtained was used to dissolve β-lactoglobulin, α-lactalbumin and BSA to give 100 g/kg protein solutions. Some of the experiments required lower protein concentrations (e.g. light scattering and some PAGE analyses). In these cases, the permeate was diluted appropriately so that the mineral contents simulated that of WPC solutions with the same protein concentrations. For example, when a 2% (w/w) α-lactalbumin solution was required, the permeate obtained from ultrafiltration of the 100 g/kg WPC solution was diluted five times.

Mixtures of β-lactoglobulin and α-lactalbumin or BSA at a ratio of 2:1 were prepared by dissolving 6.65 g of β-lactoglobulin and 3.35 g of α-lactalbumin or BSA in 90 g WPC permeate. Mixtures of β-lactoglobulin, α-lactalbumin and BSA at a ratio of 2:1:1 were prepared by dissolving 5.0 g of β-lactoglobulin, 2.5 g of α-lactalbumin and 2.5 g of BSA in 90 g WPC permeate. Mixtures of α-lactalbumin and BSA at a ratio of 1:1 were prepared by mixing 5 g of α-lactalbumin and 5 g of BSA in 90 g WPC permeate.

3.3.3. Dialysis of WPC solutions

Acid and cheese WPC solutions (120 g/kg, pH 6.8) were prepared. Aliquots (300 ml) of each solution were placed in dialysis tubing (molecular weight cut-off: 1 kDa), and then dialysed against 10 l of the other solution at ~ 4°C (i.e. 300 ml aliquots of acid WPC were placed in 10 l of cheese WPC and vice versa). An aliquot was removed after dialysing for various times (from 6 to 24 h), the mineral content was determined, and then aliquot was used for further analyses including: gel compression test, rheological measurement, size exclusion chromatography and microscopy.

3.4. Heat treatment of protein solutions

Aliquots (1.5 ml) of WPC solutions or pure protein solutions were transferred into a set of pre-weighed 2-ml round-bottomed Beckman polyallomer centrifuge tubes (0.35 mm wall thickness, 11 mm internal diameter, 34 mm height). The tubes containing the samples were then reweighed, closed with fitted lids, and then heated in a water bath thermostatically controlled at 75 or 80 °C for various times (from 30 s to 30 min; the time taken for the samples to attain 74.9 °C was 20 s). The tubes were removed and immediately placed in ice water for 5 min and then held at room temperature (~ 20 °C) for 2 h. The heated samples were used for PAGE analyses and ultracentrifugation experiments.

3.5. Ultracentrifugation of WPC solutions

In some cases, the heated WPC solutions (Section 3.4) were centrifuged at 90,000 × g for 1 h or at 45,000 × g for 2 h. The supernatants were carefully removed and the tubes

containing the pellets were turned upside down and allowed to stand on a filter paper for 20 min at room temperature to allow free flow of residual supernatant from the pellet. The pellets obtained were freeze-dried and the dry weights were determined. The supernatants were examined by PAGE (Chapter 4).

The pellets obtained from centrifugation of the unheated WPC solutions were analysed for protein composition, mineral content, and fat and phospholipid using the methods described in Section 3.2, and used for further experiments (Chapter 7).

3.6. Preparation of WPC gels for compression tests

Acid WPC and cheese WPC solutions (120 g/kg, pH 6.8), dialysed and non-dialysed, were placed in plastic tubes (20 mm diameter, 300 mm long) and the ends of the tubes were tightly tied using a rubber band. The tubes were then placed in a water bath that was thermostatically controlled at 80 °C. The tubes were removed after 1 h, placed immediately in ice water (~ 0 °C) for 30 min and left at room temperature (~ 20 °C) for 30 min before using for compression tests. The gels were cut into 20 mm cylinders, and then compressed vertically to a displacement of 10 mm, using an Instron (Model No. 4502, S. No. H3096). The force at the maximum displacement or the breaking point was recorded as a measure of gel strength for each sample.

3.7. Polyacrylamide gel electrophoresis (PAGE)

The protein samples were analyzed using the Mini-Protean II dual slab cell system (Bio-Rad Laboratories, Hercules, CA 94547, USA) discontinuous PAGE system for both the one-dimensional (1D) and two-dimensional (2D) separations.

3.7.1. One-dimensional PAGE (1D-PAGE)

One-dimensional native-PAGE

The native resolving gel was made from a mixture of 8 ml of a 30% stock solution of a 37.5:1 mixture of acrylamide and N,N'-methylene-*bis*-acrylamide (Bio-Rad Cat. No. 161-0158), 2.0 ml of resolving gel buffer (3.0 M Tris adjusted (at 20 °C) to a pH of 8.8) and 6.0 ml of water. This mixture was warmed to 20 °C, degassed by evacuation while stirring, and then kept cool. Just prior to pouring the gel, 80 μL of freshly prepared 10% (w/v) ammonium persulphate solution and 8 μL of N,N,N,'N'-tetramethylethylenediamine (TEMED) were added to the mixture. The gel-setting

gel-setting apparatus was assembled using 0.75 mm spacers and 3.3 ml of resolving gel solution was put between each pair of glass plates. About 0.5 ml of water was then placed above the resolving gel solution and the apparatus was set aside in a warm place (20-25 °C) for the gels to set; about 30 min. The water was then drained off with the aid of a paper wick. The stacking gel was made from a mixture of 1 ml of a 30% mixture of acrylamide and bis-acrylamide, 2.0 ml of stacking gel buffer (0.5 M Tris adjusted to pH 6.8) and 5 ml of water. This was degassed and 40 µl of 10% ammonium persulphate solution and 8 µl of TEMED were added. This mixture was pipetted into the gap between the glass plates and the slot-former was inserted, taking care that no air bubbles were entrained. Once the gel had set, the gel-formers and gels were often enclosed in a plastic bag which was put in the cold-room (6 °C) overnight. The electrode buffer stock solution was 0.125 M Tris, 0.95 M glycine and was adjusted to pH 8.3. In use, this solution was diluted 1:4 with water. The sample buffer comprised 20% stacking gel buffer, 0.01% bromophenol blue and 8% glycerol. The power supply was a Bio-Rad Model 1000/500 and the maximum voltage, current, power and time were set at 210 V, 70 mA, 6.5 W and 1.5 h for running two gels. Each gel was stained for 1 h in 50 ml of Coomassie dye solution (0.1% Coomassie blue R-250, 25% isopropanol, 10% acetic acid in water) in a closed 500 ml container with contiunous agitation. This was followed by two destaining steps of 1 and 19 h respectively, with 100 ml of a destaining solution (10%isopropanol, 10% acetic acid). The gels were then photographed using a 35 mm camera fitted with both green (XI) and orange (G) Hoya filters (to minimise the stray light) on to 100 ASA Kodak T-max film, which was then processed in the usual way. Band intensities were determined using an Ultrascan XL laser densitometer (LKB Produkter, Stockholm-Bromma 1, Sweden).

One-dimensional SDS-PAGE

The 1D SDS gels were prepared similarly using the recipes described by Bio-Rad in the literature supplied with the equipment and based on the descriptions of Laemmli (1970). The resolving gel buffer was 1.5 M Tris/HCl buffer, pH 8.8. The stacking gel buffer and stock acrylamide were the same as described for the native-PAGE. A stock solution of electrode buffer was prepared from 9 g of Tris-base, 43.2 g of glycine and 3 g of SDS, made up to 600 ml with deionised water, pH 8.3. The resolving gel was

prepared by adding 2.02 ml of deionised water to 2.5 M Tris/HCl, 5.3 ml of acrylamide stock and 100 μl of 10% (w/v) SDS stock. The resolving gel was degassed and 5 μl of TEMED and 50 μl of freshly made ammonium persulphate (10% w/w) were added and mixed gently. The resolving gel (3.6 ml) was poured between two glass plates and overlaid with deionised water. The resolving was then left at room temperature (~ 20 °C) for 45 min to set and the water was removed from the top of the resolving gel prior to application of the stacking gel. The stacking gel was prepared from 1.3 ml of acrylamide stock, 2.5 ml of stacking gel buffer, 100 μl of SDS stock and 6.1 ml of deionised water. After degassing, 10 μl of TEMED and 50 μl of ammonium persulphate were added and gently mixed. The stacking gel was poured on top on the resovling gel and a 10-slot comb was inserted. Before running the gel, the comb was removed and the wells, formed by the comb, were rinsed with deionised water to remove unpolymerised gel solution.

Two gels were placed into an electrode buffer chamber. The stock electrode buffer solution was diluted 1:4 with deionised water and used to fill the inner buffer chamber. A Hamilton syringe (Hamilton Company, Reno, Nevada, USA) was used to inject 10 μl of sample into a well. The gels were then run, using the same conditions as for the native gels, until the tracking dye (bromophenol blue) had run off the bottom of the resolving gel.

The staining, destaining and recording protocols followed the procedure described for the native gels. The protein bands on the resultant gels were identified by comparison with reported patterns (Basch *et al.*, 1985). The molecular weight standards were the following: phosphorylase (97,000), BSA (66,200), ovalbumin (45,000), carbonic anhydrase (31,000), soybean trypsin inhibitor (21,500) and lysozyme (14,400). They were not reduced for SDS-PAGE run without pretreatment of the samples with mercaptoethanol.

3.7.2. Reaction kinetic evaluation

The rates of the loss of β-lactoglobulin, α-lactalbumin and BSA at 75 °C, as measured by 1D-PAGE, were determined using the general reaction kinetics equations as described by Dannenberg & Kessler (1988). Each set of results was fitted to the

appropriately derived equation for n = 1, 1.5 or 2, and the reaction rate constant (k_n) was calculated using the Fig P statistical program (Biosoft, Ferguson, MO 63135, USA). The coefficients of correlation (r^2) were used to indicate how well each data set fitted the equations.

3.7.3. Two-dimensional PAGE (2D-PAGE)

2D-PAGE was used to identify the components in the protein aggregates in heated protein solutions. The heated protein solutions (plus control unheated samples) were diluted 1:30 with appropriate sample buffer prior to electrophoresis on alkaline or SDS gels, as described above. Each gel had quadruplicate samples of a heated protein solution.

After the first-dimension separation (described above), the sample wells were dried using strips of filter paper and filled with SDS stacking gel solution, and this was allowed to set (about 30 min). The gel was removed from between the glass plates and carefully cut so that each strip contained all the protein bands from each sample, including any material that did not migrate into the stacking gel. Two of the strips were stained to confirm that the separation in the "first dimension" was satisfactory. One of the unstained strips was placed in hot (94 °C) SDS sample buffer containing 0.5% of 2-mercaptoethanol for 30 s. The strip was then removed from the hot sample buffer and washed with a little water to remove excess 2-mercaptoethanol solution, and the surplus water was blotted from the strip surface with filter paper strips. The gel strip was placed on one of the glass plates and perpendicular to the spacers (0.75 mm), the second plate was placed over it and the two plates were assembled into the gel-setting equipment. SDS resolving gel solution (3.3 ml) was then carefully poured between the plates, leaving a space of about 12 mm between the top of the gel solution and the bottom of the gel strip. The gel solution was then overlaid with water in the usual fashion and the gel strip should be completely immersed in this water. This provided further washing off of excess 2-mercaptoethanol from the surface of the strip (the 2-mercaptoethanol would have inhibited the acrylamide polymerisation). After the resolving gel had set, the water was removed, the equipment was flushed with a little stacking gel solution and the space was filled with stacking gel solution, with the

equipment tilted at about 30°, so that no bubbles were trapped beneath the gel strip. A standard well-forming comb, which had most of the teeth removed, was then inserted so that the wells formed were level with the centre of the gel strip and at least 4 mm distant from the gel strip. After loading the appropriate chamber buffer and the control sample, the proteins were electrophoresed in the "second dimension". The resultant gel was then stained, destained and photographed as described above.

The WPC or pure protein solutions were diluted with the appropriate sample buffer (so that the final protein concentrations were 1.2 g/kg) and analysed using native-PAGE as described by Andrews (1983) and SDS-PAGE as described by Laemmli (1970) using Bio-Rad Mini Protean II equipment (Bio-Rad Laboratories, Richmond, CA 94804, USA).

After the gels had been stained, with Coomassie Blue, and destained, they were photographed using a 35 mm camera fitted with both a green (XI) and an orange (G) Hoya filter (to minimise the stray light) on to a 100ASA Kodak T-max film. The protein bands on the resultant gels were identified by concurrent electrophoresis of samples of BSA, α-lactalbumin, β-lactoglobulin A, β-lactoglobulin B and acid casein. Some of the other bands were identified by comparing the results with reported patterns (Basch *et al.*, 1985; N. Haggarty, 1996, pers. comm.).

The gels were scanned using an Ultrascan XL laser densitometer and the results were analysed using an LKB 2400 GelScanXL software program (LKB Produkter AB, Bromma, Sweden) to obtain quantitative results. The peak area of each protein band was reported as a % of the corresponding band in the unheated control WPC samples.

3.8. Oscillatory rheometry

The development of the WPC gel network during heating was followed using a Bohlin VOR rheometer (Bohlin Reologi AB, Lund, Sweden), operating in an oscillation mode as described by Tang *et al.* (1993) and Gezimati *et al.* (1996*a*). The protein solution (13 ml) was placed in a measuring system (C25) consisting of an oscillating cup (the outer cylinder, 22 mm diameter) and a fixed bob (the inner cylinder, 18 mm diameter), with sample contained in the annular gap between them. A torsion bar of 42.9×10^{-3} Nm was used. The solution was covered with a thin layer of parafin oil to prevent

evaporation. The frequency was set at 1 Hz, and the shear strain was at 0.0206 ± 0.0005 throughout the entire measurement range. The protein solutions were heated *in* situ from 25 to 80 °C at a rate of 2 °C/min, and held at 80 °C for 120 min. The viscoelastic properties of the gel, including the storage modulus (G′), loss modulus (G″) and the loss tangent (tan δ), were recorded every 4 min. During the measurement, the cup oscillates applying a sinusoidal deformation of defined amplitude (γ_0) and frequency (0.9 Hz for this particular test). The material applies a resistive torque which is measured by the measuring device, converted to shear stress, of amplitude τ_0, and recorded by the computer data station.

3.9. Size exclusion chromatography

The heated WPC solutions were diluted with 20 mM imidazole/HCl buffer (pH 6.8, 50 mM NaCl) to give a final protein concentration of 6 g/kg and then filtered using an Alltech cellulose acetate membrane 0.22 μm (Type HA) and a syringe filter. The samples (50 μl) were separated on a Superose 6HR 10/30 column (Pharmacia Biotech), using a GBC HPLC system (GBC Scientific Equipment Pty Ltd., Dandenong, Victoria 3175, Australia) equipped with a LI 1150 HPLC pump, a LI 1200 UV/Vis detector set at 280 nm and a LI 1440 system organiser.

The protein composition of each peak was determined by collecting peaks separately from the column outlet, freeze-drying and then analysing by native- and SDS-PAGE under non-reducing and reducing conditions.

3.10. Transmission electron microscopy

3.10.1. Sample fixation and staining

The WPC samples for transmission electron microscopy (TEM) were prepared as follows: 1.0 ml of WPC solution (120 g/kg) was fixed by adding 25% glutaraldehyde solution (0.2 ml), blending the mixture on a vortex mixer for 2-3 s, and allowing it to stand for 20 min at room temperature. The fixed sample was then mixed with an equal quantity of hot 3% agar solution, and the mixture was blended on a vortex mixer for 2-3 s. The mixture was spread on a microscopic slide, allowed to solidify and cut into 1 mm^3 blocks.

The sample blocks were transferred into Bijoux bottles and washed in a 0.2M sodium cacodylate-HC1 buffer (pH 7.2) for 30 min. The buffer was changed after 60 min, and then again after a further 60 min. The samples were then post-fixed in 1% (w/v) osmium tetroxide in cacodylate-HC1 buffer for 2 h, washed with distilled water, stained with 1% (w/v) aqueous uranyl acetate solution for 30 min and finally washed again with distilled water.

3.10.2. Sample dehydration and embedding

The stained blocks were dehydrated using a graded series (50, 70, 90%) of ethanol-water mixtures followed by absolute ethanol, and were then embedded in epoxy resin (Araldite CY212, Taab Laboratories, United Kingdom). The embedding procedure involved mixing of incomplete resin mixture (10 ml of dodecenyl succinic anhydride (DDSA) + 10 ml of epoxy resin + 1 ml of butyl phthalate) with the dehydrated blocks, placing them on rollers (speed 14 rev/min) for 6 h, then replacing the incomplete resin mixture with a complete resin mixture (incomplete resin mixture + 0.4 ml of benzyldimethylamine (BDMA)) and continuing on rollers for another 6 h in order to complete the infiltration of the resin into the blocks.

3.10.3. Sample molding and hardening

The infiltrated sample blocks were carefully placed in the plastic moulds and labelled. Freshly prepared complete resin mixture was then poured into the moulds. The moulds were placed in an oven, maintained at 60 °C for 24 h, for hardening.

3.10.4. Trimming and thin sectioning of sample blocks

The hardened blocks, after cooling to room temperature, were trimmed with a block trimmer (Reichert-Jung TM 60, C. Reichert Optische Werke AG, Wien, Austria) and carefully sectioned (70-90 nm thickness) with a diamond knife (DDK, Delaware Diamond Knives, Inc., Delaware, USA), using a ultramicrotome (Reichert-Jung Ultracut E, C. Reichert Optische Werke AG, Wien, Austria). The thin sections were then mounted on 300 mesh copper grids (Probing and Structure, Queensland, Australia) previously dipped in chloroform-cellotape mixture, stained with lead citrate for 2 min,

washed with distilled water and dried on a filter paper.

3.10.5. Viewing and development of electron micrographs

The sections were viewed under a transmission electron microscope (Philips EM 201C, The Netherlands) at an accelerating voltage of 60 kV at the Crown Research Institute (CRI), Palmerston North. The negatives were enlarged three times and the electron micrographs were developed at the CRI.

3.11. Confocal scanning laser microscopy (CSLM)

The confocal scanning laser microscope was operated in fluorescence mode as described by Lucey *et al.* (1996). The WPC solutions (120 g/kg, pH 6.8) were prepared and heated as described (Section 3.3.). The fluorescent protein dye Fast Green FCF (Merck, Darmstadt, Germany) was used to stain the protein matrix. A few grains (~ 5 mg) of Fast Green FCF were dissolved in 10 ml of water, and a few drops of this mixture were added to the heated WPC solutions, and then mixed gently before analysis. A drop of each solution was placed on a microscope slide, covered with a cover slip and then viewed under the confocal scanning laser microscope (Leica TCS 4D confocal microscope (Leica Lasertechnik GmbH, Heidelberg, Germany)) with a 100 × oil immersion objective (numerical aperture = 1.4). The microscope had an air-cooled Ar/Kr laser which was used with an excitation wavelength of 568 nm. Each sample was prepared in duplicate on two different occasions. Multiple fields were viewed and typical fields were acquired and stored as TIFF (format) files. The brightness/contrast of these files was adjusted using the Paint Shop ProTM Version 3.12 software package (Jasc Software, Inc., Eden Prairie, Minnesota, USA). Photographs were made from typical fields.

3.12. Light scattering

Heat-induced aggregation of different mixtures of β-lactoglobulin, α-lactalbumin and BSA was followed using a photon correlation spectroscopy, as described by Davis & Pinder (1993). Protein solutions (50 g/kg, pH 6.8) were prepared (Section 3.3.), and 0.5 ml of each solution was placed in an NMR tube (3 mm internal diameter), placed

in the sample holder, and heated at 75 °C *in situ* for 15 min. The scattered light intensity was measured (i.e. the number of photon detectections over a short period of 5 s was counted) at an angle of 90° every 30 s.

CHAPTER 4

EFFECTS OF WPC CONCENTRATION ON HEAT-INDUCED INTERACTIONS OF WHEY PROTEINS

4.1. Introduction

Whey protein concentrate (WPC) products are used in a wide range of food applications, not only because of their nutritional value but also because they have desirable functional properties, such as the ability to form heat-induced gels. Consequently the heat-induced gelation of WPC solutions has been the subject of many studies in recent years (Mangino *et al.*, 1987*a,b*; Tang *et al.*, 1993, 1995; Karleskind *et al.*, 1995). These have shown that the gelation properties of WPC are affected by many factors including sodium, calcium and protein concentrations, pH, heating temperature and the presence of other components.

It has been suggested that the whey proteins undergo heat-induced gelation through a series of steps involving denaturation, aggregation, strand formation and network formation (Aguilera, 1995) to give a translucent slightly rubbery gel or an opaque compressible "gel". The former type has also been called fine stranded and the latter type has been called particulate, on the basis of microscopic examination (Stading & Hermansson, 1991; Langton & Hermansson, 1992). The type of gel formed is determined by pH, mineral composition and protein concentration and type (Tang *et al.*, 1993, 1995; Bowland & Foegeding, 1995).

The gel-forming properties of the protein components of WPC have been investigated with particular emphasis on β-lactoglobulin, the major protein present. Paulsson & Dejmek (1989) reported that the shear modulus of β-lactoglobulin gels was proportional to the protein concentration. McSwiney *et al.* (1994*a*) observed that the loss of native-like β-lactoglobulin during heating was faster than the formation of disulphide-linked aggregates, which was faster than gel formation. These results

supported the hypothesis that hydrophobically associated protein aggregates were formed before a self-supporting gel network (McSwiney *et al.*, 1994*a*, *b*). Paulsson *et al.* (1986) reported that a BSA concentration of ~ 20 g/l was required for gel formation during heating compared with ~ 50 g/l for β-lactoglobulin, whereas α-lactalbumin did not form a gel even up to 200 g/l. In a recent study (Gezimati *et al.*, 1996*a*), it was found that, when a mixture of BSA and β-lactoglobulin in a buffer that mimicked the WPC environment was heated at 70 °C, there was no obvious interaction between the two proteins under some gelling conditions and they formed two separate homogeneous aggregates, strands and gel systems which intertwined to give the final gel. However, when mixtures of β-lactoglobulin and α-lactalbumin were heated, it was concluded that these proteins interacted to form heterogeneous aggregates which formed the final gel structure (Gezimati *et al.*, 1997).

The mechanisms of aggregation and gel formation in commercial WPCs could be quite different from those in these model systems which often do not contain glycomacropeptide (GMP), minor proteins, uncharacterised and diverse high molecular weight material, lactose or different levels of mineral components. Therefore, a range of preliminary experiments was conducted to investigate the effects of heat treatment on WPC solutions. A commercial rennet WPC was used for the preparation of the WPC solutions (10, 30, 60 and 120 g/kg, pH 6.8) using the method described in Section 3.3.1. Samples of each solution were heated at 75 °C for different times using the method described in Section 3.4. The loss of native protein structures from the heated samples was investigated using 1D-PAGE as described in Section 3.7.1. The nature of the protein aggregates formed during heat treatment was characterised using 2D-PAGE as described in Section 3.7.3. The degree of protein aggregation in the heated WPC solutions was followed using ultracentrifugation and size exclusion chromatography as described in Sections 3.5 and 3.9 respectively. The results of these experiments are presented in this chapter.

4.2. Nomenclature

Based on PAGE results, some key terms used to differentiate the various forms of protein in heated WPC systems were defined by Gezimati *et al.* (1996*a*). The same nomenclature is used in this thesis. "Native-like" refers to the proteins that, under alkaline PAGE conditions (pH 8.3), migrated as if they were native proteins. "SDS-monomeric" refers to those proteins that, under SDS-PAGE conditions (pH 8.3, 0.1 % SDS), gave slightly more intense bands and ran as monomers. "Total reducible" protein refers to those proteins that, under the same SDS-PAGE conditions but in the presence of a reducing agent (2-mercaptoethanol), gave even more intense bands and migrated as though they were reduced monomeric proteins. Figure 4.1 demonstrates the relationships between these different forms of proteins and their typical variations during the heat treatment of WPC solutions.

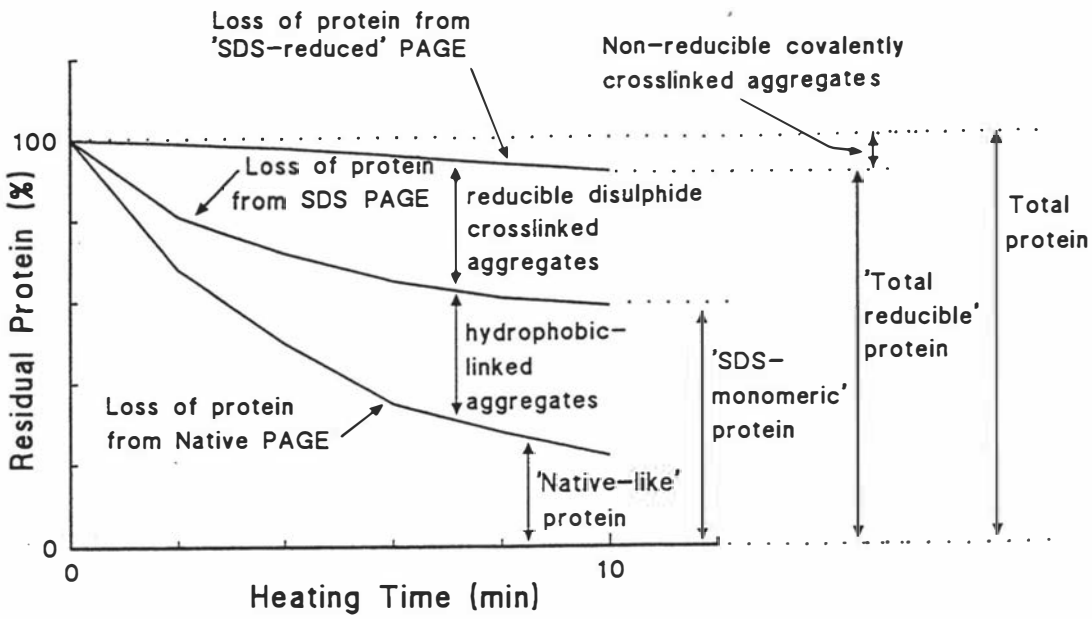


Figure 4.1. Schematic representation of the relationship between the relative amounts of different forms of proteins ("native-like", "SDS-monomeric" and "total reducible") in heated WPC solutions.

When a protein solution is heated and then analysed using native-PAGE, the amount of native-like monomeric proteins decreases with heating time (the rate of loss depends on many factors, such as temperature, concentration, pH and mineral content). The loss of native-like monomeric proteins is presumably due to the formation of aggregates linked by hydrophobic interactions and/or disulphide bonds. If a heated protein solution is analysed using SDS-PAGE, the monomeric protein bands appear to be more intense, which may be attributed to the dissociation of hydrophobically associated aggregates by SDS, resulting in a higher concentration of monomeric proteins; hence the term "SDS-monomeric". If the heated solution is treated with a reducing agent, e.g. 2-mercaptoethanol, and analysed by SDS-PAGE, the protein bands appear even more intense. In most cases, the intensities of the bands are virtually the same as those in an unheated WPC sample. In this system, in addition to the dissociation of hydrophobically associated aggregates, disulphide-linked aggregates are also disrupted, converting virtually all protein to monomers; hence the term "total reducible" proteins. In the present study, no non-reducible protein aggregates were observed in any of the heated WPC solutions. This may have been due to the mild heating conditions (75 °C, pH 6.8) used in this investigation. The formation of such aggregates, however, has been observed in other milk systems heated under more severe conditions (Singh & Creamer, 1991*a,b*).

4.3. WPC composition

Analysis showed that the WPC powder (derived from rennet whey) contained 941 g dry matter/kg of which 813 g was protein, 157 g was GMP (also measured as part of the protein), 65 g was fat and 47 g was lactose. The protein contents of the 10, 30, 60 and 120 g/kg WPC solutions (based on Kjeldahl determination) were 8.20, 24.2, 48.9 and 97.7 g/kg respectively. GMP contents of about 150 g/kg are quite common for WPCs derived from rennet or cheese whey (M. Pritchard, 1995, pers. comm.). The mineral analyses showed that the WPC powder contained 3130 μg/g of calcium, 7350 μg/g of potassium, 1560 μg/g of sodium, 2520 μg/g of phosphorus and 9390 μg/g of sulphur.

4.4. Identification of protein components in unheated WPC solutions

4.4.1. 1D-PAGE

Pure protein standards were run on native or SDS gels in order to identify the protein bands in the WPC solution (Fig. 4.2*a, b*). The unheated WPC solutions gave native-PAGE patterns (Fig. 4.3*a*) that showed bands that could be identified as β-lactoglobulin A and B, α-lactalbumin and BSA. There was also a band close to the beginning of the resolving gel and another that indicated that some proteins had not entered the gel. There was also a slightly diffuse region between the α-lactalbumin and BSA bands. This region probably consisted of several bands of caseins and other whey protein components. Similarly the major protein bands in unheated WPC solutions were identified in the SDS gels (Fig. 4.3*b*). α-Lactalbumin migrated faster than β-lactoglobulin which appeared as one band instead of two as in native-PAGE. The mobility of the proteins was different in the native and SDS gels. Under native-PAGE conditions, the mobility of a protein is primarily determined by the protein's charge-to-mass ratio, whereas, under SDS conditions, the mobility is determined mainly by molecular mass. Again there was material that did not enter the resolving gel, that only travelled a short distance into the gel and that ran between the β-lactoglobulin and BSA bands. When the samples were reduced prior to electrophoresis (Fig. 4.3*c*), the IgL, IgH and lactoferrin bands could be identified by comparison with reported patterns (Basch *et al.*, 1985). There was no material that did not enter the resolving gel although there was a light band near the top of the resolving gel and some diffuse staining between the β-lactoglobulin and BSA bands.

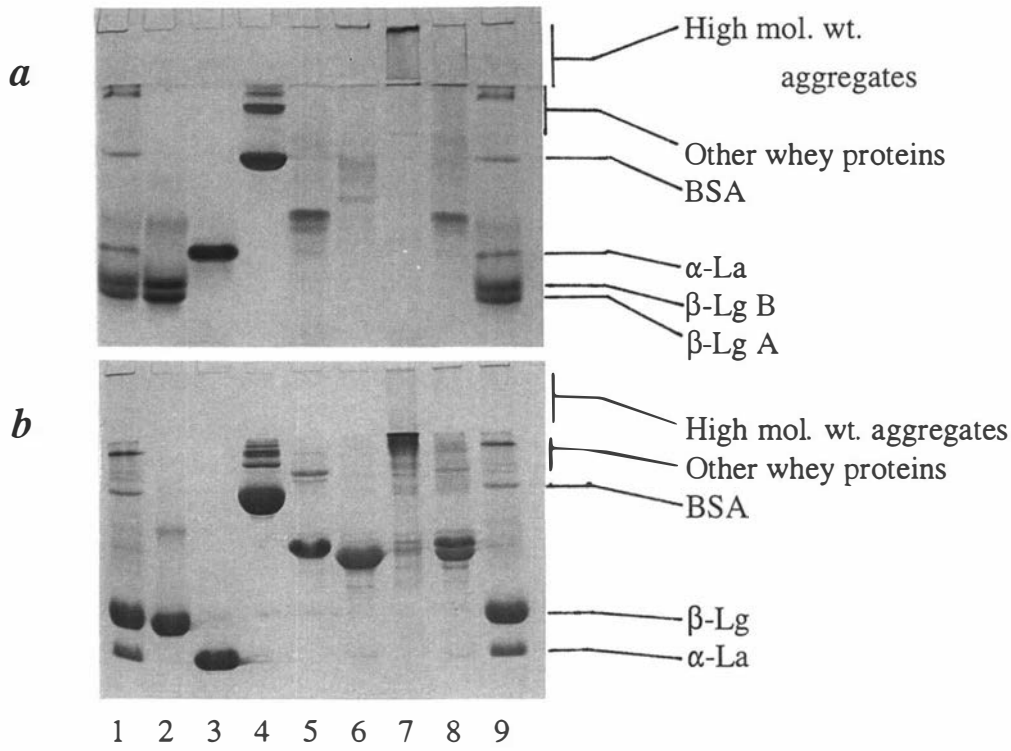


Figure 4.2. Native- (*a*) and SDS-PAGE (*b*) patterns of pure protein standards used to identify protein bands of WPC solutions. The samples are WPC solutions (slots 1 & 9), β-lactoglobulin (slot 2), α-lactalbumin (slot 3), BSA (slot 4), α_{s1}-casein (slot 5), β-casein (slot 6), κ-casein (slot 7) and commercial casein powder (slot 8). The abbreviations used: α-La - α-Lactalbumin; β-Lg - β-Lactoglobulin; mol. wt. - molecular weight.

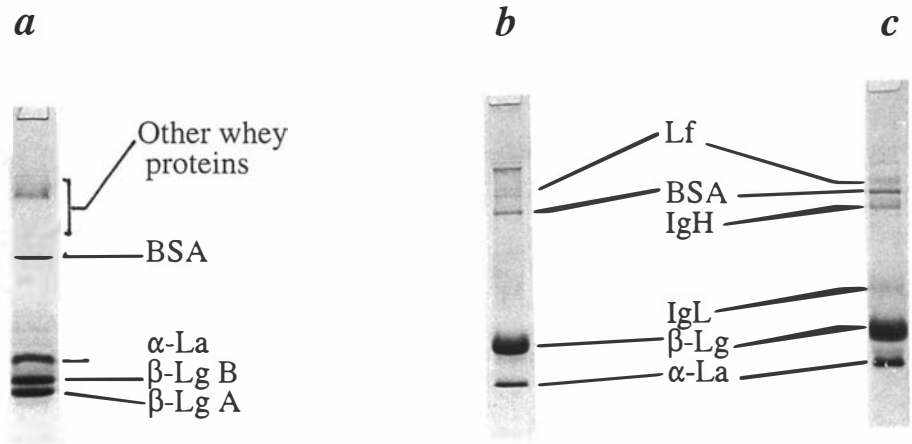


Figure 4.3. 1D-PAGE patterns of unheated WPC solutions (120 g/kg): native-PAGE (*a*), SDS-PAGE (*b*) and SDS-PAGE of the reduced samples (*c*). The abbreviations used: Lf - Lactoferrin; IgH & IgL - heavy fractions & light fractions of Immunoglobulins; α-La - α-Lactalbumin; β-Lg - β-Lactoglobulin.

4.4.2. 2D-PAGE

Typical 2D-PAGE patterns of an unheated control WPC solution can be viewed in Fig. 4.4. On top of each gel lies a stained gel strip showing the protein bands from the first-dimension separation running from left to right. Immediately underneath this strip is a second strip that contained the same protein bands as were used as samples for the second-dimension separation. On the left-hand side, an aliquot of the corresponding sample was run to help identify the protein bands. The bands that could be identified on the native and SDS 1D gels as β-lactoglobulin A and B, α-lactalbumin and BSA were readily identified as the various spots on the native-SDS 2D gel (Fig. 4.4*a*). Similarly the spots for these proteins, lactoferrin and the corresponding reduced proteins, as well as reduced IgL and IgH, were identified on the SDS 2D gel (Fig. 4.4*b*). Some other spots were identified in this way. The spot labelled X in Fig. 4.4*a* was probably glyco-α-lactalbumin (N. Haggarty, 1996, pers. comm.) and the spots labelled Y1 and Y2 in Fig. 4.4*b* were likely to be α_{S1}- and β-caseins respectively. There were a number of spots corresponding to BSA, lactoferrin, IgH and IgL, which after reduction were resolved from the material caught at the top of the stacking and resolving gels. There were other spots for which the proteins were not identified. It was not possible to identify GMP in any of these gel systems because of the staining techniques used.

4.5. 1D-PAGE patterns of heated WPC solutions

4.5.1. Native-PAGE

When the WPC solutions were heated for various times at 75 °C and examined by 1D-PAGE, the intensities of all the whey protein bands diminished for the non-reduced samples with corresponding increases in the material caught within the stacking gel and at the top of the resolving gel (Fig. 4.5*a*, *b*). The minor whey protein components were the most heat sensitive and their concentration decreased faster than that of the major proteins (indicated by the rapid decreases in the band intensities).

When the PAGE patterns of the heated 10, 60 and 120 g/kg WPC solutions were compared, some differences could be observed. The native-PAGE patterns of the 10 g/kg WPC solution (Fig. 4.5 I*a*) showed a fine band just behind the BSA band that

appeared to increase in intensity with heating time. This band probably corresponded to a complex between a BSA molecule and either a β-lactoglobulin molecule or an α-lactalbumin molecule, and was not observed in either the 60 or 120 g/kg WPC solutions that had been heated (Fig. 4.5 I*b, c*). The native-PAGE patterns of the 10 g/kg WPC solutions also showed that the diffuse region between the α-lactalbumin and BSA bands increased in intensity with heating time. This suggested that at least a portion of the protein in this region was attributable to some whey protein aggregates, most probably β-lactoglobulin dimers, that may have formed during heating. In contrast, the intensity in this region in the 60 and 120 g/kg WPC solutions decreased with heating time. Furthermore, in the 10 g/kg WPC solution, a faint band running just ahead of the BSA band was apparent for samples heated for 12 and 15 min (Fig. 4.5 I*a*, slots 8 & 9). This band probably corresponded to a higher molecular weight aggregate species (e.g. β-lactoglobulin tetramer). This band was not observed in either the 60 or 120 g/kg WPC solutions.

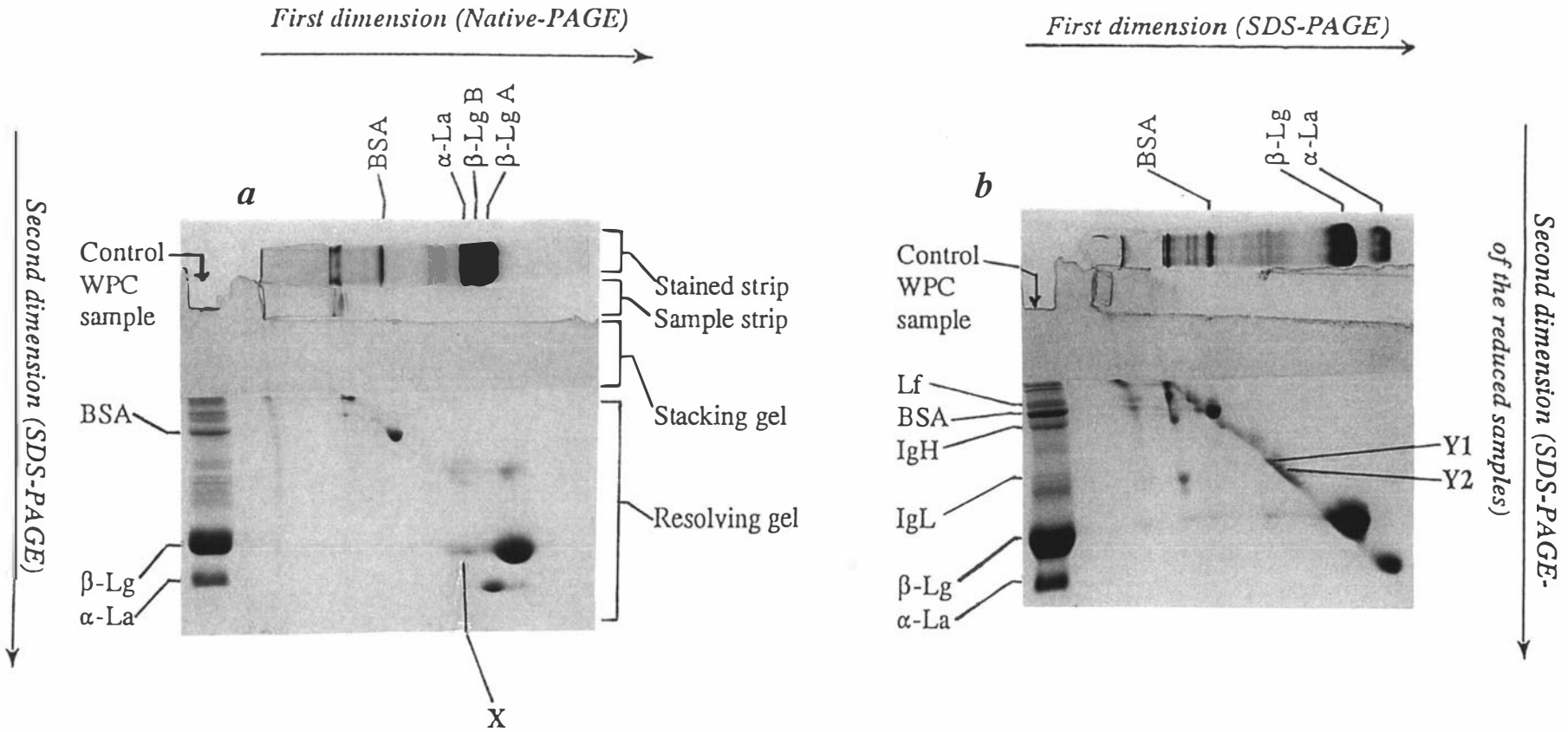


Figure 4.4. Two- dimensional (2D) electrophoretic patterns of an unheated control WPC solution (120 g/kg): *(a)* first dimension, native-PAGE; second dimension, SDS-PAGE; *(b)* first dimension, SDS-PAGE; second dimension, SDS-PAGE of samples within gel strip that were treated with 2-mercaptoethanol. Refer to Figs. 4.2 and 4.3 for abbreviations. X, Y1 & Y2 are as described in the text.

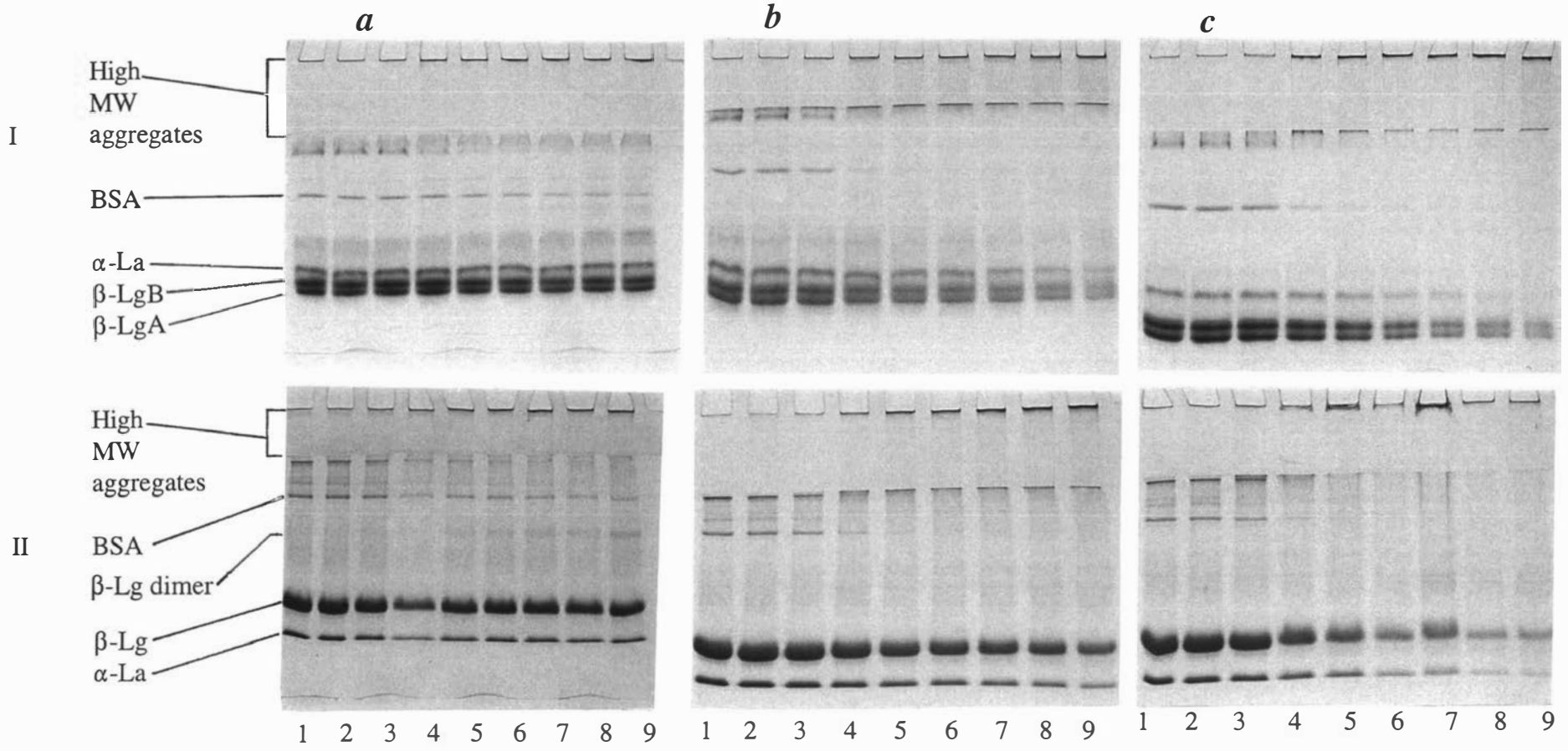


Figure 4.5. Native-PAGE (I) and SDS-PAGE (II) patterns of 10 (*a*), 60 (*b*) and 120 (*c*) g/kg WPC solutions heated at 75 °C for 0 (slots 1 & 2), 2 (slot 3), 4 (slot 4), 6 (slot 5), 8 (slot 6), 10 (slot 7), 12 (slot 8) and 16 (slot 9) min. Refer to Figs. 4.2 and 4.3 for abbreviations.

4.5.2. SDS-PAGE

The SDS-PAGE patterns of the heated WPC solutions (Fig. 4.5 II) also showed some differences. The SDS-PAGE patterns of the 10 g/kg WPC solution (Fig. 4.5 II*a*) clearly showed a discrete band between the β-lactoglobulin and BSA bands that increased in intensity with heating time. As this band showed an increase in intensity with heating time in SDS conditions, it is likely that this band corresponded to disulphide-linked polymers formed during heating. The SDS-PAGE patterns of the 60 and 120 g/kg WPC solutions (Fig. 4.5 II*b, c*) showed that the region between the β-lactoglobulin and BSA bands had several faint bands. These bands were clearer in the 60 g/kg WPC solution than in the 120 g/kg WPC solution. These results suggest that various intermediate protein species were formed during heating of the WPC solutions. The formation of intermediate species was more prominent in solutions of lower protein concentrations, i.e. in the order 10 > 60 > 120 g/kg.

Reduction of these WPC samples resulted in complete disappearance of the material within the stacking gel and that caught at the top of the resolving gel, and all the bands were essentially the same from one slot to another. Figure 4.6 shows typical SDS-PAGE patterns of the WPC solutions after reduction.

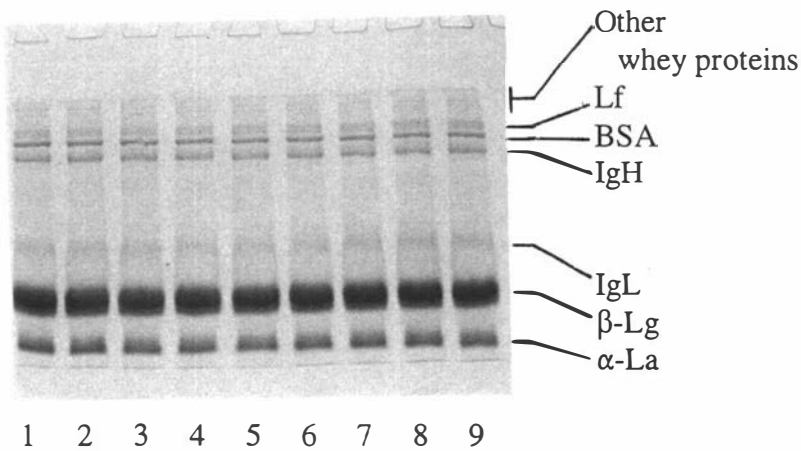


Figure 4.6. Typical SDS-PAGE patterns of the heated WPC solutions (60 g/kg) after treatment with 2-mercaptoethanol. The heating times were the same as indicated in Fig. 4.5.

4.6. Loss of proteins during heating

The PAGE results for the heated WPC solutions (Fig. 4.5) were quantified using the method described in Section 3.7.1. The concentrations of native-like or SDS-monomeric proteins remaining after heat treatment of WPC solutions were determined by comparing the corresponding band intensities of heated and unheated samples. The intensities of the protein bands in the heated WPC solutions were expressed as percentages of the corresponding band intensities of the unheated WPC solutions.

4.6.1. Loss of native-like or SDS-monomeric proteins

The amounts of residual native-like and SDS-monomeric proteins in the heated samples are shown in Fig. 4.7. Each point is the average of the results from three different experiments, and the error bars indicate variations. The results show that when 10 g/kg WPC solutions were heated, there were small decreases in the quantities of β-lactoglobulin and α-lactalbumin with heating time (Fig. 4.7*a, b*). However, the quantities of the native-like and SDS-monomeric BSA, immunoglobulin and lactoferrin (Fig. 4.7*c*) showed larger decreases with heating time. This result appeared to be inconsistent with the results of Parris *et al.* (1991) who observed a considerable degree of denaturation in freshly prepared acid and cheese whey on heating at 70 - 90 °C. When a fresh rennet whey (~ 10 g/kg whey protein) was heated under the same conditions, there were apparent losses of β-lactoglobulin and α-lactalbumin from native- and SDS-PAGE (results not shown). The minimal loss of protein from heated 10 g/kg WPC solutions may be attributed to different mineral compositions.

The loss of native-like and SDS-monomeric β-lactoglobulin and α-lactalbumin with heating time was greater from the 60 g/kg WPC solution and greatest from the 120 g/kg WPC solution (Figs. 4.5 & 4.7*a, b*). The results for the loss of native-like β-lactoglobulin from the 30 and 60 g/kg WPC solutions showed a lower initial stage, followed by more rapid loss. However, the initial slow stage was undetectable for the 120 g/kg WPC solution. In the 120 g/kg WPC solution, the quantities of SDS-monomeric β-lactoglobulin and α-lactalbumin present in each heated sample were considerably greater than the quantities of native-like β-lactoglobulin and

α-lactalbumin. However, the loss of SDS-monomeric β-lactoglobulin from the 60 g/kg WPC solutions was only slightly lower than that of the native-like proteins.

Native-like α-lactalbumin was lost (in terms of proportions of the original protein) from the 60 g/kg WPC solutions more quickly than native-like β-lactoglobulin, although the loss of the two proteins was comparable from the 120 g/kg WPC solution (Fig. 4.7). The rate of loss of SDS-monomeric α-lactalbumin appeared to be faster than that of SDS-monomeric β-lactoglobulin at all WPC concentrations. The observation that the loss of native α-lactalbumin was faster than that of native β-lactoglobulin was in agreement with the results of Hollar *et al.* (1995) obtained by heating 160 g/kg WPC solutions at 71 °C. However, it was contrary to the findings of other studies (Donovan & Mulvihill, 1987*a, b*; Dannenberg & Kessler, 1988; Singh & Creamer, 1991*b*) on the denaturation of these proteins in milk or a sweet whey environment. These differences may be due to differences in composition of the reaction medium and in the protein concentration.

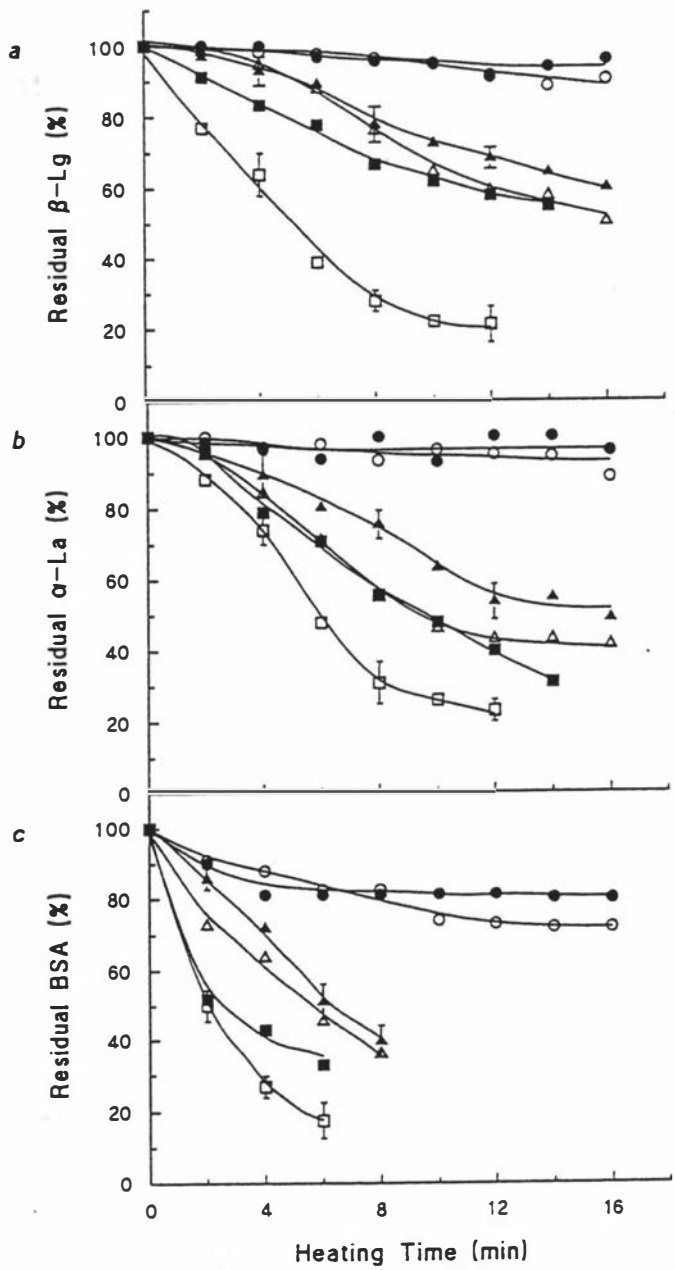


Figure 4.7. Loss of native-like (○, Δ, □) and SDS-monomeric (●, ▲, ■) β-lactoglobulin (*a*), α-lactalbumin (*b*) and BSA (*c*), on heating 10 (○, ●), 60 (Δ , ▲) and 120 (□, ■) g/kg WPC solutions at 75 °C.

4.6.2. Kinetic evaluation of the thermal loss of whey proteins

The results for the thermal loss of native-like β-lactoglobulin, α-lactalbumin and BSA at 75 °C in 10, 30, 60 and 120 g/kg WPC solutions were used to evaluate the kinetics for these reactions. The kinetics for the loss of protein structures on heating are commonly expressed using the following equation (Dannenberg & Kessler, 1988*a,b*):

$$v = -\left(\frac{dC}{dt}\right) = k_n (C)^n \qquad (1)$$

where n = order of the reaction; k_n = rate constant for the reaction; C = the concentration and v = the rate of reaction (i.e. the rate of loss of native-like proteins; the term dC/dt). If the initial concentration of the native-like protein is C_0, and the concentration after heating for time t is C_t, then the integrated form of equation (1) is:

$$\left(\frac{C_0}{C_t}\right)^{n-1} = 1 + (n - 1)\, k_n\, C_0^{(n-1)}\, t \qquad (2)$$

for $n \neq 1$; and

$$\ln\left(\frac{C_t}{C_o}\right) = -k_1\, t \qquad (3)$$

for $n = 1$. When an accurate value of n is inserted in equation (2), a plot of $(C_0/C_t)^{(n-1)}$ versus heating time will give a straight line with a slope equal to $(n-1)k_nC_0^{(n-1)}$.

The data for the loss of native-like β-lactoglobulin, α-lactalbumin and BSA were fitted to equation (2) with n = 1.5 or 2, and to equation (3) with n = 1. These graphs are shown in Fig. 4.8 for n = 1 and Figs 4.9 - 4.11 for n = 1.5 and 2. The best fit was determined by linear regression analysis, and the coefficients of determination (r^2) were compared. The results showed that, for the three proteins considered (β-lactoglobulin, α-lactalbumin and BSA), the data could be adequately described by

reaction orders between 1.0 and 2.0, but the coefficients of determination (r^2) were highest, at all concentrations, when the data were fitted to equation (2) with a reaction order of n = 1.5 (Figs 4.9 - 4.11).

No kinetic data on whey protein aggregation in heated WPC solutions have been reported in the literature. However, the value of n = 1.5 found in this study is in agreement with that reported by Kessler & Beyer (1991) for denaturation of β-lactoglobulin during the heating of skim milk at 75-90 °C.

From equation (2) an apparent rate constant k_{app} (min^{-1}) may be described by:

$$k_{app} = k_n C_0^{(n-1)} \qquad (4)$$

Irrespective of the assumed value for n ($n \neq 1$), k_{app} has the units of inverse time (min^{-1}). Comparisons of different kinetic models can be based on a determination of k_{app}, because it always has the same units irrespective of the assumed value for the reaction order. Table 4.1 shows the calculated values of k_n and k_{app} (for n = 1, 1.5 and 2) and how they were affected by the initial WPC concentrations. (Note: for n = 1, k_n = k_{app} because $C_0^{(n-1)}$ = 1.) The results show that the values of both k_n and k_{app} increased with increasing WPC concentrations. This suggests that the loss of proteins did not occur via simple first- or second-order processes despite the high r^2 values. Differences in protein and mineral concentrations in the WPC solutions may result in a change in reaction mechanism.

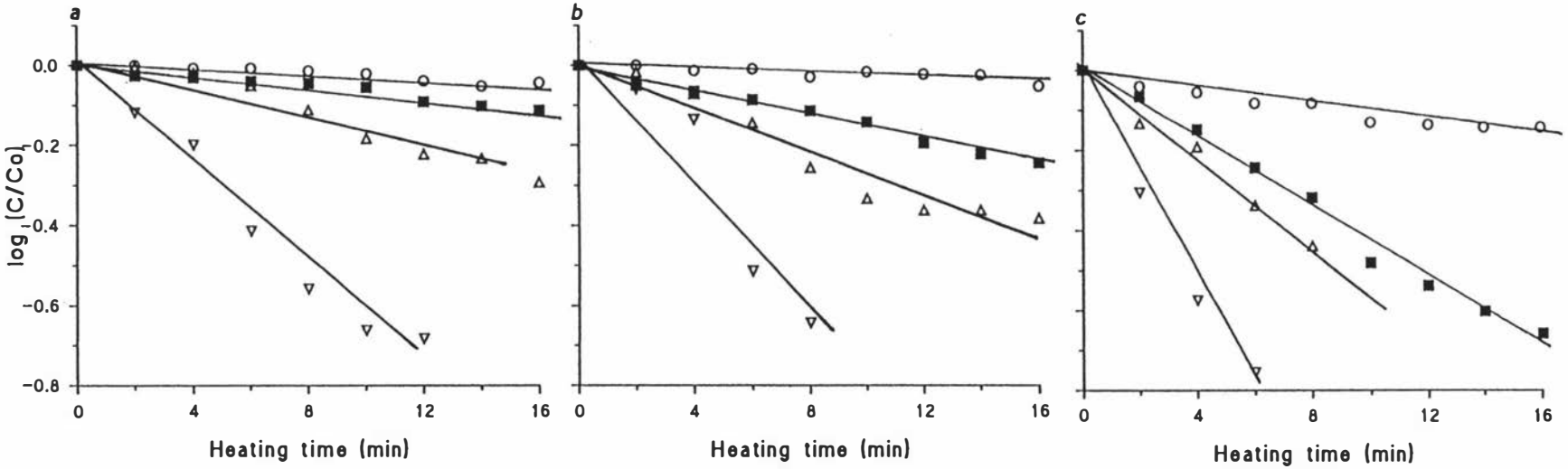


Figure 4.8. Loss of native-like β-lactoglobulin (*a*), α-lactalbumin (*b*) and BSA (*c*) at 75 °C, from 10 (○), 30 (□), 60 (Δ) and 120 (∇) g/kg WPC solutions, represented as a reaction of the order $n = 1$.

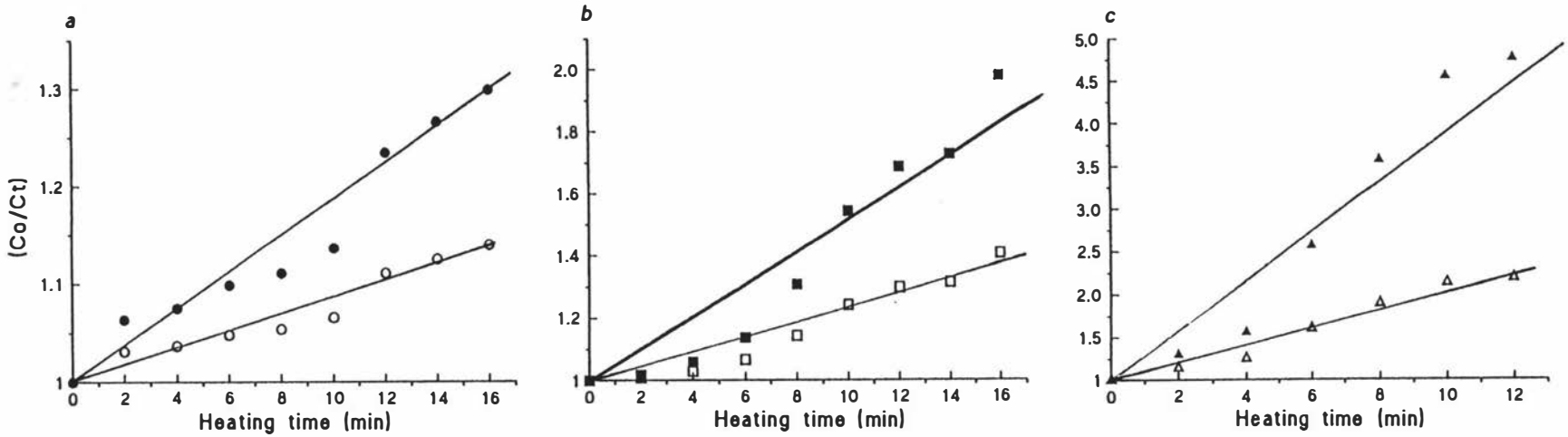


Figure 4.9. Loss of native-like β-lactoglobulin at 75 °C, from 30 (*a*), 60 (*b*) and 120 (*c*) g/kg WPC solutions, represented as a reaction of the order $n = 1.5$ (○, □, Δ) or $n = 2$ (●, ■, ▲).

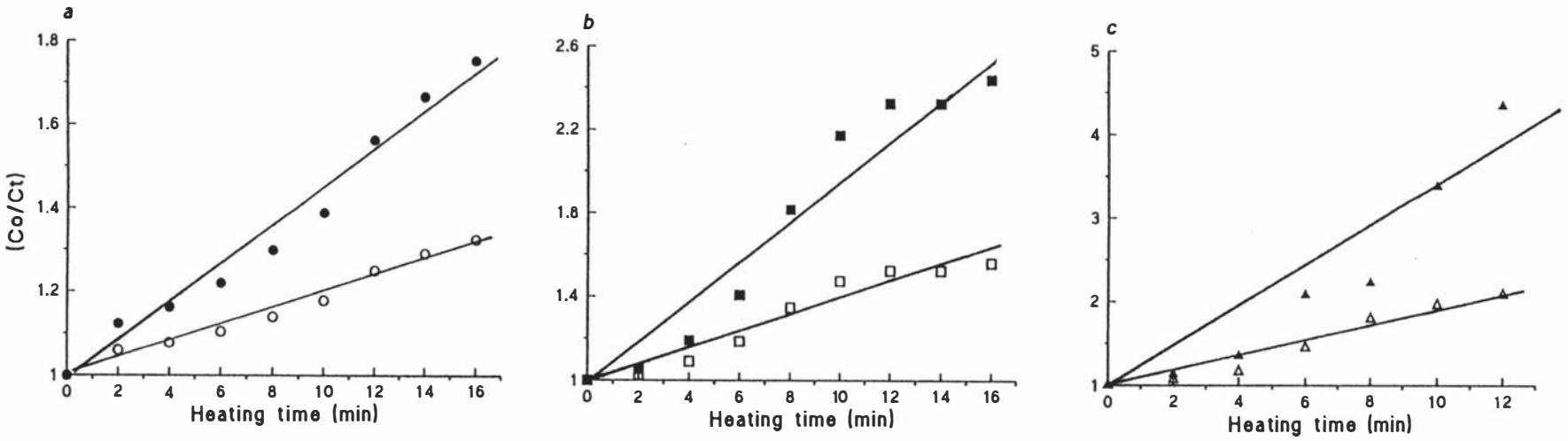


Figure 4.10. Loss of native-like α-lactalbumin at 75 °C, from 30 (*a*), 60 (*b*) and 120 (*c*) g/kg WPC solutions, represented as a reaction of the order $n = 1.5$ (○, □, Δ) or $n = 2$ (●, ■, ▲).

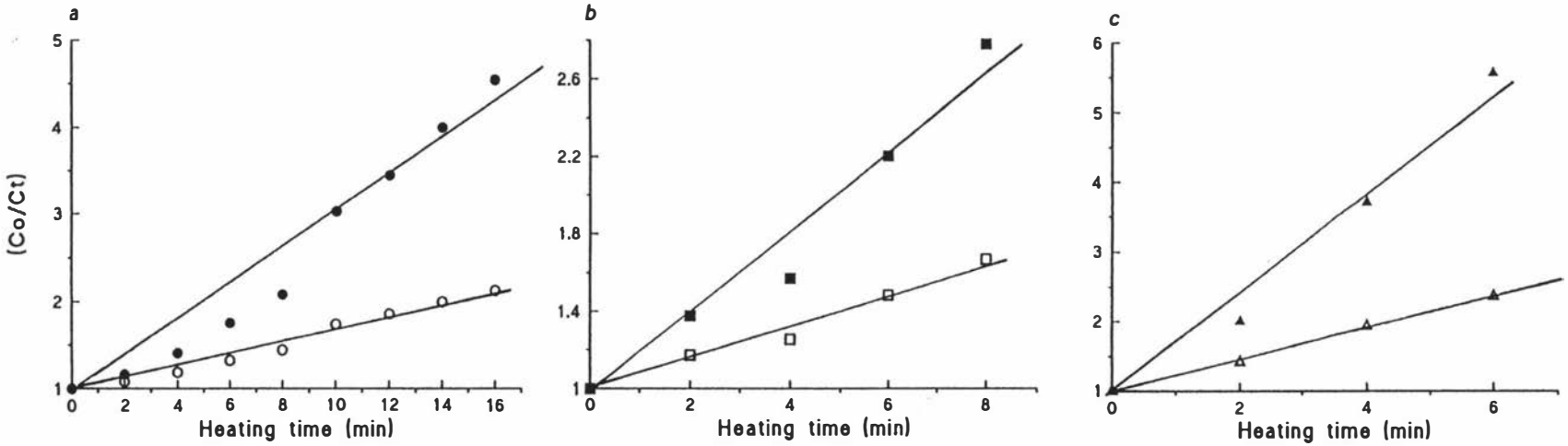


Figure 4.11. Loss of native-like BSA at 75 °C, from 30 (*a*), 60 (*b*) and 120 (*c*) g/kg WPC solutions, represented as a reaction of the order $n = 1.5$ (○, □, Δ) or $n = 2$ (●, ■, ▲).

For a typical reaction of this nature, there are two orders of reaction: the reaction order with respect to time, n_t; the reaction order with respect to concentration, n_c (van Boekel, 1996). To determine n_c (i.e. the order of reaction with respect to concentration), the initial rate of reaction, v, (i.e. the initial rate of loss of native-like protein), determined using equation (1), for each protein (β-lactoglobulin, α-lactalbumin and BSA) was derived from the data used in Figs 4.8 - 4.11. The initial rate of reaction for each data set was calculated over the first 4 min of heating. A plot of log (initial rate, v) versus log (initial concentration, C_0) (Fig. 4.12) resulted in a straight line (r^2 = 0.97 - 0.99) for all of the whey proteins studied. The slope of this plot gives the order of reaction with respect to concentration, n_c, and the intercept gives the value of log k (van Boekel, 1996). The n_c values were found to be 0.98, 0.98 and 0.81 for β-lactoglobulin, α-lactalbumin and BSA respectively. The respective k values were determined to be 0.1, 0.1 and 1.6 min^{-1}.

These results show that, for each of the proteins studied, the order of reaction with respect to concentration, n_c, was different from the order of reaction with respect to time, n_t, calculated using equation (2). The order of reaction with respect to time (n_t = 1.5) was greater than the order of reaction with respect to concentration ($n_c < 1$). This suggests that an inhibitor was formed during the course of the reactions (van Boekel, 1996). Building up the concentration of protein aggregates in heated WPC solutions probably hinders the free movement, and hence lowers the kinetic energy, of individual native-like molecules. This results in an inhibiting effect that slows the loss of native-like proteins as heating progresses.

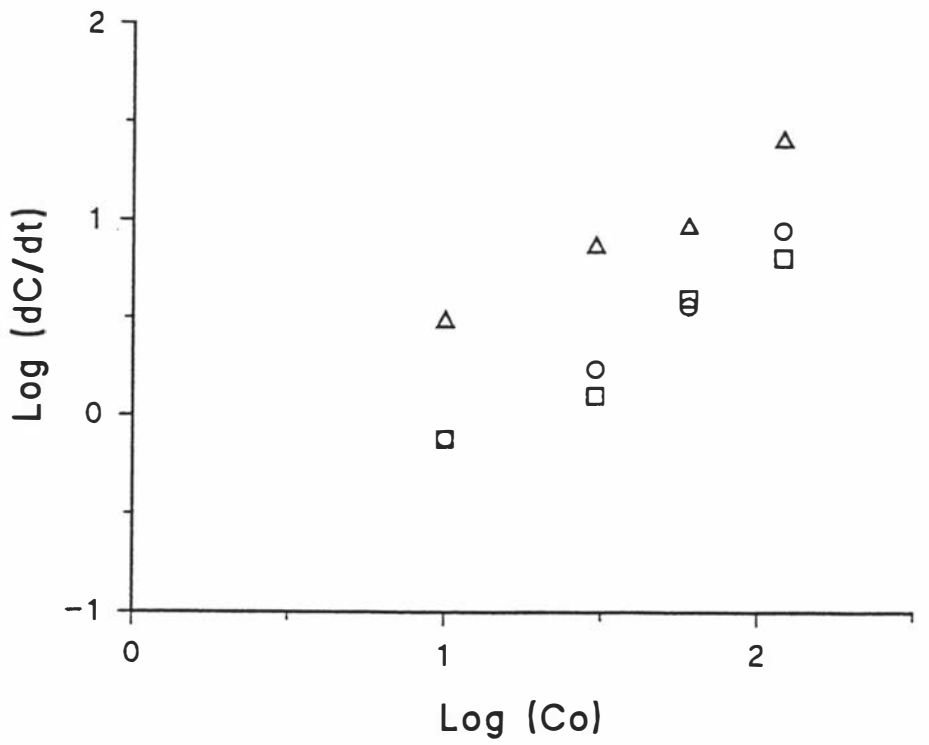


Figure 4.12. Plot of initial rate of loss (v) of native-like β-lactoglobulin (○), α-lactalbumin (□) and BSA (Δ) versus initial WPC concentrations (C_o).

Table 4.1. Effect of initial WPC concentration on the apparent rate constant (k_{app}), and on the rate constant (k_n) of the loss of native-like β-lactoglobulin, α-lactalbumin and BSA at 75 °C, with n =1, 1.5 or 2 . C_0 : initial WPC concentration, g/kg; k_{app}: in min^{-1}; k_n ($= k_{app}/C_0)^{(n-1)}$ in $(g/kg)^{-1}min^{-1}$.

		β-Lactoglobulin			α-Lactalbumin			BSA		
	C_0	k_{app}	k_n	r^2	k_{app}	k_n	r^2	k_{app}	k_n	r^2
n = 1	10	-0.0071	-0.0071	0.89	-0.0057	-0.0057	0.76	-0.0173	-0.0173	0.91
	30	-0.0071	-0.0071	0.90	-0.0263	-0.0263	0.98	-0.0784	-0.0784	0.95
	60	-0.0142	-0.0142	0.96	-0.0421	-0.0421	0.91	-0.0783	-0.0783	0.96
	120	-0.0683	-0.0683	0.93	-0.0710	-0.0710	0.94	-0.0132	-0.0132	0.89
n = 1.5	10	0.0081	0.0025	0.89	0.0061	0.0019	0.96	0.0229	0.0072	0.93
	30	0.0168	0.0031	0.95	0.0404	0.0073	0.97	0.1508	0.0275	0.98
	60	0.0544	0.0071	0.94	0.0817	0.0105	0.98	0.1644	0.0212	0.98
	120	0.2204	0.0201	0.98	0.2030	0.0185	0.96	0.4582	0.0418	1.00
n = 2	10	0.0082	0.0008	0.88	0.0063	0.0006	0.93	0.0253	0.0025	0.93
	30	0.0180	0.0006	0.88	0.0468	0.0016	0.97	0.0234	0.0028	0.96
	60	0.0643	0.0011	0.94	0.1051	0.0018	0.94	0.2191	0.0037	0.96
	120	0.3534	0.0029	0.96	0.3097	0.0026	0.94	0.7685	0.0064	0.91

4.6.3. Protein aggregate formation in heated WPC solutions

The loss of native-like or SDS-monomeric proteins during heating was accompanied by concomitant increases in the amount of protein aggregates formed, as indicated by increasing quantities of the material caught within native and SDS stacking gels with heating time (Fig. 4.5). By comparing the native- and SDS-PAGE results, it was possible to elucidate the relative proportions of proteins that were monomeric, and that were involved in forming hydrophobic or disulphide-linked aggregates. When 30 g/kg WPC solutions were heated (Fig. 4.13*a*) about 80% of the total β-lactoglobulin remained in a native-like state after 16 min heating at 75 °C. The aggregates formed were largely disulphide-linked aggregates with smaller amounts of hydrophobic aggregates present. In heated 60 g/kg WPC solutions (Fig. 4.13*b*), about 50% of the initial β-lactoglobulin remained native-like after heating for 16 min and the aggregates formed in these solutions were largely disulphide-linked. However, in heated 120 g/kg WPC solutions (Fig. 4.13*c*), β-lactoglobulin aggregation involved the formation of both hydrophobic and disulphide-linked aggregates in almost equal proportions. Therefore the extent to which β-lactoglobulin formed hydrophobic aggregates in heated WPC solutions appeared to be dependent on the WPC concentration. The higher the WPC concentration, the higher were the proportions of hydrophobically associated β-lactoglobulin aggregates.

The loss of native-like α-lactalbumin from 30, 60 and 120 g/kg WPC solutions during heating resulted largely in the formation of disulphide-linked aggregates, and relatively smaller proportions of hydrophobic aggregates (Fig. 4.14). The proportion of hydrophobic to disulphide-linked aggregates formed by α-lactalbumin in all WPC solutions appeared to be fairly similar, with no strong indication of an effect of concentration on the proportions of hydrophobic aggregates, as was the case for β-lactoglobulin (Fig. 4.13).

Unlike β-lactoglobulin or α-lactalbumin, BSA showed a considerable loss of native-like structure at all WPC concentrations including the 10 g/kg WPC solutions (Fig. 4.15). The aggregates formed were largely disulphide-linked, and the levels of hydrophobic aggregates were relatively low and somewhat variable.

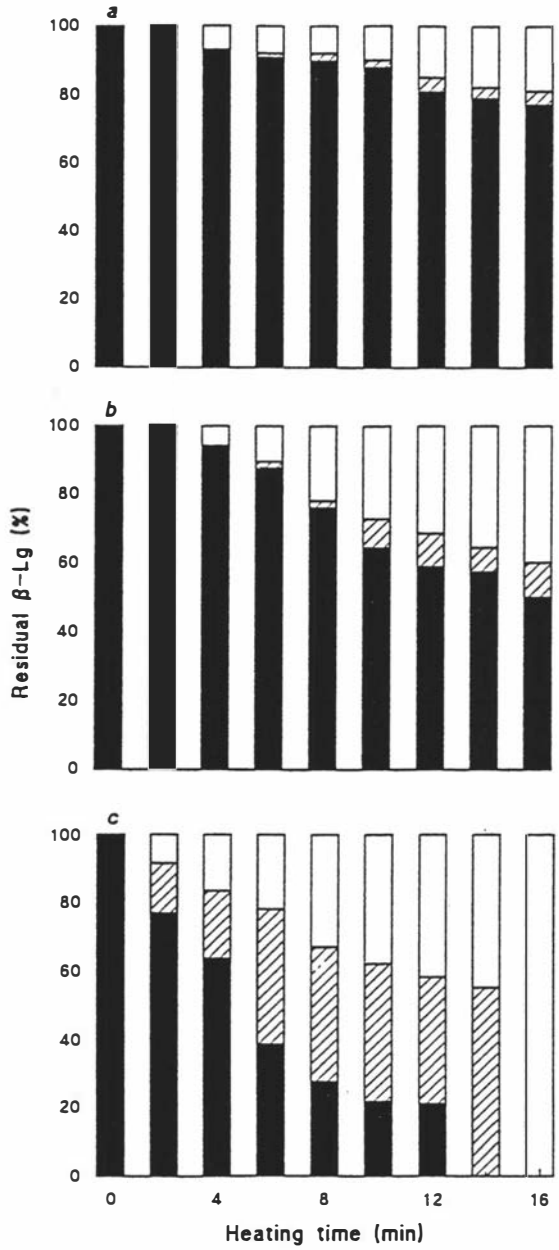


Figure 4.13. β-Lactoglobulin aggregates in 30 (*a*), 60 (*b*) and 120 (*c*) g/kg WPC solutions heated at 75 °C. β-Lactoglobulin species: native-like (■), hydrophobic aggregates (▨) and disulphide-linked aggregates (□). Note: The results for the native-like or hydrophobic aggregates for the last two samples in *c* (i.e. samples heated for 12 & 16 min) were not available because the PAGE bands were either too faint or not sharp enough to give a well-defined peak on scanning. The same reason applies to the missing results for α-lactalbumin (Fig. 4.14) and BSA (Fig. 4.15).

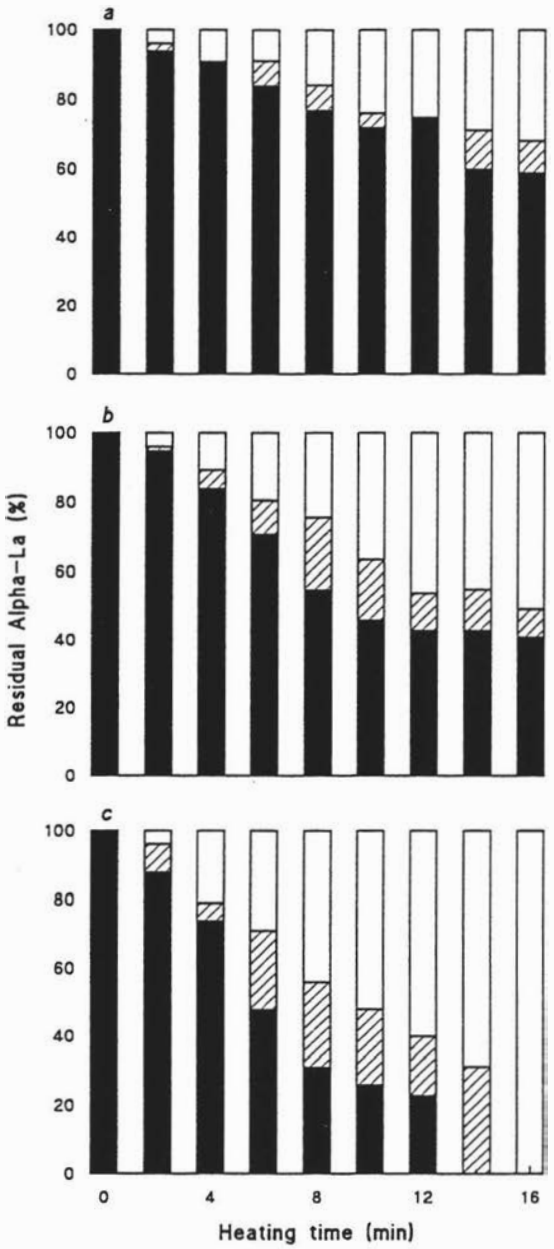


Figure 4.14. α-Lactalbumin aggregates in 30 (*a*), 60 (*b*) and 120 (*c*) g/kg WPC solutions heated at 75 °C. α-Lactalbumin species: native-like (■), hydrophobic aggregates (▨) and disulphide-linked aggregates (□). Note: The missing results were as described in Fig. 4.13.

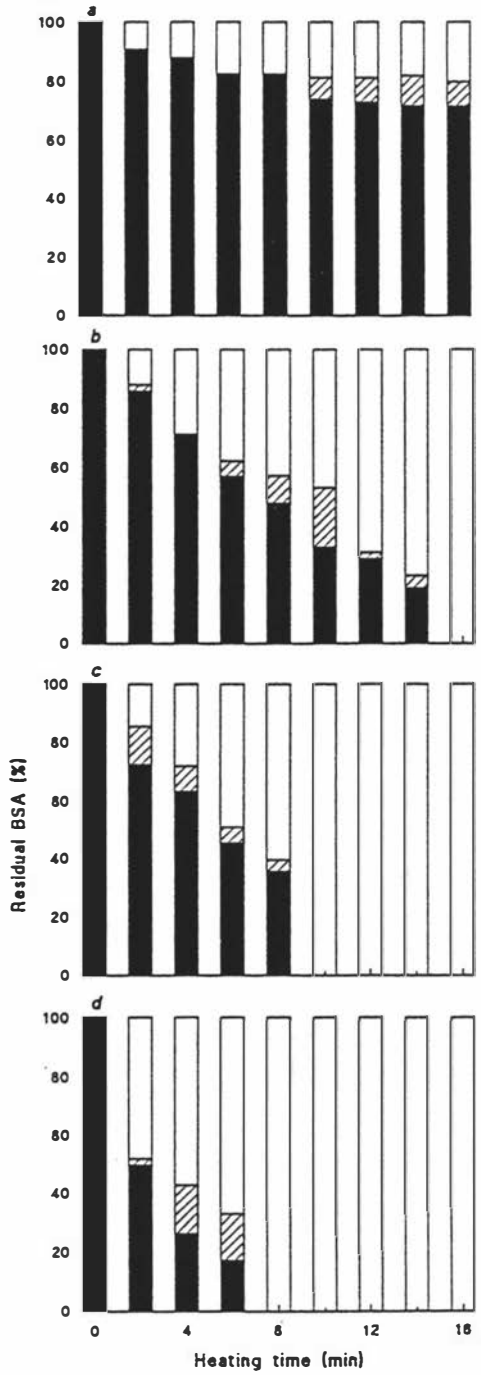


Figure 4.15. BSA aggregates in 10 (*a*), 30 (*b*), 60 (*c*) and 120 (*d*) g/kg WPC solutions heated at 75 °C. BSA species: native-like (■), hydrophobic aggregates (▨) and disulphide-linked aggregates (□). Note: The missing results were as described in Fig. 4.13.

When the involvement of β-lactoglobulin, α-lactalbumin and BSA in the formation of aggregates during heating were compared, it appeared that α-lactalbumin and BSA were involved predominantly in the formation of disulphide-linked aggregates and to a lesser degree in hydrophobic aggregation at all WPC concentrations. The behaviour of β-lactoglobulin, however, was dependent on the WPC concentration. In 30 and 60 g/kg WPC solutions, the aggregates formed were largely disulphide-linked whereas, in 120 g/kg WPC solutions, similar amounts of hydrophobic and disulphide-linked aggregates were present after heating.

It has been reported that the thermal denaturation of β-lactoglobulin proceeds via a number of steps (Sawyer, 1968; McKenzie, 1971). At pH values near 7.0, β-lactoglobulin exists predominantly in the dimeric form, but the dimer dissociates reversibly to monomers when the temperature is increased above 30 °C (McKenzie , 1971). Heating above 65 °C causes unfolding of these monomers which increases the reactivity of the single sulphydryl group in the molecule. This is accompanied by aggregation of the β-lactoglobulin molecules as a result of sulphydryl-disulphide interchange reactions and hydrophobic interactions. The present study measured the loss of native-like proteins resulting from increases in aggregation. The extent of unfolding required prior to aggregation is unknown but it is likely to be affected by the protein concentration. As the protein concentration is increased, the partially unfolded molecules interact more rapidly and produce soluble and insoluble aggregates (Nielsen *et al.*, 1996). It is likely that both disulphide bonds and non-covalent interactions are involved in forming these aggregates (McSwiney *et al.*, 1994), and the relative importance of these interactions is affected by the protein concentration (Nielsen *et al.*, 1996). Non-covalent interactions (e.g. hydrophobic) may be more important at high protein concentrations because of the close proximity of the protein molecules.

4.7. Characterisation of protein aggregates formed during heating of WPC solutions

The aggregates observed using native- and SDS-PAGE (Fig. 4.5) were characterised using 2D-PAGE (Fig. 4.16). Examination of the "sample" gel strip in Fig. 4.16*a, c, e* showed that the material that had been caught in the sample well and the material at the

top of the resolving gel had not moved out of the sample strip. In contrast, the material caught in the sample gel strip in Fig. 4.16*b, d, f* resolved completely and migrated into the 2D gel, giving rise to various spots corresponding to the monomeric proteins. Comparison of the native-SDS 2D-PAGE pattern (Fig. 4.16*a*) of the 10 g/kg WPC sample heated for 30 min at 75 °C with the pattern for the unheated control sample (Fig. 4.4*a*) indicated a number of new spots. The material that had moved a few millimetres into the gel was only partially eluted from the sample gel. The region labelled A had a moderately well-defined spot which corresponded to the bands in the region between BSA and α-lactalbumin in the native gel strip and in the SDS gel on the left-hand-side of Fig. 4.16*a*. A distinct spot, labelled C, was observed in the native-SDS 2D gel (Fig. 4.16*a*) and had slightly lower molecular weight than the β-lactoglobulin dimer. Upon reduction, this spot resolved to give monomeric α-lactalbumin (labelled C', in Fig. 4.16*b*) and a faint β-lactoglobulin spot, indicating the presence of disulphide-linked α-lactalbumin dimers and/or disulphide-linked α-lactalbumin-β-lactoglobulin 1:1 complex in the heated 10 g/kg WPC solution. There was also a faint horizontal comet-tail of intensity between the β-lactoglobulin spot on the 2D gel and the β-lactoglobulin band at the left-hand side. Comparison of the SDS 2D-PAGE pattern (Fig. 4.16*b*) with the control pattern (Fig. 4.4*b*) showed that most of the material caught in the sample well travelled into the gel and that all the other material in the sample gel travelled into the 2D gel. After reduction, the material caught in the sample well resolved into a series of spots corresponding to many of the proteins in the reduced sample in the left-hand channel. The relative band intensities were different, the most notable being the absence of α-lactalbumin, and the presence of little β-lactoglobulin and BSA, which were less intense than IgH and lactoferrin. The material that had been caught at the top of the resolving gel also gave a number of spots. In this case, the BSA spot was more intense than any other. Between the β-lactoglobulin spot and the β-lactoglobulin band in the left-hand channel, there were a number of spots and an almost continuous horizontal line up to the point corresponding to the material caught at the top of the resolving gel. An intense spot (Fig. 4.16*b*) could well correspond to a disulphide-bonded β-lactoglobulin dimer, based on the position of the unreduced protein on the SDS gel. Also apparent were a number

of faint α-lactalbumin spots across the gel (i.e. between the α-lactalbumin spot and the corresponding band in the left hand channel) indicating the presence of disulphide-bonded α-lactalbumin in the aggregates.

Comparison of the 2D-PAGE results from the heated 120 g/kg WPC sample (Fig. 4.16*e*, *f*) with those from the 10 g/kg WPC sample (Fig. 4.16*a*, *b*) showed some interesting differences. For the native-SDS 2D-PAGE pattern (Fig. 4.16*e*), BSA, casein, β-lactoglobulin, α-lactalbumin and glyco-α-lactalbumin were all present but the dimeric spot (A in Fig. 4.16*a*) was markedly diminished in intensity. Most of the material that had been caught in the sample slot and at the top of the resolving gel did not travel into the SDS gel, as was the case for the 10 g/kg WPC sample (Fig. 4.16*a*). However, some of this material travelled down the SDS gel, indicating that some of it was dissociated from high molecular weight aggregates by the SDS buffer. It is quite clear that SDS dissociated monomeric β-lactoglobulin from a number of aggregates to show up as a faint line and a series of spots between the β-lactoglobulin spot and the β-lactoglobulin band in the left-hand channel. A number of faint spots, which were apparently of slightly lower mobility than β-lactoglobulin, were observed in the region labelled B (Fig. 4.16*a*). The protein components that corresponded to these spots could not be identified. Comparable spots were also apparent for BSA.

Comparison of the non-reduced and reduced SDS 2D-PAGE pattern (Fig. 4.16*f*) with that of the equivalent 10 g/kg WPC pattern (Fig. 4.16*b*) showed substantial differences in the way that β-lactoglobulin formed disulphide-bonded aggregates at the two different concentrations, with less dimer at the higher concentration. A number of α-lactalbumin spots across the gel were also observed in the heated 120 g/kg WPC sample, although the intensities of these spots appeared to be much lower than those observed in the heated 10 g/kg WPC solutions (Fig. 4.16*b*).

The patterns for the heated 60 g/kg WPC samples (Fig. 4.16*c, d*) were intermediate between the 10 and 120 g/kg WPC patterns.

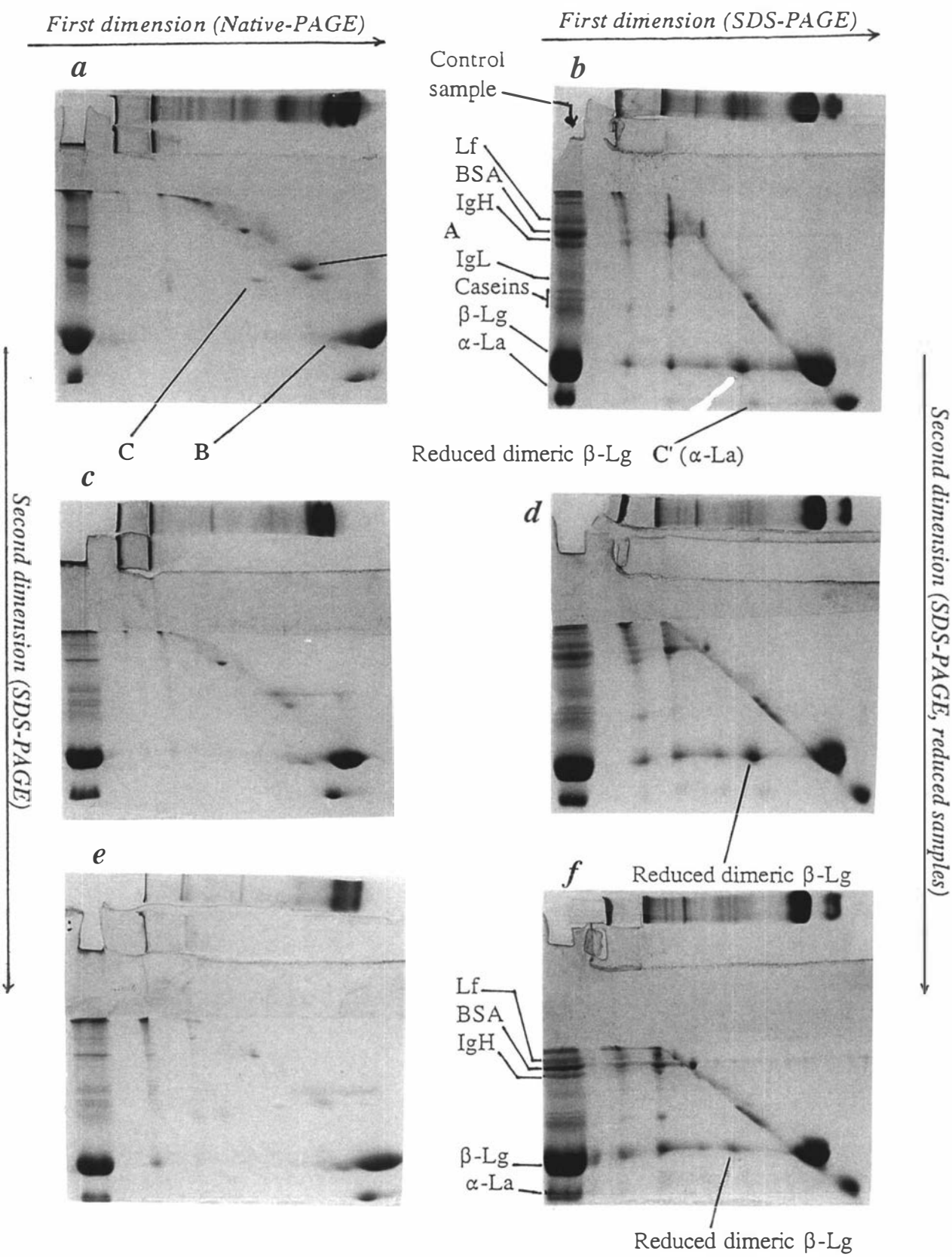


Figure 4.16. 2D-PAGE patterns of 10 (*a, b*), 60 (*c, d*) and 120 (*e, f*) g/kg WPC solutions, heated at 75 °C for 30, 8 and 4 min respectively. Samples were appropriately diluted with sample buffer and then analysed as described in the Fig. 4.4 legend. Refer to Figs 4.2 and 4.3 for abbreviations. A, B, C & C' are as described in the text.

4.8. Analysis of heated WPC solutions by size exclusion chromatography

The loss of native-like proteins on heating was also investigated using size exclusion chromatography (SEC) on a Superose 6HR column. The elution profile of unheated WPC solutions showed four peaks (Fig. 4.17*a*,i). The molecular weights of the material corresponding to each peak were estimated by comparing the eluting time of each peak with that of known molecular weight standards. The molecular weights corresponding to these peaks were approximately $>1x10^6$, $\sim7x10^4$, $\sim2x10^4$ and $\sim 5x10^4$ Da, for peaks 1, 2, 3 and 4 respectively. The composition of the proteins in these peaks was determined for a sample of 60 g/kg WPC solution heated for 4 min. Fractions were collected and analysed using PAGE (Fig. 4.18). Peak 1 represented "soluble" protein aggregates defined as those aggregates that were able to pass through a 0.22 μm filter paper (Fig. 4.18, slot 3). The PAGE results (Fig. 4.18*a, b*) showed that this peak contained no monomeric proteins migrating into the resolving gels of the native and SDS gels. It appears that some material was caught on top and within the stacking gels. The material that eluted between peaks 1 and 2 (Fig. 4.18*a, b*, slot 4) showed a similar patterns to that of peak 1. When the material corresponding to peak 1 and that eluting between peaks 1 and 2 was reduced and then run on an SDS gel (Fig. 4.18*c*), monomeric protein bands were observed. Peak 1 contained mainly β-lactoglobulin and α-lactalbumin. The material that eluted between peaks 1 and 2 contained almost all of the whey protein components. It is clear from these results that the material corresponding to peak 1 and that eluting between peaks 1 and 2 was disulphide-linked aggregates, as indicated by protein bands showing up on the SDS gel after reduction of the samples (Fig. 4.18*c*), but not on native or SDS gels (Fig. 4.18*a, b*). Peak 2 (Fig. 4.18, slot 5) contained mainly BSA and immunoglobulin, whereas peak 3 (Fig. 4.18, slot 6) was made up mainly of β-lactoglobulin and peak 4 (Fig. 4.18, slot 7) contained both β-lactoglobulin and α-lactalbumin.

The size of peaks 2-4 decreased progressively with heating time indicating continuous loss of native-like protein on heating. The elution profiles of 10 g/kg WPC solutions (Fig. 4.17*a*) showed small decreases in the sizes of peaks 2-4 during heating. The elution profiles of 120 g/kg WPC solutions (Fig. 4.17*c*) showed relatively larger decreases in the sizes of peaks 2-4 with heating time, indicating greater loss of native-like protein with heating time. The decreases in the sizes of peaks 2-4 of heated

60 g/kg WPC solutions (Fig. 4.17*b*) were intermediate between those of 10 and 120 g/kg WPC solutions.

For all the heated WPC solutions, the decrease in peak 2 was faster than that of peak 3 or 4. These results were consistent with the electrophoresis results i.e. the loss of native-like BSA was faster than that of either β-lactoglobulin or α-lactalbumin.

The relative decreases in peaks 2, 3 and 4 can be used as an indication of the rates of loss of native proteins in heated 10, 60 and 120 g/kg WPC solutions. This rate was found to increase with increasing WPC concentration, which confirms the electrophoresis results (Figs 4.5 & 4.7). It is difficult, however, to relate the changes in peak 1 (corresponding to aggregates) to the extent of aggregation at different concentrations, as there was no consistent trend in changes in the size of peak 1. When an aliquot of a solution of solutes with a continuous range of molecular size is passed down a column of a size-exclusion resin matrix with ideal properties, an artefact peak is apparent at the void volume of the column where all the molecules with molecular sizes greater than the exclusion volume of the column matrix elute as a single peak. The width of this peak is related to diffusion characteristics of the solutes. The observed curves are the superposition of this void volume peak with the fore-shortened distribution of quantity versus molecular size (Singh & Creamer, 1991*b*). The absorbance of large molecules and complexes is a combination of light-scattering (measured as turbidity), which increases very rapidly with molecular size, and true chromatophore absorbance. Thus, the size of the early peaks (peak 1, Fig. 4.17) may not truly represent quantity (Singh & Creamer, 1991*a,b*). In addition, differing amounts of aggregated material could be removed by the pre-analysis filtration step used before injecting samples into the column.

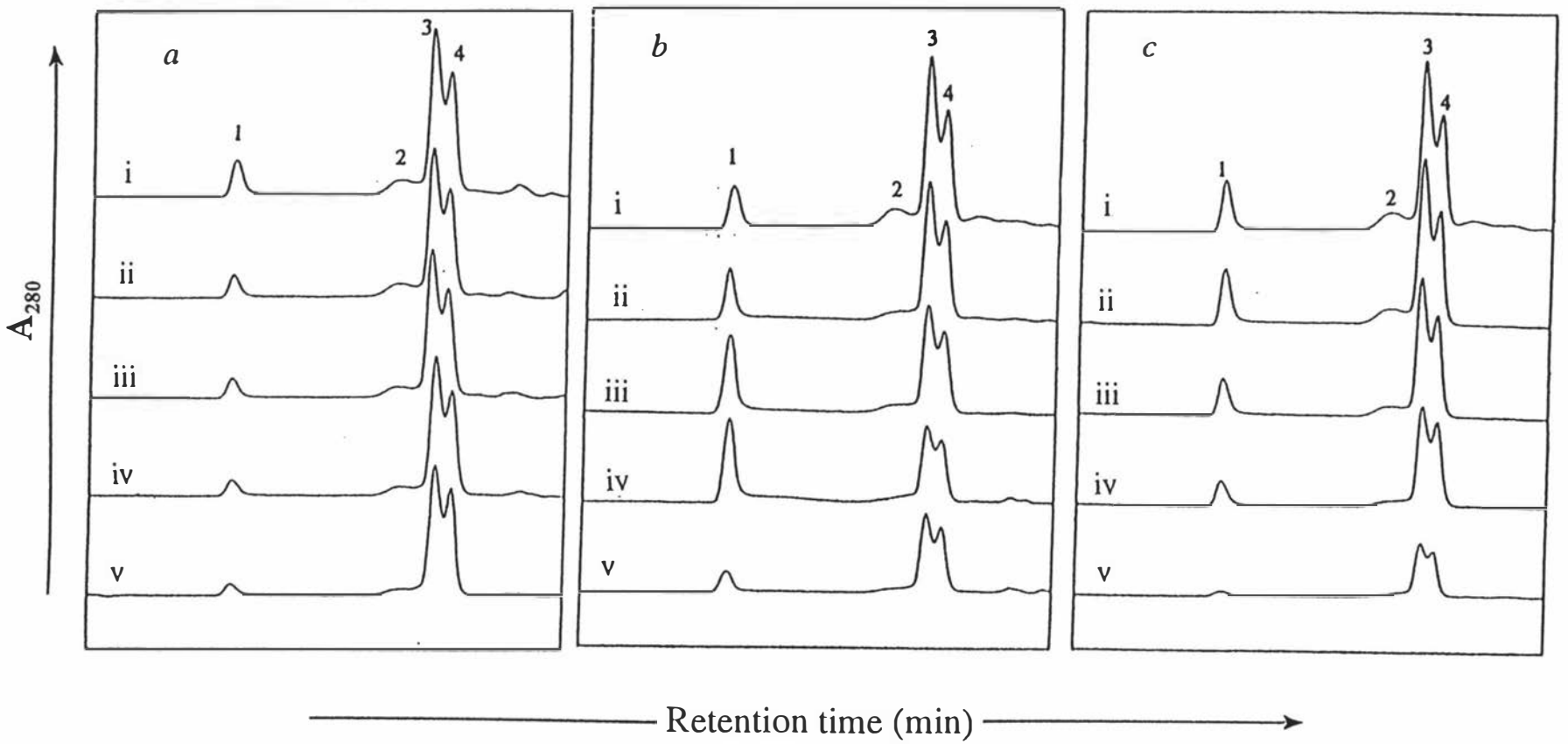


Figure 4.17. SEC elution profiles of heated WPC solutions. Superose 6HR 10/30 column, flow rate 0.4 ml/min, eluent: 20 mM imidazole buffer (pH 6.9, 50 mM NaCl). 10 (*a*), 60 (*b*) and 120 (*c*) g/kg WPC solutions, heated at 75 °C for 0 (i), 2 (ii), 4 (iii), 6 (iv) and 10 (v) min. The samples were diluted with the buffer so that the final WPC concentration was 0.002 g/kg, and filtered through a 0.22 μm filter before analysis.

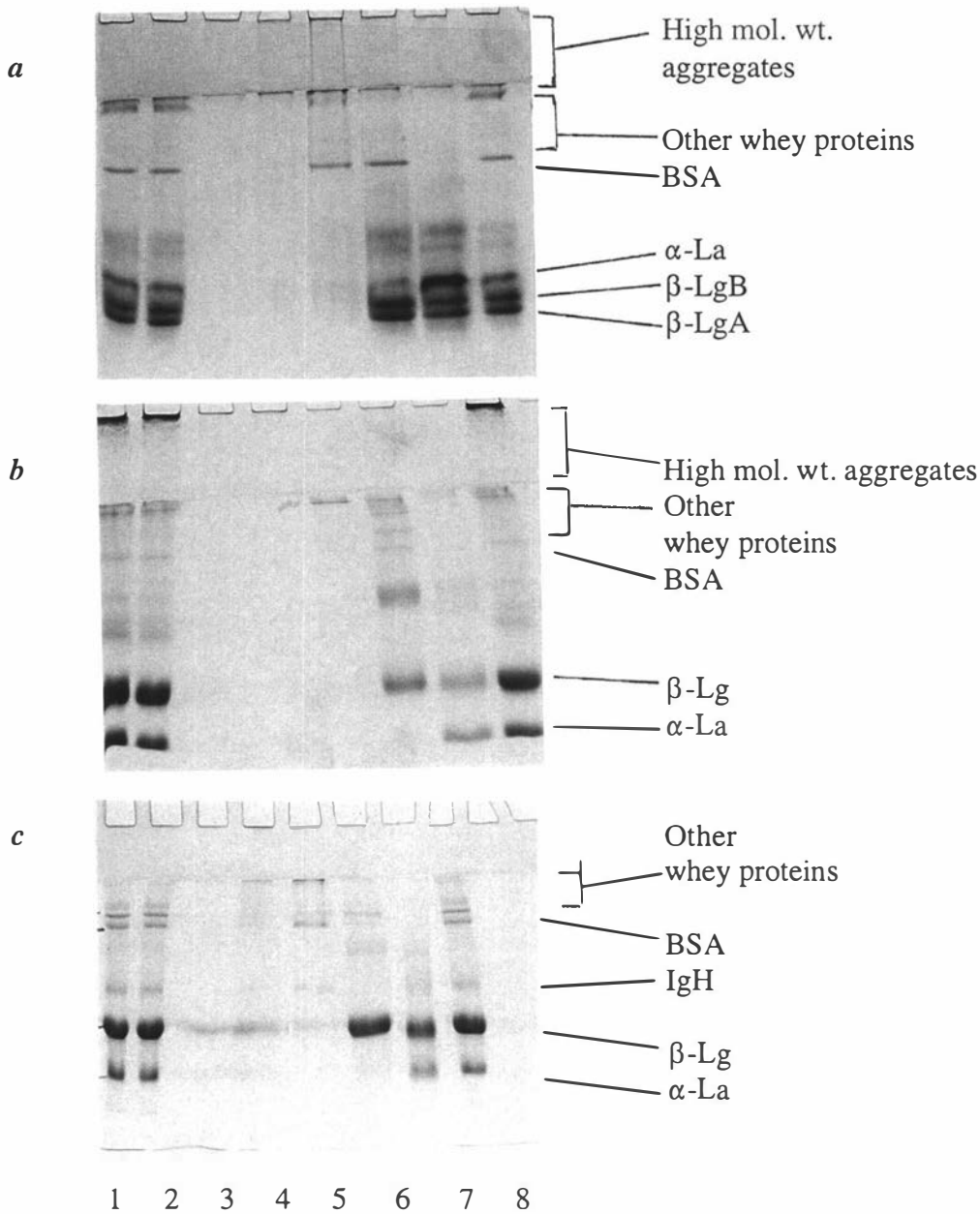


Figure 4.18. Electrophoretic patterns of the SEC peaks obtained from 60 g/kg WPC solutions, heated for 4 min (Fig. 4.17*b*, iii). The samples were collected and analysed using native- (*a*) and SDS-PAGE before (*b*) and after (*c*) reduction. The samples were: control (i.e. heated 60 g/kg WPC solution - slots 1, 2 & 8); peak 1 (slot 3); material that eluted between peaks 1 and 2 (slot 4); peak 2 (slot 5); peak 3 (slot 6) and peak 4 (slot 7). Refer to Figs 4.2 and 4.3 for abbreviations.

4.9. Analysis of heated WPC solutions by ultracentrifugation

Heated WPC solutions were ultracentrifuged at 90,000 × g for 60 min in order to remove large "insoluble" protein aggregates or gelled material (Section 3.4). The supernatants obtained were analysed using 1D-PAGE. The native- and SDS-PAGE patterns of the supernatants were similar to those of the corresponding WPC solutions before centrifugation. Figure 4.19 shows the native- and SDS-PAGE patterns of the supernatants obtained from centrifugation of the 120 g/kg WPC solutions (see Figs 4.5 I*c* and II*c* for PAGE patterns before centrifugation). From comparison of the native-PAGE patterns of the heated WPC solutions (Fig. 4.5 I*c*) with those of the corresponding supernatant samples (Fig. 4.19*a*) it is clear that the patterns were generally similar in both cases, except that the supernatant samples had less material caught on top of the resolving gel and within the stacking gel. This showed that a large proportion of aggregates, presumably the larger ones, were sedimented under the centrifugation conditions used. The SDS-PAGE patterns of the supernatant (Fig. 4.19*b*) also showed similar patterns to those of the corresponding WPC samples (Fig. 4.5 II*c*).

The SDS-PAGE of the reduced supernatant samples (Fig. 4.19*c*), however, showed different patterns from those of their corresponding heated WPC solutions (Fig. 4.6). As discussed earlier (Section 4.5), typical SDS-PAGE patterns of the reduced samples of heated WPC solutions showed that virtually all of the previously aggregated material was dispersed and entered the gels as monomeric protein and the band intensities did not alter with heat treatment (Fig. 4.6). The SDS-PAGE patterns of the reduced supernatant samples (Fig. 4.19*c*) showed that the band intensities decreased with heating time at a similar rate to that shown by the SDS-PAGE patterns of the corresponding WPC solutions before reduction (Fig. 4.5 II*c*) but not after reduction (Fig. 4.6). However, it was noted that aggregated material could not be seen in the stacking gels (Fig. 4.19*c*) and that the protein bands were slightly more intense than those in the non-reduced samples. This suggested that the aggregated material in the supernatant (seen in the slots in Fig. 4.19*b*) was disulphide-linked. The SDS-PAGE patterns of the supernatant samples also showed that the minor protein components (BSA, IgH and lactoferrin) disappeared almost completely after heating for 6 min (Fig.

4.19*b*, slot 5). Upon reduction of the same supernatant samples (Fig. 4.19*c*) the aggregated material that was caught on top of the resolving gel and within the stacking gel (Fig. 4.19*a*) resolved to give bands that corresponded to BSA and IgH in all heated samples, even in samples that were heated for 15 min. This indicates that BSA and IgH were involved in the formation of disulphide-linked aggregates that were not removed by centrifugation.

Quantitation of the PAGE results for the supernatants showed that the loss of native-like β-lactoglobulin was similar to that of SDS-monomeric protein at all WPC concentrations. However, the loss of reducible proteins was slower from the 120 g/kg WPC solutions than from the 60 g/kg solutions (Fig. 4.20). The results for α-lactalbumin and BSA (not shown) showed similar trends. After a given heat treatment, the amounts of total reducible protein in the supernatant were slightly higher than the amounts of SDS- or native-like monomers.

It appears that both β-lactoglobulin and α-lactalbumin were equally involved in the formation of large sedimentable aggregates. Both proteins seemed to be removed from solution by centrifugation in the same way. This is probably attributable to these proteins complexing with each other to form large aggregates. It was interesting to observe that there were few hydrophobic aggregates in the supernatant, even in 120 g/kg WPC solutions. This suggests that the hydrophobically associated material largely contributed to the formation of the large aggregates, supporting the conclusions from the 2D-PAGE analysis (Fig. 4.16*f*).

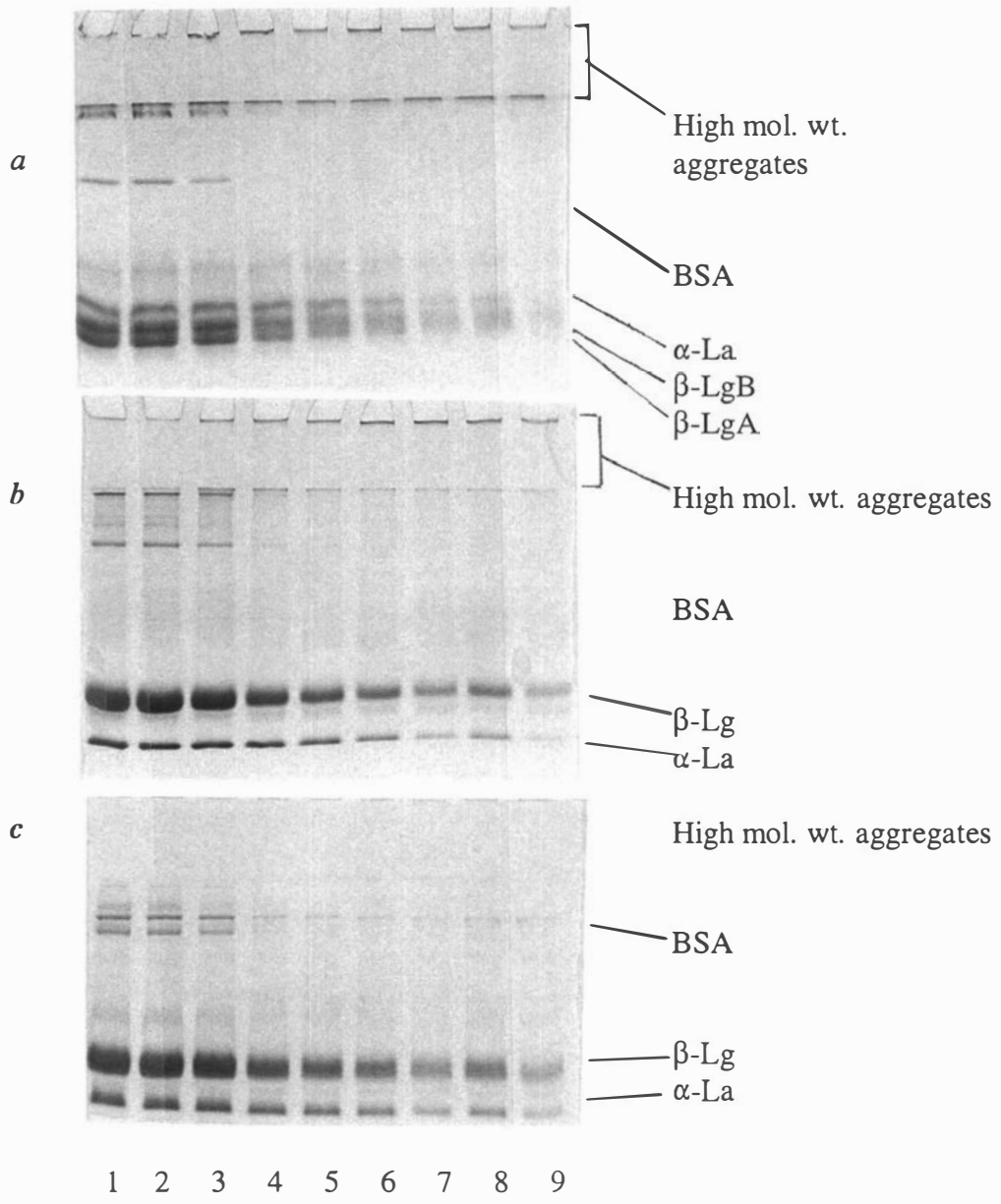


Figure 4.19. Native- (*a*) and SDS-PAGE (non-reduced samples, *b*; reduced samples, *c*) patterns of the supernatants obtained from centrifugation of heated 120 g/kg WPC solutions. WPC solutions were heated at 75 °C for 0 (slots 1 & 2), 2 (slot 3), 4 (slot 4), 6 (slot 5), 8 (slot 6), 10 (slot 7), 12 (slot 8) and 15 (slot 9) min. The samples were the same as those analysed in Fig. 4.5*c*.

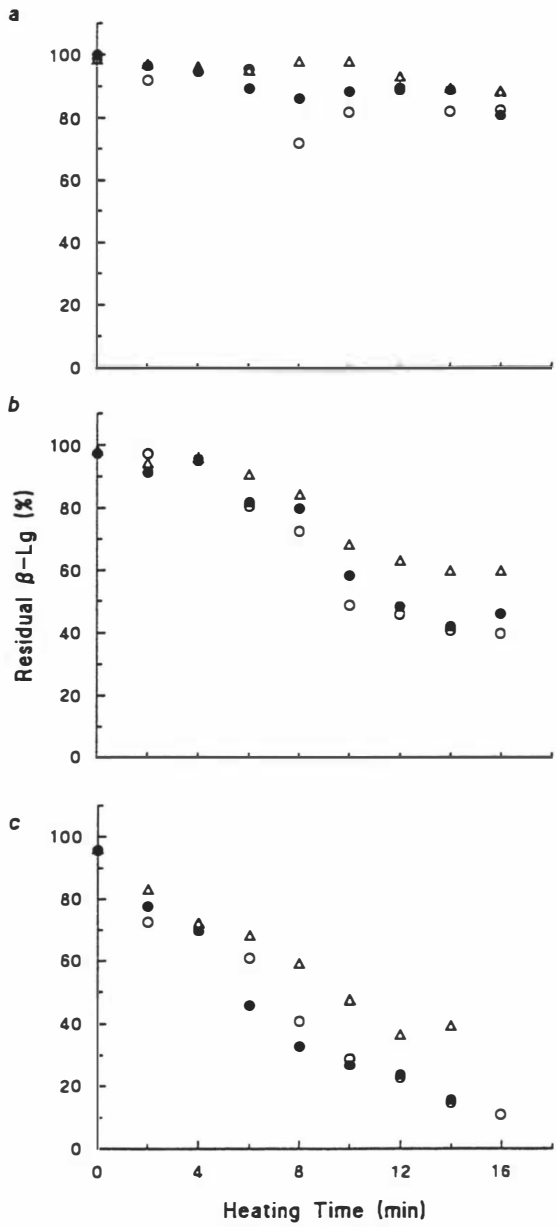


Figure 4.20. Loss of native-like (●), SDS-monomeric (○) and total reducible (Δ) β-lactoglobulin from the supernatant obtained from centrifugation of 10 (*a*), 60 (*b*) and 120 (*c*) g/kg WPC solutions.

Although it was possible to elucidate the composition and nature of the soluble aggregates that remained in the supernatant after centrifugation, it was not easy to estimate their likely size ranges. The protein aggregates in a WPC solution grow in size with heating time (Steventon *et al.*, 1994). Under the conditions used for the centrifugation, it was estimated that particles with a sedimentation coefficient of less than 12S would remain in the supernatant after centrifugation for 60 min. The sedimentation coefficient is determined by many factors such as the density of the particle, the viscosity of the medium and the centrifugal force. Some of these parameters were unknown and hence an accurate determination of the cut-off particle size was not possible. However, the native-PAGE results (Fig. 4.19*a*) indicate that the supernatant contained aggregates that could not enter the stacking gel and hence had a molecular weight of ~ 10^6 Da.

4.10. Insoluble aggregates

Ultracentrifugation of the WPC solutions (both heated and unheated) resulted in the separation of the large protein aggregates as pellets at the bottom of the centrifuge tubes. In this section, the composition of these aggregates, which are referred to as insoluble aggregates, is described and discussed.

4.10.1. Unheated WPC solutions

When the unheated WPC solutions were centrifuged (90,000 × *g*), a considerable amount (about 8-10% of the total solid content of the WPC solution) of material was sedimented (Fig. 4.22). SDS and SDS-reduced PAGE (Fig. 4.21) analyses showed that the pellet contained all the whey protein components, but the minor components (immunoglobulin, BSA and lactoferrin) and caseins were present at relatively higher concentrations. Comparison of the SDS-PAGE patterns of the unheated 120 g/kg WPC solution (Fig. 4.21, slots 1 & 2) with its pellet (Fig. 4.21, slots 3 & 4), indicated that the pellet showed a considerable amount of material caught on the top and within the stacking gel. When the samples were reduced before electrophoresis (Fig. 4.21, slots 7 & 8), the material that was caught in the stacking gel resolved almost completely, intensifying the monomeric protein bands. This suggests that the pellet obtained from

the unheated WPC solution contained aggregated proteins that were linked by disulphide bonds. It also showed that a small amount of material could not enter the resolving gel even after reduction (Fig. 4.21, slots 7 & 8), indicating that there may have been some protein aggregates linked by non-reducible covalent bonds. It was interesting to see the presence of a κ-casein band in the reduced samples (Fig. 4.21, slots 7 & 8) but not in the non-reduced samples (Fig. 4.21, slots 3 & 4). Some of this κ-casein was complexed with whey proteins, e.g. β-lactoglobulin, by disulphide bonds as well as being in disulphide bonded κ-casein aggregates. These whey protein/κ-casein complexes were probably formed by the heat treatment used during the manufacture of the whey that was used to make the WPC.

Steventon *et al.* (1994) reported that most commercial WPC powders exhibit between 30 and 50 g/kg of insoluble material consisting of both lactose and aggregated protein. This reported range is much higher than the range (8-10 g/kg of insoluble material) determined for the WPC product used in this study.

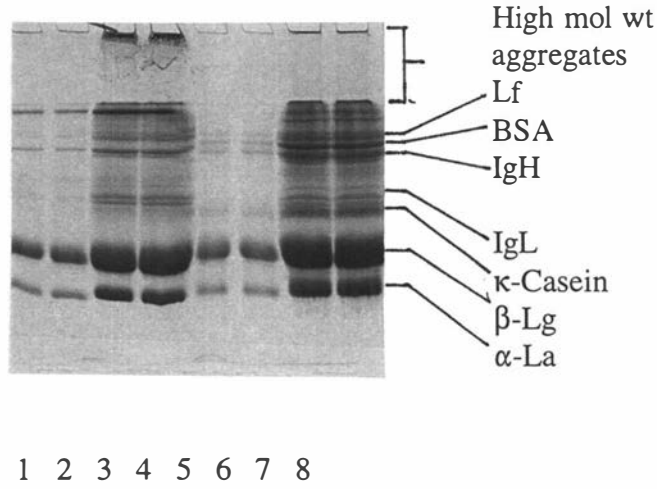


Figure 4.21. SDS-PAGE patterns of 120 g/kg WPC solutions and the pellets from the same solutions. Slots 1 & 2, unheated 120 g/kg WPC solution; slots 3 & 4, pellet from unheated 120 g/kg WPC solution; slots 5 & 6, unheated reduced 120 g/kg WPC solution; slots 7 & 8, unheated reduced pellet from 120 g/kg WPC solution.

4.10.2. Heated WPC solutions

Accumulation of insoluble aggregates during heating

The quantities of insoluble protein aggregates formed during the heating of WPC solutions were determined using ultracentrifugation as described in Section 3.5. When 10 g/kg WPC solutions were heated at 75 °C, the dry weight of pellet, i.e. insoluble aggregates, did not appear to change significantly with heating time, indicating that no extensive aggregation had taken place (Fig. 4.22). In 60 g/kg WPC solutions, the dry weight of the insoluble aggregates increased slowly with heating time and reached about 30% after 35 min of heating. In 120 g/kg WPC solutions, there was a relatively rapid increase in the dry weight of the insoluble aggregates during the first 10 min of heating. After this the increase was relatively slow. This may suggest that two different types of aggregation reactions had taken place during heating of the 120 g/kg WPC solution.

The PAGE results (Figs 4.5 & 4.7) showed that the native-like BSA (and other minor whey protein components) was lost almost completely after heating for about 6 min and the loss of these protein components resulted in the formation of predominantly disulphide-linked aggregates (Fig. 4.15). The loss of β-lactoglobulin and α-lactalbumin became more apparent after heating for 6 min. It appears, therefore, that the initial rapid increase in the pellet dry weight was mainly due to the formation of disulphide-linked aggregates by the BSA and the other minor protein components, plus small aggregates (probably hydrophobic, Fig. 4.13) formed by β-lactoglobulin and α-lactalbumin. Hoffmann *et al.* (1997) studied the weight-averaged molecular masses of aggregates formed in 10, 30, 50, 75 and 100 g/l β-lactoglobulin solutions during heating at 65 °C. They observed that in β-lactoglobulin solutions other than 100 g/l there were very few changes in the weight-averaged molecular masses of β-lactoglobulin during heating up to 24 h. In the 100 g/l β-lactoglobulin solutions, however, there was a large increase in the weight-averaged molecular masses of aggregates, reaching a plateau similar to that observed in Fig. 4.22. In the 120 g/kg WPC solutions, the weight-averaged molecular masses of protein aggregates probably increased in a similar way to that observed by Hoffmann *et al.* (1997).

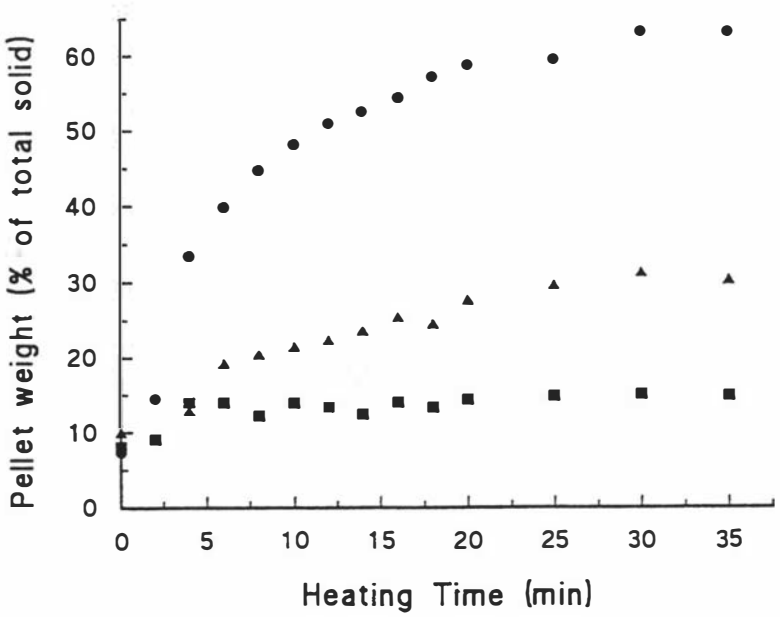


Figure 4.22. The dry weight (% total solid) of the insoluble aggregates formed during heating of 10 (■), 60 (▲) and 120 (●) g/kg WPC solutions at 75 °C.

Variations in the composition of protein aggregates during heating

Centrifugation of the heated WPC solutions resulted in two main fractions: the soluble and insoluble fractions. The soluble fraction consisted mainly of monomeric proteins and relatively small aggregates that remained in the supernatant after centrifugation, as discussed in Section 4.9. The insoluble fraction, i.e. the sedimented pellet, consisted of relatively large protein aggregates (some of which were present in the WPC powder), gelled protein in some of the heated samples (e.g. 120 g/kg WPC samples formed a gel after heating for 8 min at 75 °C) and a small amount of soluble protein that was trapped in the pellet. Figure 4.23 illustrates the different components of these two fractions.

Soluble fraction (supernatant)
- monomeric protein
- small protein aggregates

Insoluble fraction (pellet)
- aggregates that exist in WPC
- large protein aggregates
- gelled protein
- soluble protein

Figure 4.23. Components of the soluble and insoluble fractions of the heated WPC solutions after centrifugation.

In order to elucidate how individual whey protein components become incorporated into the insoluble fraction during heating, the change in the composition of the insoluble aggregates with heating time was estimated from the SDS-PAGE results for the reduced supernatant samples obtained from heated 120 g/kg WPC solutions. Quantitation of the protein bands on the SDS gel shown in Fig. 4.19 gave the relative quantities of each protein component remaining in the supernatant. The rest of the proteins were assumed to have been incorporated into the insoluble fraction. For the purpose of this estimation, the following assumptions were made.

- Based on the pellet weights for samples of unheated WPC solutions (Fig. 4.22), the amount of protein aggregates that were already present in the unheated WPC solution, i.e. x-component, was assumed to be 10% of the total protein content of the WPC solution. The PAGE results (Fig. 4.21) showed that the x-component consisted of all whey protein components and casein. For the purpose of this discussion, this material is considered as only one component.
- β-Lactoglobulin and α-lactalbumin were assumed to consist of 50 and 25% respectively of the total protein content of the WPC solution. BSA and other minor whey protein components (e.g. immunoglobulin and lactoferrin) were

assumed to make up the other 25% of the total protein content of the WPC solution.

- Based on PAGE results (Fig. 4.19), it was assumed that the loss of other minor whey protein components occurred at the same rate as that of BSA. The calculation of their relative proportions present in the insoluble fraction was based solely on the quantitation of the loss of BSA.
- It was also assumed that the amount of soluble protein trapped in the pellet was negligible, and was therefore ignored in the calculations.

Based on these assumptions and quantitation of the PAGE results for the reduced supernatant samples, an attempt was made to elucidate the relative proportions of each protein component in the pellet. Figure 4.24 shows the relative proportion of the protein components in the pellet.

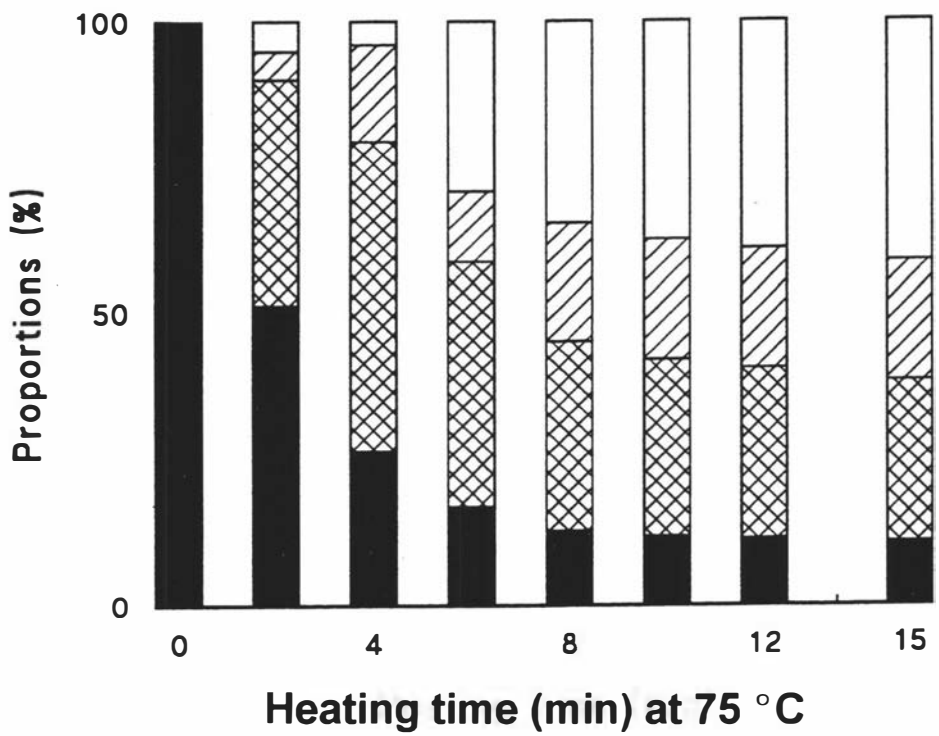


Figure 4.24. Relative proportions of each whey protein component in the sedimented pellets obtained from centrifugation of heated WPC solutions. Components: x-component, ■; BSA and the other minor whey protein components, ⊠; α-lactalbumin, ▨; β-lactoglobulin, □.

The pellet obtained from centrifugation of the unheated WPC solution consisted of the x-component only (Fig. 4.24). The contribution of the x-component to the overall composition of the pellet decreased to ~ 50% after heating for 2 min, and continued to decrease with further heating, reaching a minimum of 10% after heating for about 8 min. The pellet obtained from centrifugation of the WPC solution heated for 2 min consisted of ~ 50% heat-induced aggregates. These aggregates consisted largely of BSA and the other minor whey protein components with very minimal contribution from either β-lactoglobulin or α-lactalbumin. With further heating (up to 6 min), the proportion of heat-induced aggregates in the pellet continued to increase and the composition was largely made up of BSA and the other minor whey protein components with an increasing contribution from both β-lactoglobulin and α-lactalbumin (Fig. 4.24). With further heating (after 8 min), the proportion of heat-induced aggregates remained at a maximum of ~ 90%, but the proportions of each protein component appeared to be changing. The proportions of β-lactoglobulin and α-lactalbumin in the pellet continued to increase with heating time, resulting in decreases in the proportions of BSA and the other minor whey proteins.

These results showed that, when the WPC solutions were heated, the initial insoluble aggregates formed at the early stages of heating, up to ~ 4 min, consisted primarily of BSA and the other minor whey protein components. β-Lactoglobulin and α-lactalbumin had very little involvement in the aggregate formation at this stage. They became more involved at the later stages of heating, i.e. after 6 min. β-Lactoglobulin became the predominant pellet component at the later stages of heating (after ~ 8 min). These results are consistent with the PAGE results (Figs 4.5 & 4.7) where the BSA and other minor whey protein components were shown to have become almost completely lost from the SDS-PAGE gel after heating for 6 min.

It was observed that 120 g/kg WPC samples heated for 8 min at 75 °C formed a gel. It was estimated, based on the PAGE results, that, after heating a 120 g/kg WPC solution for 8 min, more than 60% of the total protein of the WPC solution was incorporated in the pellet. This figure agrees well with the results for the dry weight of the pellets (Fig. 4.22) where ~ 57% of the total dry weight of the WPC solutions was recovered in the pellet. The protein components of the pellet included ~ 52% of the

total β-lactoglobulin, ~ 60% of the total α-lactalbumin and almost all (~ 97%) of the BSA and other minor whey protein components. Figure 4.13 showed that, in the WPC sample heated for 8 min, ~ 50% of the β-lactoglobulin that had lost the native-like state was involved in the formation of hydrophobic aggregates. From this estimation, only ~ 26% (half of the estimated 52%) of the β-lactoglobulin had formed disulphide-linked aggregates. Thus, the formation of a gel by the 120 g/kg WPC solution after heating for 8 min at 75 °C required a significant contribution from β-lactoglobulin but it appears that half of the β-lactoglobulin was monomeric and involved in hydrophobically associated aggregates.

4.11. Discussion

In this chapter, electrophoresis, size exclusion chromatography and sedimentation analysis were used to characterise the effects of heating on WPC solutions. Both native-PAGE and size exclusion chromatography measured the loss of native proteins from the system (Figs. 4.5, 4.7 & 4.17) whereas sedimentation analysis measured the accumulation of "final" products from the polymerisation reactions. SDS-PAGE measured the amounts of hydrophobic and disulphide linked aggregates formed in WPC solutions during heating. The 2D-PAGE characterised the intermediate species. The aggregation was shown to start from as early as 2-4 min of heating at 75 °C, as indicated by the appearance of aggregate bands on native- and SDS-PAGE gels (Fig. 4.5 - on top of stacking gel, slots 3 & 4). These aggregates were shown to consist of β-lactoglobulin, α-lactalbumin, BSA and immunoglobulin polymerised via hydrophobic interaction and disulphide cross-linkages as indicated by 2D PAGE (Fig. 4.16).

The possible protein interactions leading to aggregation and gelation during the heating of WPC were examined by 2D-electrophoretic methods. Such methods have been previously used in dairy research to gain greater resolution of complex mixtures such as the peptides in cheese or the minor components of milk (Shimazaki & Sukegawa, 1984; Holt & Zeece, 1988). The differentiation of peptides or proteins cross-linked by disulphide bonds from other peptides has been done using 2D SDS-PAGE with an intermediate reduction step (Zeece *et al.*, 1989), but combinations of native- and SDS-PAGE systems have not been used to distinguish hydrophobically

associated protein aggregates from native-like proteins or from disulphide-bonded aggregates in a heat-treated mixture of proteins.

The present studies clearly show that, when WPC solutions were heated at 75 °C, aggregates, consisting of β-lactoglobulin, α-lactalbumin, BSA, caseins and minor whey proteins, were formed and that both hydrophobic interactions and disulphide cross-linkages were involved in forming these aggregates. The extent of aggregation and the nature of the stabilising forces involved in the formation of the aggregates were affected by the WPC concentration at heating. The amount of hydrophobically associated β-lactoglobulin (the protein that is stable under the conditions of separation in native-PAGE, i.e. ~ 20 °C and ~ pH 8.5, but not in SDS-PAGE) increased with concentration, especially between 60 and 120 g/kg WPC (Fig. 4.5*c*). However, the quantity of hydrophobically associated α-lactalbumin increased to a smaller extent with concentration. It was estimated that, when 120 g/kg WPC was heated at 75 °C for 12 min, about 80% of the total β-lactoglobulin was aggregated, of which about 50% was associated hydrophobically (Fig. 4.7*a*). By comparison, about 78% of the total α-lactalbumin was present as aggregates, of which 25% was linked by hydrophobic interactions (Fig. 4.7*b*).

It was clear that, when the WPC solutions were heated at 75 °C, 1:1 disulphide-linked complexes of α-lactalbumin and β-lactoglobulin were observed predominantly in the 10 g/kg WPC solution (Fig. 4.16*a, b*), but not in the 60 g/kg and 120 g/kg WPC solutions. The various spots observed in region B (Fig. 4.16*a*) may be attributable to various partially unfolded forms of β-lactoglobulin (Manderson *et al.*, 1998). Such forms of β-lactoglobulin have been implicated in heated solutions of pure protein using differential scanning calorimetry (Qi *et al.*, 1995) and light scattering techniques (Griffin *et al.*, 1993).

The protein content of the WPC used in this study was 810 g/kg and the GMP content was 160 g/kg, so that the "whey protein" content was probably 650 g/kg. The β-lactoglobulin content of the WPC was probably about 600 g/kg, i.e. ~ 72 g/kg solution for the 120 g/kg WPC solution. This compares with concentrations of about 100 g/l of solution for many of the other studies, e.g. McSwiney *et al.* (1994*a, b*), involving β-lactoglobulin alone. When Gezimati *et al.* (1996*a*, *b*, 1997) examined

mixtures of β-lactoglobulin and BSA or α-lactalbumin, they found that β-lactoglobulin and BSA, but not α-lactalbumin, were able to form stable hydrophobic aggregates when the pure protein was heated at 75 °C in a buffer that simulated the WPC environment. When α-lactalbumin was heated with β-lactoglobulin (and probably BSA), it formed stable hydrophobic aggregates (Gezimati *et al.* 1996*b.*, 1997). The results from the 1D-PAGE experiments, which confirmed the differences between the loss of native-like proteins and the loss of SDS-monomeric proteins from the heated 120 g/kg WPC solutions (Fig. 4.5), were as expected from the earlier results involving mixtures of pure proteins (Gezimati *et al.*, 1996*a, b,* 1997). Therefore, it is likely that the protein-protein interactions that lead to the formation of aggregates in heated 120 g/kg WPC solutions are similar to those observed in the model systems used by Gezimati *et al.* (1996*a, b,* 1997).

CHAPTER 5

THE EFFECT OF WPC COMPOSITION ON HEAT-INDUCED AGGREGATION OF WHEY PROTEINS

5.1. Introduction

The manufacturing processes used for the production of whey protein concentrate (WPC) powders are described in Chapter 2. Because of the different treatments given to the milk when processing various cheese and casein products, the compositions of the resultant wheys have been reported to be different (Sienkiewicz & Riedel, 1990). Morr & Foegeding (1990), for example, showed that the protein, lactose and mineral compositions of rennet and lactic acid wheys were different. These differences were related not only to seasonal and geographical factors but also to technological factors. Consequently, WPC powders made by the same process will have different compositions, and properties, because of the starting material. In addition, the use of various membrane technologies to remove lactose and/or minerals from whey during WPC manufacture may contribute to varied compositions among different WPC powders. The differences in composition are likely to influence the functional properties of different WPCs.

Two commercial WPC powders were used in this investigation. ALACEN 342, referred to as "acid WPC", was derived from sulphuric acid whey that was obtained after the manufacture of casein by precipitation with sulphuric acid. ALACEN 392, referred to as "cheese WPC", was derived from "sweet" whey that was obtained after cheese manufacture from whole milk. This chapter describes the protein and mineral compositions, and thermal aggregation behaviour, of the acid and cheese WPCs. The differences in aggregation behaviour are discussed in relation to differences in mineral composition.

5.2. WPC Composition

The WPC powders were analysed for total protein, moisture, fat and mineral contents (Section 3.2). The concentrations of β-lactoglobulin, α-lactalbumin and bovine serum albumin (BSA) were estimated using 1D-PAGE (Section 3.2).

Analysis showed that the acid and cheese WPC powders had similar total protein contents, i.e. 845 and 837 g/kg respectively (Table 5.1). The acid WPC contained more β-lactoglobulin (456 g/kg) and α-lactalbumin (160 g/kg) than the cheese WPC (416 and 149 g/kg respectively). The fat content of the cheese WPC (68 g/kg) was higher than that of the acid WPC (55 g/kg). This was probably because the cheese WPC was derived from whole milk, as opposed to skim milk for the acid WPC. The cheese WPC contained a considerable amount of glycomacropeptide (GMP) (172 g/kg), also measured as part of the total protein, compared with the acid WPC (< 5 g/kg). The GMP content of the cheese WPC was similar to that of rennet WPC, 157 g/kg (Section 4.3).

Mineral analysis showed that there were differences between the mineral contents of the acid and cheese WPC powders (Table 5.1). The amount of Ca in the cheese WPC (3968 mg/kg) was more than double that in the acid WPC (1661 mg/kg). The acid WPC contained almost twice as much K (13,835 mg/kg) as the cheese WPC (7666 mg/kg). The amount of Na in the cheese WPC (2252 mg/kg) was almost three times that in the acid WPC (823 mg/kg). The amount of Mg in the cheese WPC (440 mg/kg) was about four times that in the acid WPC (97 mg/kg). Overall, the cheese WPC had higher Ca, Na, P and Mg contents whereas the acid WPC had a higher K content. The relatively high K content of the acid WPC was due largely to the use of KOH for neutralisation after ultrafiltration/diafiltration (UF/DF) during manufacture. The generally higher mineral content of the cheese WPC was due largely to the higher pH (pH 6.0-6.6) of the starting raw whey, compared with that (pH 4.5) of acid whey. At higher pH values, the whey proteins are negatively charged and the positively charged minerals are bound to them. Because they are bound, they are not removed during the UF/DF processes; hence the high mineral content of the cheese WPC (see Fig. 2.2).

Table 5.1. Composition of acid and cheese WPC powders

Component	Acid WPC (g/kg dry weight)	Cheese WPC (g/kg dry weight)
Total protein	845	837
β-Lactoglobulin	456	416
α-Lactalbumin	160	149
BSA	52	62
GMP	< 5	172
Fat	55	68
Phospholipids	17	21
Mineral content (mg/kg)		
Ca	1661	3968
K	13,835	7666
Na	823	2252
Mg	97	440

Lactose contents were not determined for these products, but, according to the manufacturers, the lactose contents were < 42.2 and 54.7 g/kg for the acid and cheese WPCs respectively. The relatively low lactose content of the acid WPC powder was due mainly to extensive use of UF and DF during manufacture.

5.3. PAGE Analysis of the unheated WPC solutions

Acid and cheese WPC solutions (120 g/kg, pH 6.8) were prepared as described in Section 3.3, and sub-samples were diluted with native or SDS sample buffers, and then analysed using 1D- or 2D-PAGE.

5.3.1. 1D-PAGE

The 1D-PAGE results (Fig. 5.1) showed that the two WPC solutions had similar patterns. The β-lactoglobulin and α-lactalbumin bands in the acid WPC solution (Fig. 5.1*a*, *b*, slot 1) appeared to be more intense than the corresponding bands in the cheese

WPC solution (Fig. 5.1*a, b,* slot 2), whereas the BSA band in the cheese WPC solution appeared to be more intense than that in the acid WPC solution. This observation supported the protein analysis results (Table 5.1) showing that the acid WPC contained more β-lactoglobulin and α-lactalbumin than the cheese WPC whereas the cheese WPC contained more BSA. The region between BSA and α-lactalbumin (Fig. 5.1*a*) or β-lactoglobulin (Fig. 5.1*b*) appeared to contain more material, probably caseins, in the acid WPC than in the cheese WPC. In both native- and SDS-PAGE patterns, there was some material caught on top of the stacking and resolving gels, indicating the presence of high molecular weight disulphide-linked aggregates in both WPC solutions.

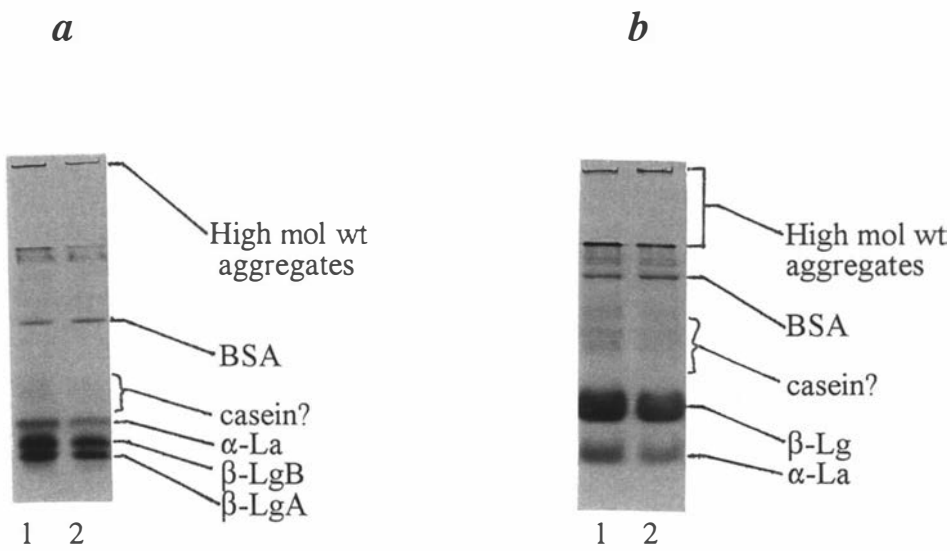


Figure 5.1. 1D-PAGE patterns of unheated 120 g/kg WPC solutions: Native-PAGE (*a*), SDS-PAGE (*b*). The abbreviations were as described in Fig. 4.3. Samples: acid WPC (slot 1) and cheese WPC (slot 2) solutions.

5.3.2. 2D-PAGE

The unheated WPC solutions were also analysed using 2D-PAGE. The native-SDS 2D-PAGE patterns of the unheated WPC solutions (Fig. 5.2 I*a, b*) were similar. In both WPC solutions, some faint spots corresponding to BSA and minor whey protein components were dissociated from the material that was caught on top of the stacking and resolving gels (of the sample gel strip), indicating the presence of some hydrophobically-associated aggregates. The acid WPC solution (Fig. 5.2 I*a*) showed

a faint region that corresponded to the material between BSA and α-lactalbumin in the sample gel strip. This region, which probably corresponded to casein fractions, was faint in the cheese WPC (Fig. 5.2 I*b*). The faint region on the left-hand side of the β-lactoglobulin spot in both 2D gels (Fig. 5.2 I*a, b*) was attributed to glyco-α-lactalbumin, which was also observed in rennet WPC solution (Fig. 4.4).

The SDS 2D-PAGE patterns of the unheated acid WPC solution (Fig. 5.2 II*a*) showed that the region between BSA and β-lactoglobulin of the sample gel strip resolved to give spots that corresponded to α_{s1}-, β- and κ-caseins. The material that was caught on top of the resolving gel (sample gel strip) resolved to give clear spots that were identified as lactoferrin, IgH, BSA and κ-casein. These observations suggest that disulphide-linked aggregates involving these proteins were present in the acid WPC. A faint spot (labelled x) corresponding to β-lactoglobulin appeared to have resolved from the material in the region between BSA and β-lactoglobulin. This probably resolved from disulphide linked β-lactoglobulin dimers that were present in the WPC powders or β-lactoglobulin that may have been complexed with κ-casein.

The SDS 2D-PAGE patterns of the unheated cheese WPC solution (Fig. 5.2 II*b*) showed somewhat similar features to those observed for the acid WPC solution, suggesting that similar types of aggregates existed in the WPC powders. However, the protein spots that resolved from the aggregated material in the sample gel strip of the cheese WPC (Fig. 5.2 II*b*) were very faint. There was no κ-casein in the cheese WPC solution, as κ-casein would have been converted to para-κ-casein and GMP during the cheese manufacture.

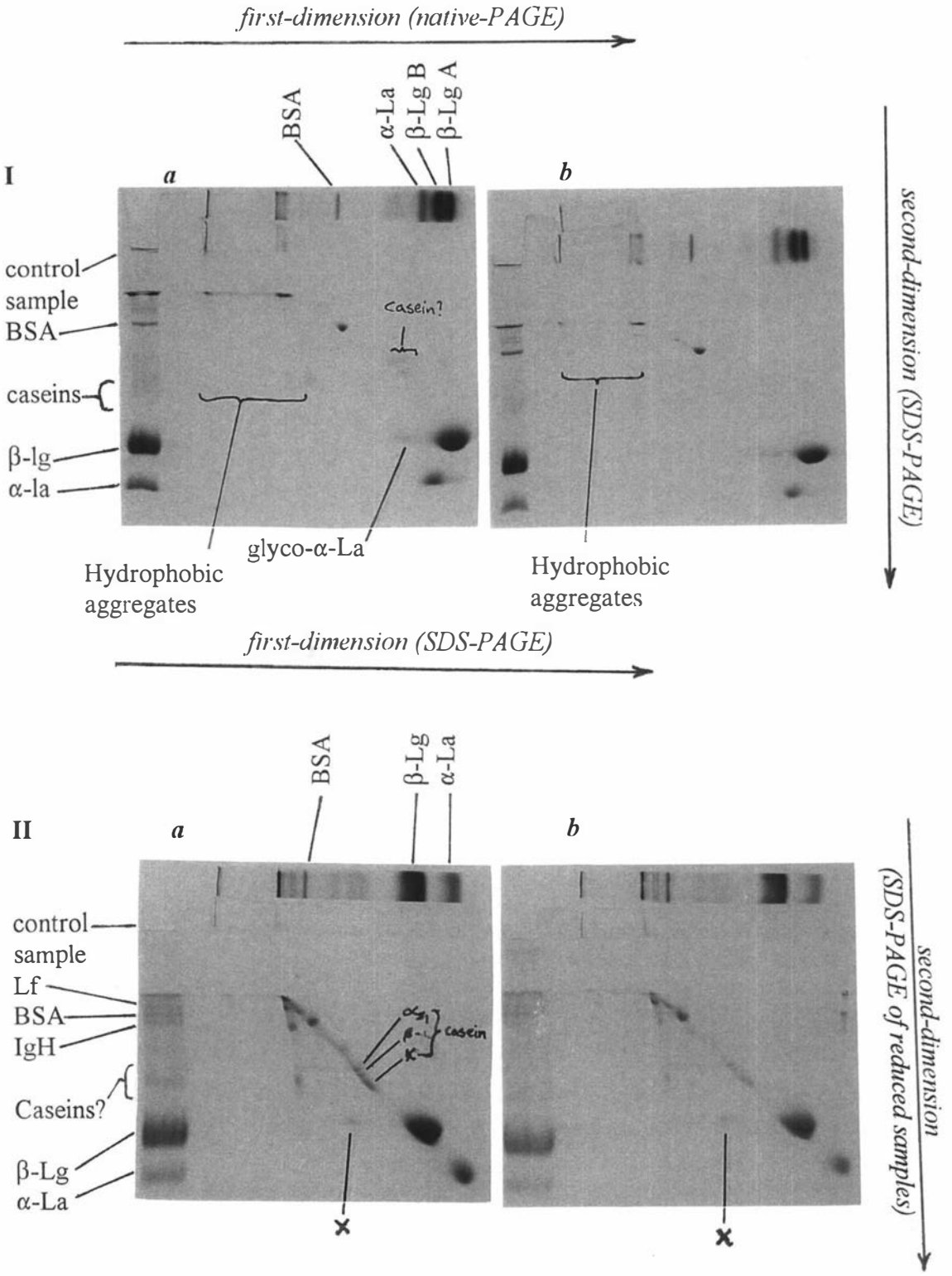


Figure 5.2. 2D-PAGE patterns of unheated acid WPC (*a*) and cheese WPC (*b*) solutions. I. First dimension: native-PAGE, second dimension: SDS-PAGE. II. First dimension: SDS-PAGE, second dimension: SDS-PAGE of the reduced samples. Abbreviations are as described in Fig 4.4.

5.4. Effect of heat treatment on WPC solutions

5.4.1. Loss of protein during heating of WPC solutions

Samples of acid and cheese WPC solutions (120 g/kg, pH 6.8) were heated for different times using the method described in Section 3.3.2. The heated WPC solutions were analysed using native- and SDS-PAGE (Section 3.5.1), ultracentrifugation (Section 3.4) and size exclusion chromatography (Section 3.8).

1D-PAGE patterns of heated acid and cheese WPC solutions

The two WPC solutions showed very similar native-PAGE patterns (Fig. 5.3 I*a, b*). The protein band intensities decreased with increased heating time at 75 °C, indicating continuous loss of native-like proteins, accompanied by concomitant accumulation of high molecular weight aggregates that were caught on top of the stacking and resolving gels. In both WPC solutions, the loss of BSA (and other minor whey proteins) was faster than that of β-lactoglobulin or α-lactalbumin. The intensity of the diffuse region between BSA and α-lactalbumin, particularly in the acid WPC solution (Fig. 5.3 I*a*), appeared to decrease with heating time. In both WPC solutions, the loss of α-lactalbumin was faster than the loss of β-lactoglobulin; the same observation was also made in heated rennet WPC solutions (Section 4.6.1). There were two main differences between the native-PAGE patterns of the heated acid and cheese WPC solutions: the loss of β-lactoglobulin, α-lactalbumin and BSA appeared to be faster from the cheese WPC solutions (Fig. 5.3 I*b*) than from the acid WPC solutions (Fig. 5.3 I*a*) and the accumulation of material on top of the stacking and resolving gels appeared to be more prominent in the heated acid WPC solutions than in the heated cheese WPC solutions.

The SDS-PAGE patterns of the two WPC solutions (Fig. 5.3 II*a, b*) showed some of the features observed on the native gels. The loss of SDS-monomeric proteins was faster from the cheese WPC solutions than from the acid WPC solutions. The accumulation of material on top of the stacking and resolving gels was more prominent in the acid (Fig. 5.3 II*a*) than in the cheese (Fig. 5.3 II*b*) WPC solutions. The region between BSA and β-lactoglobulin was quite different for the two WPC solutions. In the acid WPC solutions (Fig. 5.3 II*a*), this region appeared to increase in intensity with

heating time. The samples heated beyond 4 min showed several distinctive bands in this region which were probably "intermediate" protein species (e.g. β-lactoglobulin dimers) that were formed during heating. These bands, however, were not seen in the SDS gel of the heated cheese WPC solutions (Fig. 5.3 II*b*). The amount of material in this region appeared to decrease with heating time in the cheese WPC solutions.

In conclusion, these results showed that on heating at 75 °C the loss of proteins from the cheese WPC solutions was faster than that from the acid WPC solutions. The formation of intermediate protein species and the accumulation of protein aggregates on top of the stacking and resolving gels during heating were more prominent in the acid WPC solutions than the cheese WPC solutions.

Quantitation of the loss of protein from acid and cheese WPC solutions during heating

The loss of protein from heated WPC solutions as measured by native- and SDS-PAGE was quantified using the method described in Section 3.5.1. Figure 5.4 shows the loss of native-like and SDS-monomeric proteins during heating. Each data point is an average of three different measurements (i.e. results from three different replicate experiments). The error bars indicate the variations in the measured values. The loss of native-like β-lactoglobulin, α-lactalbumin and BSA (Fig. 5.4*a, c, e*) from the heated cheese WPC solutions was considerably faster than the loss of the same proteins from the acid WPC solutions. The loss of SDS-monomeric proteins, however, was only slightly faster from the cheese WPC solutions (Fig. 5.4*b, d, f*). This suggests that, although the loss of native-like proteins was considerably faster from the cheese WPC solutions than the acid WPC solutions, the extent to which disulphide-linked aggregates were formed during heating was probably similar in the two WPC solutions. More hydrophobically-associated aggregates were probably formed in the heated cheese WPC solutions, as indicated by faster rates of loss of native-like protein.

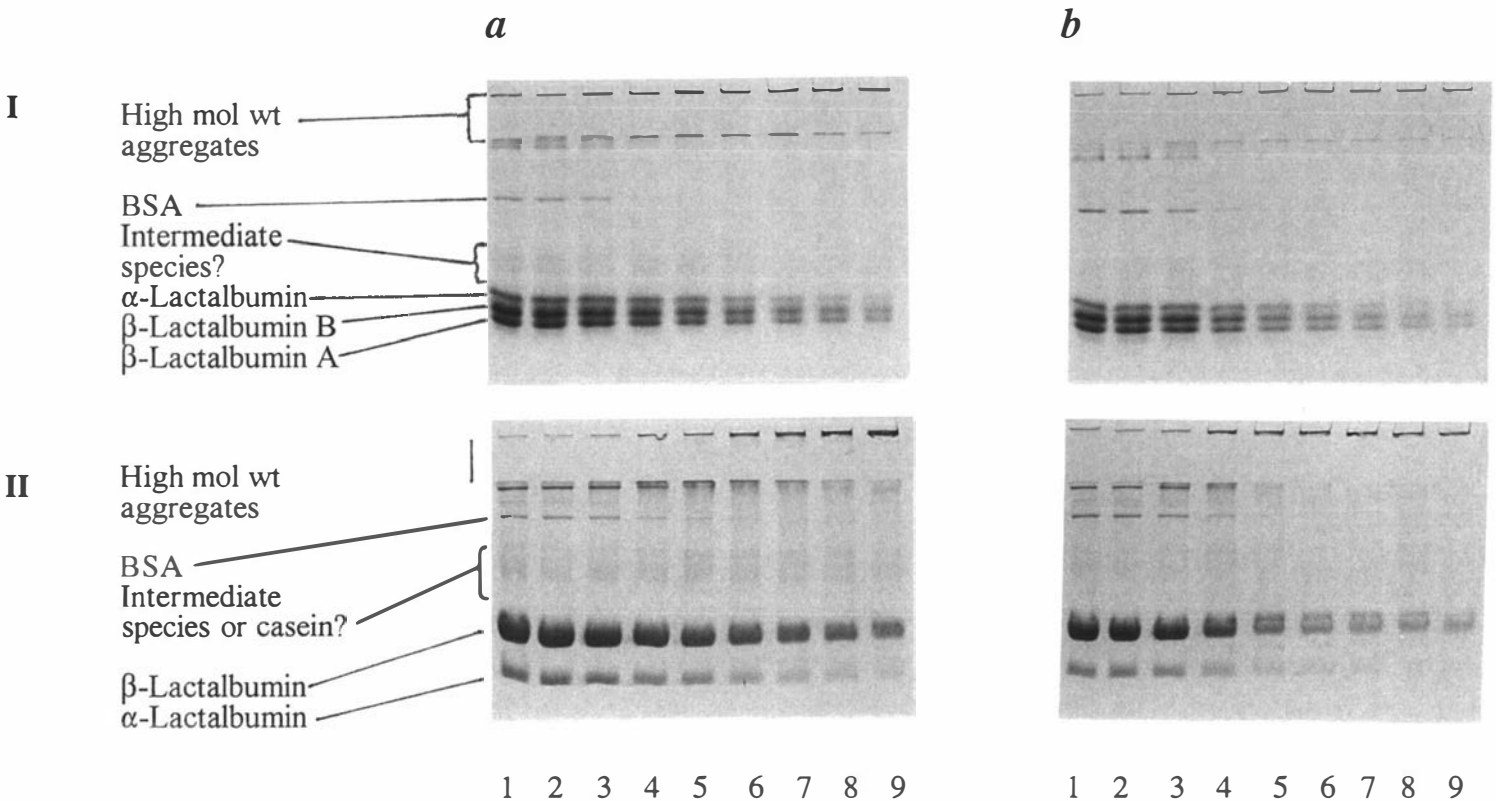


Figure 5.3. Native-PAGE (I) and SDS-PAGE (II) patterns of acid WPC (*a*) and cheese WPC (*b*) solutions (120 g/kg, pH 6.8) heated at 75 °C for 0 (slots 1 & 2), 2 (slot 3), 4 (slot 4), 6 (slot 5), 8 (slot 6), 10 (slot 7), 12 (slot 8), and 15 (slot 9) min.

Kinetics of the loss of whey proteins from heated WPC solutions

The PAGE results for the loss of native-like and SDS-monomeric β-lactoglobulin, α-lactalbumin and BSA from heated 120 g/kg WPC solutions (Fig. 5.4) were used to determine the kinetics of these reactions. Using equations (1)-(4), (discussed in Section 4.6.2), the rate constant (k_n) and the apparent rate constant (k_{app}) for each data set were calculated for an assumed reaction order n = 1, 1.5 or 2. The best fit lines were determined by linear regression analysis, and the coefficients of determination (r^2) were compared. Table 5.2 shows the calculated values of k_n and k_{app} for the loss of native-like β-lactoglobulin, α-lactalbumin and BSA from the heated acid and cheese WPC solutions. Table 5.3 shows the calculated values for the loss of SDS-monomeric proteins.

The results showed that the loss of native-like β-lactoglobulin from the heated acid or cheese WPC solutions could be adequately described by n = 1 or 1.5 whereas the loss of native-like α-lactalbumin was better described by a reaction order n = 1 (Table 5.2). This was generally in agreement with the earlier finding for the loss of these proteins from 120 g/kg rennet WPC solutions (Section 4.6.2) under the same heating conditions. The loss of BSA from these WPC solutions could be adequately modelled by a reaction order between 1 and 2 (Table 5.2). The loss of native-like BSA from heated 120 g/kg rennet WPC solutions was also shown to be adequately modelled by reaction orders between 1 and 2 (Section 4.6.2). The loss of native-like proteins from the heated acid and cheese WPC solutions followed the order BSA >> β-lactoglobulin > α-lactalbumin. These results suggest that the mechanisms that govern the loss of the native-like proteins on heating different WPC (rennet, acid and cheese) solutions are probably the same.

Comparison of the values of k_{app} and k_n for the loss of native-like proteins (β-lactoglobulin, α-lactalbumin and BSA) from the acid and cheese WPC solutions (Table 5.2) showed that the values obtained for cheese WPC were always greater (by a factor of at least 2) than the values obtained for acid WPC. This confirms that the loss of native-like proteins was faster from the heated cheese WPC solutions than from the heated acid WPC solutions.

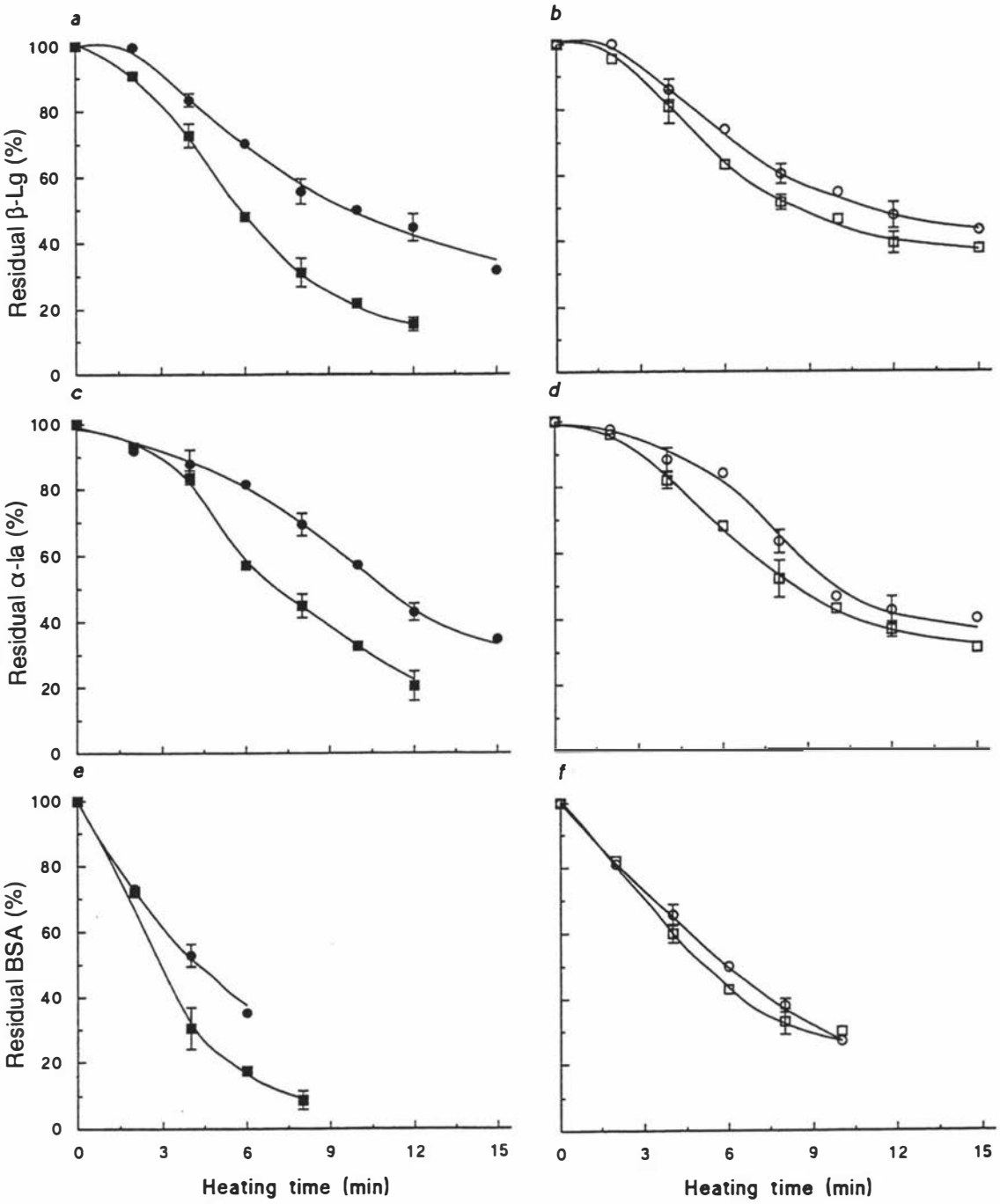


Figure 5.4. Loss of native-like (●, ■) and SDS-monomeric (○, □) β-lactoglobulin (*a, b*), and α-lactalbumin (*c, d*), and BSA (*e, f*) from 120 g/kg acid WPC (●, ○) or cheese (□, ■) WPC solutions during heating at 75 °C.

The kinetics for the loss of SDS-monomeric β-lactoglobulin, α-lactalbumin and BSA (Table 5.3) showed that the values of r^2 for the calculated rate constant k_n were similarly high when an assumed order of reaction n = 1, 1.5 or 2 was used. This suggests that the loss of SDS-monomeric proteins from either acid or cheese WPC solutions can be adequately described by a reaction order, n, between 1 and 2. The values of k_{app} and k_n for the loss of SDS-monomeric proteins were lower for the acid WPC solutions that the cheese WPC solutions. The r^2 values for the calculated k_n for the loss of SDS-monomeric proteins were very similar in both WPC solutions.

Table 5.2. Calculated apparent rate constants (k_{app}) and rate constants (k_n) for the loss of native-like β-lactoglobulin, α-lactalbumin and BSA from heated 120 g/kg acid and cheese WPC solutions, assuming n = 1, 1.5 or 2. Units of k_{app} and k_n were as described for Table 4.1 (Section 4.6).

		β-Lactoglobulin			α-Lactalbumin			BSA		
	n	k_{app}	k_n	r^2	k_{app}	k_n	r^2	k_{app}	k_n	r^2
Acid WPC	1	-0.0491	-0.0491	0.97	-0.0462	-0.0462	0.98	-0.1070	-0.1070	0.99
	1.5	0.1042	0.0095	0.96	0.0944	0.0086	0.91	0.2254	0.0206	0.98
	2	0.1399	0.0012	0.92	0.1245	0.0010	0.88	0.3010	0.0025	0.95
Cheese WPC	1	-0.0774	-0.0774	0.97	-0.0711	-0.0711	0.98	-0.1191	-0.1191	0.93
	1.5	0.2664	0.0243	0.94	0.1952	0.0178	0.90	0.6006	0.0548	0.95
	2	0.4521	0.0038	0.89	0.2985	0.0025	0.83	1.1690	0.0106	0.94

Table 5.3. Calculated apparent rate constants (k_{app}) and rate constants (k_n) for the loss of SDS-monomeric β-lactoglobulin, α-lactalbumin and BSA from heated 120 g/kg acid and cheese WPC solutions, assuming n = 1, 1.5 or 2. Units of k_{app} and k_n were as described for Table 4.1 (Section 4.6).

		β-Lactoglobulin			α-Lactalbumin			BSA		
	n	k_{app}	k_n	r^2	k_{app}	k_n	r^2	k_{app}	k_n	r^2
Acid WPC	1	-0.0440	-0.0044	0.96	-0.0462	-0.0462	0.93	-0.1070	-0.1070	0.99
	1.5	0.0788	0.0024	0.98	0.0916	0.0084	0.92	0.1808	0.0165	0.97
	2	0.0985	0.0038	0.97	0.0731	0.0010	0.91	0.2596	0.0022	0.93
Cheese WPC	1	-0.0470	-0.0470	0.93	-0.0521	-0.0521	0.96	-0.0732	-0.0732	0.95
	1.5	0.0974	0.0089	0.97	0.1660	0.0110	0.98	0.1792	0.0164	0.98
	2	0.1282	0.0110	0.97	0.1589	0.0013	0.97	0.2537	0.0021	0.98

5.5. Protein aggregate formation in heated WPC solutions

5.5.1. Analysis by 1D-PAGE

The loss of native-like or SDS-monomeric proteins during the heating of WPC solutions was also used to elucidate the relative proportions of proteins that were native-like and that were involved in the formation of hydrophobic or disulphide-linked aggregates (Fig. 5.5) as described earlier (Section 4.6.3). It is clear that the loss of native-like β-lactoglobulin, α-lactalbumin and BSA during the heating of the acid WPC solutions (Fig. 5.5*a, c, e*) resulted predominantly in the formation of disulphide-linked aggregates. Few hydrophobically-associated aggregates were formed under the heating conditions used. By contrast, the loss of β-lactoglobulin, α-lactalbumin and BSA from the cheese WPC solutions resulted in the formation of considerable amounts of hydrophobically-associated aggregates in addition to disulphide-linked aggregates. It was observed that the cheese WPC samples that were heated for ≥ 8 min formed gels (the same observation was made for heated 120 g/kg rennet WPC solutions, Section 4.10.2.2). The acid WPC samples, however, did not form a gel even after heating for 15 min.

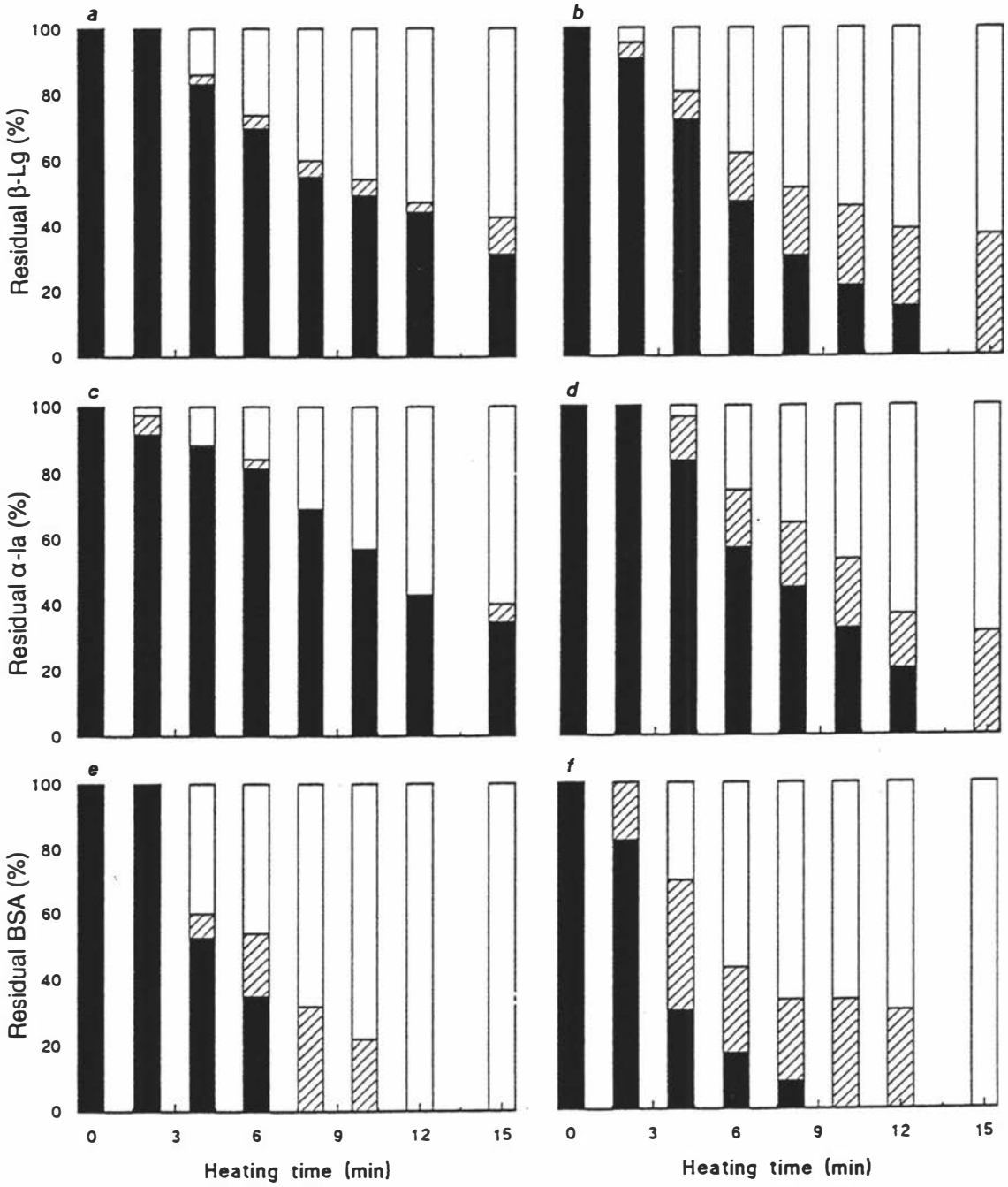


Figure 5.5. β-Lactoglobulin (*a, b*), α-lactalbumin (*c, d*) and BSA (*e, f*) aggregates in heated 120 g/kg acid WPC (*a, c, e*) and cheese WPC (*b, d, f*) solutions heated at 75 °C. Protein species: native-like (■), hydrophobic aggregates (▨) and disulphide-linked aggregates (□). Note: The results for the native-like or hydrophobic aggregates for some of the samples were not available because the PAGE bands were either too faint or not sharp enough to give a well defined peak on scanning.

5.5.2. Characterisation of protein aggregates by 2D-PAGE

The protein aggregates observed using native- and SDS-PAGE (Fig. 5.3) were characterised using 2D-PAGE (Fig. 5.6). The native-SDS 2D-PAGE patterns of the heated acid WPC solutions (Fig. 5.6 I*a*) showed that some faint spots, corresponding to the minor whey protein components, had dissociated from the material caught on top of the resolving and stacking gels of the sample gel strip. This could not be seen in the cheese WPC solutions (Fig. 5.6 I*b*). This was contrary to the conclusions drawn from the 1D-PAGE analyses (Figs 5.4 & 5.5), where it was shown that the loss of native-like proteins from the cheese WPC solutions during heating resulted in the formation of considerable amounts of hydrophobically-associated aggregates. In the heated acid WPC solutions, however, it was shown that the aggregates formed during heating were largely disulphide linked with minimal hydrophobically-associated aggregates present. The contradictory observations made using 2D-PAGE could be attributed to the quantity of protein aggregates that were caught on top of the stacking and resolving gels. The quantity of aggregates caught on top of the stacking and resolving gels was greater in the acid WPC solutions than in the cheese WPC solutions (Fig. 5.3), although the loss of native-like proteins was faster from the cheese WPC solutions than from the acid WPC solutions during heating.

The accumulation of material on top of the stacking and resolving gels in 1D-PAGE (Fig. 5.3) did not necessarily reflect the amount of native-like proteins lost during heating. The protein aggregates had to be within a specific size range in order to be caught on top of the stacking and resolving gels. If the protein aggregate was larger than the pore size of the stacking gel, it would have stayed on top of the stacking gel and would have been washed away during staining or destaining of the gels. The aggregates that were caught on top of the stacking gel (or resolving gel) partly eluted into the gel, and therefore were not washed away during the staining or destaining process. It is possible that majority of the aggregates produced by heating the cheese WPC solutions were larger than the pore size of the stacking gel; hence they were not observed in the 2D-PAGE system.

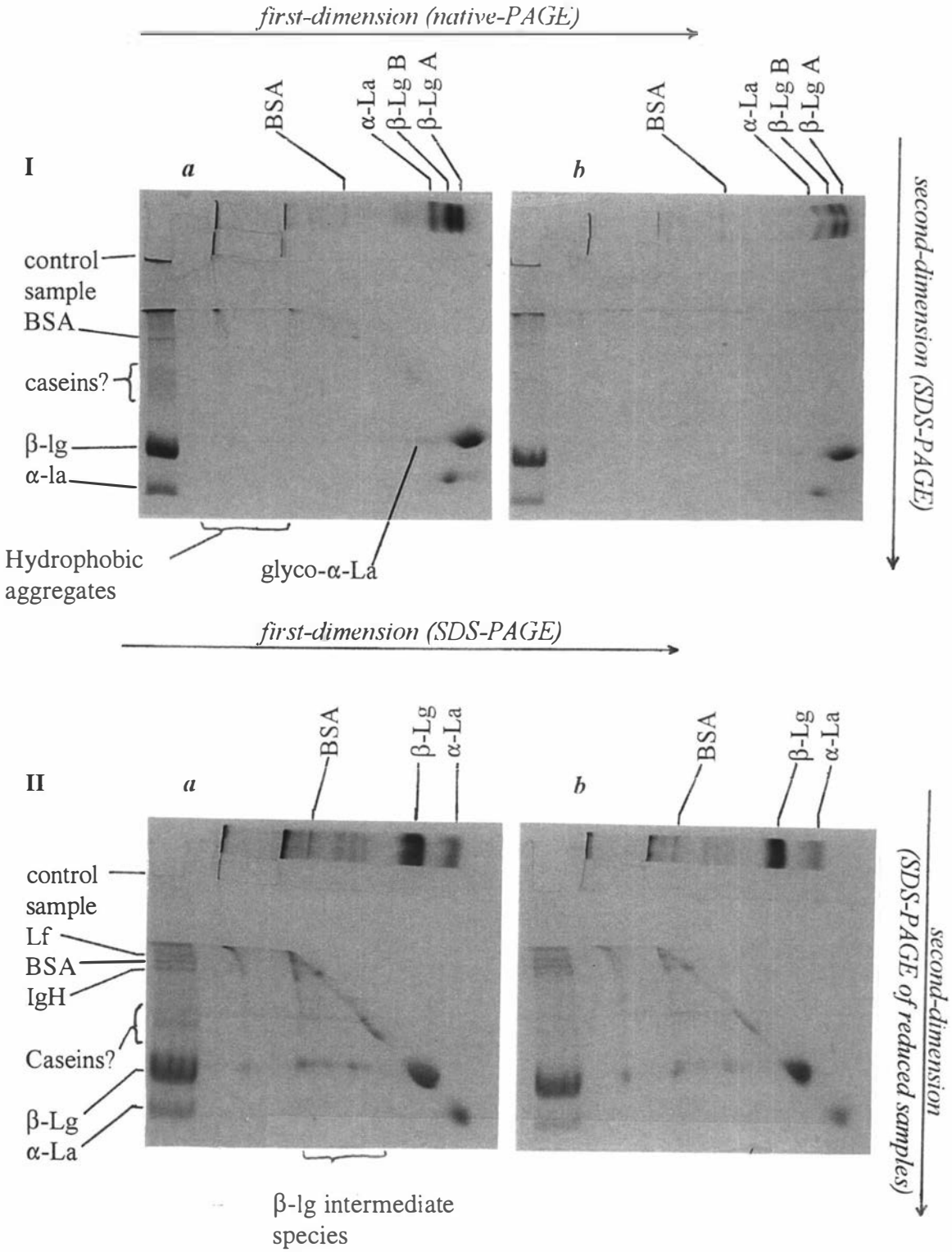


Figure 5.6. 2D-PAGE patterns of heated (75 °C for 6 min) acid (*a*) and cheese (*b*) WPC solutions. I. First dimension: native-PAGE, second dimension: SDS-PAGE. II. First dimension: SDS-PAGE, second dimension: SDS-PAGE of the reduced samples. Abbreviations are as described in Fig 4.4.

The SDS 2D-PAGE patterns of the heated acid and cheese WPC solutions were similar (Fig. 5.6 II*a, b*). In both gels, the material that was caught on top of the stacking and resolving gels of the sample gel strips resolved to give spots that corresponded to β-lactoglobulin, α-lactalbumin, BSA and other minor whey protein components. The region between BSA and β-lactoglobulin (sample gel strip) resolved to give spots that corresponded to β-lactoglobulin, supporting the earlier suggestion that there could be whey protein intermediate species in this region (Section 5.4.1.1). In addition, faint bands corresponding to casein fractions also migrated from this region. These bands were more intense in the acid WPC solutions (Fig. 5.6II*a*) than in the cheese WPC solutions (Fig. 5.6II*b*).

5.5.3. Analysis of heated WPC solutions by size exclusion chromatography

The heated WPC solutions were also examined using size exclusion chromatography (SEC) (Section 3.8). The elution profiles of the unheated WPC samples showed four main peaks (Fig 5.7). The molecular weights and protein compositions of the material corresponding to each peak were determined to be the same as for rennet WPC (Section 4.8); i.e. $> 1 \times 10^6$, $\sim 7 \times 10^4$, $\sim 2 \times 10^4$ and $\sim 1.5 \times 10^4$ Da, for peaks 1, 2, 3 and 4 respectively. Peak 1 represented soluble aggregates, and peak 2 contained mainly BSA and immunoglobulin. Peak 3 contained mainly β-lactoglobulin, and peak 4 contained mainly α-lactalbumin. Peaks 2-4 decreased progressively with heating time at 75 °C, indicating continuous loss of native-like proteins. The decrease in size of peak 2 was faster than that of peaks 3 and 4, indicating that the losses of BSA and the other minor whey proteins were faster than the loss of either β-lactoglobulin or α-lactalbumin. The decreases in the size of peaks 2- 4 were faster for the cheese WPC solutions than the acid WPC solutions, supporting the earlier suggestion that the loss of native-like proteins was faster from the cheese WPC solutions than from the acid WPC solutions, as indicated by 1D-PAGE (Section 5.4).

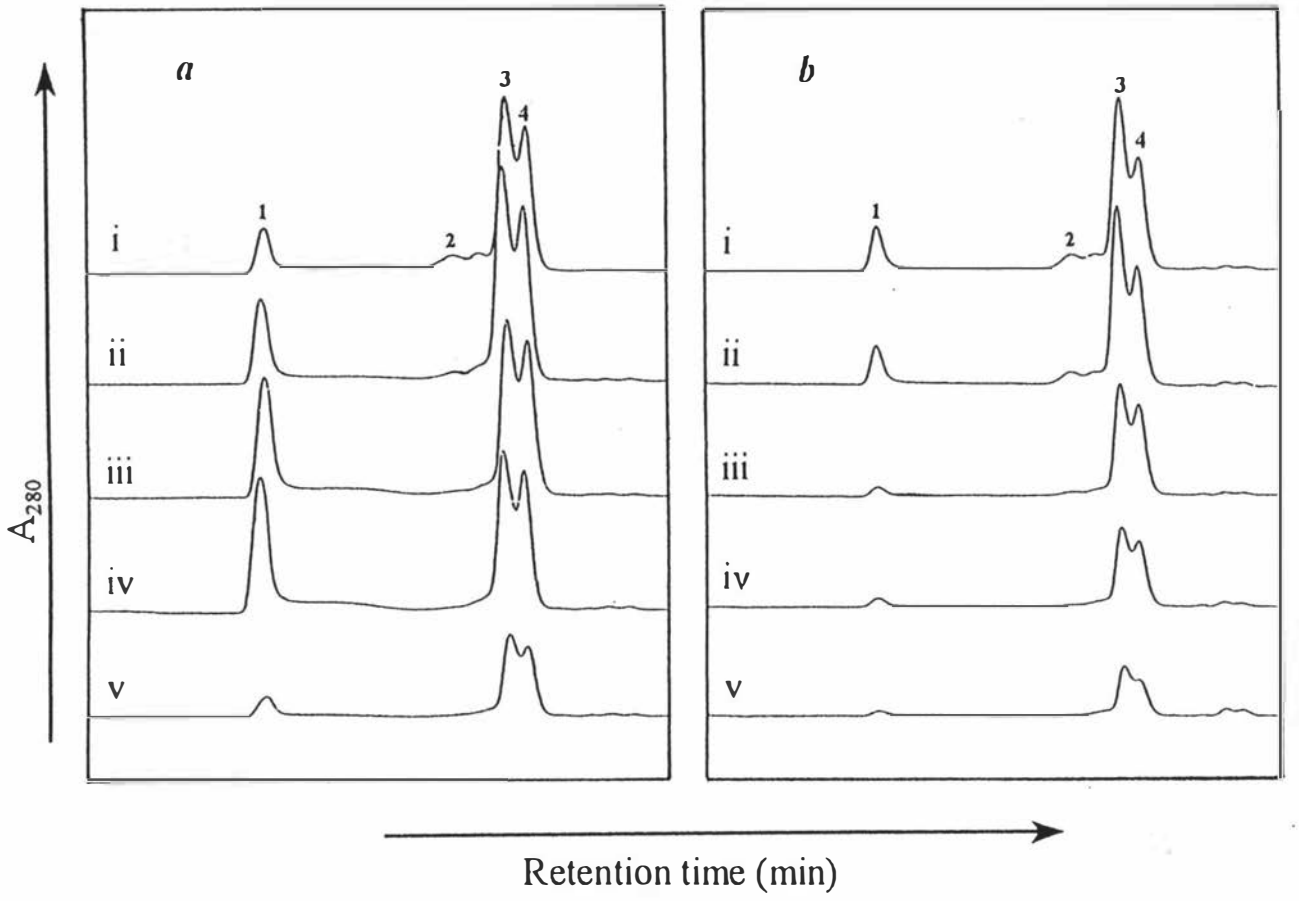


Figure 5.7. The SEC elution profiles of acid WPC (*a*) and cheese WPC (*b*) solutions. Suprose 6HR 10/30 column, flow rate 0.4 ml/min, eluent: 20 mM immidazole buffer (pH 6.9, 50 mM NaCl). 120 WPC solutions heated at 75 °C for 0 (i), 2 (ii), 4 (iii), 6 (iv) and 10 (v) min. The samples were diluted with the buffer so that the final WPC concentration was 0.002 g/kg, filtered through a 0.22 μm filter before analysis.

The main difference between the heated acid and cheese WPC solutions was that peak 1 increased in size with heating time up to 6 min in the heated acid WPC solution, but decreased in size with heating time in the heated cheese WPC solution. In sample preparation for size exclusion analysis, the WPC solutions were filtered through a 0.22 μm filter before the samples were injected into the column (Section 3.9). This filtration step would remove particles of size > 0.22 μm. The results (Fig. 5.7), therefore, suggest that, when the acid WPC solutions were heated, there was an accumulation of small protein aggregates (< 0.22 μm) with heating time up to 6 min. After heating for 10 min, these small aggregates probably interacted to form larger aggregates which were removed by the filtration process, resulting in a decrease in the size of peak 1 (Fig. 5.7*a*, v). On the other hand, in the heated cheese WPC solutions, the aggregates formed were probably larger than 0.22 μm and consequently were removed by the filtration procedure, and hence peak 1 decreased continuously with heating time.

Overall, when an acid WPC solution was heated, small aggregates were formed which accumulated over time up to a certain point and then probably interacted to form larger aggregates. On the other hand, when a cheese WPC solution was heated, large aggregates were formed immediately. Again, these results are in close agreement with those obtained using electrophoresis (Sections 5.5.1 & 5.5.2).

5.5.4. Analysis of heated WPC solutions by transmission electron microscopy (TEM)

The heated WPC solutions were also analysed using TEM (Section 3.10). The WPC solutions were prepared, heated, sectioned and stained appropriately as described in Section 3.10, and then viewed under an electron microscope.

The TEM micrograph of the acid WPC solution heated at 75 °C for 2 min (Fig. 5.8*a*) showed a homogeneous fine mesh of tiny protein aggregates. The micrograph of the sample heated for 6 min (Fig. 5.8*b*) showed a similar particle size distribution but a higher degree of "hairiness" (Langton & Hermansson, 1996). The micrograph of the acid WPC gel (i.e. sample heated for 20 min, Fig. 5.8*c*) again showed a similar size distribution but more uniform aggregate structure and porosity. (Note: the more intense dark spots/clusters were probably localised concentrated dye that was not mixed well during sample preparation.) These results suggest that, when a solution of acid WPC

was heated, very fine aggregates, << 0.5 μm, were formed. Further heating caused the formation of a homogeneous gel with very fine structure and the details were not clear even at 10,000 × magnification. The gel structure of the acid WPC gels was probably fine-stranded (Stading & Hermansson, 1991).

The micrographs of the heated cheese WPC solutions showed very different aggregate structures from that of the acid WPC solutions. After heating for 2 min (Fig. 5.9*a*), relatively large (> 10 μm) irregularly shaped clusters were formed (Langton & Hermansson, 1996), with large spaces between them. It appears that these clusters consisted of conglomerates (Langton & Hermansson, 1996) that were linked together by intertwined fibrils (Van Kleef, 1986) or hairy-like structures (Langton & Hermansson, 1996). The hairy structures also appeared to extend from the surfaces of the clusters. The micrograph of the sample heated for 4 min (Fig. 5.9*b*) showed that clusters of similar size were present but they appeared to be joined more closely to each other by the hairy structures. The cheese WPC gel (i.e. sample heated for 10 min, Fig. 5.9*c*) showed that the clusters were distributed more evenly and most of the intervening spaces were filled with the hairy structures. The gel network seemed to have a more uniform porosity. Compared with the acid WPC gel (Fig. 5.8*c*), the cheese WPC gel had a greater range of porosity. The cheese WPC gel structure was probably a combination of fine-stranded (Stading & Hermansson, 1991) and particulate (Langton & Hermansson, 1996) structures.

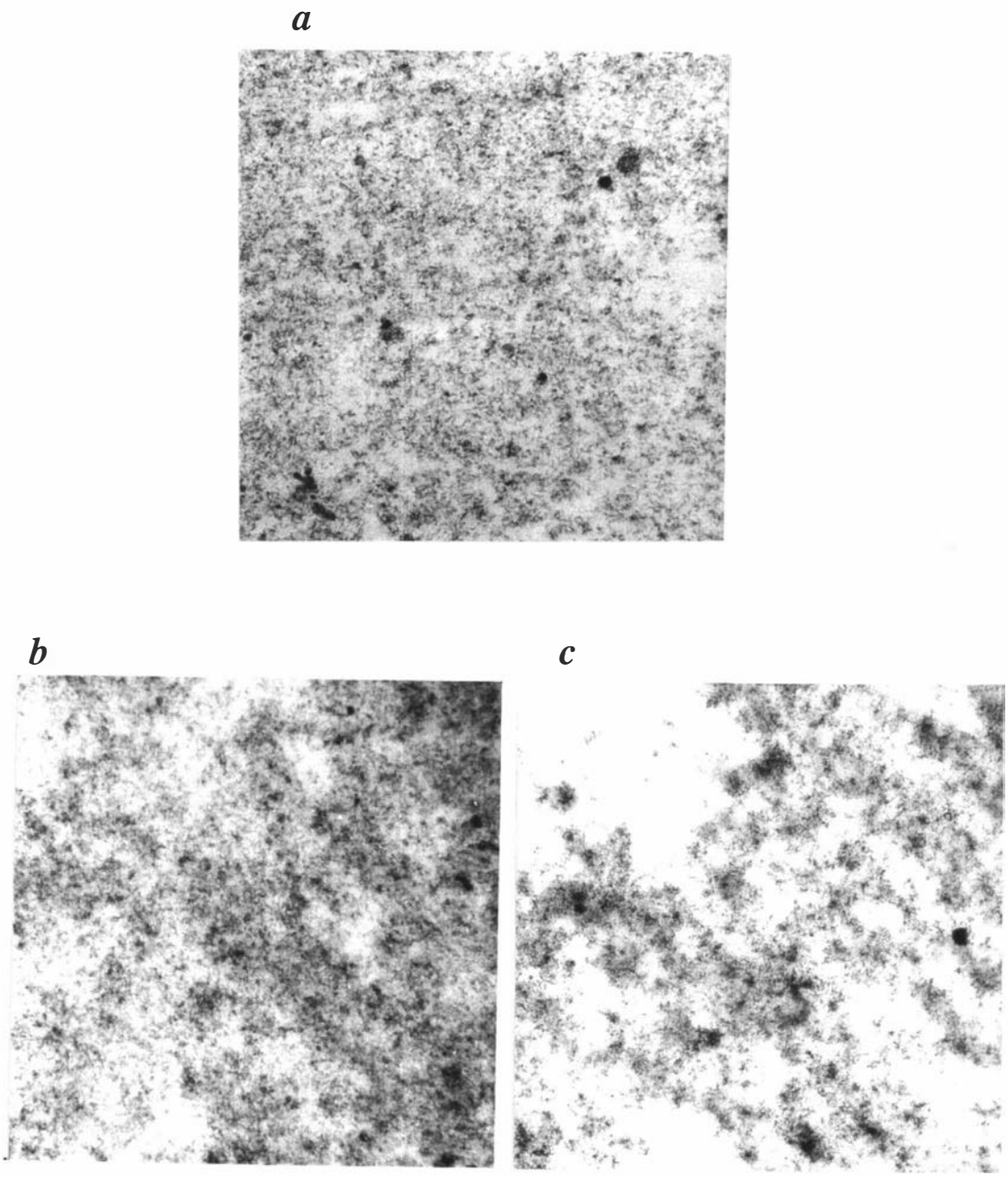


Figure 5.8. TEM micrographs of acid WPC aggregates. 120 g/kg WPC solutions (pH 6.8) were heated at 75 °C for 2 (*a*), 6 (*b*) and 20 (*c*) min before analysis. Magnification: 72,000. See Section 3.10 for sample preparation.

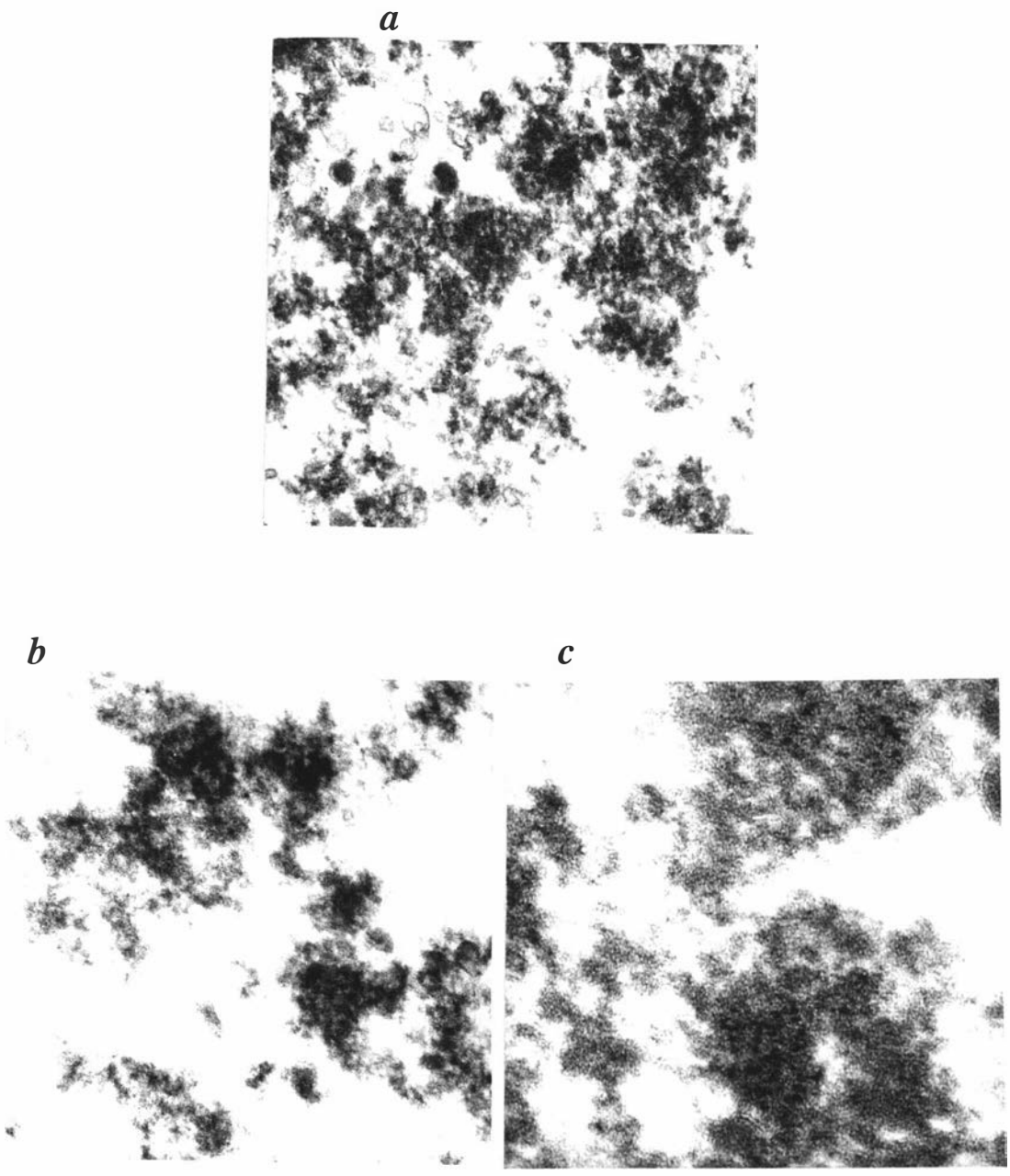


Figure 5.9. TEM micrographs of cheese WPC aggregates. 120 g/kg WPC solutions (pH 6.8) were heated at 75 °C for 2 (*a*), 4 (*b*) and 8 (*c*) min before analysis. Magnification: 72,000. See Section 3.10 for sample preparation.

5.6. Discussion

The composition analyses (Table 5.1) showed that the two WPC powders used in this investigation had some major differences in protein and mineral compositions. The cheese WPC powder had a significantly higher GMP content. GMP is formed during the early phase of the enzymatic coagulation of milk as a result of hydrolysis of κ-casein (Sharma *et al.*, 1993). It was not possible to detect or to follow the heat-induced changes in GMP using the techniques of the current study. Because it is present at a relatively high concentration in cheese WPC, its behaviour during thermal treatments needs to be investigated. Very little literature information is available on how GMP affects the heat-induced aggregation or gelation of WPC (Dalgleish, 1992). Because it is very soluble (Richardson *et al.*, 1992) and contains no cysteine residues, it could act as a diluent; therefore the effective functional protein concentration in the cheese WPC product may be less than expected on the basis of the total protein as determined by the Kjeldahl method.

The electrophoresis results (Figs 5.1, 5.2 & 5.3) showed that the acid WPC solutions contained more casein components than the cheese WPC solutions. This may arise from the presence of casein "fines" during acid precipitation of milk. These fines could subsequently become concentrated during UF, and end up in the WPC. It is not clear what role casein fines played in the heat-induced aggregation of WPC. It is possible that the casein fines had a nucleating effect that gave rise to the formation of small aggregates in acid WPC solutions during heating. There is no literature information on the likelihood of this possibility. The effect of casein fines on the heat-induced aggregation of WPC should be taken into account in future investigations.

The different mineral environments would have certainly influenced the heat-induced aggregation of the WPCs. The different aggregate sizes formed by the two WPC solutions during heating may be related to their calcium contents. The effect of calcium on the heat-induced aggregation of whey proteins has been studied by many workers. Hollar *et al.* (1995) reported that, when a WPC solution (16% total solids) was dialysed against simulated milk ultrafiltrate containing from 0 to 9 mmol calcium and heated at 66 °C, the mixture progressively became more denatured and formed less soluble aggregates and more insoluble precipitates as the calcium content increased.

Barbut (1995) reported that increasing the calcium level (10-180 mmol/l) of whey protein isolate solutions (100 g/l, pH 7.0) changed the aggregate structure from fine protein strands to larger thicker strands. Parris *et al.* (1993) observed that the amount of insoluble protein aggregates in heated sweet whey increased with increasing calcium levels. The results of these studies support the view that the larger, more particulated aggregate structures formed in the cheese WPC solutions during heating were probably attributable to the high calcium content of the WPC powder (Table 5.1). However, differences in other minerals (e.g. Na, K, Mg) may also have been responsible for the observed differences in the aggregation behaviour of the acid and cheese WPCs.

Langton & Hermansson (1996) reported that addition of salt (0.1 mol/m^3 NaCl) to the WPC solution before heating increased cluster size, conglomerate size, hairiness and porosity but reduced string of beads. It appears that the differences between the aggregate/gel structures of the two WPCs could be attributed to the different mineral contents. The general WPC gel network structure is based predominantly on disulphide bonding, with some involvement of other non-specific types of interactions (hydrogen, hydrophobic and ionic) that are mediated by calcium (Morr, 1979). The 1D-PAGE results (Figs 5.4 & 5.5) suggested that upon heating, predominantly disulphide-linked aggregates were formed in the acid WPC solutions, whereas considerable amounts of hydrophobic aggregates were formed in the cheese WPC solutions. Analysis showed (Table 5.1) that the cheese WPC had relative large quantities of sodium and calcium. It appears therefore that the high degree of cluster, conglomerate and hairiness structure formation in the cheese WPC solutions was probably mediated by calcium and/or sodium ions. Some of these features probably involved hydrophobic aggregations.

In conclusion, the acid and cheese WPCs had similar protein contents as determined by the Kjeldahl method. The protein content of the cheese WPC included a considerable amount of GMP. The cheese WPC had higher Ca, Na, P and Mg contents whereas the acid WPC had a higher K content. When the WPC solutions were heated at 75 °C, the loss of native-like proteins was faster from the cheese WPC solutions than from the acid WPC solutions. The loss of these proteins from the acid WPC solutions resulted predominantly in the formation of disulphide-linked aggregates, whereas the loss of the same proteins from the cheese WPC solutions resulted in the

formation of considerable amounts of hydrophobically-associated aggregates in addition to disulphide-linked aggregates. The aggregate structures formed by the acid WPC solutions during heating were described to be mainly fine stranded whereas those formed by the cheese WPC solutions were described to be a combination of fine-stranded and particulate structures.

CHAPTER 6

THE EFFECT OF THE MINERAL ENVIRONMENT ON THE AGGREGATION AND GELATION OF WPC SOLUTIONS

6.1. Introduction

WPCs are used as functional ingredients in many foods such as processed meat, bakery and dairy products (Kinsella & Whitehead, 1989). Commercial uses of WPC, however, are limited because of large variations in the functional properties. These variations are due largely to variations in compositions and processing treatments (Xiong, 1992). Morr & Foegeding (1990) demonstrated that WPCs obtained from different commercial sources had considerably different mineral contents, including calcium, sodium and phosphorus. Hence, better control of the mineral contents of WPC products may increase their commercial value as functional ingredients. The properties of the heat-induced protein gel network depend on the type of interactions between the denatured protein molecules. The protein gel network is held together predominantly by inter- and intramolecular disulphide bonds (Gupta & Reuter, 1992) formed via sulphydryl-disulphide interchange or sulphydryl oxidation reactions (Monahan *et al.*, 1995). In addition, other non-covalent interactions, such as hydrophobic and ionic interactions, also contribute to the formation of the overall gel network (Mangino, 1992).

The presence of mineral ions can alter the net charge on the protein molecules and hence the types of interaction that are involved in the formation of the overall gel network. Most of the studies on the effects of minerals on WPC gelation have considered only one or two mineral ions at a time. Many workers, for example, have studied the effect of calcium concentration on WPC gel strength and texture (Schmidt *et al.*, 1979; Johns & Ennis, 1981; Schmidt *et al.*, 1984; Mangino, 1984; Tang *et al.*, 1995). Other workers have studied the effects of sodium (Kuhn & Foegeding, 1990; Xiong, 1992; Tang *et al.*, 1995) on WPC gel properties. Limited information is

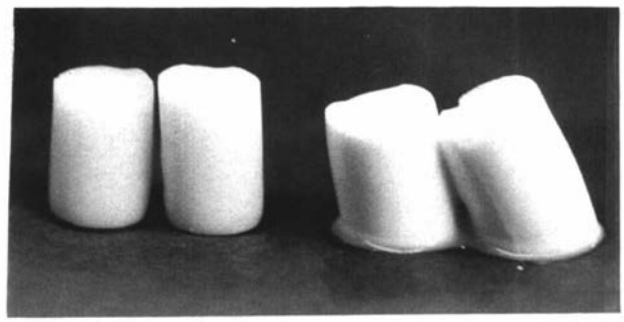
available on the possible combined effects of the ions present in the WPC solutions that are likely to influence the protein interactions that lead to gel formation. Consequently, a series of experiments was conducted to investigate how variations in the overall mineral compositions of WPC solutions affect the gel properties. The mineral environment of the WPC solutions was modified by dialysis. The effect of this modification on the formation and properties of heat-induced WPC gels was measured by either compression tests or oscillatory rheometry. The loss of proteins and the formation of aggregates during gel formation were analysed using PAGE, HPLC and confocal laser microscopy.

6.2. Acid and cheese WPC gels

Acid and cheese WPC solutions, 120 g/kg, pH 6.8 (Section 3.3), were used for preparing gel cylinders for compression tests using Instron (Section 3.6) or measuring the development of gels during heating, using oscillatory rheometry (Section 3.8).

6.2.1. Compression test

Figure 6.1 shows a photograph of cylindrical WPC gel samples formed by heating WPC solutions at 80 °C. The acid WPC gels (Fig. 6.1*a*) were firmer and more transparent than the cheese WPC gels (Fig. 6.1*b*). The cheese WPC gels were opaque and "soft"; the gel cylinders could hardly support their own weight and a considerable amount of fluid was expelled from the gels over a period of 3-5 min.



a *b*

Figure 6.1. Photograph of acid WPC (*a*) and cheese WPC (*b*) gel cylinders formed by heating 120 g/kg WPC solutions (pH 6.8) at 80 °C for 60 min.

These gel cylinders were used for compression tests using an Instron universal testing equipment. When the samples were compressed, the force at maximum deformation or the first breaking point was taken as the reference point for each gel sample. Figure 6.2 shows the typical force-displacement profiles obtained when compressing different samples of the same gel; the reference point (P) for each sample is also indicated. The acid WPC gels had a higher force (4.7 N) at maximum deformation than the cheese WPC gels which had broken on compression at a smaller force (2 N). These results show that the acid WPC gels were stronger (higher compression forces at maximum deformation) and apparently more elastic, as the gel cylinders did not break even when compressed to 50% of their initial height. The cheese WPC gels were obviously weaker and more brittle, and they broke easily on compression at much smaller forces.

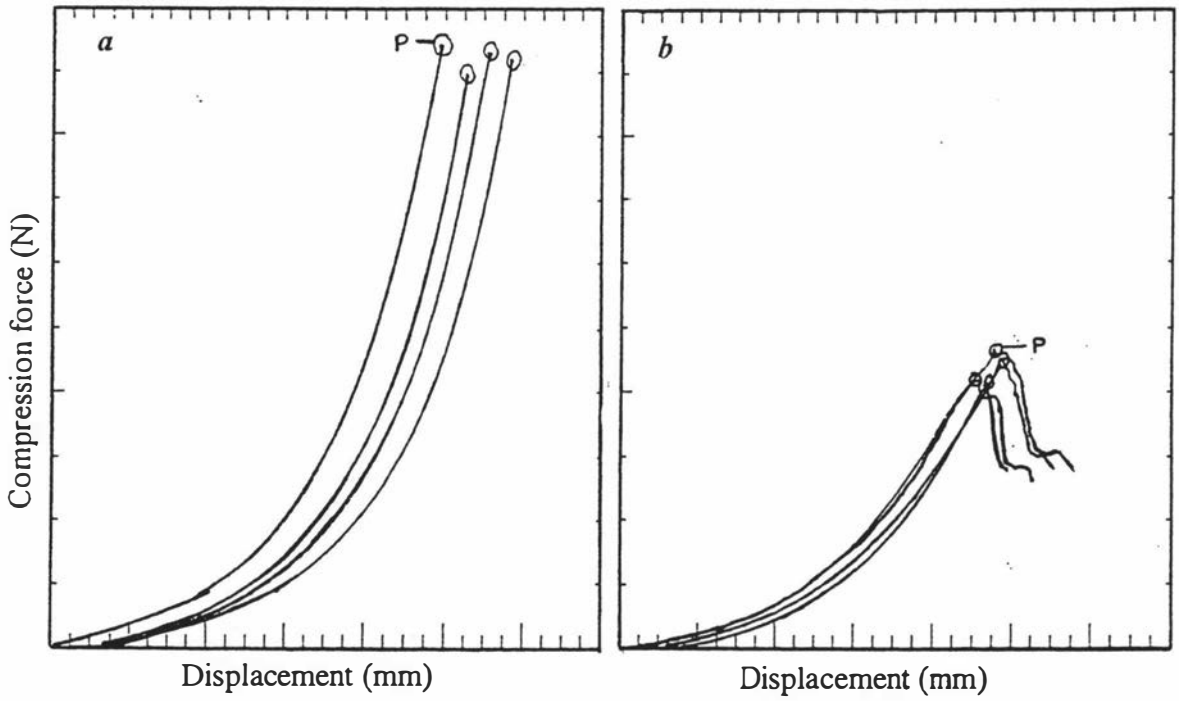


Figure 6.2. Force-displacement profiles recorded during compression of acid WPC (*a*) and cheese WPC (*b*) gel cylinders. WPC solutions (120 g/kg, pH 6.8) were heated in 20 mm diameter plastic tubes at 80 °C for 60 min, kept at 4 °C overnight, cut into 20 mm lengths and then used for the compression tests.

6.2.2. Oscillatory rheometry

The WPC solutions were heated in the Bohlin C25 measuring system as described in Section 3.8. The changes in the storage modulus (G′) of the WPC solutions during heating at 75 or 80 °C were followed. When the WPC solutions were heated at 75 °C, there was no apparent increase in G′ until ~ 17 min for the cheese WPC solution, and ~ 34 min for the acid WPC solution (Fig. 6.3). When the same WPC solutions were heated at 80 °C, the cheese WPC solution started to show an increase in G′ once 80 °C was reached, whereas the acid WPC solution did not show any increase in G′ until ~ 8 min of heating. At both heating temperatures, the cheese WPC solution showed a relatively fast increase in G′ initially but G′ tended to increase more slowly at the later stages of heating. For the acid WPC solution, on the other hand, G′ started to increase slowly and became faster with heating time with no indication of reaching a plateau.

After heating for 120 min at 75 °C, both acid and cheese WPC solutions had reached a similar G′ value, whereas at 80 °C, the final G′value (after 120 min) for the acid WPC solution was considerably higher than that of the cheese WPC solution. These results are consistent with the compression test results obtained after heating the WPC solutions at 80 °C for 60 min (Fig. 6.2).

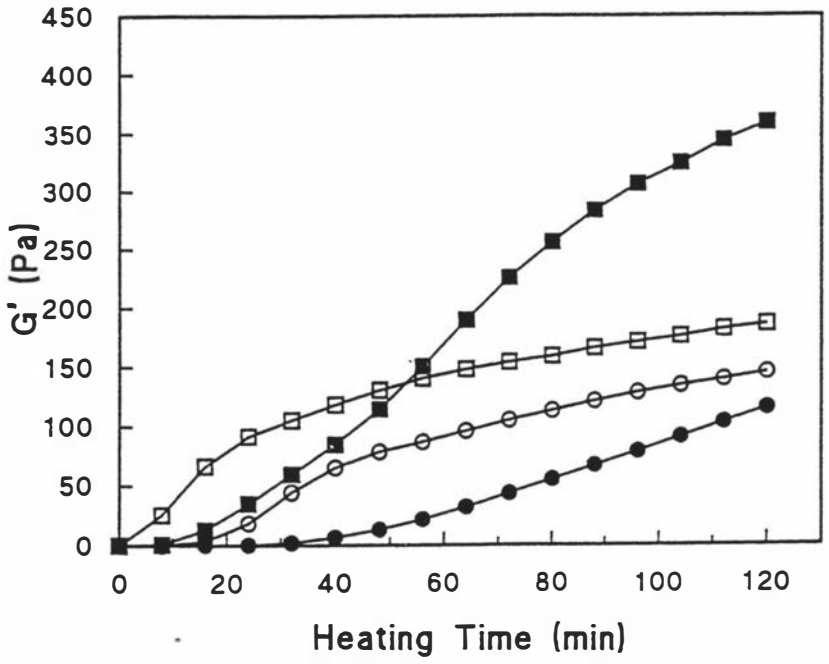


Figure 6.3. Changes in the storage modulus (G′) of 120 g/kg cheese WPC (□, ■) or acid WPC (○, ●) solutions during heating at 75 (○, □) or 80 (●, ■) °C.

6.3. Effect of dialysis on gel strength

Samples (300 ml) of acid and cheese WPC solutions were dialysed for different times against large quantities (10 l) of cheese and acid WPC solutions respectively. The dialysed samples were used for preparing gel cylinders for compression tests (Section 3.6). Figure 6.4 shows the changes in the WPC gel strength with dialysis time. Gel strength refers to the force at maximum deformation or the first breaking point as described earlier (Section 6.2.1).

The gel made from the control cheese WPC solution (i.e. non-dialysed WPC solution) had a gel strength of ~ 2 N. When samples of cheese WPC solution were

dialysed against acid WPC solution, the gel strength increased with increasing dialysis time up to 24 h. There was a slow increase during the first 12 h (from ~ 2 to ~ 2.6 N), followed by a faster increase during the next 12 h (from ~ 2.6 to 4.5 N). After dialysis for 24 h, the gel strength was more than double (4.5 N) the initial gel strength, a value comparable with that of the control acid WPC gel (4.7 N).

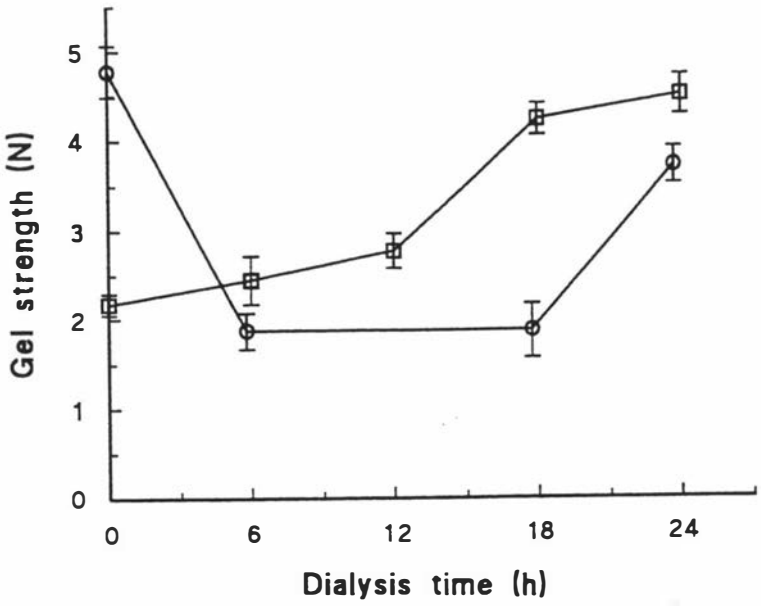


Figure 6.4. Effect of dialysis (4 °C) on the gel strength of cheese WPC (□) or acid WPC (○) solutions. See Fig. 6.2 for sample preparation. Every data point is an average of at least four measurements. The error bars indicate the differences between the smallest and largest measured values.

The gel made from the control acid WPC solution (Fig. 6.4) had a strength of 4.7 N, which was more than double the gel strength of gels made from the control cheese WPC solution (~ 2 N, Fig. 6.4). When the acid WPC solution was dialysed against cheese WPC solution, the gel strength decreased from 4.7 N to ~ 2 N during the first 6 h of dialysis. The gel strength did not change during a further 12 h of dialysis, but then increased to ~ 3.5 N after dialysis for a further 6 h.

It is interesting to note that, during the first 6 h of dialysis, the acid WPC gel quickly approached the initial strength of the control cheese WPC gel (2 N, Fig. 6.4) and then remained at this level for a further 12 h. This trend appeared to coincide with

the initial increase in gel strength of the cheese WPC solution (Fig. 6.4), where the gel strength reached levels that were comparable with the initial strength of the acid WPC gels (4.7 N) during the 24 h of dialysis.

Visual inspection of the gels made from the dialysed WPC solutions indicated that the acid WPC gels became more opaque with increasing dialysis time. The samples that were dialysed for 6 or 18 h formed gels that were apparently "weak", curd-like and very similar to the gels made from the control cheese WPC solution. The gels made from the acid WPC samples that were dialysed for 24 h were firm and apparently stronger than the gels made from the acid WPC solutions that were dialysed for 6 or 18 h. The cheese WPC gels became more transparent and firmer with increasing dialysis time. Unlike the gels from the control cheese WPC solution, the gels made from the dialysed samples did not break upon compression.

6.4. Effect of dialysis on gel formation - oscillatory rheometry

Samples of the dialysed cheese WPC solutions were heated at 80 °C, and the development of gels during heating was followed by measuring the changes in G′. The control cheese WPC solution started to form a gel by the time the heating temperature (80 °C) was reached (Fig. 6.5), as indicated by the increase in the storage modulus G′ at 0 heating time. The value of G′ increased rapidly during the first 20 min followed by a slow increase with further increases in heating time. At the end of the 120 min heating, the value of G′ had reached a maximum value of ~ 400 Pa.

Dialysis of cheese WPC resulted in a gradual decrease in the initial slope (first 20 min) of the G′ vs time curves. Beyond 20 min, the slope of the G′ vs time curve increased with increasing dialysis time. The final G′ values after heating for 120 min also increased with increasing dialysis time, except that the WPC sample that had been dialysed for 24 h had a lower final G′ value (~ 500 Pa) than that (~ 600 Pa) of the sample that had been dialysed for 12 h.

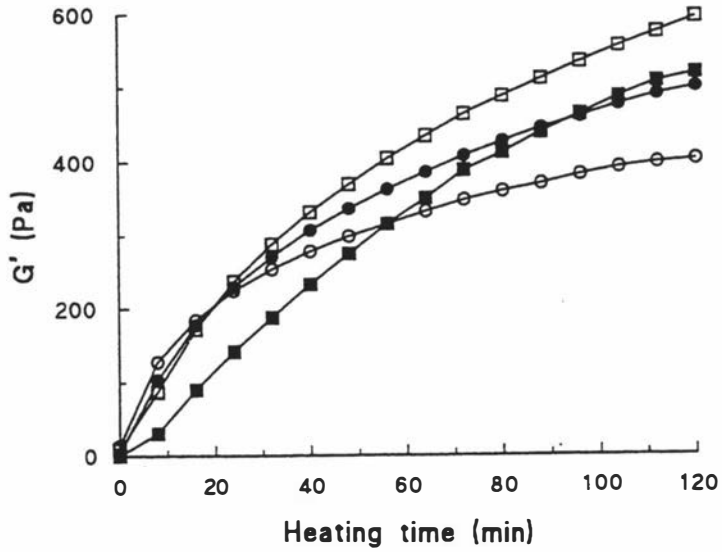


Figure 6.5. Effect of dialysis time on the development of storage modulus (G′) as a function of heating time at 80 °C of cheese WPC solutions. Cheese WPC solution (120 g/kg, pH 6.8) was dialysed against large quantities of acid WPC solution for 0 (control ○), 6 (●) 12 (□) and 24 (■) h prior to heat treatment.

The control acid WPC solution (Fig. 6.6) showed a lag phase of up to 8 min during which no recognisable increase in G′ occurred. Then G′ increased rapidly with increases in heating time, reaching a final value of ~ 600 Pa after 120 min. Dialysis of the acid WPC solution against cheese WPC solution for up to 17 h prior to heating resulted in marked decreases in the final G′ values. These results suggest that, when cheese WPC solution was dialysed against acid WPC, its gelling behaviour became more like that of acid WPC solution, and vice versa.

The final G′ values (after heating for 120 min at 80 °C) as a function of dialysis time are plotted in Fig. 6.7. The control cheese WPC solution had a G′ value of ~ 400 Pa which increased to ~ 600 Pa after dialysis for 12 h; this value was comparable with that observed for the control acid WPC gel (580 Pa). The sample that was dialysed for 24 h had a slightly lower G′ value (460 Pa).

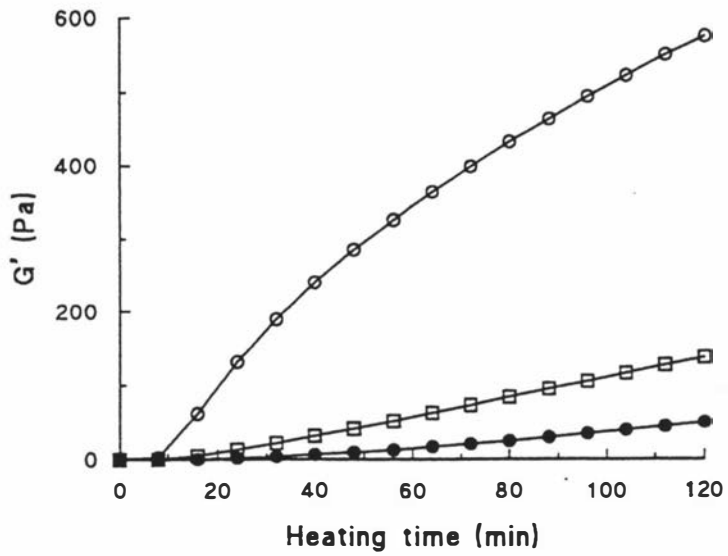


Figure 6.6. Effect of dialysis time on the development of storage modulus (G′) as a function of heating time at 80 °C of acid WPC solutions. Acid WPC solution (120 g/kg, pH 6.8) was dialysed against excess cheese WPC solution for 0 (control , ○), 5 (●) and 17 (□) h prior to heat treatment.

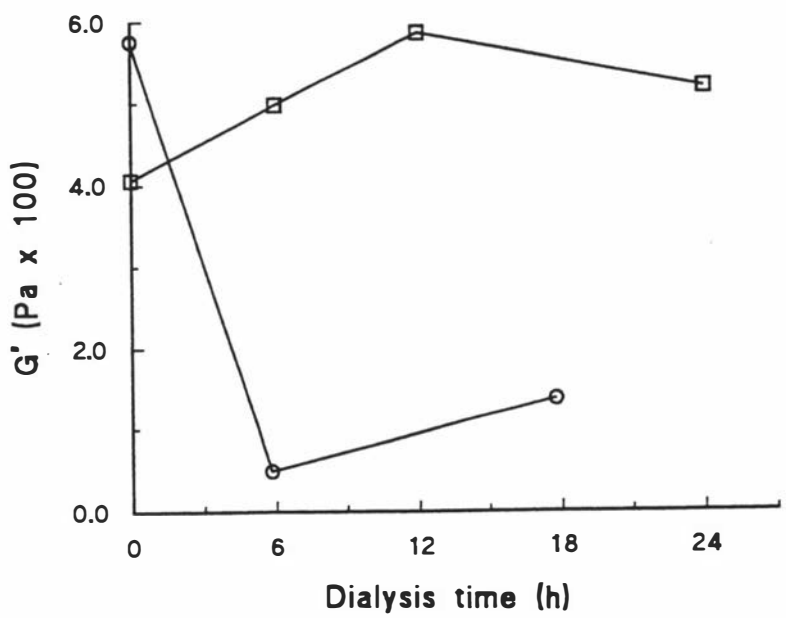


Figure 6.7. Effect of dialysis on the G′ values after heating for 120 min at 80 °C of cheese WPC (□) and acid WPC (○) gels.

The control acid WPC solution had a final G′ value of ~ 580 Pa. After dialysis for 5 h, the final G′ value decreased markedly to ~ 50 Pa. Further dialysis for 17 h slightly increased the final G′ value to ~ 120 Pa. These trends were consistent with those obtained using the compression tests (Fig. 6.4).

6.5. Effect of dialysis on the mineral content of WPC solutions

Analysis showed that the Na content of cheese WPC solution decreased by ~ 40% during 24 h of dialysis (Table 6.1). The K content on, the other hand, increased to more than double its initial level after dialysis for 6 h, and then increased gradually. Both the Ca and Mg contents decreased by ~ 50% during 24 h of dialysis. The P content decreased by ~ 27% during the 24 h dialysis time.

The Na content of acid WPC solution increased to more than double its initial level during the first 5 h of dialysis, and then increased gradually (Table 6.2). The K content dropped considerably (50%) after 5 h of dialysis and then decreased gradually. The Ca content increased gradually during the 24 h of dialysis.

Table 6.1. Variations in the mineral content of cheese WPC solutions with dialysis time

	Mineral content (mmol/kg of solution)			
Mineral	0 h	6 h	12 h	24 h
Na	91	85	67	57
K	183	404	455	477
Ca	142	109	96	70
Mg	21	20	17	12

Figure 6.8 shows the effect of dialysis on the concentrations of monovalent cations (Na and K), individually and in combination. It is clear that the changes in K concentration in acid and cheese WPC solutions with dialysis time (Fig. 6.8*a*) were much larger than the changes in Na concentration. For example, dialysing a small sample of cheese WPC against acid WPC for 24 h resulted in a decrease of ~ 30

mmol/kg in Na concentration but a relatively large increase (~ 300 mmol/kg) in K concentration. When the changes in both K and Na were combined (Fig. 6.8*b*), the trends were very similar to those of the changes in K alone (Fig. 6.8*a*).

Table 6.2. Variations in the mineral content of acid WPC solutions with dialysis time.

	Mineral content (mmol/kg of solution)			
Mineral	0 h	5 h	17 h	24 h
Na	20	47	50	53
K	424	212	192	201
Ca	39	49	57	67
Mg	2	4	5	6

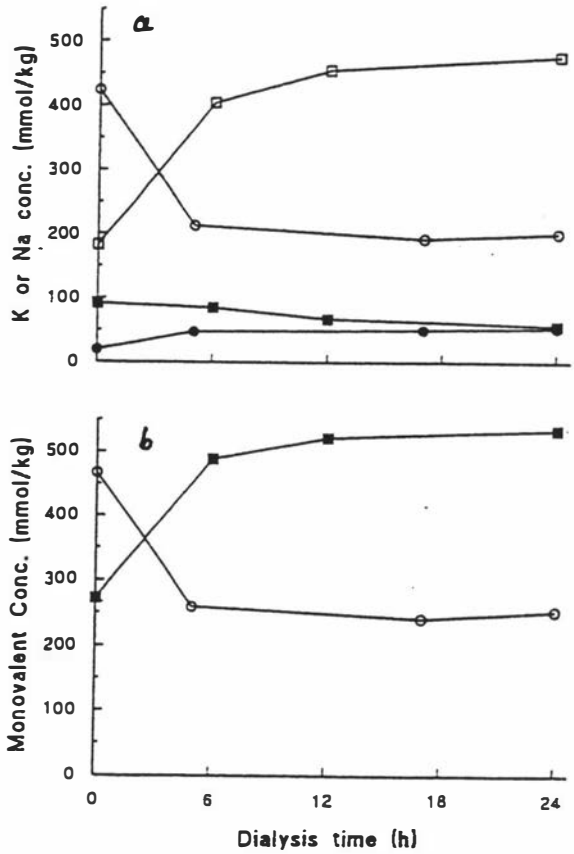


Figure 6.8. *a:* Effect of dialysis on the residual concentrations of Na (●, ■) and K (□, ○) in cheese WPC (□, ■) and acid WPC (○, ●) solutions. *b:* Effect of dialysis on the combined residual concentrations of Na plus K in cheese WPC (■) and acid WPC (○) solutions.

Figure 6.9 shows the effect of dialysis on the concentrations of the divalent cations (Ca and Mg), individually and in combination. The changes in the Mg concentration in both acid and cheese WPC solutions during dialysis were much smaller than the changes in the Ca concentration (Fig. 6.9*a*). When the changes in both Ca and Mg were combined (Fig. 6.9*b*), the trends were very similar to those of the changes in Ca alone (Fig. 6.9*a*).

It appears that dialysis changed the concentrations of monovalent cations relatively quickly so that there was an equilibrium between the two WPC solutions (Fig. 6.8). When a sample of cheese WPC solution was dialysed against excess acid WPC solution, the monovalent ion concentration of the cheese WPC sample increased, within 6 h, to levels that were comparable with that of the acid WPC solution (Fig. 6.8*b*). An opposite trend was observed when a sample of acid WPC was dialysed against excess cheese WPC solution; the monovalent ion concentration of the acid WPC sample decreased, within 6 h, to levels that were comparable with that of the cheese WPC solution. These results suggest that the monovalent ions were present in a fully "soluble" state in the WPC solutions. During dialysis, these ions could freely migrate through the dialysis membrane until an equilibrium concentration was reached between the two WPC solutions.

The changes in the divalent cation concentrations, on the other hand, appeared to approach an intermediate level with increasing dialysis time (Fig. 6.9*b*). The concentration of divalent cations was higher in the cheese WPC solution than in the acid WPC solution. After dialysis for 24 h, the divalent cation concentrations in either WPC solution reached levels that were somewhat intermediate between the initial concentrations in the acid WPC solution and those in the cheese WPC solution. These results suggest that the interchange of the divalent cations between the two WPC solutions was limited, which was probably due to binding of a fraction of the divalent cations to the proteins, indicating that the bound fraction could not migrate through the dialysis membrane.

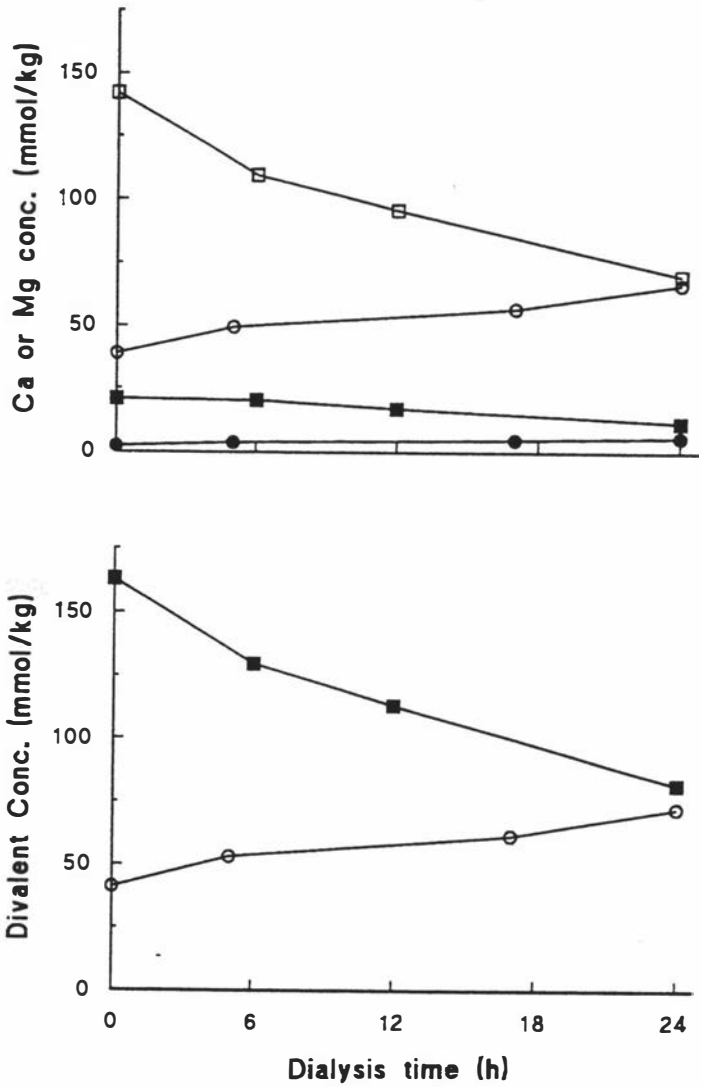


Figure 6.9. *a:* Effect of dialysis on the concentrations of Ca (■, ●) and Mg (□, ○) in cheese WPC (□, ■) and acid WPC (○, ●) solutions. *b*: Effect of dialysis on the combined concentrations on Ca and Mg in cheese WPC (■) and acid WPC (○) solutions.

6.6. Effect of dialysis on the aggregation of whey proteins

The dialysed acid and cheese WPC solutions were heated and then analysed using 1D-PAGE (Section 3.7), size exclusion chromatography (Section 3.9) and confocal laser microscopy (Section 3.11). The WPC samples that showed significant changes in gel strength after dialysis were analysed. Acid WPC samples dialysed for 14 and 24 h, and cheese WPC samples dialysed for 24 h were used for these analyses. In the following discussion, the dialysed WPC samples are denoted by either acid or cheese,

followed by the number of hours for which the samples had been dialysed. Thus, the acid WPC samples that had been dialysed for 14 h and 24 h are referred to as acid 14 and acid 24; cheese 24 refers to the cheese WPC sample that had been dialysed for 24 h. The non-dialysed WPC solutions are referred to as "control" WPC solutions.

6.6.1. Loss of proteins analysed by PAGE

The dialysed samples (acid 14, acid 24 and cheese 24) were heated (Section 3.4), diluted with sample buffer and then analysed using 1D native- or SDS-PAGE. Figure 6.10 shows the native- and SDS-PAGE patterns of heated dialysed WPC solutions. The PAGE patterns obtained for the dialysed solutions were very similar to those of the control WPC solutions (i.e. non-dialysed WPC solutions, Fig 5.1). However, quantitation of these patterns showed that the rates of loss of whey proteins from the dialysed solutions were different from the rates of loss of the same proteins from the control WPC solutions. To illustrate this point, the calculated rate constants for the loss of native-like and SDS-monomeric β-lactoglobulin, α-lactalbumin and BSA during heating from the dialysed WPC solutions, using a reaction order of $n = 1.5$, are shown in Table 6.3.

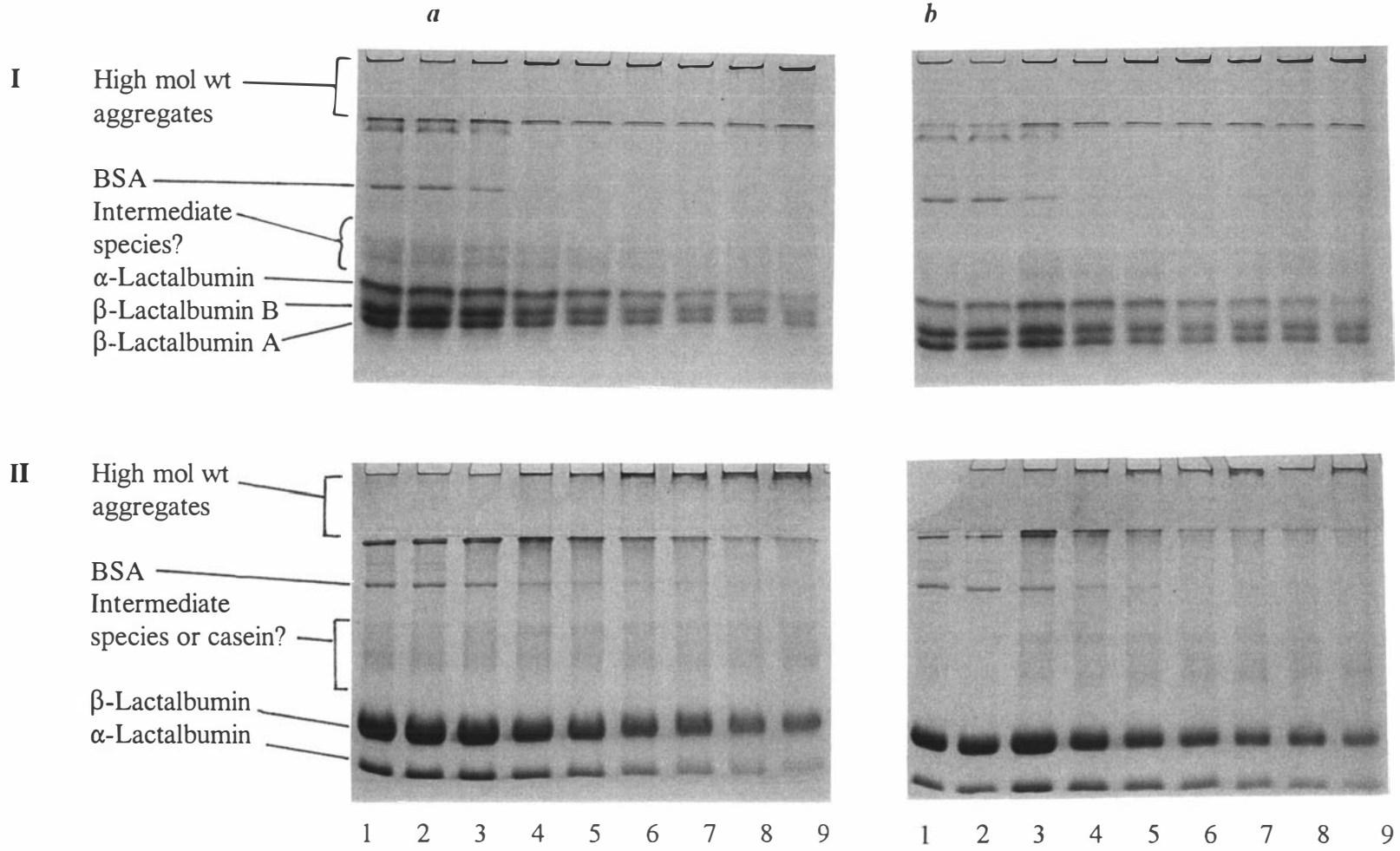


Figure 6.10. Native- (I) and SDS-PAGE (II) patterns of acid 14 (*a*), acid 24 (*b*) and cheese 24 (*c*) WPC solutions heated at 75 °C for 0 (slots 1 & 2), 2 (slot 3), 4 (slot 4), 6 (slot 5), 8 (slot 6), 10 (slot 7), 12 (slot 8) and 15 (slot 9) min. (Note: (*c*) is on the following page).

c

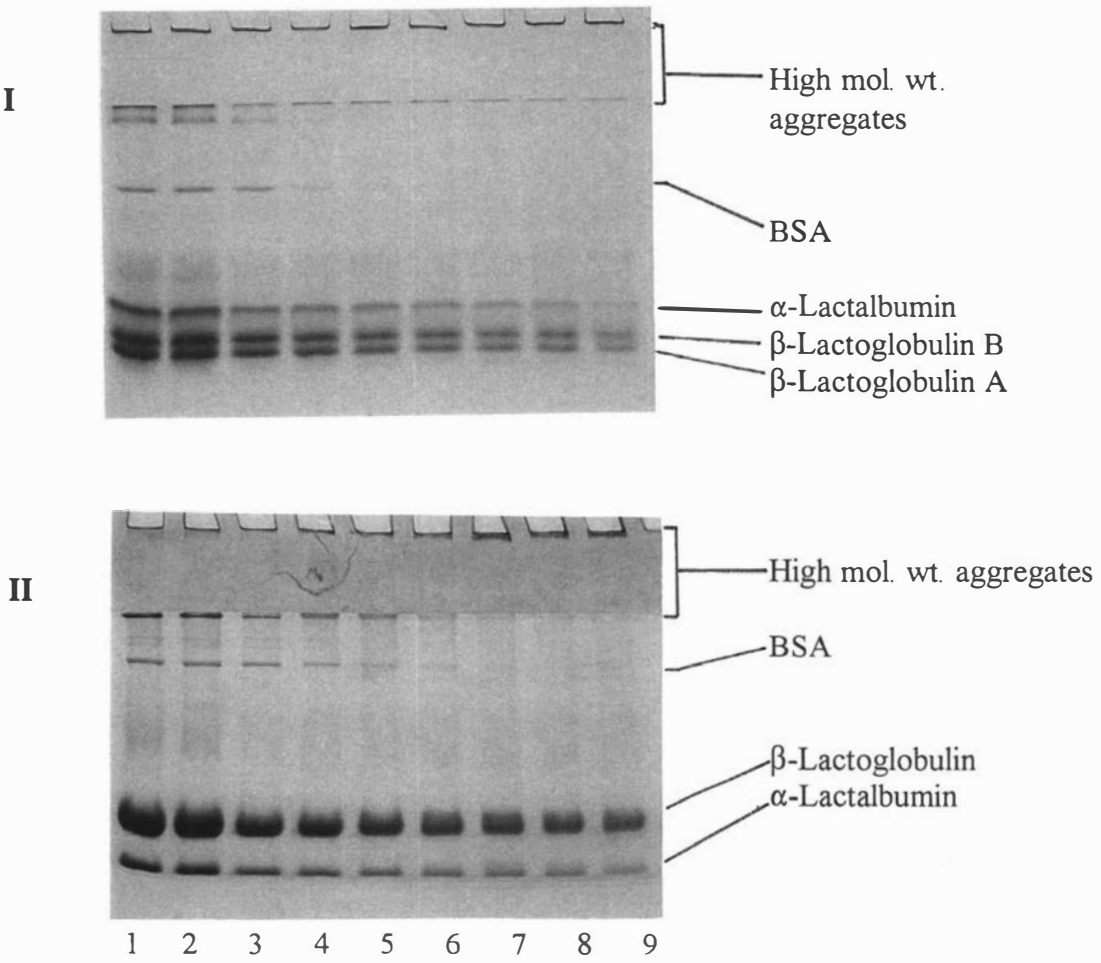


Figure 6.10. (Continue). Native- (I) and SDS-PAGE (II) patterns of cheese 24 (*c*) WPC solutions heated at 75 °C for 0 (slots 1 & 2), 2 (slot 3), 4 (slot 4), 6 (slot 5), 8 (slot 6), 10 (slot 7), 12 (slot 8) and 15 (slot 9) min.

Table 6.3. Calculated rate constants (k_n, $min^{-0.5}$) and coefficients of determination (r^2) for the loss of native-like and SDS-monomeric β-lactoglobulin, α-lactalbumin and BSA from dialysed WPC solutions during heating, using a reaction order n = 1.5

Loss of native-like	**β-Lactoglobulin**		**α-Lactalbumin**		**BSA**	
Samples	k_n	r^2	k_n	r^2	k_n	r^2
Acid WPC control*	0.0095	0.96	0.0086	0.91	0.0206	0.98
Acid 14	0.0286	0.99	0.0226	0.97	0.0669	0.98
Acid 24	0.0107	0.96	0.0096	0.99	0.0822	0.96
Cheese WPC control*	0.0243	0.94	0.0178	0.90	0.0548	0.95
Cheese 24	0.0172	0.99	0.0121	0.98	0.0655	0.98
Loss of SDS-monomeric	**β-Lactoglobulin**		**α-Lactalbumin**		**BSA**	
Acid WPC control**	0.0024	0.98	0.0084	0.92	0.0165	0.97
Acid 14	0.0124	0.98	0.0160	0.96	0.0298	0.95
Acid 24	0.0117	0.97	0.0127	0.90	0.0399	0.90
Cheese WPC control**	0.0089	0.97	0.0110	0.98	0.0164	0.98
Cheese 24	0.0111	0.96	0.0122	0.93	0.0284	0.98

* Results also presented in Table 5.2. ** Results also presented in Table 5.3.

The coefficients of determination (r^2) for the calculated values of k_n were reasonably high for each set of data, indicating that each set of data could be adequately modelled by a reaction order (n) = 1.5. The values of k_n for the loss of native-like β-lactoglobulin, α-lactalbumin and BSA from the dialysed acid WPC solutions (acid 14 and acid 24) were higher than the values for the loss of the same proteins from the control samples (Table 6.3). This showed that dialysis increased the rate of loss of proteins from the acid WPC solutions during heating. The k_n values for the loss of the native-like proteins from cheese 24 were lower than the values for the loss of the same proteins from the control cheese WPC solution (Table 6.3). Therefore, it appears that dialysis resulted in a decrease in the rate of loss of proteins from the cheese WPC solution.

The k_n values for the loss of SDS-monomeric proteins from the dialysed WPC solutions were higher than those for the loss of the same proteins from the control WPC solutions.

By comparing the native- and SDS-PAGE results for the loss of proteins from the dialysed WPC solutions, the relative proportions of proteins that were monomeric, and that were involved in the formation of hydrophobic or disulphide-linked aggregates, were determined (Fig. 6.11) as described in Section 4.6.3. Both β-lactoglobulin and α-lactalbumin formed relatively large quantities of hydrophobically associated aggregates in acid 14 (Fig. 6.11*a, d*), and relatively smaller quantities in acid 24 (Fig. 6.11*b, e*) and cheese 24 (Fig. 6.11*c, f*). It is clear that the relatively high rates of loss of native-like β-lactoglobulin and α-lactalbumin coincided with the formation of considerable amounts of hydrophobically associated aggregates in acid 14. The loss of the same proteins from acid 24 and cheese 24, which occurred at relatively slow rates, coincided with the formation of predominantly disulphide-linked aggregates.

It was observed that acid 14 formed gels after heating for 8 min (the same gelling time as that of the control cheese WPC solution). Acid 24, on the other hand, did not form a gel even after heating for 15 min.

These results show that dialysis of the acid WPC solution against excess cheese WPC solutions for 14 h increased the loss of native-like proteins and that the loss was comparable with the loss of the same proteins from the non-dialysed cheese WPC solution. This increase was associated with the accumulation of considerable amounts of hydrophobically associated aggregates.

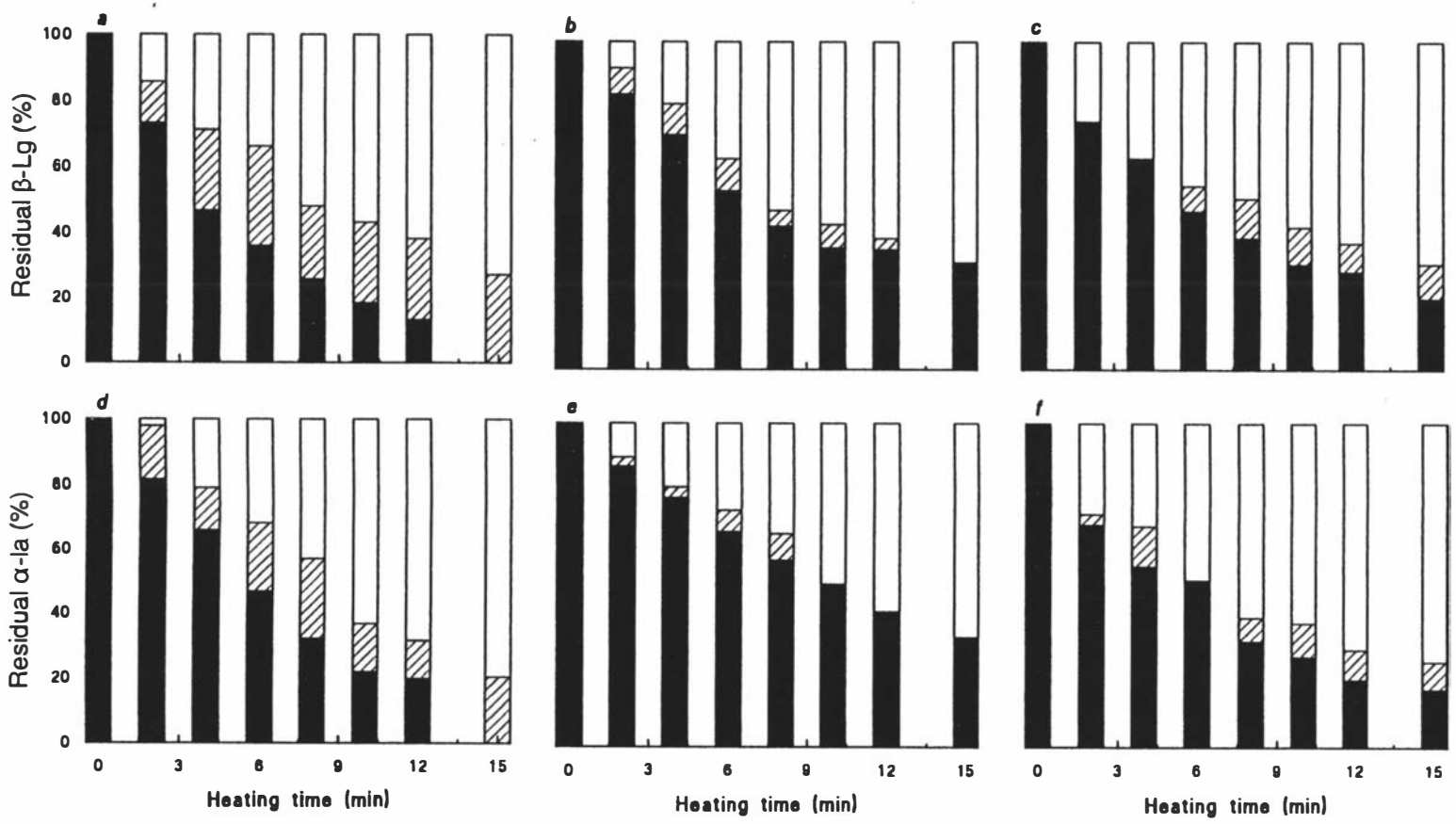


Figure 6.11. β-Lactoglobulin (*a, b, c*) and α-lactalbumin (*d, e, f*) aggregates in heated acid 14 (*a, d*), acid 24 (*b, e*) and cheese 24 (*c, f*) WPC solutions. Protein species: native-like (■), non-covalently linked aggregates (▨) and disulphide-linked aggregates (□). Note: The results for the native-like or non-covalently linked aggregates for some of the samples were not available because the PAGE bands were either too faint or not sharp enough to give a well defined peak on scanning.

6.6.2. Loss of proteins measured by size exclusion chromatography

The SEC profiles of the heated dialysed acid WPC solutions (Fig. 6.12*a, b*) were quite different from those of the control acid WPC solutions (Fig. 5.7*a*). Heated samples of acid 14 showed no accumulation of small (< 0.22 μm) aggregates (Fig. 6.12*a*, peak 1), as indicated by the decreasing size of peak 1 with heating time. The heated samples of acid 24 (Fig. 6.12*b*) showed an increased amount of small aggregates for the sample heated for 2 min. However, heating for more than 2 min showed decreasing amounts of small aggregates (i.e. decreases in the size of peak 1 with heating time). This indicates that the small aggregates quickly formed larger aggregates (> 0.22 μm) that were removed by the pre-analysis filtration step. The heated samples of cheese 24 showed that considerable quantities of small aggregates (< 0.22 μm) were formed (Fig. 6.12*c*). Even in the sample that was heated for 10 min, there was still a considerable quantity of fine aggregates, indicated by the size of peak 1 (Fig. 6.12*c*, v). It is clear that dialysis resulted in changing the aggregation of the cheese WPC from the formation of relatively large aggregates that were removed by the pre-analysis filtration (Fig. 5.7*b*) to the formation of considerable quantities of small (< 0.22 μm) aggregates, as indicated by the size of peak 1 (Fig. 6.12*c*).

6.6.3. Confocal laser microscopy

Control WPC solutions (i.e. non-dialysed acid and cheese WPC solutions) and the dialysed samples (acid 14, acid 24 and cheese 24) were heated for 8 min and then viewed under a confocal laser microscope. Figure 6.13 shows the photomicrographs of the acid WPC solutions. The control acid WPC solution (Fig. 6.13*a)* showed tiny aggregates that appeared to be homogeneous, with a similar appearance to that observed using TEM (Fig. 5.8*a*). Although the samples of heated acid 14 and acid 24 solutions appeared to have aggregates that were larger (Fig. 6.13*b, c*), the structures were not very clear.

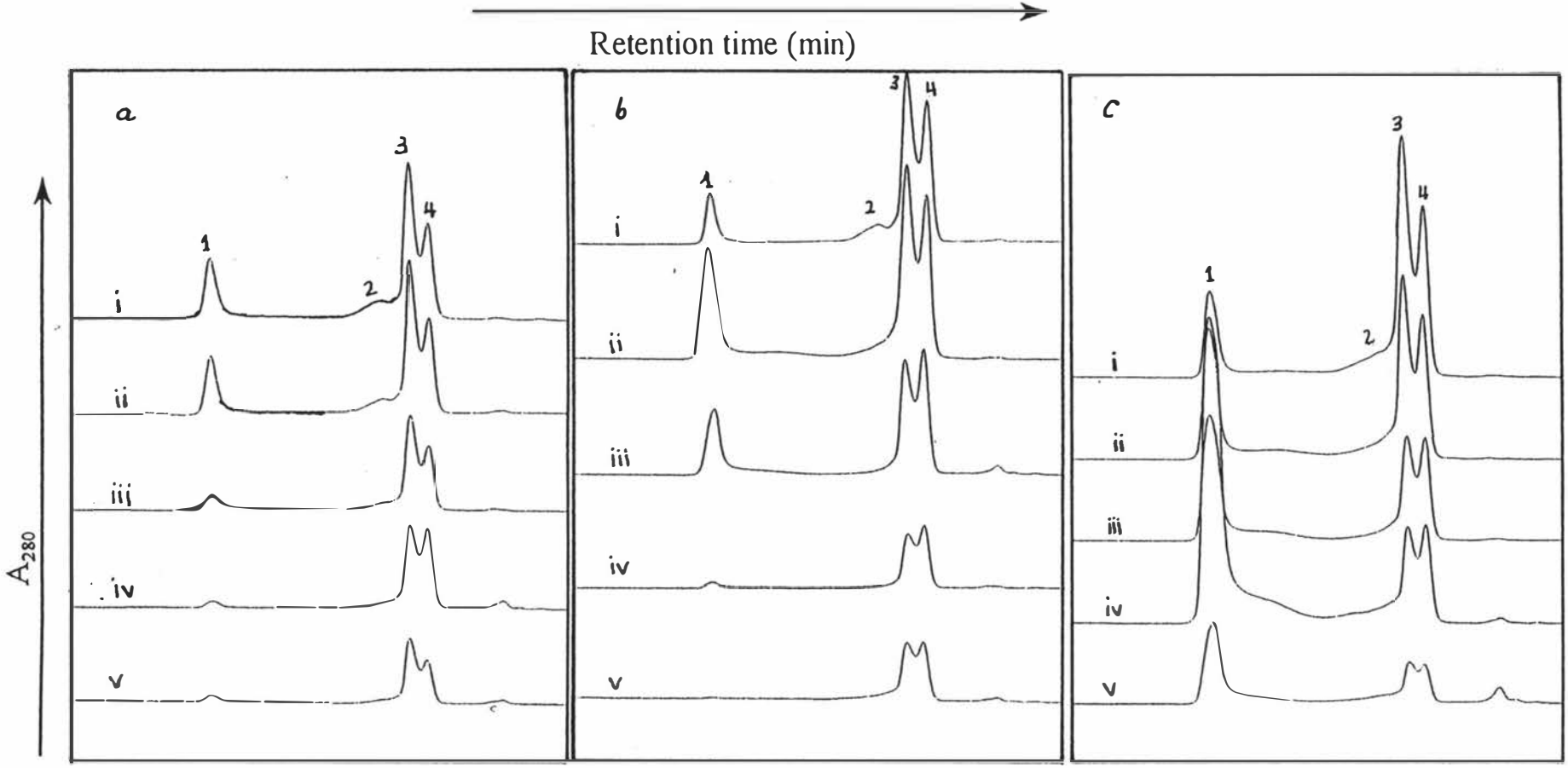


Figure 6.12. SEC elution profiles of 120 g/kg (pH 6.8) acid 14 (*a*), acid 24 (*b*) and cheese 24 (*c*) WPC solutions heated at 75 °C for 0 (i), 2 (ii), 4 (iii), 6 (iv) and 10 (v) min. Superose 6HR 10/30 column, flow rate 0.4 ml/min, eluent - 20 mM imidazole buffer (pH 6.9, 50 mM NaCl).

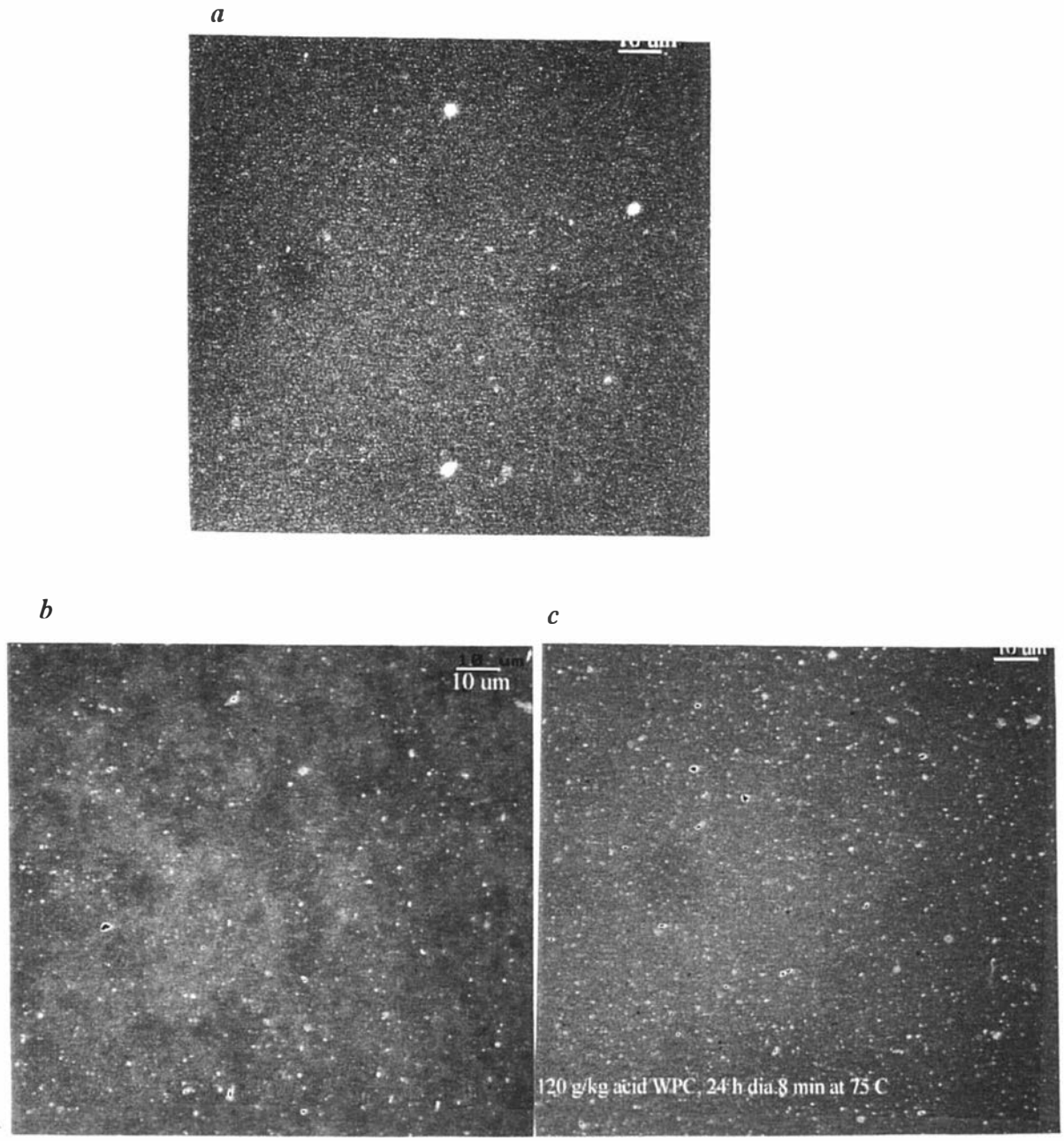


Figure 6.13. Confocal micrographs of 120 g/kg (pH 6.8) acid (*a*), acid 14 (*b*) and acid 24 (*c*) WPC solutions heated at 75 °C for 8 min, and viewed under a confocal laser microscope. Magnification: 10,000.

The confocal micrograph of the heated control cheese WPC solution (Fig. 6.14*a*) showed similar aggregate structure to that viewed using TEM (Fig. 5.8*b*). The

structure was not uniform but contained large pores and the aggregates contained a number of large particles. Dialysing the cheese WPC solution for 24 h (cheese 24) before heating changed the aggregate structure dramatically (Fig. 6.14*b*). The aggregates appeared to be small, uniformly distributed and homogeneous; this aggregate structure was very similar to that of the control acid WPC (Fig. 6.13*a*).

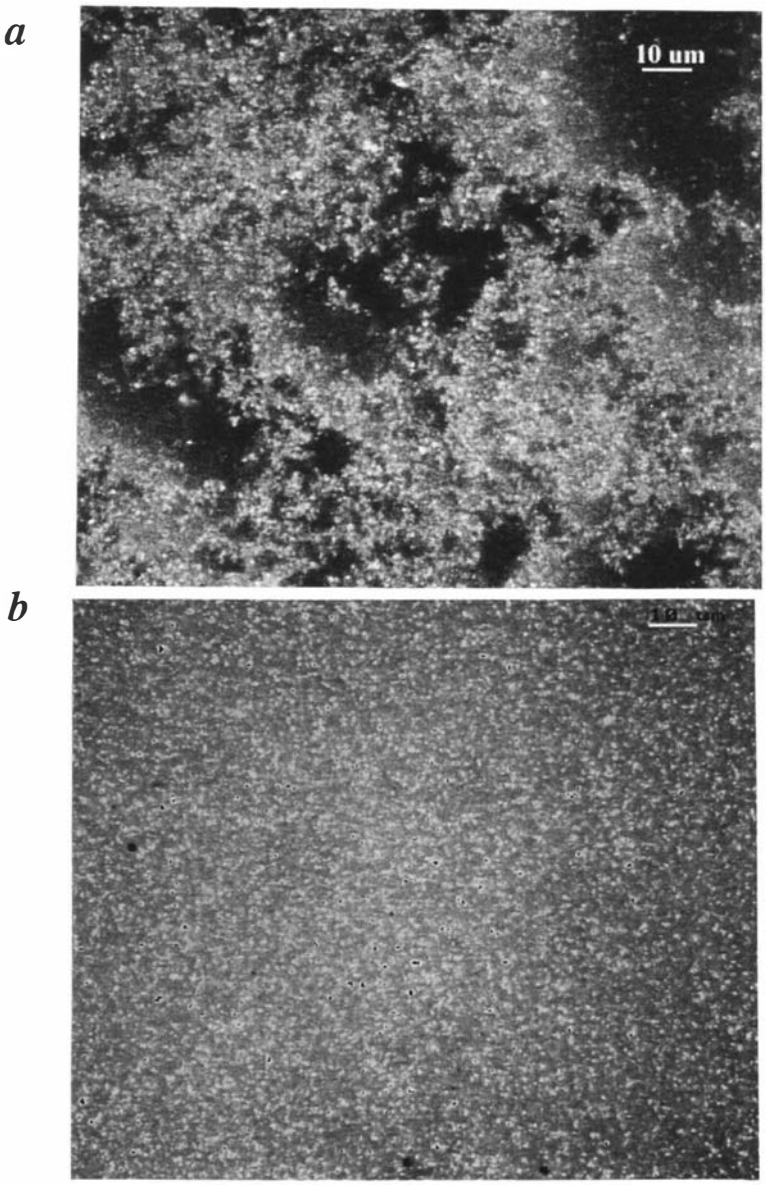


Figure 6.14. Confocal micrographs of 120 g/kg (pH 6.8) control cheese (*a*) and cheese 24 (*b*) WPC solutions heated at 75 °C for 8 min, and viewed under a confocal laser microscope. Magnification: 10,000.

6.7. Discussion

It is generally accepted that two basic protein gel structures are commonly found in food systems (Doi, 1993). The first is a "particulated" gel that is composed of relatively large particles bound to one another to form a network. These gels are usually opaque and have poor water-holding capacity. The second is a "fine-stranded" gel produced by the association of small diameter particles or strands to form a network. These gels are usually translucent and have good water-holding capacity (Stading & Hermansson, 1991; Langton & Hermansson, 1992). Variations between these two types of gel exist and sometimes mixed gels are formed (Barbut, 1995). These two types of gel have also been observed in WPC gels (Aguilera, 1995). The formation of one or the other type is determined largely by the differences between the rate of protein denaturation and the rate of aggregation of the denatured proteins (Taylor & Fryer, 1992), and the relative proportions of protein-protein attractive and repulsive interactions (Egelandsdal, 1980; Clark & Lee-Tuffnell, 1986; Mulvihill & Kinsella, 1987; Gault & Fauquant, 1992). The formation of a fine-stranded gel requires that protein denaturation takes place at a faster rate than protein aggregation, and that there is a balance between the protein-protein attractive and repulsive interactions. Particulated gels are formed when the aggregation of proteins takes place at faster rates than protein denaturation, and the protein-protein attractive and repulsive interactions are not in balance. Excessive attraction results in a randomly aggregated coagulum or even a precipitate, whereas excessive repulsion results in weak or non-existent aggregation (Tang *et al.*, 1995).

The results of this study clearly showed that the heat-induced gels produced from acid and cheese WPCs were very different. The cheese WPC gels were opaque, had low gel strength and showed high syneresis, i.e. poor-water holding capacity (Fig. 6.1). These gels were composed of large protein aggregates (Figs 5.9 & 6.14). These observations suggest that the cheese WPC gels probably fit the description of the "particulated" gels. The acid WPC gels, on the other hand, were more transparent (Fig. 6.1), had higher gel strength (Figs 6.2, 6.3 & 6.4) and probably had good water-holding capacity, because no syneresis was observed. These gels were composed of small aggregates (Figs 5.6, 5.7 & 6.13). The acid WPC gels could, therefore, be described as "fine-stranded" gels. The two WPCs used also had very different mineral compositions

(Table 5.6) which were largely responsible for the different gel types and strengths. The most significant differences in the mineral composition of the WPCs were the relatively high concentrations of Ca and Mg in cheese WPC, and a higher concentration of K in acid WPC. When the mineral composition of the WPC solutions was altered by dialysis, the WPC gel strength was also altered (Figs. 6.4, 6.5 & 6.7). The results also indicated that the proportions of non-covalently linked (hydrophobic and ionic associated) aggregates in the heated WPC solutions were also altered as a result of the dialysis (Fig. 6.11).

Kuhn & Foegeding (1991) reported that WPI gels with low levels of added NaCl (25-30 mM) were translucent and gelatin-like with low shear stress and high shear strain values, whereas WPI gels formed with low levels of added $CaCl_2$ (7.5 mM) were opaque and curd-like with low shear stress (gel strength) and shear strain values. Increasing the levels of either salt caused a sharp increase in the shear stress of the gel to a maximum at 50-75 mM NaCl or at 20 mM $CaCl_2$. Increasing $CaCl_2$ also caused an increase in gel shear strain to a maximum at 50-75 mM $CaCl_2$, whereas increasing NaCl caused a decrease in shear strain to a minimum at 50-75 mM NaCl. The different effects of NaCl and $CaCl_2$ were reported to be general monovalent (Na, L, K, Rb, Cs) or divalent (Ca, Mg, Ba) cation effects. When both monovalent and divalent ($KCl/CaCl_2$) cations were added to the WPI solution, the divalent cation determined the shear strain (Khun & Foegeding, 1991).

Tang *et al.* (1995) studied the effect of pH and added NaCl and $CaCl_2$ on gel development in WPC solutions using oscillatory rheometry. They observed that, at pH 7 and 8, the storage modulus, G′, increased to a maximum and then decreased with increasing ionic strength. They concluded that the changes in both pH and salt concentration affected the gel development by altering the balance between attractive and repulsive protein-protein interactions. Xiong (1992) observed, that on heating (60-95 °C) WPI solution (1.2 mg/ml) at pH ≥ 6.5 and zero ionic strength, the protein-protein interaction was largely suppressed. However, the addition of NaCl, and NaCl in conjunction with sodium phosphate, promoted protein-protein interaction.

The divalent cations (Ca or Mg) induce protein aggregation in three different ways: (1) electrostatic shielding, (2) ion-specific hydrophobic interactions and (3) crosslinking of adjacent anionic molecules by forming protein-Ca-protein bridges (Kinsella *et al.*, 1989; Wang & Damodaran, 1991; Ju & Kilara, 1998). de Wit (1981) demonstrated that the amount of Ca that was necessary to induce the aggregation of β-lactoglobulin was equivalent to the net negative charge of the β-lactoglobulin. Similarly, Zittle *et al.* (1957) reported that the amounts of Ca bound to β-lactoglobulin were stoichiometrically equivalent to the net charge of the protein, and were independent of the protein concentration and its denatured state.

The relatively high divalent cation content of the cheese WPC used in the current investigation was probably due to the high pH (i.e. 6.0-6.6) of the starting raw whey used in WPC manufacture (see Fig. 2.2). This pH range is well above the isoelectric points of the major whey proteins (β-lactoglobulin, 5.13; α-lactalbumin, 4.2-4.5; BSA, 4.7-4.9). At this pH range, the protein molecules are negatively charged and would bind a considerable amount of cations. Because these cations are bound to proteins, they would not be removed during the UF/DF stages of the manufacturing process; hence the high concentration of these cations in the cheese WPC. The acid WPC, on the other hand, is manufactured from raw whey at pH 4.5. At this pH, the proteins are positively charged; hence the cations, including Ca and Mg, remain soluble rather than binding to proteins, and are removed during the UF/DF stages. The relatively high K content of the acid WPC was due largely to the use of KOH for neutralisation of the UF retentate during WPC manufacture.

There is probably an optimum concentration of cations that gives rise to the formation of gels with maximum gel strength (Tang *et al.*, 1995); this concentration was not defined for either cheese WPC or acid WPC. The fact that the gel strength of the cheese WPC solutions increased after altering the mineral content (Fig. 6.7) indicated that the initial levels of cations were outside the optimum levels (probably too high). The high concentration of cations, particularly Ca and Mg, would result in excessive ionic interactions of protein via the three different ways stated above, which would promote the aggregation process and give rise to the formation of considerable proportions of non-covalently linked aggregates in the cheese WPC solutions (Fig.

6.11). The fast rates of protein aggregation probably gave rise to the formation of the large aggregates observed for these WPC solutions (Figs 6.12 & 6.14).

Morr & Josephson (1968) suggested that the heat-induced aggregation of whey proteins in the presence of Ca involved the formation of large aggregates, which was dependent on the Ca concentration. Li *et al.* (1994) suggested that Ca binding during the initial steps of polypeptide unfolding stabilises the unfolded form and that inter- and or intramolecular crosslinks between ionic carboxyl groups (and possibly histidine ligands) are bridged by divalent ions during the gel formation process. A decrease in the Ca concentration would be expected to reduce the occurrence of Ca bridges in the gel, and would favour the formation of fine stranded gels (Li *et al.*, 1994). More recently, Ju & Kilara (1998) investigated the effect of added $CaCl_2$ on the aggregation behaviour of WPI at 45 °C. They reported that high $CaCl_2$ concentrations (from 30 to 50 mM) resulted in quicker initial increases and higher maximum turbidity values after 5 h of incubation. At 50 mM $CaCl_2$, large aggregates were formed which sedimented during incubation, whereas at < 30 mM $CaCl_2$, the aggregates formed were stable colloidal particles that did not sediment. The results of the current study are consistent with the findings of these workers.

The monovalent cations affect the WPC gelation in two different ways. (1) They reduce the electrostatic repulsive forces between the negatively charged protein molecules by masking the charged residues; hence the reactive groups of the unfolded molecules can come into close proximity with each other. (2) They reduce the extent of divalent cation bridges between molecules; hence disulphide bonds are the predominant linkages between protein molecules. The monovalent cations bind on to the unfolded protein molecules in same way as described for the divalent cations; however, because of the single positive charge, they do not form bridges between the protein molecules but simply mask the negatively charged groups, hence reducing the repulsive forces between the protein molecules. This allows the molecules to come closer to each other, giving rise to the formation of non-covalent associations between the exposed hydrophobic groups or covalent disulphide bonds between molecules. This study showed that the acid WPC gels were formed predominantly by disulphide-linked bonds with little contribution from non-covalently linked aggregates (Fig. 5.5). The

high concentration of K in the acid WPC system resulted in masking of the negatively charged groups, hence reducing the binding of divalent cations and the formation of divalent bridges between the molecules. Because of the limited number of reactive sites (-SH and S-S) on the protein molecules, the formation of disulphide bonds between the molecules is a relatively slow reaction. This is probably the main cause of the lower reaction rates and the formation of smaller protein aggregates observed for the heated acid WPC solutions. Overall, the monovalent cations had a positive effect, to a certain level, on WPC gelation.

It was mentioned earlier (Section 6.1) that the protein gel network is held together predominantly by inter- and intramolecular disulphide bonds with some contributions from non-covalent interactions. The proportions of proteins that were linked by disulphide bonds or non-covalent interactions in the heated WPC solutions were estimated in this study using a combination of native- and SDS-PAGE. The latter was estimated as the proportion of monomeric proteins that were not native but dissociated from protein aggregates in the presence of SDS. Presumably, these proteins had interacted via hydrophobic or ionic associations. The results of the current investigation indicated that, the higher the proportions of non-covalently linked proteins in the heated WPC solutions, the weaker and generally "poorer" the gel properties would be. This implies that, when the denatured proteins in a heated WPC solution are linked predominantly by disulphide bonds, a stronger gel is formed. The results showed that the "poor" gelling properties of the cheese WPC was due largely to the high content of divalent cations (Ca and Mg) in the product. As mentioned earlier, this high level was due to cations being bound to proteins at the relatively high pH of the raw whey used in cheese WPC manufacture. This finding is significant because it provides a means of improving the gelation properties of cheese WPC, simply by reducing the pH of the raw whey before UF.

In conclusion, the heat-induced WPC gel properties are affected by the mineral environment. High concentrations of divalent cations can result in the formation of considerable proportions of non-covalently linked aggregates, which is manifested in low gel strength and poor gelling properties. Monovalent cations promote the formation of disulphide linked aggregates, which favours the formation of fine-stranded gels. The poor gelling properties of cheese WPC can be explained largely by the excessive levels of divalent cations. Reduction of these levels during manufacture could result in the production of cheese WPC with gelling properties comparable with those of acid WPCs.

CHAPTER 7

INSOLUBLE AGGREGATES IN WPC POWDERS: COMPOSITION AND EFFECTS ON HEAT-INDUCED GELATION

7.1. Introduction

The manufacture of WPC (see Chapter 3) involves a variety of steps including thermal processing, ultrafiltration, evaporation and spray drying (Modler, 1985; Sherwin & Foegeding, 1997). The temperatures used during processing may cause denaturation and aggregation of proteins which can alter their functional properties. Denatured and aggregated proteins are largely insoluble and hence cannot be used in foods to produce effects heavily dependent on solubility (Fox & Mulvihill, 1982). The effect of various unit processes during WPC manufacture on the functionality of the product is cumulative (Schmidt *et al.*, 1984). Kohnhorst and Mangino (1985) reported that the solubility of 11 commercial WPCs ranged from 25.4 to 82.4%. Morr (1985) reported that commercial WPCs may contain 20 to 50% denatured protein, however the extent of protein denaturation has not been reported in most studies of functionality. Dunkerley and Zadow (1988) suggested that WPCs could be 'tailor-made' for certain applications by controlling the solubility of the protein. In contrast, Dybing & Smith (1991) suggested that it is desirable to avoid protein denaturation during manufacture in order to retain maximum WPC functionality.

The extent of heat-induced denaturation and aggregation of protein during WPC manufacture is also affected by other factors such as pH and ionic strength (de Wit & de Boer, 1975). Heating of whey protein solutions at pHs close to the protein isoelectric points (pH 4.3-5.0) generally results in a measurable reduction in protein solubility (Morr *et al.*, 1973) and the presence of calcium has been shown to reduce protein solubility upon heating (de Wit & van Kessel, 1996).

It was shown in Chapter 4 that when an unheated 120 g/kg WPC solution was centrifuged at 90,000 × g for 1 h , considerable amounts (up to 100 g/kg) of aggregates were sedimented. Although, these aggregates contained all of the whey proteins, there were disproportionately high concentrations of BSA and the other minor whey protein components. It was important to characterize these aggregates and to determine how they may affect the gelling properties of the WPC powders. Hence we investigated the nature of these aggregates and how they affect the gelling properties of acid and cheese WPC solutions.

7.2. Composition of the pellet

The pellets obtained from centrifugation (45,000 × g, for 2 h at 20 °C) of unheated WPC solutions (120 g/kg, pH 6.8) were freeze-dried and then analyzed for total protein, fat, phospholipid and mineral content (Table 7.1). The β-lactoglobulin, α-lactalbumin and BSA contents were determined by quantitative native- or SDS-PAGE, as described earlier (Section 5.1). The composition of the WPC powders presented in Table 5.1 is also shown in Table 7.1 for comparison with the pellets.

When the WPC solutions were centrifuged, the quantities of the pellets obtained were 67 and 100 g/kg dry weight for acid and cheese WPCs, respectively. The acid WPC pellet had slightly higher contents of total protein, β-lactoglobulin, α-lactalbumin and BSA than that the cheese WPC pellet (Table 7.1). The cheese WPC pellet, however, had higher contents of fat and phospholipid. The total protein concentrations of the pellets were similar to that of the original WPCs, but the concentrations of β-lactoglobulin and α-lactalbumin were slightly less in the pellets. The BSA concentrations of the pellets were much higher than that of the WPCs. The fat content of the acid WPC pellet was similar to that of the original WPC whereas the phospholipid content was higher in the pellet. The cheese WPC pellet, on the other hand, had much higher contents of fat and phospholipid than that of the original WPC. It was not possible to determine the GMP contents of the pellets because of the low solubility of the pellet in the elution buffer for HPLC analyses (Section 3.2). However, when the supernatant obtained by centrifugation of the 120 g/kg cheese WPC solutions was analysed, the GMP content of the supernatant (168 g/kg) was similar to that of the

WPC solutions (172 g/kg). Therefore, it appears that the GMP was not preferentially incorporated into the pellet.

Table 7.1. Composition of insoluble aggregates and WPC powders.

	Concentration (dry weight)			
	Acid WPC		Cheese WPC	
Component	Powder*	Pellet	Powder*	Pellet
	g/kg	g/kg	g/kg	g/kg
Total protein	845	851	837	827
β-Lactoglobulin	436	387	422	379
α-Lactalbumin	158	145	149	136
BSA	39	81	51	76
GMP	<5	ND	172	ND
Fat	55	52	68	109
Phospholipid	17	28	21	57
Mineral content (g/kg dry weight)				
Ca	1.7	0.9	4.0	2.8
K	13.8	6.4	7.7	3.0
Na	8.0	0.4	2.3	0.6
P	2.5	3.0	3.0	6.0
Mg	0.01	0.08	0.4	0.2
Total (g/kg) **	**919**	**914**	**922**	**949**

The amounts of dry pellet obtained from centrifugation of 120 g/kg WPC solutions were 67 g/kg and 100 g/kg (dry weight) respectively.
ND - not determined.
* Data also presented in Table 5.1.
** For estimation of the total quantities, note that the individual protein components and GMP were also measured as parts of the total protein contents (i.e. when determining total protein content by Kjeldahl, GMP was also included) and the phospholipid were also measured as parts of the fat content (i.e. determining the total fat content, phospholipid was also included).

The mineral contents of the pellets were less than that of the original WPCs, except for phosphorus (P) which was much higher in the pellets; this was probably due to higher concentration of phospholipid material in the pellet.

The β-lactoglobulin, α-lactalbumin and BSA accounted for 72 and 71% of the total protein of the acid and cheese WPC pellets, respectively. The protein fractions which were not accounted for probably included immunoglobulin, lactoferrin, caseins, proteose peptones and other minor whey proteins. The analysed components (Table 7.1) accounted for ~ 92 - 95% of the total dry weight of either WPCs or pellets. The remaining material probably consisted of lactose and other minerals and possibly some bacterial debris. The high BSA content of the pellets was probably due to BSA aggregation during the manufacture of WPC. BSA and the other minor whey proteins (e.g. immunoglobulin and lactoferrin) are heat sensitive and could have easily denatured during the pasteurization and evaporation processes used in the manufacture of WPC and therefore be present in WPCs as large aggregates. It is interesting to note that the pellets contained considerable amounts of fat, and this fat contained a high proportion of phospholipid. It is possible that the fat globules and phospholipids had interacted with the proteins (probably BSA and other minor whey proteins) during WPC manufacture to form dense aggregates that sedimented on centrifugation.

7.3. Characterisation of pellet proteins using PAGE

7.3.1. 1D-PAGE

The pellets were dispersed in water and the solutions analysed using 1D-PAGE. The native-PAGE patterns of the WPC pellet (Fig. 7.1*a*) were similar to those of the original WPCs; but the protein bands in the pellet samples were less intense than those of the WPC samples. The concentrations of native-like proteins (β-lactoglobulin, α-lactalbumin and BSA) in the WPC pellets were estimated, using standard curves (see Sections 3.2 and 5.1). The results showed that the concentrations of β-lactoglobulin, α-lactalbumin and BSA in the acid WPC pellet were higher than in the cheese WPC pellet (Table 7.2). These native proteins were probably trapped in the pellets during centrifugation, since the pellets were not washed. The pellets contained considerable amounts of covalently-linked high mol wt aggregates that were not dissociated under

SDS conditions, but remained on top of the stacking gels (Fig. 7.2, slots 3). Upon reduction, there appeared to be some non-reducible material that remained on top of the stacking gels (Fig. 7.2, slots 4). This material was probably bacterial cell wall and membrane.

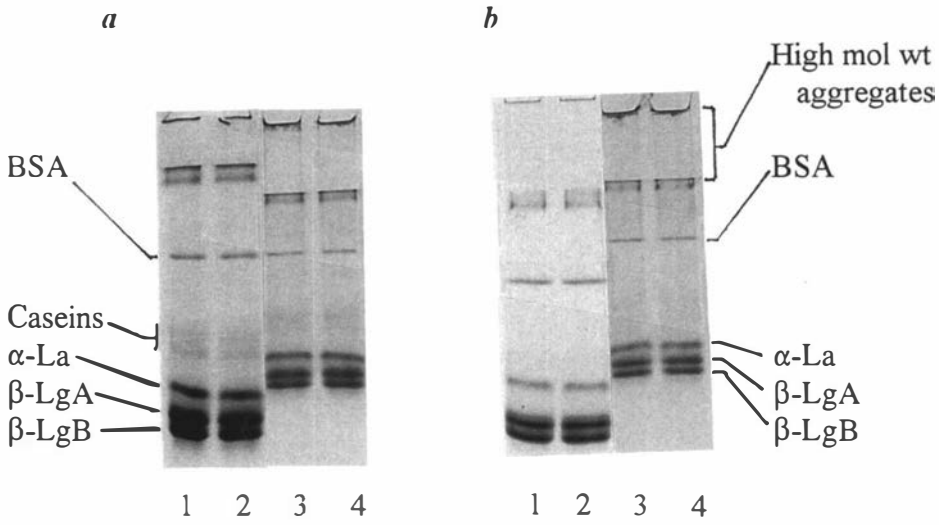


Figure 7.1. Native-PAGE patterns of acid (*a*) and cheese (*b*) WPC (slots 1 & 2) and pellets (slots 3 & 4).

Table 7.2. The concentrations of native-like β-lactoglobulin, α-lactalbumin and BSA in WPC pellets. The proportion of each protein that is native is also given, in parenthesis, as percentages of the total amount present in the pellet (Table 7.1).

	g/kg dry weight		
Samples	β-Lactoglobulin	α-Lactalbumin	BSA
Acid WPC pellet	296 (76%)	106 (73%)	33 (41%)
Cheese WPC pellet	216 (57%)	61 (45%)	26 (34%)

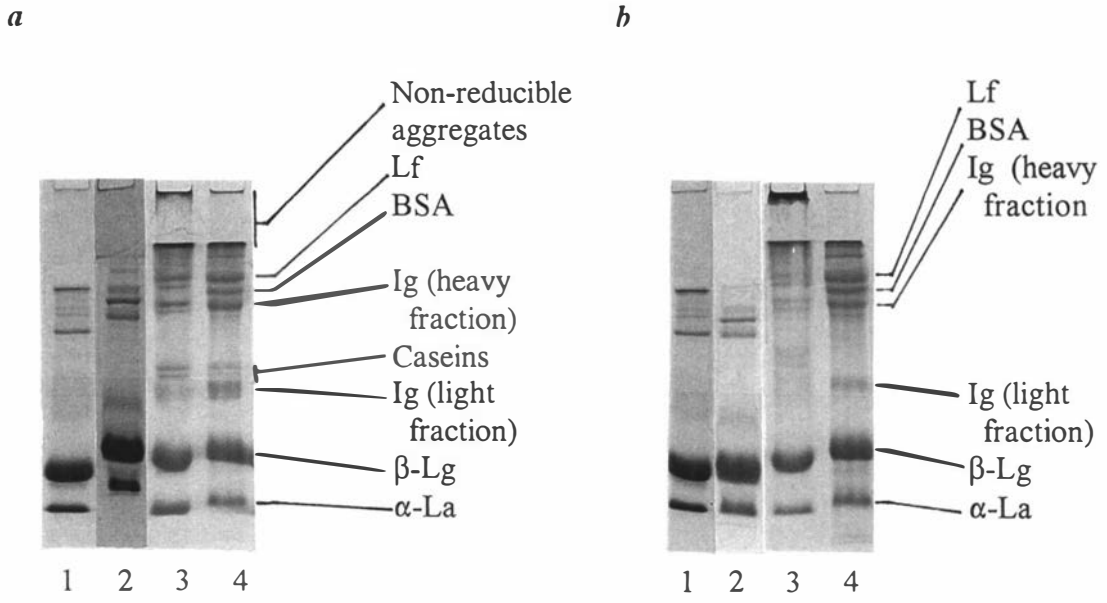


Figure 7.2. SDS-PAGE patterns of acid (*a*) and cheese (*b*) unheated WPC (slots 1 & 2) and pellets (slots 3 & 4). Slots 2 and 4 are reduced versions of slots 1 and 3, respectively.

The SDS-PAGE patterns of the WPCs and pellets are shown in Fig. 7.2. The intensity of β-lactoglobulin and α-lactalbumin bands of the pellets was less than that of the corresponding WPCs. Comparing the SDS-PAGE patterns of the acid WPC and cheese WPC pellets (Fig. 7.2*a,b*, slots 3), it was clear that the acid WPC pellet contained more casein. Also, the amount of aggregated material caught at the top of the stacking gel was greater in the cheese WPC than in the acid WPC. Reduction of the WPC samples resulted in the disappearance of the material caught on top of and within the stacking gel (Fig. 7.2, slots 2). By contrast, reduction of the WPC pellet samples did not completely resolve the material caught within the stacking gel (Fig. 7.2, slots 4), indicating that the non-reducible aggregates that were undetectable in the WPC powders were concentrated into the WPC pellets. These non-reducible aggregates were probably bacterial debris and phospholipid-protein complexes, as suggested earlier. The acid WPC and pellet samples showed bands corresponding to casein fractions (Fig. 7.2*a*, slots 3 & 4). These bands were not apparent in cheese WPC pellet (Fig. 7.2*b*, slots 3 & 4). The BSA and the minor whey protein components (Ig & Lf) were more intense in the reduced samples than in the non-reduced samples. This suggests that a

higher proportion of Ig, Lf and the other minor whey proteins is in the insoluble aggregates than in the soluble portion of the WPC.

Overall, these PAGE results showed that the pellets obtained from the WPC products contained both reducible and non-reducible aggregates. Although the composition of the non-reducible fraction was not clear, the reducible fraction contained a high proportion of the minor whey protein components. The acid WPC pellet contained considerable amounts of casein which may have formed complexes with whey proteins.

7.3.2. 2D-PAGE

The WPC pellets were also analysed using 2D-PAGE. The native-SDS 2D-PAGE patterns of the two WPC pellets (Fig. 7.3*a*) were generally similar to each other and to the pattern for the corresponding WPCs. Some of the material caught on top of the stacking and resolving gels of the sample strip (labelled as high mol wt aggregates in Fig. 7.3*a* and *b*) dissociated to give spots (labelled as "dissociated hydrophobic aggregates in Fig. 7.3) on the 2D gels that corresponded to all of the whey protein components. The spots corresponding to BSA and the minor protein components (Ig & Lf) appeared more intense than those of β-lactoglobulin and α-lactalbumin. This suggests that there were hydrophobically associated aggregates present in the pellet and these aggregates were made up predominantly of the minor protein components. There were also spots dissociated from material that travelled into the resolving gels (sample strip) which corresponded mainly to β-lactoglobulin and α-lactalbumin (appeared as horizontal streaks). These were probably involved in low molecular weight aggregates that were linked by hydrophobic association.

When the WPC pellets were analysed using SDS 2D-PAGE, some differences between the patterns of the acid and the cheese WPC pellets were seen (Fig. 7.4). The material caught in the stacking gel and on top of the resolving gel of the sample strip did not resolve completely, as mentioned earlier, indicating the presence of non-reducible protein aggregates. The bands of the non-reducible aggregates were more intense for the acid WPC pellet (Fig. 7.4*a*) than for the cheese WPC pellet (Fig. 7.4*b*). The material caught in the stacking gel and on top of the resolving gel sample strip of

the acid WPC pellet were resolved to give a number of spots corresponding to caseins, BSA and the minor protein components with very little β-lactoglobulin and no α-lactalbumin. In contrast, the same material in cheese WPC pellet resolved to give spots corresponding to all whey protein components, including β-lactoglobulin and α-lactalbumin.

Overall these 2D-PAGE results showed that when the pellets from acid WPC were suspended in native buffer, the solution contained native β-lactoglobulin, α-lactalbumin and BSA and caseins as well as high mol wt aggregates (Fig 7.3*a*). Inclusion of SDS in the mixture dissociated some minor proteins from these aggregates. Reduction of the intact large aggregates released some casein and substantial quantities of BSA, Ig and Lf. In contrast the intact large aggregates in cheese WPC released large quantities of β-lactoglobulin and α-lactalbumin as well as Lf, Ig and BSA.

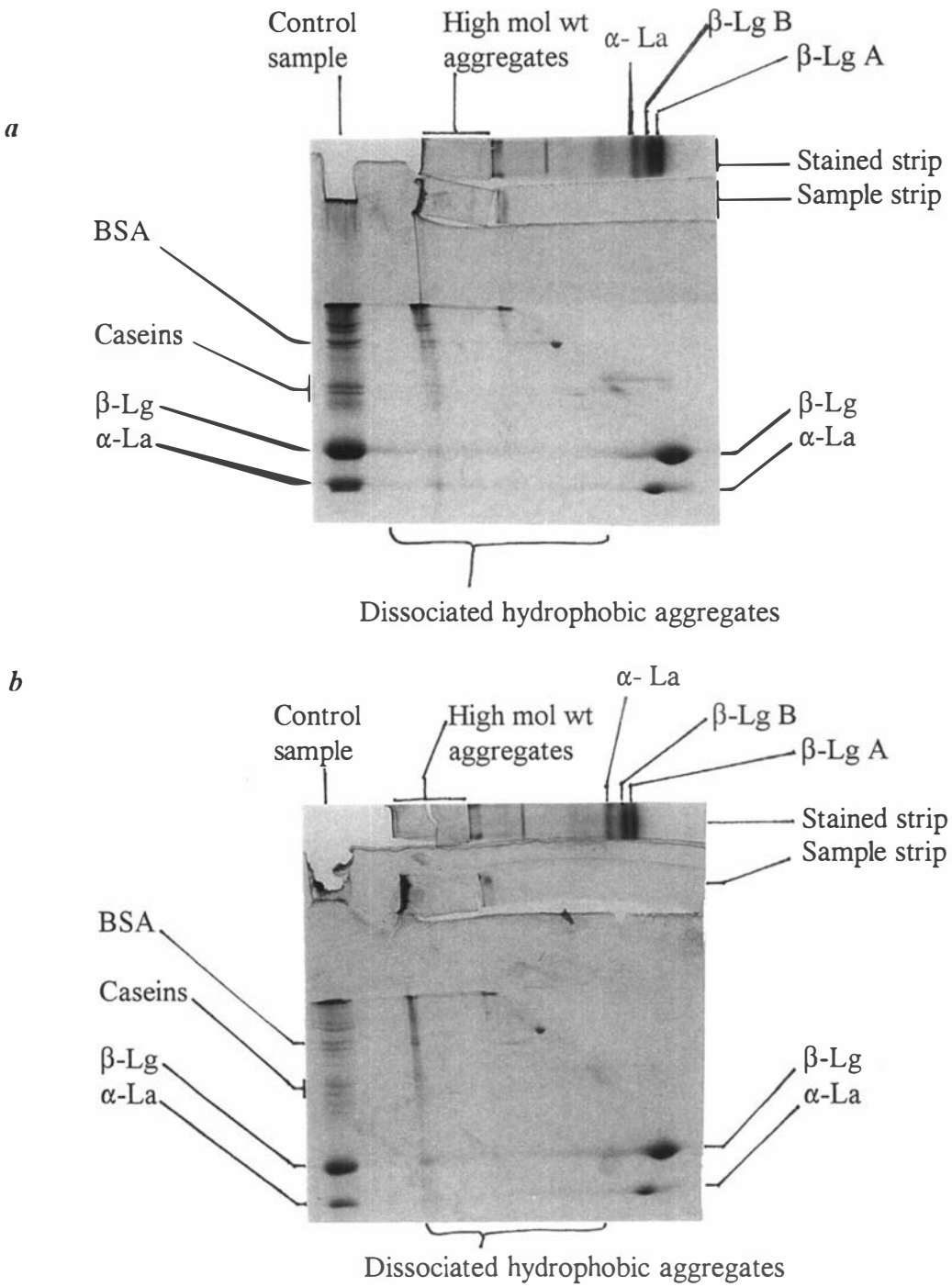


Figure 7.3. Native-SDS 2D-PAGE patterns of acid WPC pellet (*a*) and cheese WPC pellet (*b*). First dimension separation: left to right - native-PAGE; second dimension separation: top to bottom - SDS-PAGE.

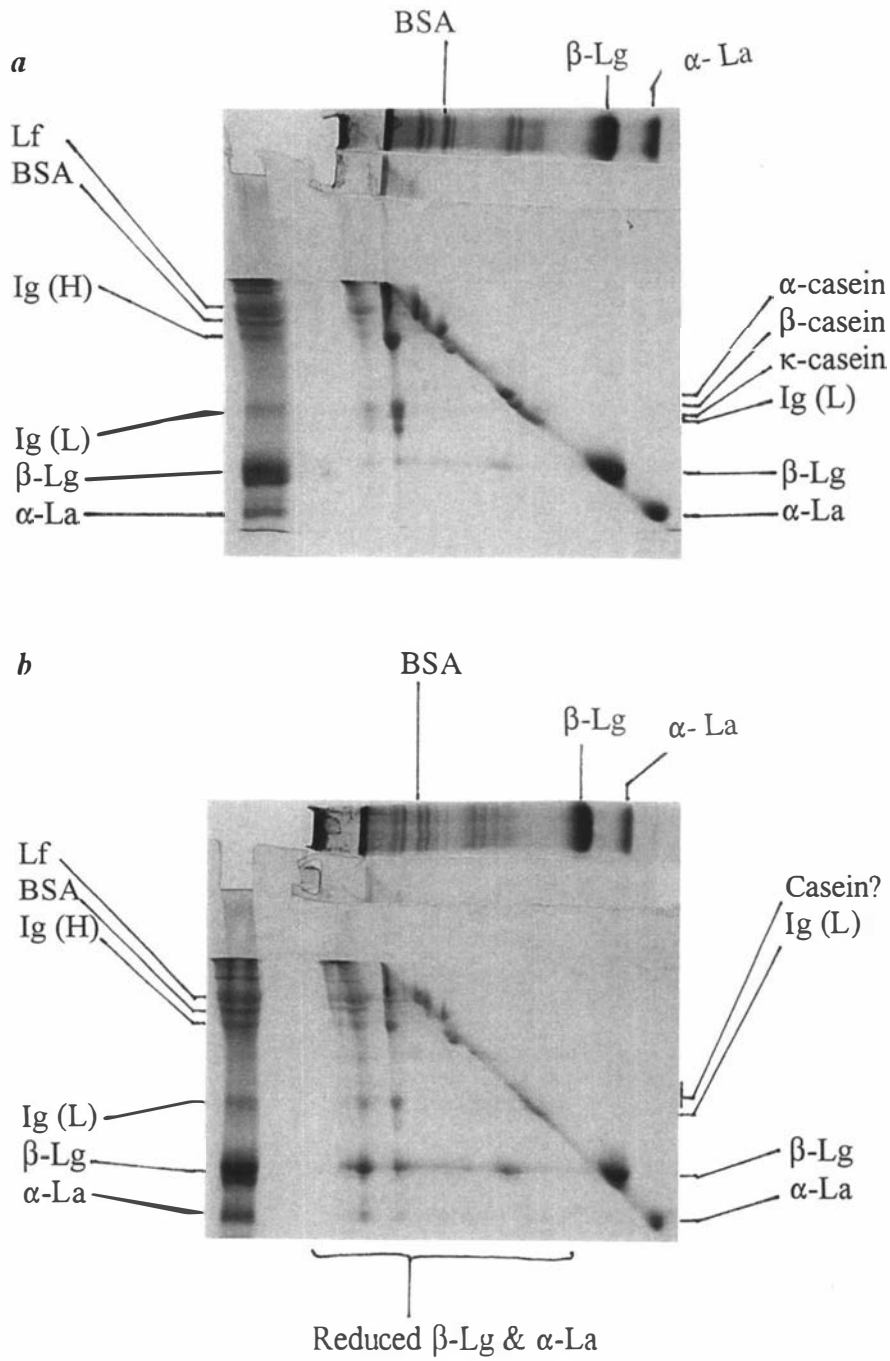


Figure 7.4. SDS 2D-PAGE patterns of acid WPC pellet (*a*) and cheese WPC pellet (*b*). First dimension separation: left to right - SDS-PAGE; second dimension separation - top to bottom - SDS-PAGE of the reduced samples.

7.4. Effects of insoluble aggregates on WPC gels

The effects of insoluble aggregates on WPC gelation were investigated. Different quantities of insoluble aggregates (pellet) were resuspended in supernatant so that a series of suspensions were produced that had equal volumes but had increasing concentrations of insoluble aggregates. Samples of these suspensions were heated at 80 °C, and the changes in the storage modulus, G′, were followed the using a Bohlin rheometer in the oscillation mode (Section 3.8). For each type of WPC, 25 g aliquots of 120 g/kg WPC solution were centrifuged at 45,000 × g for 2 h. The supernatants were poured off and put aside. An empty centrifuge tube and four containing pellets were assembled, and additional pellet material was added to some of the tubes and supernatant was added to all of the tubes so that each tube contained the same weight of sample. This resulted in 5 tubes containing: no pellet, one pellet, two pellets, three pellets and four pellets respectively. The final protein content was determined for each sample using the Kjeldahl method and the results are shown in Table 7.3.

Two control solutions were prepared. Control 1 was prepared by dissolving enough WPC in water so that the resultant solution had the same protein content as that of the supernatant (tube 1). Control 2 was prepared by dissolving enough WPC in water so that the resultant solution had the same protein content as the solution containing 4 pellets (Tube 5).

Table 7.3 shows the total protein contents of the pellet solutions. For both acid and cheese pellet solutions, the supernatant samples had similar protein contents to that of control 1, as intended. Control 2 had similar protein content to that of the solution containing 4 pellets as intended (Table 7.3). The protein contents of the WPC solutions increased with increasing levels of insoluble aggregates, with increases in the protein content in cheese WPC solutions being slightly higher. This was because the quantities of insoluble aggregates in cheese WPC were higher than that in acid WPC (Table 7.1).

Table 7.3. Protein contents of the WPC solutions containing different levels of insoluble aggregates.

Acid WPC		Cheese WPC	
Insoluble aggregates levels	Protein contents g/kg	Insoluble aggregate levels	Protein contents g/kg
1) Supernatant only	87.0	1) Supernatant only	87.1
2) Control 1 (WPC)	86.1	2) Control 1 (WPC)	86.2
3) Supernatant + 1 pellet*	89.6	3) Supernatant + 1 pellet	91.8
4) Supernatant + 2 pellets	92.2	4) Supernatant + 2 pellets	97.6
5) Supernatant + 3 pellets	97.6	5) Supernatant + 3 pellets	103.6
6) Supernatant + 4 pellets	104.3	6) Supernatant + 4 pellets	110.7
7) Control 2 (WPC)	103.6	7) Control 2 (WPC)	111.2

* 1 pellet is the amount of sedimented pellet obtained from centrifugation of 25 g aliquot of 120 g/kg WPC solution, at 45,000 × g, 2 h, see text.

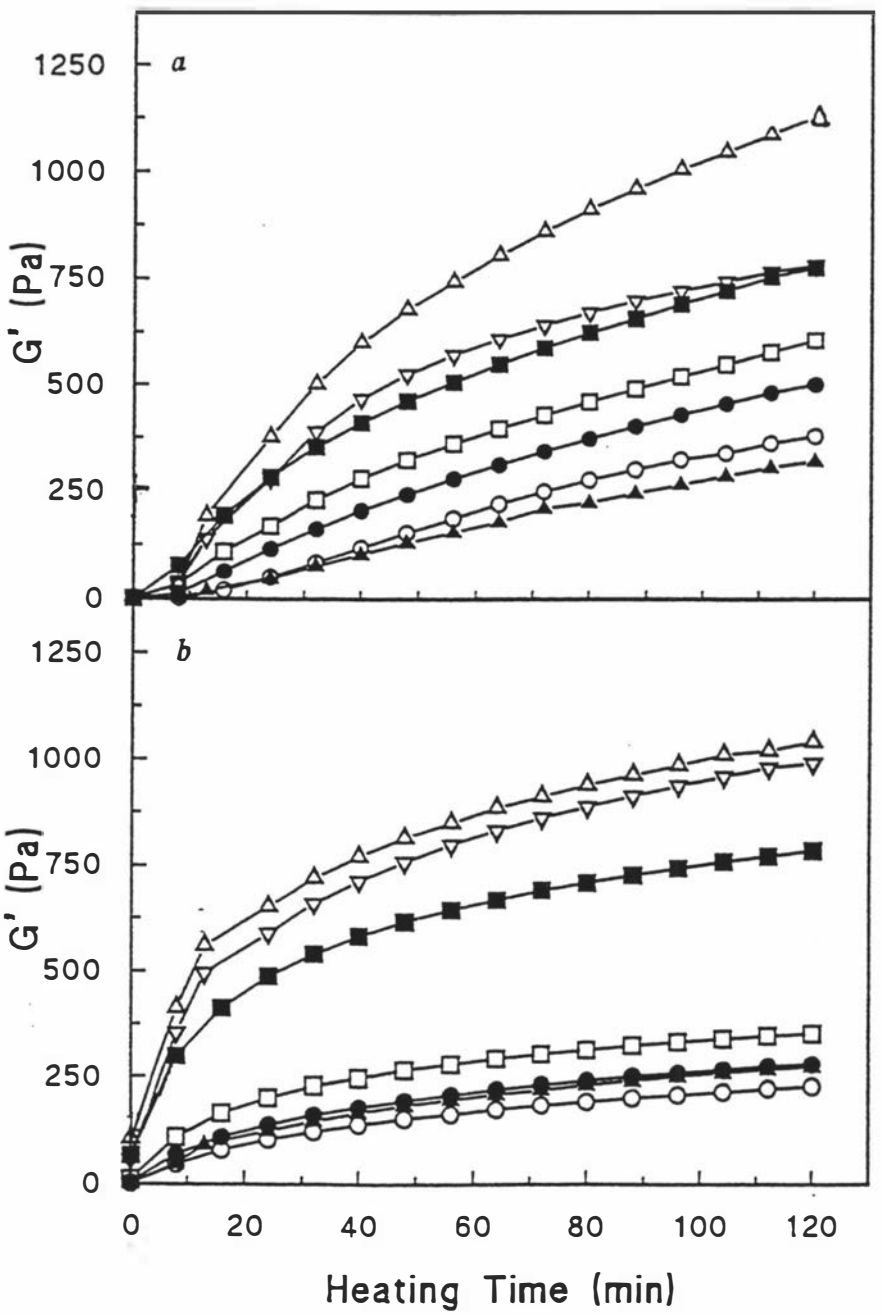


Figure 7.5. Development of storage modulus, G′, of acid WPC pellet solutions (*a*) and cheese WPC pellet solutions (*b*) during heat treatment at 80 °C. Supernatant (○), supernatant + 1 pellet (●), supernatant + 2 pellets (□), supernatant + 3 pellets (■), supernatant + 4 pellets (Δ), Control 1 (▲), control 2 (▽).

Heating acid WPC solutions with various levels of pellet material (Fig. 7.6*a*) showed lag phases of up to ~ 8 min, where there were no recognisable increases in G′, followed by a steady increase in G′ with heating time. As the level of insoluble aggregates increased, the lag phase decreased and G′ increased faster with heating time. Heating cheese WPC solutions (Fig. 7.6*b*) showed no lag phase, and initially there were fast increases in G′ followed by slower increases.

When the acid WPC solutions containing different levels of insoluble aggregates were heated, the final values of G′(after 2 h heating at 80 °C, Fig. 7.6*a*) increased with increasing levels of insoluble aggregates and protein contents (Table 7.3). The changes in G′ of the supernatant during heating were very similar to that of control 1 (control WPC, Fig 7.6*a*). Comparing the G′ profiles of the WPC solution containing 4 pellets with control 2 (both with similar protein contents), it was clear that control 2 had a lower G′ values at all heating times. Control 2 had a G′ versus time profile that was similar to that of the solution containing 3 pellets. This indicated that the increases in G′ values of the acid WPC solutions were affected by the levels of insoluble aggregates rather than protein concentration alone.

Comparing control 2 in for the acid WPC pellet solutions (Fig. 7.6*a*) to that for the cheese WPC pellet solutions, it is clear that control 2 for acid WPC solution had a G′ profile that was very similar to that of the sample containing 3 pellets, although it had similar protein content to that of the pellet solutions containing 4 pellets (Table 7.3). By contrast, control 2 for cheese WPC pellet solutions showed very similar G′ profile to that of the pellet solution with 4 pellets, as intended (Fig. 7.6*b*). It appears, therefore, the casein, which was high in pellet solution with 4 pellets, probably had an enhancing effect on the acid WPC gelation.

Overall, the presence of aggregated material in commercial WPCs does not appear to affect the gelation characteristics of cheese WPC but has positive effect on that of acid WPC.

7.5. Discussion

In this chapter, a centrifugal method was used to recover the 'insoluble' aggregates present in the WPCs. The results showed that the cheese WPC contained higher amounts of insoluble aggregates than acid WPC, and that the composition of aggregates in acid WPC was different from those in cheese WPC. The insoluble aggregates obtained from acid WPC contained considerably higher amounts of native-like β-lactoglobulin and α-lactalbumin, and relatively high concentration of caseins. These aggregates in cheese WPC, on the other hand, had higher concentrations of fat and phospholipids, and the β-lactoglobulin and α-lactalbumin present were largely denatured and aggregated.

The results of the current study clearly demonstrate that the insoluble aggregates consist predominantly of BSA and the other minor whey protein components. It is known that these proteins are more sensitive to heat and are readily denatured and aggregated under relatively mild heating conditions (~ 60 °C). These aggregates were probably generated during heat-treatment processes used in the manufacture of WPC.

The cheese WPC pellet contained a considerable amount of fat which was due to the high fat content of the original cheese WPC (Tables 5.1 & 7.3). The lipid phase of milk systems is stabilized in part by a protective layer of proteins forming a coat on the fat globule surface (Walstra, 1983). When the milk systems are exposed to high shear in processes, such as homogenization or pumping, the fat/water interfacial area increases, and there is an increase in the adsorption of proteins to the lipid surface. While some of the proteins of the cheese WPC may have associated with the fat in this way, it appears that most of the proteins were linked via disulphide bonds (Fig.7.4*a*). In heated milk systems, the denatured whey proteins associate with casein micelles or interact with whey proteins that are adsorbed to the surface of the fat globules (Singh *et al.*, 1996). It appears that the same interactions take place during WPC manufacture, resulting in formation of dense fat-protein complexes which sedimented on centrifugation.

It was also shown that aggregated material in acid WPC appeared to have a similar effect on gel stiffness as a similar quantity of WPC (Fig. 7.5). However, the aggregated material in cheese WPC was less effective in maintaining the gel stiffness

of the heated product (Fig. 7.6*b*). This effect can be ascribed partly to the high fat content and partly to the presence of aggregated/denatured proteins. The cheese WPC was shown in Chapter 5 to produce heat-induced gels with lower gel strength than that produced from acid WPC under the same gelling conditions. It appears that the presence of fat in the WPC pellet contributes significantly to the poor gel strength of the cheese WPC.

The acid WPC pellet, on the other hand, had a positive impact on the gel strength of the product. This could be attributable to the high content of native-like proteins in the pellet. The aggregated β-lactoglobulin and α-lactalbumin were shown to be linked largely by hydrophobic association (Fig. 7.3*a*) with minimal involvement in disulphide linkages and it appears that most of the proteins in the pellet were functional. Upon heating, they contribute significantly to the formation of the gel network. The cheese WPC pellet on the other hand had high Ca and P content compared to that of acid WPC pellet. This probably contributed to the higher proportions of β-lactoglobulin and α-lactalbumin in the cheese WPC pellet.

In conclusion, both acid WPC and cheese WPC contained considerable quantities of insoluble aggregates that were sedimented into pellets, although there were more sedimented from the cheese WPC. In both cases, the insoluble aggregates contained greater proportions of Ig, Lf, BSA and other minor proteins. 2D-PAGE analysis showed that the pellets from cheese WPC contained disulphide-linked β-lactoglobulin and α-lactalbumin, whereas those from acid WPC contained considerable amounts of caseins. The presence of insoluble aggregates did not appear to affect the gelling characteristics of cheese WPC but had positive effect on that of acid WPC.

CHAPTER 8

HEAT-INDUCED INTERACTIONS OF β-LACTOGLOBULIN, α-LACTALBUMIN AND BSA

8.1. Introduction

Whey proteins are highly functional food proteins and are extensively used in foods as gelling ingredients. Consequently, the heat-induced gelation of whey protein isolates and concentrates has been studied extensively over the last few years (Tang *et al.*, 1993; 1995; Aguilera, 1995). The major protein components of whey include β-lactoglobulin (50%), α-lactalbumin (20%) and BSA (5%), and it is generally accepted that the characteristics of the major protein, β-lactoglobulin, dominate the behaviour of the whey protein concentrates and isolates. The heat-induced protein-protein interactions that precede gelation have been studied extensively in different buffers at low protein concentrations using a wide range of techniques (Griffin *et al.*, 1993; Elofsson *et al.* 1996; Hoffmann and van Mill, 1997; Boye *et al.*, 1997) with some studies also at higher concentrations (Matssuura, 1994; Hollar *et al.*, 1995. Qi *et al.*, 1995). It is generally accepted that thiol-disulphide interchange reactions, leading to the formation inter-molecular disulphide bonds, play a major role in the heat-induced aggregation and gelation of β-lactoglobulin. McSwiney *et al.* (1994*a, b)* showed that in addition to the intermolecular disulphide bonds, stable non-covalently linked aggregates were also formed prior to gel formation in heated solutions of 10% β-lactoglobulin.

Since whey proteins are a heterogenous group of proteins with different amino acid compositions and molecular structures, it is likely that the gelation behaviour of an individual protein would be altered by the presence of the others. A number of studies have shown that soluble aggregates of whey proteins are formed during the early stages of heat-induced gel formation, and that subsequent polymerization results in the formation of a rigid gel network (Baer *et al.*, 1976; Paris *et al.*, 1993). It is possible that gel properties may be influenced by the formation of specific soluble aggregates between different whey proteins in the early stages of heating. For example,

addition of BSA or α-lactalbumin to a solution of β-lactoglobulin increases the rigidity of the resultant gels and disulphide-bonded complexes are formed between β-lactoglobulin and BSA or α-lactalbumin (Matsudomi *et al.*, 1992; 1993; 1994; Gezimati *et al.*, 1996*a, b*, 1997). Recent studies on the aggregation and gelation of mixtures of β-lactoglobulin and BSA (Gezimati *et al.*, 1996*a*) or α-lactalbumin (Gezimati *et al.*, 1996*b*; 1997; Dalgleish *et al.*, 1997) indicated that non-covalently linked aggregates were also formed in these mixtures at the early stages of gel formation.

The results presented in Chapter 4 showed that when whey protein concentrate (WPC) solutions were heated and then examined by 2D-PAGE, there was evidence of the formation of specific intermediate aggregates between various whey proteins. The present investigation extends that study by examining in greater detail the intermediate protein aggregates formed during the interactions between β-lactoglobulin and BSA or α-lactalbumin in the early stages of gel formation in an ionic environment similar to that existing in whey protein concentrate solutions.

8.2. Heat treatment of individual protein solutions

The individual protein (β-lactoglobulin, α-lactalbumin or BSA) solutions (100 g/kg, pH 6.8) were prepared by dissolving appropriate amounts of dry protein in WPC permeate. The WPC permeate was prepared by ultrafiltration of 120 g/kg acid WPC solution (Section 3.3). Samples of each solution were heated at 75 °C for different times and then analysed using 1D-PAGE. These experiments was repeated at least twice.

8.2.1. Heat treatment of β-lactoglobulin solutions

The native-PAGE pattern of the unheated β-lactoglobulin sample (Fig. 8.1*a*, slot 1) showed the typical bands corresponding to the monomeric A and B variants. Running behind these bands were two other somewhat faint bands, probably corresponding to β-lactoglobulin dimer and trimer. This indicates that the β-lactoglobulin sample used in this analysis contained some polymeric (dimer and trimer) material. The native-PAGE patterns of the heated samples (Fig. 8.1*a*, slots 2-9) showed that the polymeric bands as well as the monomeric bands diminished with heating time at 75

°C. The loss of intensity of these bands could be attributed to their involvement in the formation of higher molecular weight aggregates. The decreasing intensities of these bands were accompanied by concommitant increasing intensities of the material caught on top of the resolving gel and within the stacking gel (Fig. 8.1*a*). The accumulation of this material started as early as after 1 min of heating.

The SDS-PAGE patterns of the unheated β-lactoglobulin solution (Fig. 8.1*b*, slot 1) showed a very intense monomeric β-lactoglobulin band, a less intense band corresponding to β-lactoglobulin dimers (~ 38 kDa) and a faint fine band running ahead of the β-lactoglobulin monomeric band. The latter was probably some α-lactalbumin present as an impurity in the product used. There was also a faint region running behind the β-lactoglobulin dimeric band. This region corresponded to a molecular weight of ~ 58 kDa, which is consistent with the molecular weight of β-lactoglobulin trimer.

The SDS-PAGE patterns of the heated samples (Fig. 8.1*b*, slots 2-9) showed that the intensity of the monomeric β-lactoglobulin and α-lactalbumin bands decreased with heating time. SDS-PAGE (Fig. 8.1*b*) showed three major differences when compared with the native-PAGE (Fig. 8.1*a*). (i) The dimeric and trimeric bands increased in intensity with heating time, instead of decreasing (Fig. 8.1*a*). (ii) The samples that were heated for ≥ 4 min (Fig. 8.1*b*, slots 6-9) showed a series of bands behind the trimeric band, probably corresponding to higher molecular weight polymers, that increased in intensity with heating time. (iii) There was no material caught on top or within the stacking gel, suggesting that the material caught on top and within the stacking gel in native-PAGE (Fig 8.1*a*) had been dissociated completely in the SDS gel and had migrated into the resolving gel. It appears, therefore, that the material caught within the native-PAGE stacking gel (Fig. 8.1*a*) was probably aggregated proteins, linked by hydrophobic bonds.

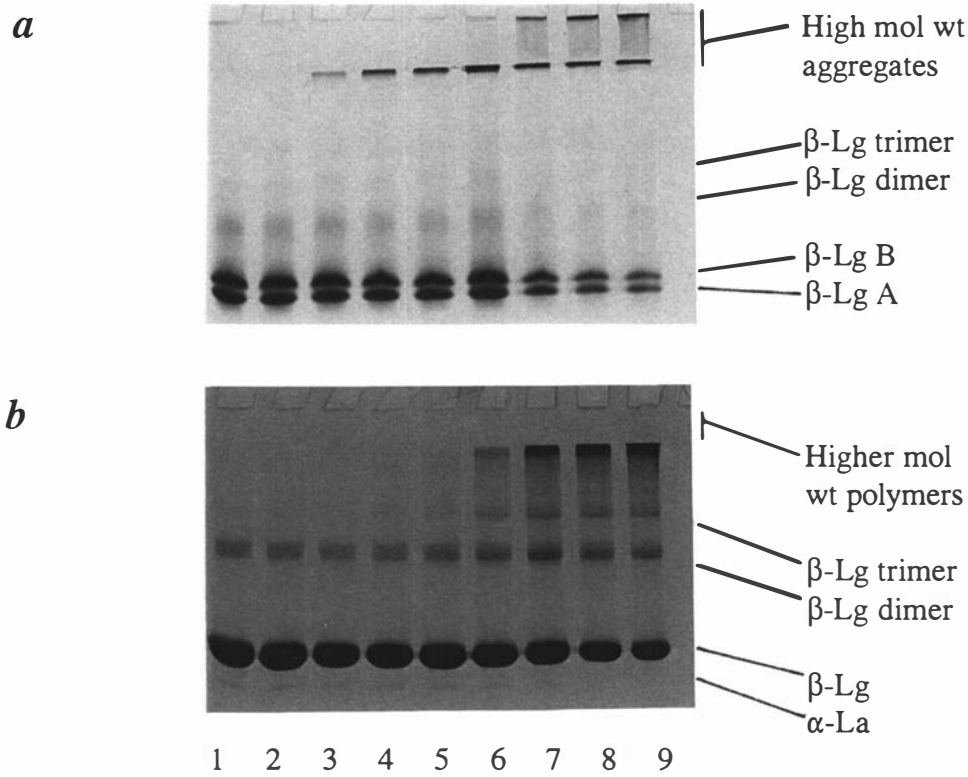


Figure 8.1. Native- (*a*) and SDS- (*b*) PAGE patterns of β-lactoglobulin (β-Lg) solution (100 g/kg, pH 6.8) heated at 75 °C for 0 (slot 1), 0.5 (slot 2), 1 (slot 3), 1.5 (slot 4), 2 (slot 5), 4 (slot 6), 6 (slot 7), 8 (slot 8) and 10 (slot 9) min.

8.2.2. Heat treatment of α-lactalbumin solutions

The native-PAGE patterns of the unheated α-lactalbumin solution (Fig. 8.2*a*) showed an intense monomeric band and faint regions running both ahead and behind. These regions, probably heat-induced altered forms of monomeric α-lactalbumin, appeared to be more intense in the heated sample (Fig. 8.2*a*, slot 2) than in the unheated sample. The native-like monomeric band did not appear to change in intensity after heating for 10 min at 75 °C. Unlike the heated β-lactoglobulin, there was no aggregated material caught on top of the stacking or resolving gel in the native-PAGE patterns (Fig. 8.1*a*),

The SDS-PAGE patterns of the unheated (Fig. 8.2*b*, slot 1) and heated (slot 2) α-lactalbumin sample were similar with an intense monomeric α-lactalbumin band and another faint fine band running behind. The latter corresponded to a molecular weight

of ~ 28 kDa which is consistent with the molecular weight of α-lactalbumin dimer. There was no loss of SDS-monomeric α-lactalbumin during heating up to 10 min.

8.2.3. *Heat treatment of BSA solutions*

The native- and SDS-PAGE patterns of the unheated BSA solution showed an intense monomeric BSA band and a series of fine bands that travelled short distances into the resolving gel (Fig. 8.3*a, b,* slot 1). These bands corresponded to higher molecular weight components of whey protein (e.g. lactoferrin) which were probably present in the BSA product used. When the BSA solutions were heated at 75 °C for 2 min, the monomeric BSA disappeared almost completely (Fig. 8.3*a*, slot 2). The SDS-PAGE patterns showed that a considerable amount of SDS-monomeric BSA was present in the heated sample (Fig. 8.3*b*, slot 2), indicating that significant amounts of hydrophobically-associated BSA aggregates were formed during heating. The samples heated for longer than 2 min formed gels, and were, therefore, difficult to analyse.

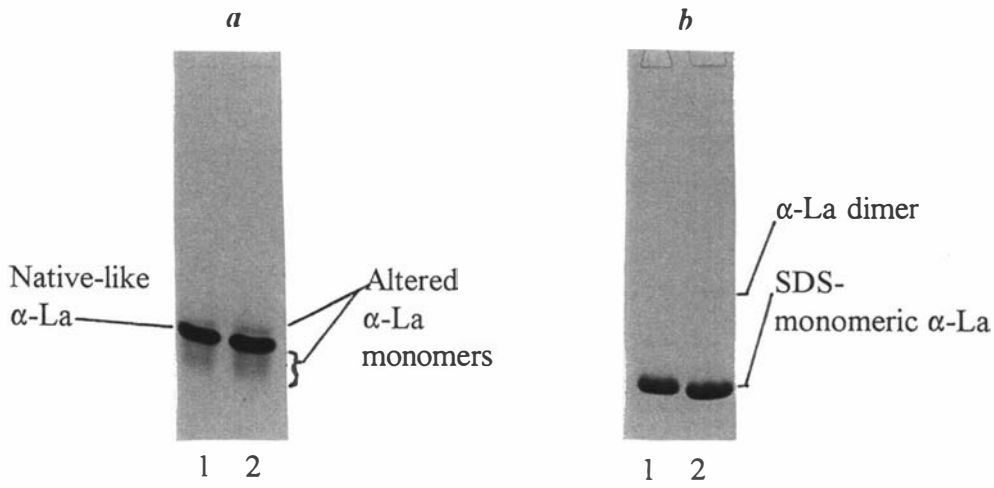


Figure 8.2. Native- (*a*) and SDS- (*b)* PAGE patterns of 100 g/kg α-lactalbumin (α-La) solutions heated at 75 °C for 0 (slot 1) or 10 (slot 2) min.

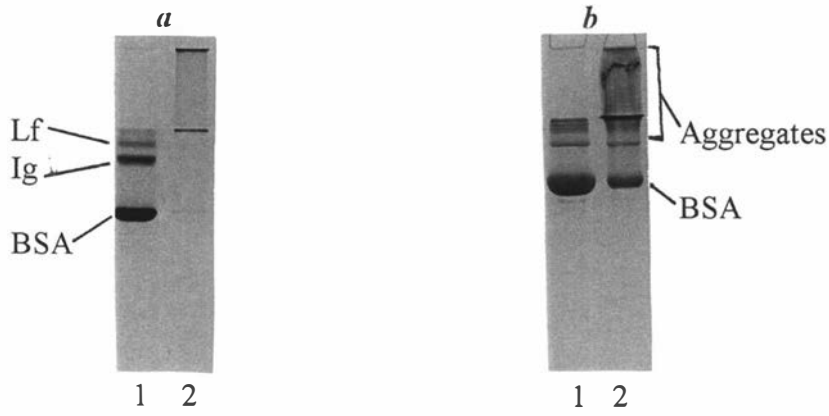


Figure 8.3. Native- (*a*) and SDS- (*b*) PAGE patterns of 100 g/kg BSA solutions heated at 75 °C for 0 (slot 1) or 2 (slot 2) min. Lf - Lactoferrin, Ig - Immunoglobulin.

8.3. Heat treatment of binary protein mixtures

Mixtures of β-lactoglobulin and α-lactalbumin or BSA at a ratio of 2:1 were prepared by mixing 2 parts β-lactoglobulin and 1 part α-lactalbumin or BSA. The final mixtures contained ~ 66.5 g/kg β-lactoglobulin and ~ 33.5 g/kg α-lactalbumin or BSA and the total protein content was ~ 100 g/kg. A mixture of α-lactalbumin and BSA at a ratio of 1:1 was also prepared. The final mixture contained ~ 50 g/kg of each protein, and the total protein content was ~ 100 g/kg. Aliquots of the mixtures were heated at 75 °C and then analysed using 1D-PAGE (Section 3.7).

8.3.1. Heat treatment of mixtures of β-lactoglobulin and α-lactalbumin

The native-PAGE pattern of the unheated mixture of β-lactoglobulin and α-lactalbumin (Fig. 8.4*a*, slot 1) showed bands corresponding to the β-lactoglobulin A and B variants and α-lactalbumin and a somewhat faint region (probably β-lactoglobulin dimers). When the protein solutions were heated at 75 °C (Fig. 8.4*a*, slots 2-9), these bands diminished with heating time. In contrast to the heated solution of α-lactalbumin alone (Fig. 8.2*a*), the monomeric α-lactalbumin band diminished with heating time, indicating the loss of native-like α-lactalbumin on heating in mixtures with β-lactoglobulin. This indicates that the loss of native α-lactalbumin in the heated solution was probably facilitated by the presence of β-lactoglobulin.

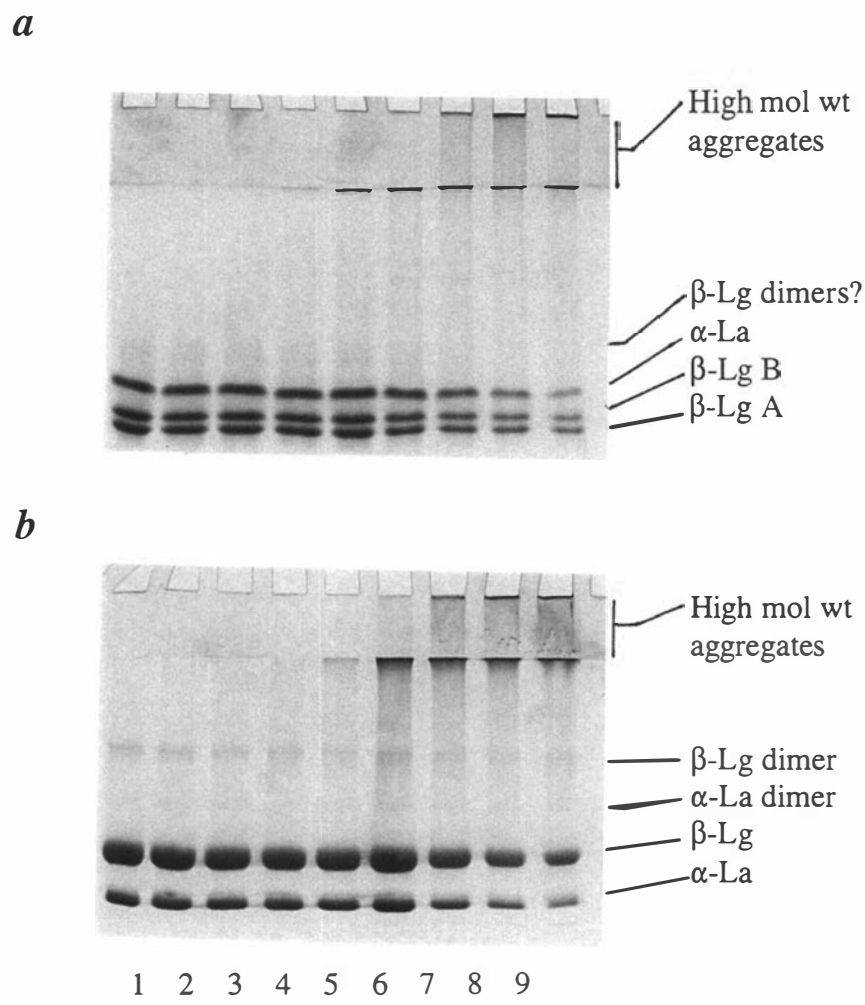


Figure 8.4. Native- (*a*) and SDS- (*b*) PAGE patterns of 2:1 mixtures of ß-lactoglobulin and α-lactalbumin (100 g/kg, pH 6.8) heated at 75 °C for 0 (slot 1), 0.5 (slot 2), 1 (slot 3), 1.5 (slot 4), 2 (slot 5), 4 (slot 6), 6 (slot 7), 8 (slot 8) and 10 (slot 9) min.

The SDS-PAGE patterns of the heated mixtures of β-lactoglobulin and α-lactalbumin (Fig. 8.4*b*, slots 7-9) showed that there was material caught on top and within the stacking gel, in contrast to the patterns for β-lactoglobulin alone (Fig. 8.1*b*), where no such material was observed for the heated samples. This material was probably consisted of heterogeneous polymers formed by disulphide linkages between β-lactoglobulin and α-lactalbumin molecules. A series of very faint bands ran behind the monomeric β-lactoglobulin band, and did not seem to diminish with heating time. These bands were probably various forms of aggregates with different molecular

weights. The β-lactoglobulin dimeric band appeared to diminish slowly with heating time, in contrast to the increasing intensity observed for the same band in the heated solution of β-lactoglobulin alone (Fig. 8.1*b*). It appears that when β-lactoglobulin was heated alone, higher proportions of lower molecular weight polymers (e.g. dimers and trimers) were formed and migrated into the resolving gel instead of being caught within the stacking gel. By contrast, in the mixture of β-lactoglobulin and α-lactalbumin, larger aggregates, which did not migrate into the resolving gel but were caught within the stacking gel, were formed. It is also possible that the interactions between β-lactoglobulin and α-lactalbumin were more favourable than the interactions of β-lactoglobulin with itself.

8.3.2. Heat treatment of mixtures of β-lactoglobulin and BSA

The PAGE patterns of the heated mixtures of β-lactoglobulin and BSA are shown in Fig. 8.5. The native-like monomeric bands of both β-lactoglobulin and BSA diminished with heating time (Fig. 8.5*a*). The loss of native-like BSA was relatively faster than that of β-lactoglobulin. BSA and the higher molecular weight whey protein components (e.g. lactoferrin) disappeared almost completely after heating for 4 min (Fig. 8.5*a*, slot 6). A fine band running just behind the BSA monomeric band appeared after heating for 30 s and increased in intensity with heating time up to 1.5 min, but then decreased and disappeared completely after heating for 2 min. This band was not seen when a solution of BSA was heated alone (Fig. 8.3). This band was probably β-lactoglobulin-BSA dimer complex. The accumulation of material on top of the resolving gel started as early as 30 s, and became more apparent after heating for 2 min.

The SDS-PAGE patterns of heated mixtures of β-lactoglobulin and BSA (Fig. 8.5*b*) showed that the β-lactoglobulin monomeric band diminished with heating time relatively slowly. The band running between the β-lactoglobulin and BSA bands was probably β-lactoglobulin dimer. It slowly diminished in intensity with heating time. The BSA band was present even in the sample that was heated for 10 min (Fig. 8.5*b*, slot 9), in contrast to the native gel (Fig. 8.5*a*), where the monomeric BSA band disappeared completely after heating for 2 min at 75 °C. This shows that

hydrophobically-associated aggregates involving BSA were formed during heating. These aggregates were dissociated under SDS-PAGE conditions and migrated into the resolving gel, as observed. A fine band, β-lactoglobulin-BSA dimer complex, running behind the BSA band again appeared after heating for 30 s. Material started to accumulate within the stacking gel after heating for 1 min (Fig. 8.5*b*, slot 3) and increased in intensity thereafter.

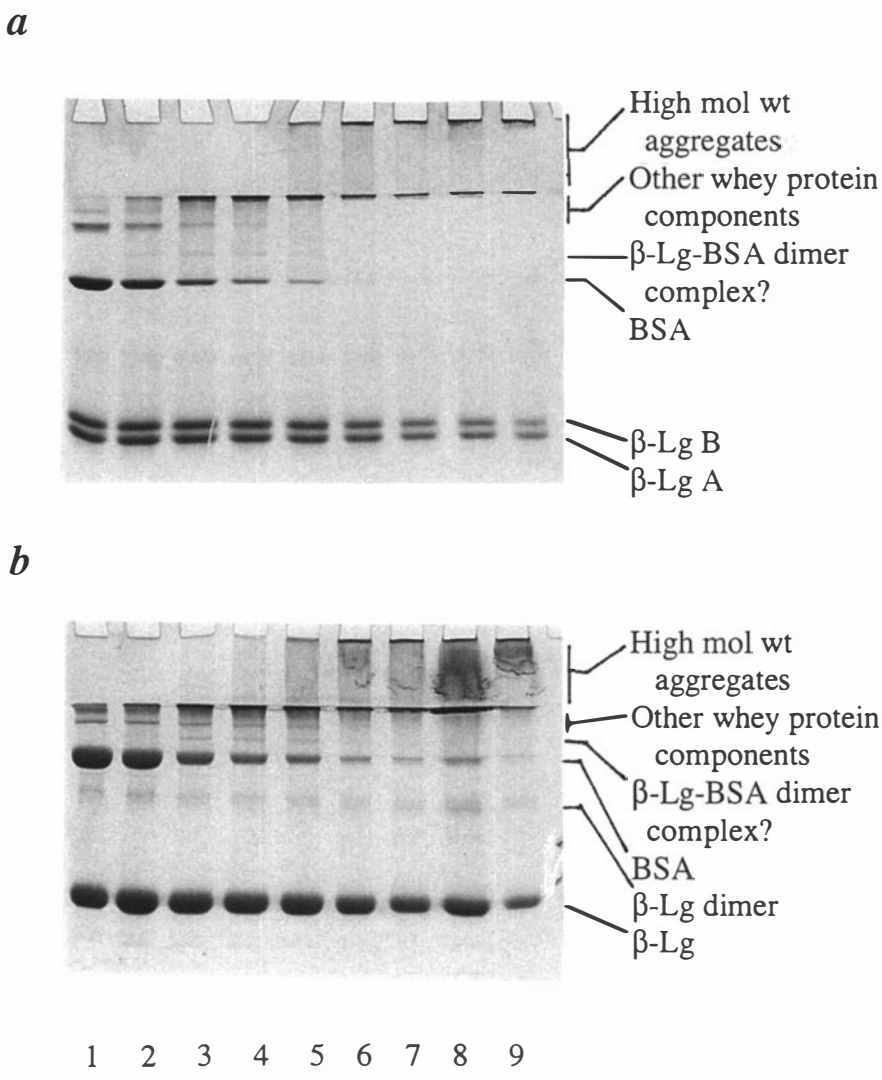


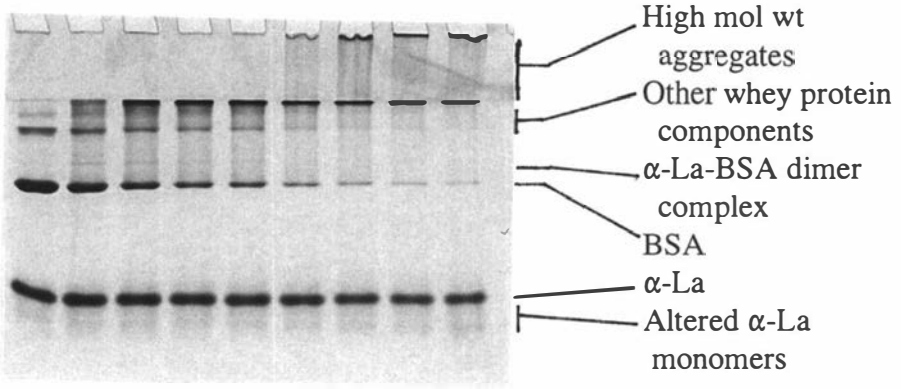
Figure 8.5. Native- (*a*) and SDS- (*b*) PAGE patterns of 2:1 mixtures of β-lactoglobulin and BSA (100 g/kg, pH 6.8) heated at 75 °C for 0 (slot 1), 0.5 (slot 2), 1 (slot 3), 1.5 (slot 4), 2 (slot 5), 4 (slot 6), 6 (slot 7), 8 (slot 8) and 10 (slot 9) min.

8.3.3. Heat treatment of mixtures of α-lactalbumin and BSA

The native-PAGE pattern of the unheated mixture of α-lactalbumin and BSA (Fig. 8.6*a*, slot 1) showed intense bands of monomeric α-lactalbumin, BSA and some fine bands corresponding to other minor whey protein components. There were also some faint regions running ahead of and behind the α-lactalbumin band. Upon heating, the α-lactalbumin band diminished slowly with heating time whereas the BSA band diminished relatively rapidly. On comparing the loss of native-like BSA from this heated solution (Fig. 8.6*a*) with the loss of the same protein from the mixture with β-lactoglobulin (Fig. 8.5*a*) it is clear that the loss of native-like BSA in the mixture with α-lactalbumin was slower than the loss of the same protein from the heated mixture with β-lactoglobulin. The loss of other whey proteins was relatively slow compared with the loss of the same proteins when heated in mixture with β-lactoglobulin (Fig. 8.5*a*). A fine band running just behind the BSA band, which appeared after heating for 30 s (Fig. 8.6*a*, slot 2), was probably an α-lactalbumin-BSA dimer complex. The faint regions running before and after the α-lactalbumin band probably corresponded to heat-induced altered forms of monomeric α-lactalbumin.

The SDS-PAGE pattern of the unheated mixture of BSA and α-lactalbumin (Fig. 8.6*b*, slot 1) showed bands corresponding to α-lactalbumin, BSA and other minor whey protein components and a faint band running between BSA and α-lactalbumin. The latter corresponded to a molecular weight of ~ 28 kDa, which is consistent with the molecular weight of α-lactalbumin dimer. The α-lactalbumin dimeric band slowly increased in intensity with heating time. There was a decrease in intensity of the α-lactalbumin monomeric band after heating in combination with BSA, as opposed to the unchanged behaviour observed after heating a solution of α-lactalbumin alone (Fig. 8.2). This decrease was accompanied by an increase in the intensity of the α-lactalbumin dimeric band. Another fine band running just ahead of BSA monomer (Fig. 8.6*b*) appeared after 8 min of heating and corresponded to a molecular weight of ~ 42 kDa which is consistent with the molecular weight of an α-lactalbumin trimer. This suggests that the loss of the α-lactalbumin monomers resulted in the formation of dimers, trimers and possibly other aggregates.

a



b

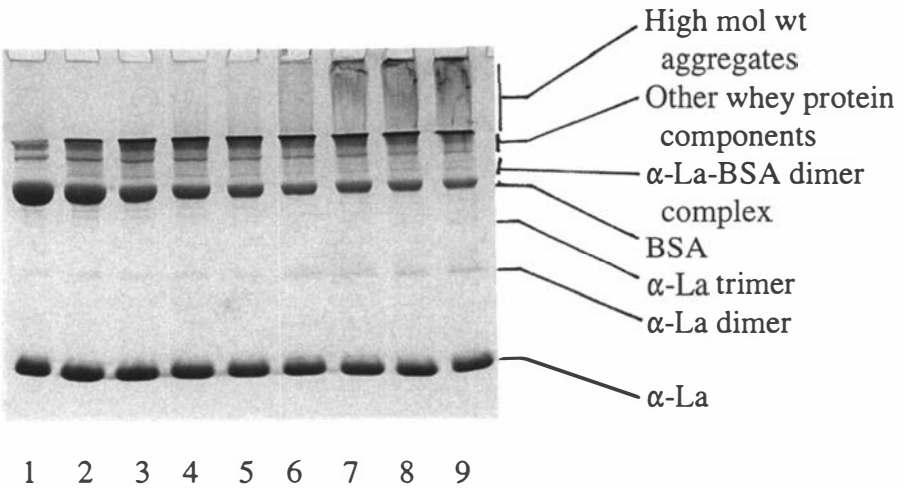


Figure 8.6. Native- (*a*) and SDS- (*b*) PAGE patterns of 1:1 (100 g/kg, pH 6.8) mixtures of α-lactalbumin and BSA heated at 75 °C for 0 (slot 1), 0.5 (slot 2), 1 (slot 3), 1.5 (slot 4), 2 (slot 5), 4 (slot 6), 6 (slot 7), 8 (slot 8) and 10 (slot 9) min.

When the heated mixture of BSA and α-lactalbumin was treated with 2-mercaptoethanol and then run on SDS-PAGE (Fig. 8.7, slot 3), the material caught within the stacking gel and on top of the resolving gel as well as the α-lactalbumin dimeric band completely disappeared, resulting in an increase in the intensities of the BSA and α-lactalbumin monomeric bands. The fact that the α-lactalbumin dimeric

band completely disappeared after 2-mercaptoethanol treatment suggests that α-lactalbumin dimers were linked by disulphide bonds.

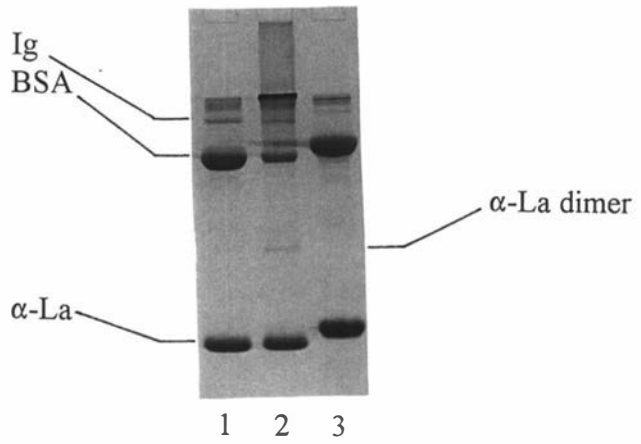


Figure 8.7. SDS-PAGE patterns of a 1:1 mixture (100 g/kg) of α-lactalbumin and BSA heated at 75 °C for 0 (slot 1) or 10 (slot 2) min. Slot 3 represents the sample in slot 2, after being treated with 2-mercaptaethanol.

In order to see whether α-lactalbumin dimers and trimers could be seen in greater quantities, further experiments were conducted using dilute solutions; a 20 g/kg 1:1 mixture of α-lactalbumin and BSA was prepared by dissolving appropriate amounts of the proteins in appropriately diluted WPC permeate. Samples of this solution were heated at 75 °C for up to 32 min and then analysed using PAGE. Figure 8.8 shows the native- and SDS-PAGE patterns of the heated samples. The native-PAGE confirms some of the observations made earlier. The series of bands (or regions) running ahead of and behind the α-lactalbumin monomeric band increased in intensity with heating time, confirming that these regions corresponded to some heat-induced altered forms of α-lactalbumin. The α-lactalbumin dimeric and trimeric bands could be seen more clearly and they increased in intensity with heating time. The series of fine bands running behind the BSA band, corresponding to α-lactalbumin-BSA complexes, was much clearer.

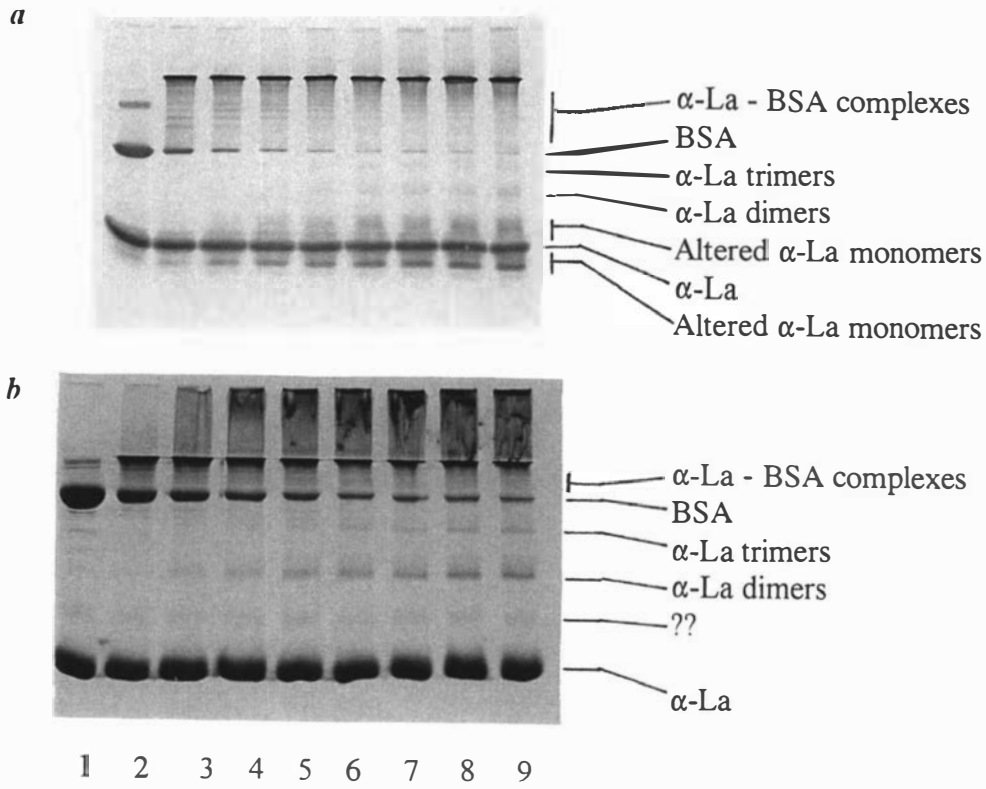


Figure 8.8. Native- (*a*) and SDS- (*b*) PAGE patterns of a 10 g/kg 1:1 mixture of α-lactalbumin and BSA heated at 75 °C for 0 (slot 1), 4 (slot 2), 8 (slot 3), 12 (slot 4), 16 (slot 5), 20 (slot 6), 24 (slot 7), 28 (slot 8) and 32 min (slot 9).

The SDS-PAGE (Fig. 8.8*b*) of these samples showed the same features as the native-PAGE. In addition, a faint region running behind the α-lactalbumin monomeric band did not seem to change in intensity with heating time. It is not clear what corresponds to this region. There were some faint regions following both dimeric and trimeric bands, which probably correspond to α-lactalbumin aggregates formed by the altered forms of the protein.

The heated samples (20 g/kg mixtures) were also analysed using size exclusion chromatography, as described in Section 3.9.

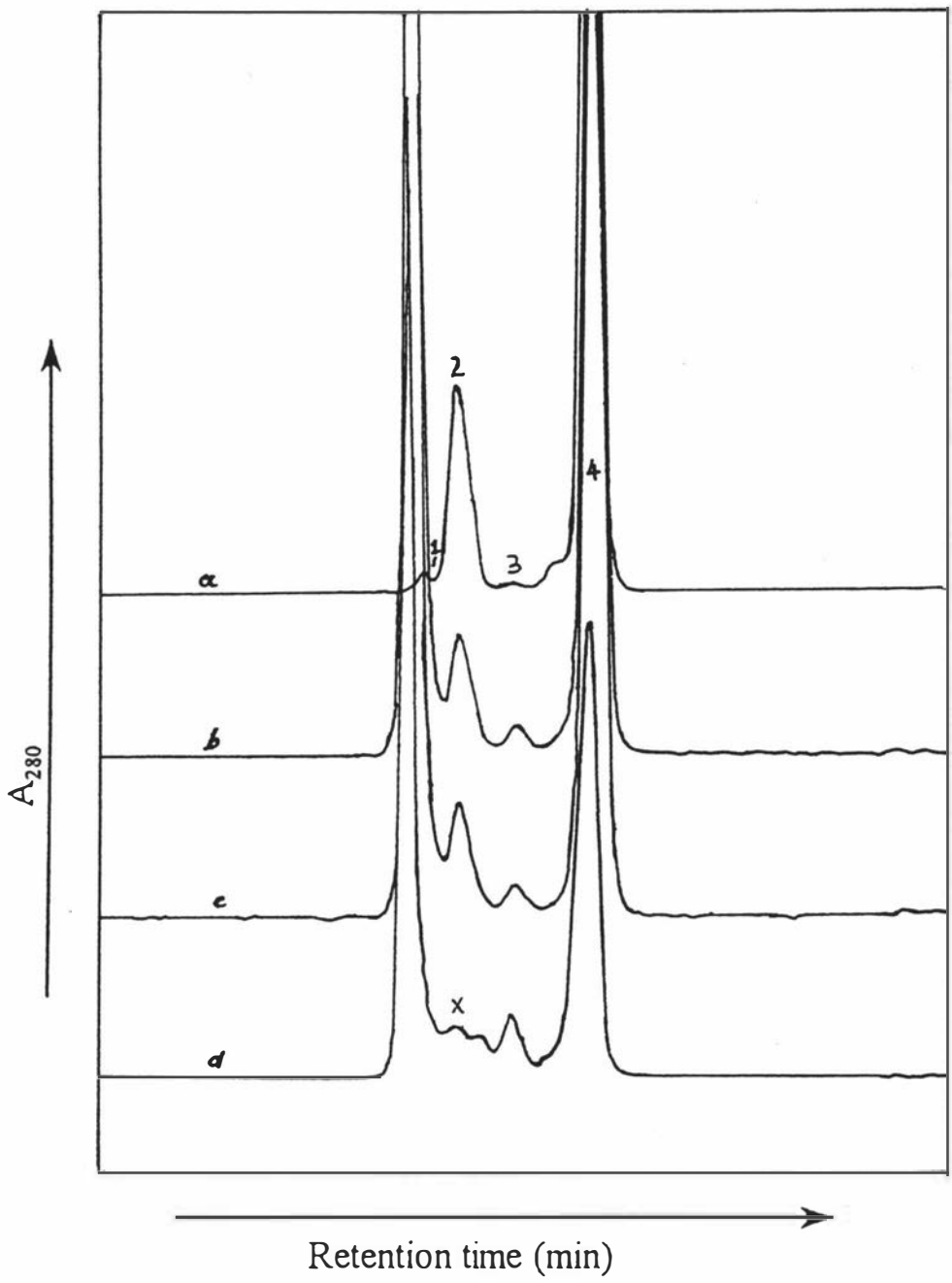


Figure 8.9. Size exclusion chromatogram of a 20 g/kg 1:1 mixture of an α-lactalbumin and BSA solution heated at 75 °C for 0 (*a*), 8 (*b*), 16 (*c*) and 24 (*d*) min.

The elution profile of the unheated sample (Fig. 8.9*a*) showed two major peaks (peaks 2 & 4) corresponding to BSA (~ 67 kDa) and α-lactalbumin (~ 14 kDa). A small peak (peak 1) corresponding to high molecular weight aggregates (> 100 kDa) was observed close to the void volume of the column. A small peak (peak 3) between the BSA and α-lactalbumin peaks (peaks 2 & 4) corresponded to α-lactalbumin dimer (~ 28 kDa). A small shoulder in front of the α-lactalbumin peak was not identified. The elution profiles of the heated samples (Fig. 8.9*b-d*) showed that the quantity of the high molecular weight aggregates (size of peak 1) increased whereas monomeric α-lactalbumin and BSA (sizes of peaks 2 & 4) decreased with heating time. The quantity of α-lactalbumin dimer increased slowly with heating time. The sample that was heated for 24 min (Fig. 8.9*d*) showed a new peak (labelled "x") just before the α-lactalbumin dimeric peak. This peak probably corresponded to α-lactalbumin trimer (~ 42 kDa).

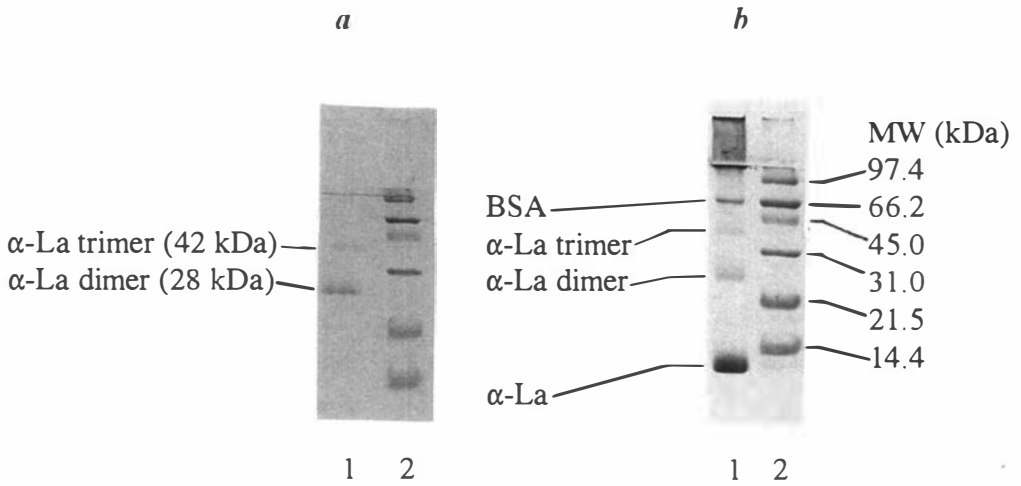


Figure 8.10. SDS-PAGE patterns of the material corresponding to peak 3 in Fig. 8.9, α-lactalbumin dimer (*a*) (slot 1), collected from the size exclusion column while running a 20 g/kg 1:1 mixture of α-lactalbumin and BSA heated for 24 min at 75 °C, and (*b*, slot 1) of the heated mixture. Slot 2 shows the SDS-PAGE patterns of the reduced molecular weight standards used for determining the molecular weights of the protein bands.

Samples corresponding to peak 3, i.e. between 17 and 22 min elution times, were collected (Fig. 8.9*d*), dialysed and freeze-dried. The dried sample was dissolved in SDS-buffer and then run on SDS-PAGE. The SDS-PAGE patterns showed two bands that corresponded to both α-lactalbumin dimer and trimer (Fig. 8.10*a*, slot 1). These were compared with the reduced SDS-PAGE of molecular weight standards - BioRad Lot No. 74177.

8.4. Heat treatment of mixtures of β-lactoglobulin, α-lactalbumin and BSA

Figure 8.11 shows the native- and SDS-PAGE patterns of the heated 100 g/kg 2:1:1 mixtures of β-lactoglobulin, α-lactalbumin and BSA. Both gels showed that the intensity of the monomeric proteins decreased with heating time at 75 °C. The intermediate aggregates formed by β-lactoglobulin and α-lactalbumin during heating in other protein mixtures (Figs. 8.1, 8.4 - 8.8) were not apparent in these gels. The fine bands following the BSA bands in both the native and SDS gels became apparent after heating (75 °C) for 1.5 min. These bands probably corresponded to various heat-induced β-lactoglobulin- and α-lactalbumin-BSA complexes, which were also observed in Figs. 8.5-8.8.

8.5. Loss of proteins during heating

The 1D-PAGE gels were scanned using the method described in Section 3.7. The band intensities were used to estimate quantities of different protein species in the heated protein solutions. The results for the loss of native-like and SDS-monomeric proteins were used to determine the reaction kinetics as described earlier (Section 4.7.2).

8.5.1. Loss of β-lactoglobulin from different protein mixtures

The loss of native-like and SDS-monomeric β-lactoglobulin from different protein mixtures during heating (75 °C) is shown in Fig. 8.12. It appears that the loss of native-like β-lactoglobulin from the 10% β-lactoglobulin solution and from the 2:1 mixture with α-lactalbumin followed similar patterns, but the loss from the 2:1 mixture with BSA was slower and that from the mixture with both BSA and α-lactalbumin was

faster (Fig. 8.12*a*).

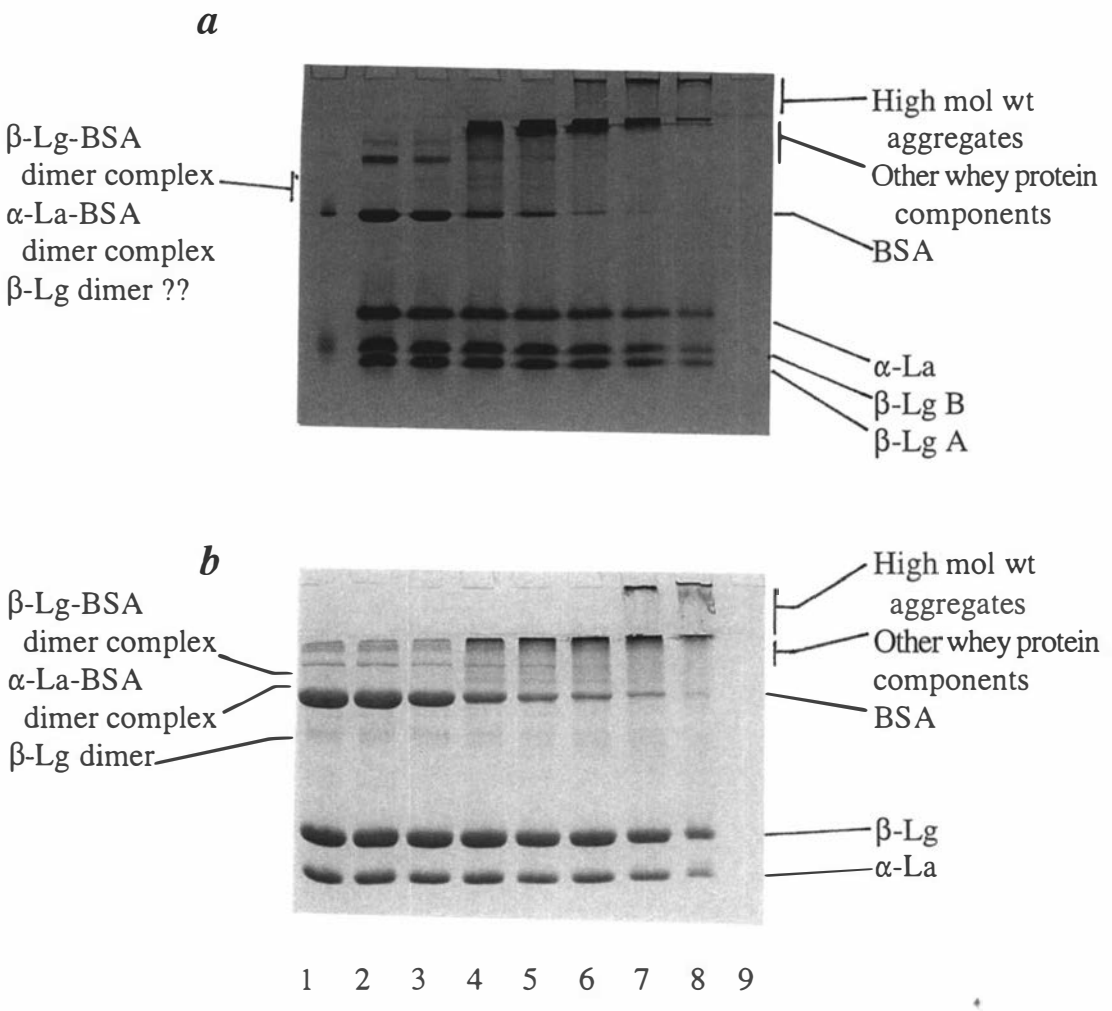


Figure 8.11. Native- (*a*) and SDS- (*b*) PAGE patterns of 2:1:1 mixtures of β-lactoglobulin, α-lactalbumin and BSA solutions (100 g/kg, pH 6.8) heated at 75 °C for 0 (slot 1), 0.5 (slot 2), 1 (slot 3), 1.5 (slot 4), 2 (slot 5), 4 (slot 6), 6 (slot 7) and 8 (slot 8) min. (Note: the control, non-heated sample, slot 1, native gel was not injected).

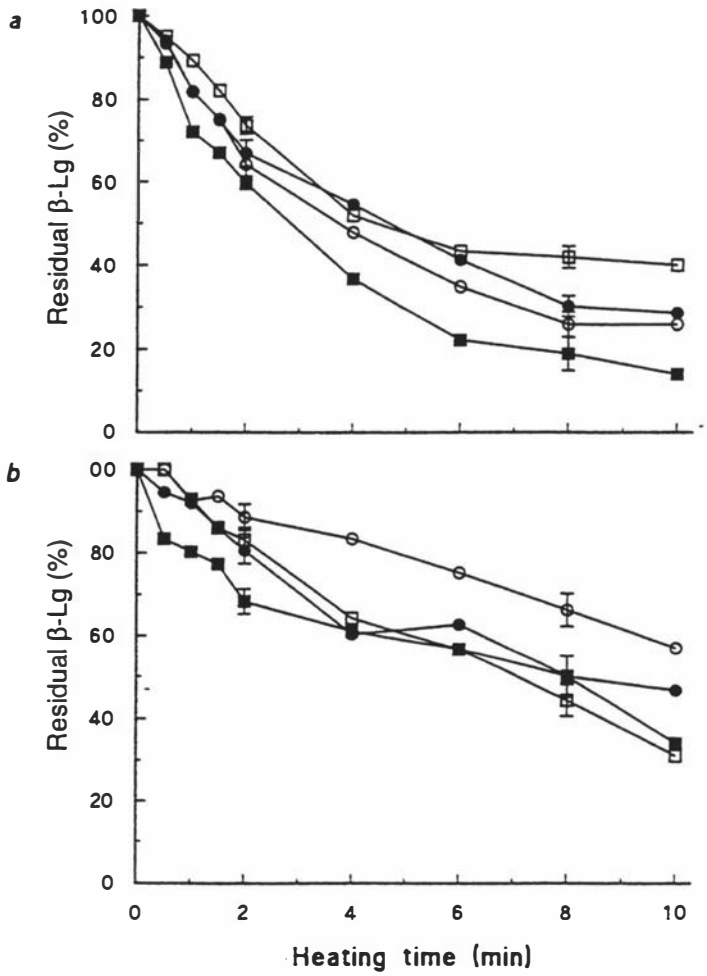


Figure 8.12. Loss of native-like (*a*) and SDS-monomeric (*b*) β-lactoglobulin from 100 g/kg protein solutions heated at 75 °C: β-Lactoglobulin solution (○), 2:1 mixture with α-lactalbumin (●) or BSA (□) and 2:1:1 mixture with both α-lactalbumin and BSA (■). Each point is an average of duplicate measurements. Error bars indicate the variation between results.

The loss of SDS-monomeric β-lactoglobulin from heated mixtures with α-lactalbumin, BSA or both showed similar trends whereas the loss from the 10% β-lactoglobulin solution was relatively slower (Fig. 8.12*b*).

The difference between the loss of native-like and SDS-monomeric β-lactoglobulin was largest when solutions of β-lactoglobulin alone or mixture with both α-lactalbumin and BSA were heated, indicating higher concentrations of hydrophobically-associated β-lactoglobulin aggregates in these solutions. These results suggested that the extent to which β-lactoglobulin was involved in the formation of hydrophobically-associated aggregates during heating was probably different in different protein mixtures.

8.5.2. Loss of α-lactalbumin from different protein mixtures

When heating a solution of α-lactalbumin alone, there was no loss of monomeric structure (Fig. 8.13), in agreement with many other studies (Matsudomi *et al.*, 1992, 1993; Hines & Foegeding, 1993; Gezimati *et al.*, 1996*a, b*). The loss of the α-lactalbumin from different heated protein mixtures (β-lactoglobulin, BSA or both) occurred at different rates. The loss of α-lactalbumin from a mixture with both β-lactoglobulin and BSA was fastest, followed by the loss from the mixture with β-lactoglobulin and then a relatively slow loss from the mixture with BSA (Fig. 8.13). These results suggest that α-lactalbumin may interact with different proteins via different mechanisms.

8.5.3. Loss of BSA from different protein mixtures

The loss of native-like BSA when heated in mixtures with α-lactalbumin was relatively slow compared with the loss from other mixtures. Native-like BSA was still observed by native-PAGE in the samples that were heated for 8 min (Fig. 8.14*a*), whereas in other mixtures, BSA disappeared completely after heating at 75 °C for 2 or 4 min.

The loss of SDS-monomeric BSA from different protein mixtures appeared to be different (Fig. 8.14*b*). The loss of SDS-monomeric BSA from the solutions of BSA alone could not be measured beyond 2 min of heating because the solutions had already

formed gels after heating. The loss from mixtures with other proteins was fastest from the mixture with β-lactoglobulin, by the mixture with both β-lactoglobulin and α-lactalbumin and the loss was slowest from the mixture with α-lactalbumin. In the mixture with α-lactalbumin, ~ 20% SDS-monomeric BSA remained after heating for 10 min. These results suggest that α-lactalbumin had an inhibiting effect on the loss of BSA structure.

It was clear that the loss of BSA, both native-like (Fig. 8.14*a*) and SDS-monomeric (Fig. 8.14*b*), took place in two separate stages, fast during the first 2 min of heating and relatively slow thereafter.

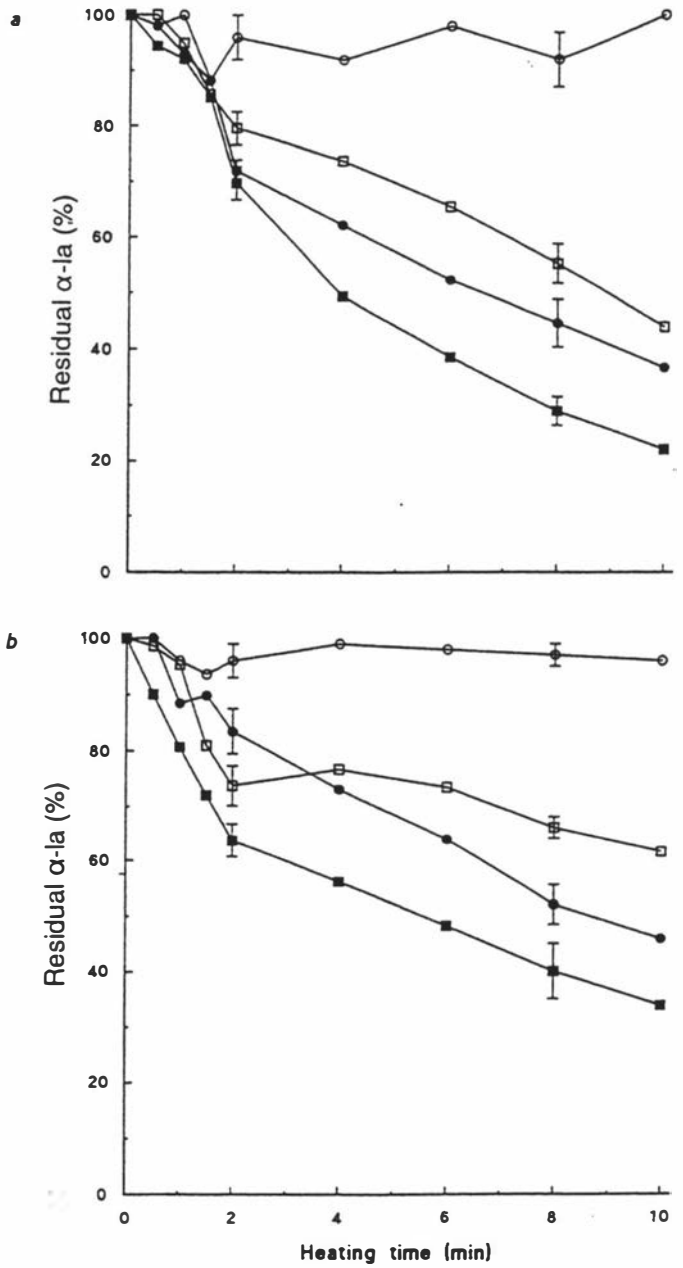


Figure 8.13. Loss of native-like (*a*) and SDS-monomeric (*b*) α-lactalbumin from 100 g/kg protein solutions heated at 75 °C: α-lactalbumin solution (○), 1:2 mixture with β-lactoglobulin (●), 1:1 mixture with BSA (□) and 1:2:1 mixture with both β-lactoglobulin and BSA (■). Each point is an average of duplicate measurements. The error bars indicate the variation between the results.

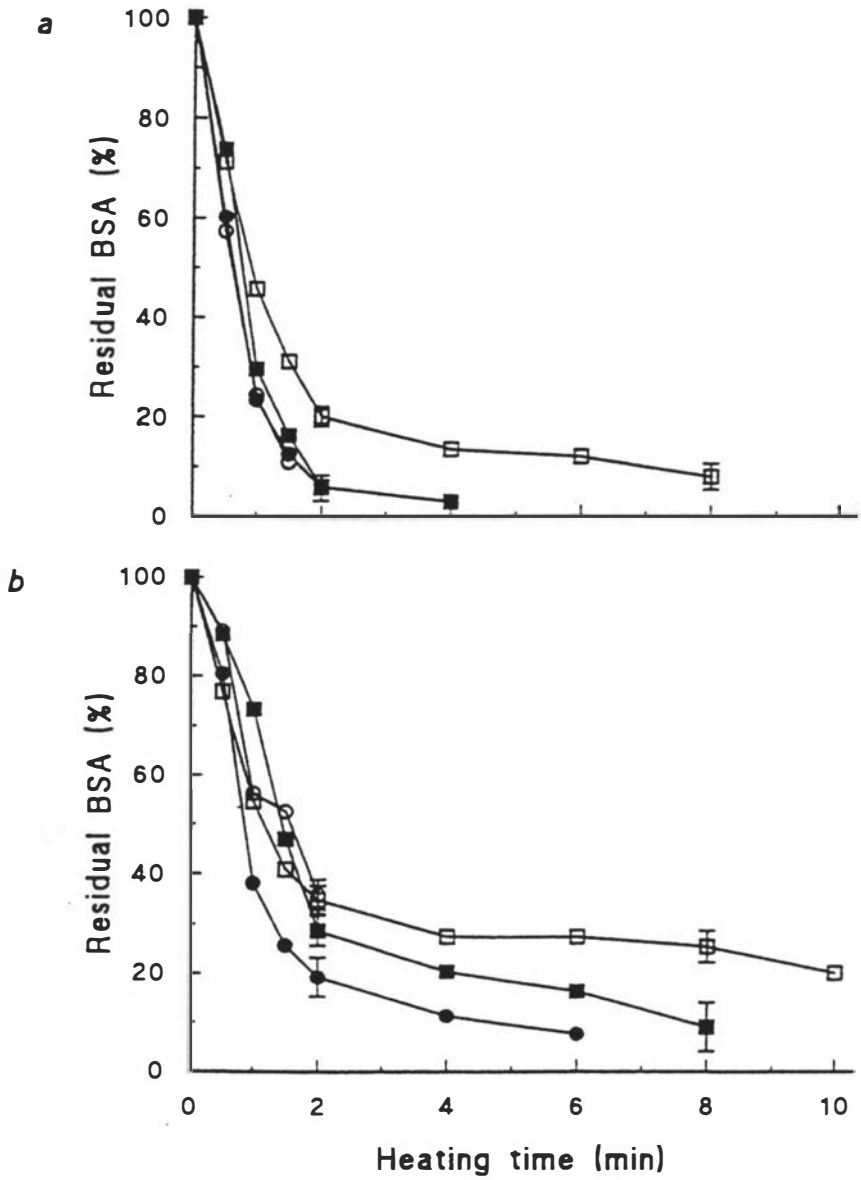


Figure 8.14. Loss of native-like (*a*) and SDS-monomeric (*b*) BSA from 100 g/kg protein solutions heated at 75 °C: BSA-solution (○), 1:2 mixture with β-lactoglobulin (●), 1:1 mixture with α-lactalbumin (□) and 1:2:1 mixture with both β-lactoglobulin and α-lactalbumin (■). Each point is an average of duplicate measurements. The error bars indicate the variation between the results.

8.5.4. Kinetic evaluation of the loss of proteins from different mixtures

The kinetics parameters for the loss of each protein (β-lactoglobulin, α-lactalbumin and BSA) from different mixtures were calculated using the equations (2) or (3) (Section 4.7.2) and the reaction rate constants, k_n , were calculated, assuming a reaction order of n = 1, 1.5 or 2. The best fit was determined for each set of data by linear regression and the coefficients of determination, r^2, were compared. The results showed that the coefficient of determination was highest for each data set when n = 1.5 was used. The calculated k_n values for the loss of both native-like and SDS-monomeric proteins, assuming a reaction of order n = 1.5, are shown in Table 8.1.

The k_n for the loss of native-like β-lactoglobulin was highest when heated in mixtures with both α-lactalbumin and BSA, and lowest when heated in mixture with BSA (Table 8.1). The k_n values for the loss of β-lactoglobulin from the solution of β-lactoglobulin heated alone or in mixture with α-lactalbumin were intermediate between those for the other two systems. The k_n value for the loss of SDS-monomeric β-lactoglobulin was highest when heated in mixture with both α-lactalbumin and BSA, and lowest when heated alone. The k_n values for the loss of SDS-monomeric β-lactoglobulin were considerably lower than those for the loss of native-like β-lactoglobulin. The difference between the k_n values for the loss of native-like and SDS-monomeric β-lactoglobulin was greatest when heated in mixture with both α-lactalbumin and BSA or in a solution of β-lactoglobulin alone, suggesting higher concentrations of hydrophobically-associated aggregates in these solutions, in agreement with observations made earlier (Figs. 8.1 & 8.12).

The k_n value for the loss of both native-like and SDS-monomeric α-lactalbumin was highest when heated in mixture with both β-lactoglobulin and BSA, but lowest when heated in mixture with BSA, and somewhat intermediate when heated in mixture with β-lactoglobulin. The k_n value for the loss of the same protein when heated by itself was not calculated as no loss of protein was observed.

The k_n values for the loss of native-like and SDS-monomeric BSA may not be reliable because the calculation was based on only a few data points for each set of heated protein systems. However, the k_n value for the loss of native-like BSA followed

the order: mixture with both β-lactoglobulin and α-lactalbumin > mixture with β-lactoglobulin only > BSA alone >> mixture with α-lactalbumin. The k_n value for the loss of SDS-monomeric BSA followed the order: mixture with β-lactoglobulin > BSA alone > mixture with both β-lactoglobulin and α-lactalbumin > mixture with α-lactalbumin. It is clear that the k_n value for the loss of both native-like and SDS-monomeric BSA was slowest when in mixture with α-lactalbumin. These results again support the suggestion that α-lactalbumin has an inhibiting effect on the loss of BSA structures.

Table 8.1. Calculated rate constants, k_n ($min^{-0.5}$) and coefficients of determination, r^2, for the loss of native-like and SDS-monomeric β-lactoglobulin, α-lactalbumin and BSA from different mixtures during heating at 75 °C, assuming a reaction of order n = 1.5

Loss of β-Lactoglobulin	**Native-like**		**SDS-monomeric**	
Protein mixtures	k_n	r^2	k_n	r^2
β-Lg alone	0.1097	0.98	0.0308	0.98
β-Lg + α-la	0.0915	0.99	0.0476	0.96
β-Lg + BSA	0.0647	0.93	0.0445	0.97
β-Lg + α-la + BSA	0.1700	0.99	0.0578	0.93
Loss of α-Lactalbumin	**Native-like**		**SDS-monomeric**	
α-La alone	-	-	-	-
α-La + β-lg	0.0651	0.99	0.0486	0.99
α-La + BSA	0.0392	0.97	0.0250	0.84
α-La + β-lg + BSA	0.1141	0.99	0.0673	0.98
Loss of BSA	**Native-like**		**SDS-monomeric**	
BSA alone	1.3525	0.95	0.2927	0.98
BSA + β-lg	1.5668	0.96	0.4466	0.96
BSA + α-la	0.6342	0.94	0.1055	0.83
BSA + β-lg & α-la	1.7612	0.97	0.2830	0.96

8.6. Protein aggregate formation in heated protein solutions

To elucidate the relative amounts of proteins that were monomeric, or involved in forming hydrophobic or disulphide-linked aggregates, the native- and SDS-PAGE results were compared in the same way as described earlier (Section 4.6.3). PAGE analysis of the reduced samples was not carried out. Given the amounts of native-like and SDS-monomeric protein present in a heated sample, it was assumed that the rest of the residual protein was disulphide-linked.

8.6.1. β-Lactoglobulin aggregates

The β-lactoglobulin aggregates formed in different heated protein mixtures are shown in Fig. 8.15. It is clear that considerable amounts of hydrophobically-associated aggregates were formed by heating β-lactoglobulin at 75 °C (Fig. 8.15*a*) or in mixture with both α-lactalbumin and BSA (Fig 8.15*d*). There was relatively little involvement of β-lactoglobulin in the formation of hydrophobically-associated aggregates when it was heated in mixture with either α-lactalbumin (Fig. 15*b*) or BSA (Fig. 8.15*c*). The aggregates in these heated solutions appeared to be largely disulphide-linked.

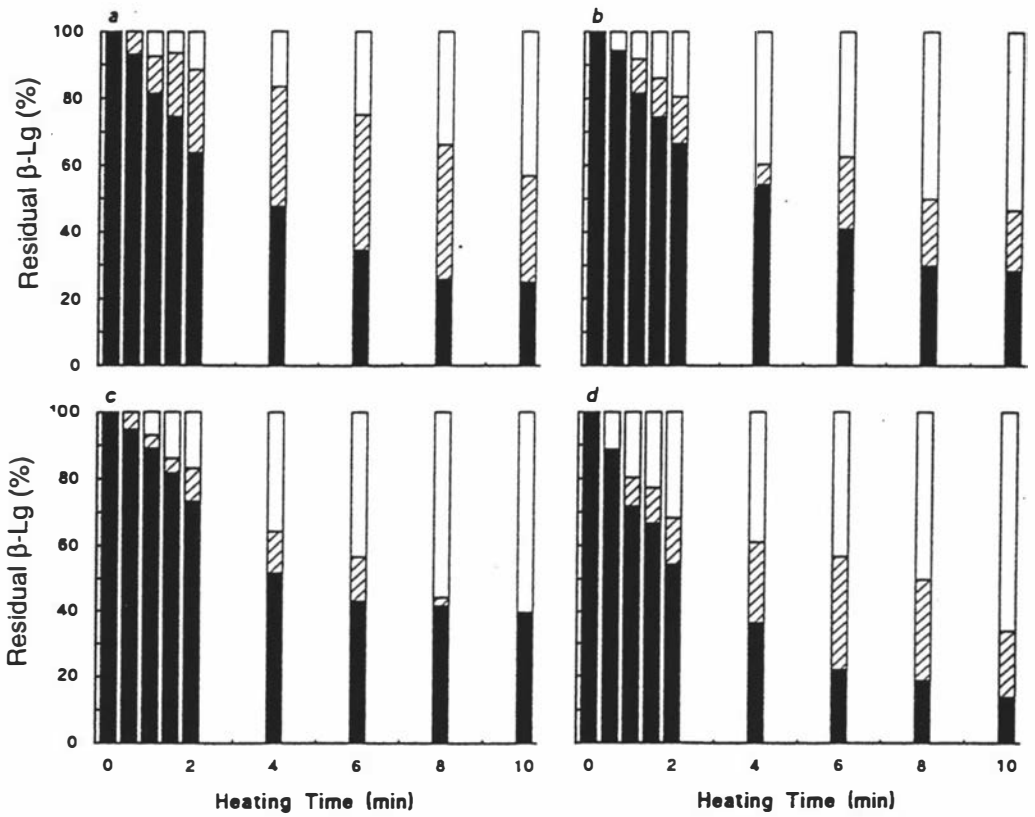


Figure 8.15. β-Lactoglobulin aggregates in 100 g/kg protein solutions heated at 75 °C: alone (*a*), in 2:1 mixture with α-lactalbumin (*b*) or BSA (*c*) and in 2:1:1 mixture with both α-lactalbumin and BSA (*d*). β-Lactoglobulin species: native-like (■), hydrophobic aggregates (▨) and disulphide-linked aggregates (□).

8.6.2. α-Lactalbumin aggregates

α-Lactalbumin had very little involvement in the formation of hydrophobically-associated aggregates in any of the protein mixtures (Fig. 8.16). The aggregates formed by α-lactalbumin were, therefore, largely disulphide-linked.

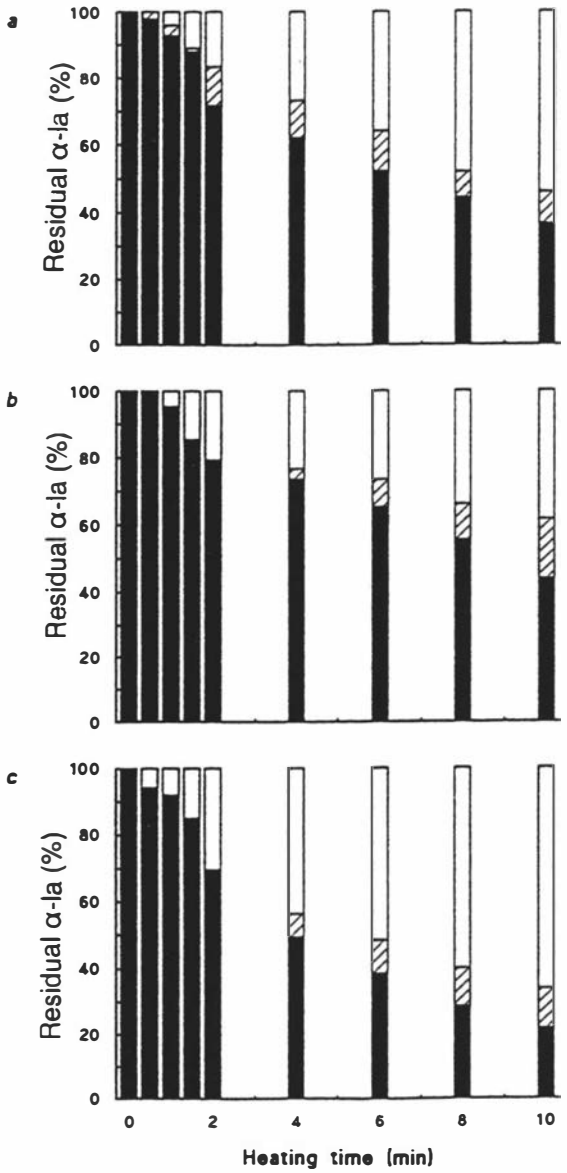


Figure 8.16. α-Lactalbumin aggregates in 100 g/kg solutions heated at 75 °C in 1:2 mixture with β-lactoglobulin (*a*), 1:1 mixture with BSA (*b*) and 1:2:1 mixture with both β-lactoglobulin and BSA (*c*). α-Lactalbumin species : native-like (■), hydrophobic aggregates (▨) and disulphide-linked aggregates (□).

8.6.3. BSA aggregates

Figure 8.17 shows the BSA aggregate species formed in different heated protein mixtures. When BSA was heated alone (Fig. 8.17*a*) the quantities of hydrophobically-associated aggregates were comparable with those of disulphide-linked aggregates formed in samples for which analysis was practicable (as samples heated for > 2 min formed gels, analysis was not done). When heated in mixtures with other proteins (Figs. 8.17*b-d*), the BSA aggregates were largely disulphide-linked. In these mixtures, the solutions remained liquid after heating at 75 °C for longer periods (up to 10 min); therefore, analysis was carried out on these samples. Considerable quantities of hydrophobically-associated aggregates were formed in these samples especially in heated mixtures with α-lactalbumin (Fig. 8.17*c*).

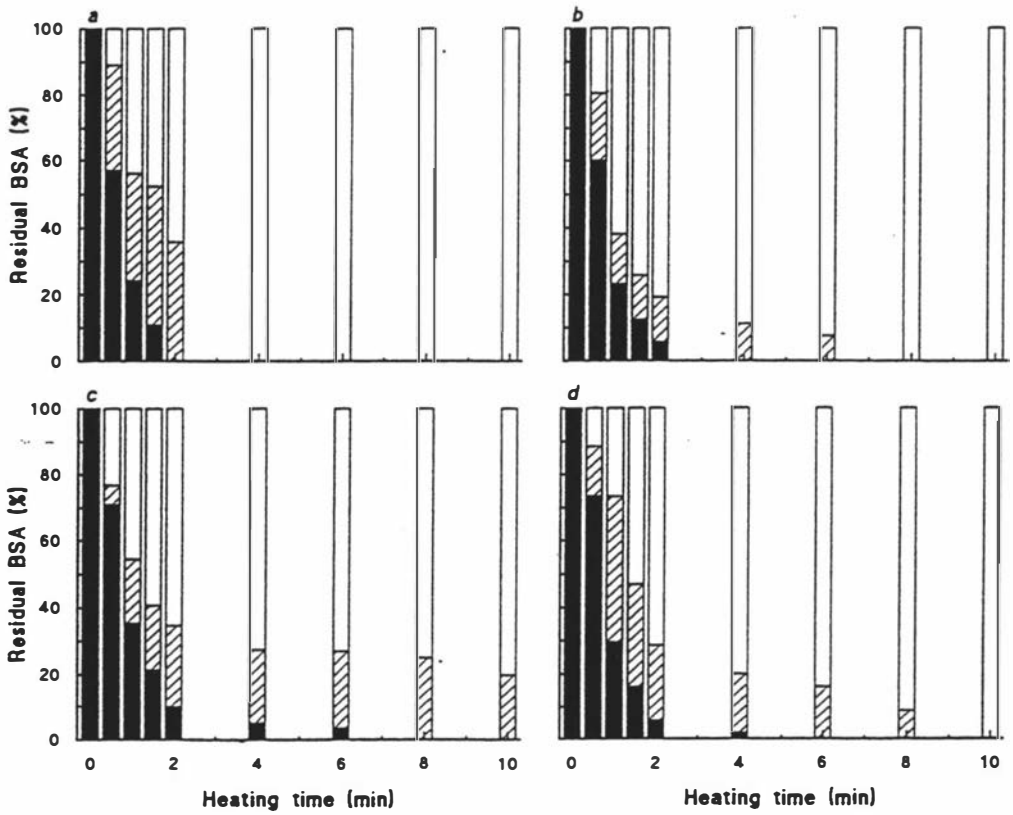


Figure 8.17. BSA aggregates in 100 g/kg solutions heated at 75 °C alone (*a*), in 1:2 mixture with β-lactoglobulin (*b*), in 1:1 mixture with α-lactalbumin © and in 1:2:1 mixture with both β-lactoglobulin and α-lactalbumin (*d*). BSA species: native-like (■), hydrophobic aggregates (▨) and disulphide-linked aggregates (□).

8.7. Protein aggregation determined using light scattering

Heat-induced aggregation of different mixtures of β-lactoglobulin, α-lactalbumin and BSA was followed using a light scattering method as described in Section 3.12 Protein solutions (50 g/kg, pH 6.8) were prepared; 0.5 ml of each solution was placed in an

NMR tube (3 mm internal diameter), and was heated at 75 °C *in situ* for 15 min. The scattered light intensity was measured at an angle of 90° every 30 s. The changes in scattered light intensity during the heating of different protein solutions are shown in Fig. 8.18.

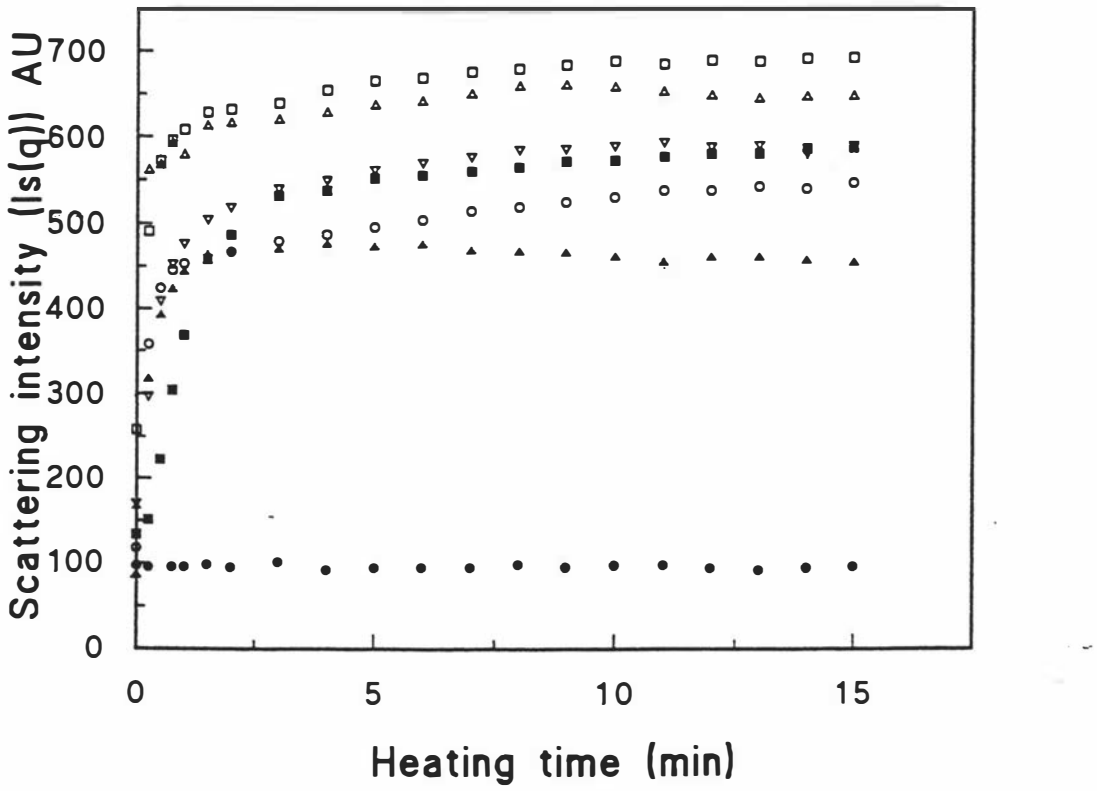


Figure 8.18. Effect of *in situ* heat treatment, 75 °C, of protein solutions (50 g/kg, pH 6.8) on scattered light intensity ($I_s(q)$), measured during static light scattering. β-Lactoglobulin (○), α-lactalbumin (●), BSA (□), 2:1 mixture of β-lactoglobulin and α-lactalbumin (■), 2:1 mixture of β-lactoglobulin and BSA (Δ), 1:1 mixture of α-lactalbumin and BSA (▲) and 2:1:1 mixture of β-lactoglobulin, α-lactalbumin and BSA (▽).

According to light scattering theory (e.g. Hoffmann *et al.*, 1996), the so-called Rayleigh ratio, $R_\theta(q)$, which is related to the particle volume and molecular mass, is proportional to the scattered light intensity, if the intensity of the incident light, I_0, the volume of the illuminated light seen by the detector, V_s , and the distance from the sample detector, d, are fixed. These conditions were held for the current experiment, and it was considered that, as the protein aggregates in the heated sample grew in size, the scattered intensity increased.

The scattered intensity did not change when a solution of α-lactalbumin was heated alone (Fig. 8.18), confirming the earlier observations (Figs. 8.2). The other protein solutions showed very similar trends, with a fast increase in scattered intensity during the first 1 or 2 min of heating followed by a plateau. The rates of initial increase in scattered light are related to the rate of aggregation in the heated samples (Hoffmann *et al.*, 1996). It appears that the initial rates of increase were different for different protein solutions. The initial increase in scattered intensity solution was fastest for the BSA and slowest for the mixture of β-lactalbumin and α-lactalbumin. It can also be seen that the levels at which the plateau occurred were different for different protein solutions. These levels were probably related to the average particle sizes in the heated samples. It appears that the aggregates formed in heated BSA solutions were largest, followed by the aggregates in the heated mixture of BSA and β-lactalbumin. The plateau for the heated mixture of BSA and α-lactalbumin occurred at the lowest level. The plateaus for the heated β-lactalbumin solution and the mixture of β-lactalbumin and α-lactalbumin were at intermediate levels between those of the BSA and the mixture of α-lactalbumin and BSA.

8.8. Characterisation of protein aggregates by 2D-PAGE

The aggregates formed during heat treatment of the protein mixture were characterised using 2D-PAGE, as described in Section 3.7.

8.8.1. Individual protein solutions

β-Lactoglobulin, α-lactalbumin and BSA solutions (100 g/kg, pH 6.8) were heated at 75 °C and analysed both by native-SDS and SDS 2D-PAGE.

Heated β-lactoglobulin solutions

The native-SDS 2D-PAGE patterns of a 100 g/kg β-lactoglobulin solution heated at 75 °C for 8 min (Fig. 8.19*a*) showed a vertical streak of a faint region dissociated from the aggregated material that was caught on top of the resolving gel of the sample gel strip. This indicates the presence of hydrophobically-associated aggregates in the heated sample. The control sample running on the left hand side showed that the aggregated materials was resolved into to more defined bands corresponding to β-lactoglobulin dimer and trimer.

The SDS 2D-PAGE of the same sample showed an almost continuous horizontal line corresponding to β-lactoglobulin monomers (Fig. 8.19*b*). There were three distinct spots along this line that corresponded to different bands in the sample strip. These spots were dissociated from the respective bands corresponding to high molecular weight material, β-lactoglobulin trimers and β-lactoglobulin dimers. The control sample, running vertically on the left hand side, showed a β-lactoglobulin monomeric band and two other slower moving bands which corresponded to β-lactoglobulin dimer and trimer. It was clear that some of the material dissociated from the dimeric and the higher molecular weight bands in the sample strip corresponded to the dimeric band of the control sample. These results suggest that a proportion of the β-lactoglobulin existed as dimers linked by non-disulphide covalent bonds. These were not reduced by 2-mercaptoethanol and had probably existed in the β-lactoglobulin product used in this study. Gezimati *et al.* (1996*b*) observed the same form of β-lactoglobulin in mixtures of β-lactoglobulin and α-lactalbumin heated at 75 and 80 °C.

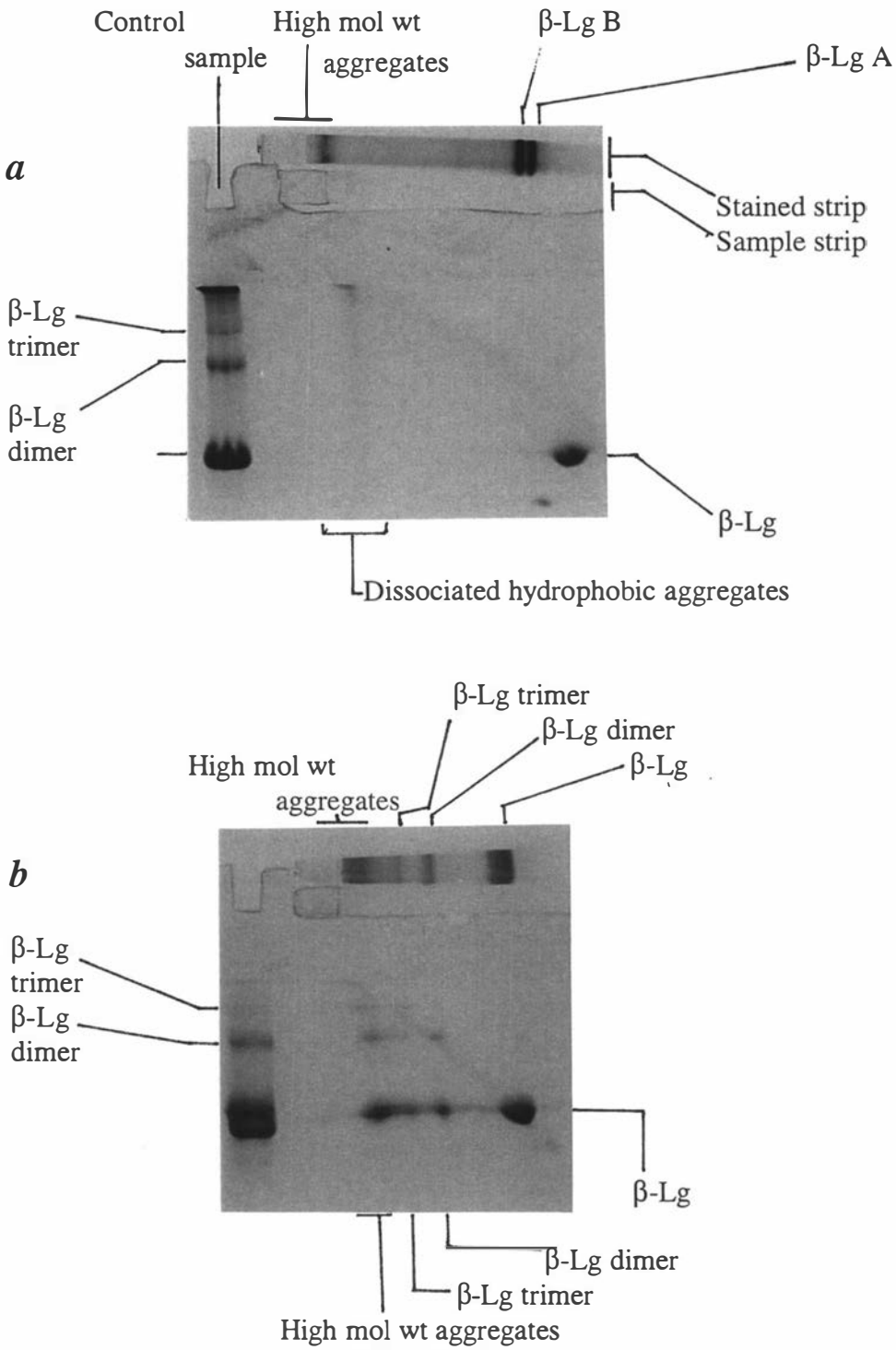


Figure 8.19. Native-SDS (*a*) and SDS (*b*) 2D-PAGE patterns of 100 g/kg β-lactoglobulin solution heated at 75 °C for 8 min.

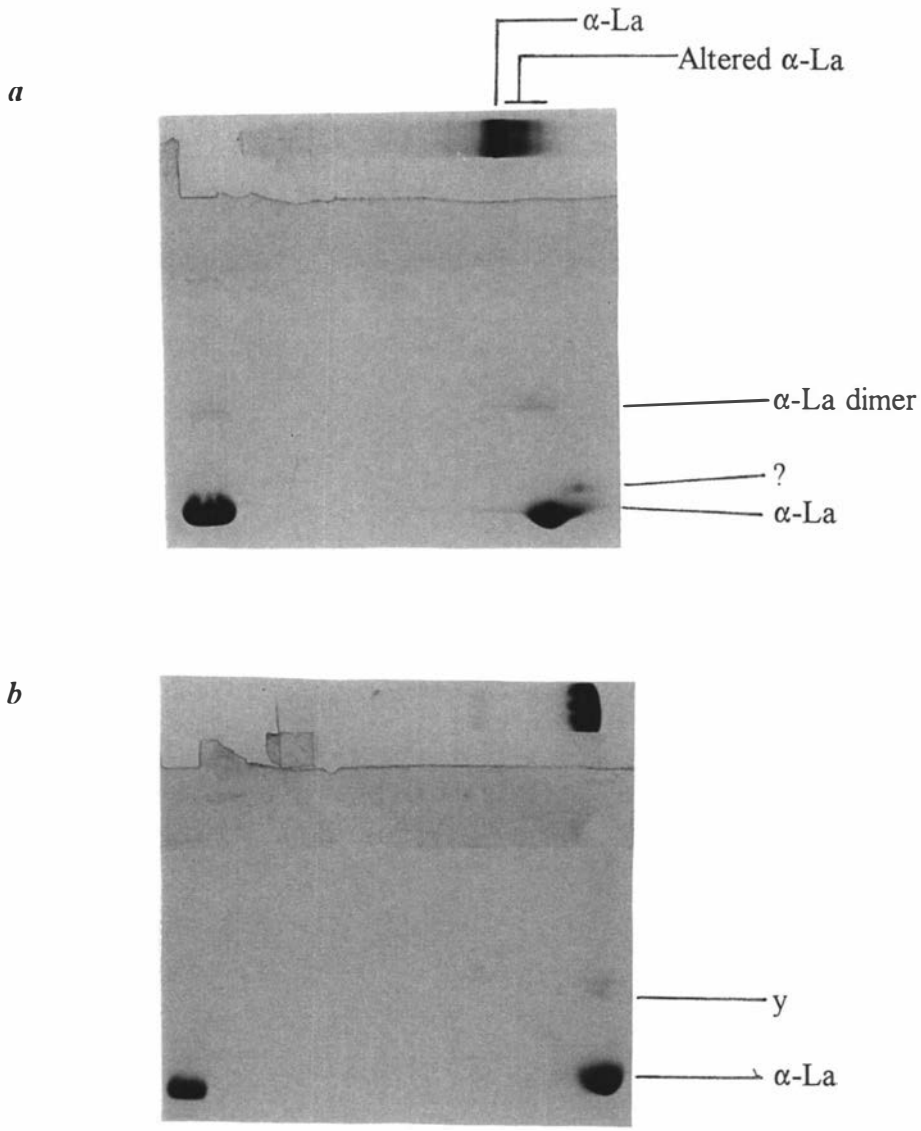


Figure 8.20. Native-SDS (*a*) and SDS (*b*) 2D-PAGE patterns of a 100 g/kg α-lactalbumin solution heated at 75 °C for 10 min.

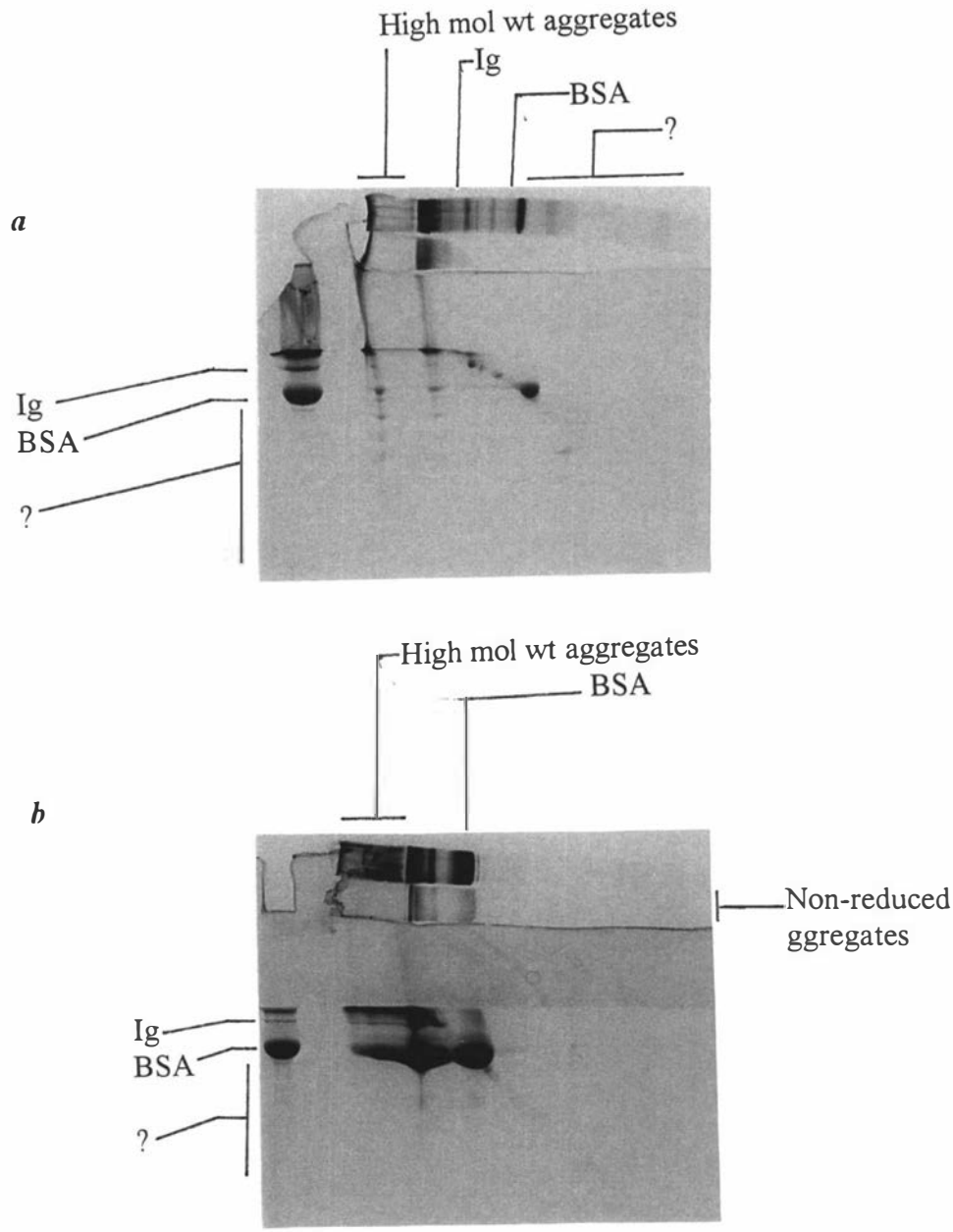


Figure 8.21. Native-SDS (*a*) and SDS (*b*) 2D-PAGE patterns of 100 g/kg BSA solution heated at 75 °C for 1 min.

Heated α-lactalbumin solutions

α-Lactalbumin solution (100 g/kg) heated for 10 min at 75 °C was used for this analysis. Figure 8.20 shows the native-SDS and SDS 2D-PAGE patterns of the heated α-lactalbumin. The control sample on the left-hand side of the native-SDS gel (Fig. 8.20*a*) showed a faint α-lactalbumin dimeric band running behind the monomeric band. In both cases, no aggregates dissociated from the sample strip. However, material eluted from the monomeric bands (labelled "y") appeared to travel the same distances as the α-lactalbumin dimeric bands. This material was not identified.

Heated BSA solutions

The native-SDS 2D-PAGE pattern of BSA solution (100 g/kg, pH 6.8) heated for 1 min at 75 °C showed spots that corresponded to BSA and other minor whey protein components (Fig. 8.21*a*). These spots dissociated from the material that was caught on top of both the stacking and resolving gels of the sample strip. They were probably involved in the formation of hydrophobically-associated aggregates. Some other spots eluted from the same material travelled longer distances into the resolving gels. It is not clear what these spots were.

SDS 2D-PAGE (Fig. 8.21*b*) showed bands corresponding to BSA and the other minor whey proteins. There were some spots that travelled ahead of BSA. These spots were probably due to some impurities present in the BSA samples used. Some material remained in the sample strip, suggesting the presence of non-disulphide covalently linked aggregates.

8.8.2. Heated mixture of β-lactoglobulin and α-lactalbumin

The native-SDS 2D-PAGE pattern of a 100 g/kg 2:1 mixture of β-lactoglobulin and α-lactalbumin heated at 75 °C for 8 min (Fig. 8.22*a*) showed that the monomeric protein bands and a faint vertical streak dissociated from the material caught on top of the resolving gel (sample strip) and travelled into the resolving gel of the 2D gel. The control sample showed an intense band corresponding to β-lactoglobulin dimer, and some aggregated material caught within the stacking gel. The spot labelled "y" was

again not identified.

The SDS 2D-PAGE (Fig. 8.22*b)* pattern showed some new spots and different features compared with when the two proteins were heated individually. The material that was caught within the stacking gel of the sample strip dissociated into bands corresponding to monomeric β-lactoglobulin and α-lactalbumin. There were also spots corresponding to β-lactoglobulin dimer and trimer that were not dissociated into monomers after reduction with 2-mercaptoethanol. The series of bands running behind the β-lactoglobulin band in the sample gel strip gave almost continuous horizontal lines that corresponded to β-lactoglobulin and α-lactalbumin.

Careful analysis of these lines showed that they consisted of various spots which are represented diagrammatically in Fig. 8.22*c*. Some of the monomeric spots of the two proteins appeared to be in "pairs" whereas others largely represented one of the proteins. The most intense pair of spots (labelled "I"), corresponded to the material caught on top of the resolving gel. These spots were generated from the disulphide-linked aggregates which were relatively large in size and they either travelled short distances or could not migrate through the resolving gel of the sample strip. These aggregates were probably a mixture of homogeneous and heterogeneous aggregates of the two proteins. The next pair of spots (labelled "II" in Fig. 8.22*c*) appeared to be dissociated from a band that corresponded to a molecular weight of ~ 62 kDa (this may represent β-lactoglobulin-α-lactalbumin complex tetramers). A complex formed by two β-lactoglobulin and two α-lactalbumin molecules would have a molecular weight of ~ 65 kDa. The other pair of spots (labelled "III" in Fig. 8.22*c*) probably corresponded to β-lactoglobulin-α-lactalbumin dimer complexes. These two spots ran just behind the β-lactoglobulin band that was generated by the reduction of β-lactoglobulin dimers (36 kDa), labelled on Fig. 8.22*c*. There were two other spots that corresponded to α-lactalbumin: one corresponded to α-lactalbumin trimers (42 kDa), whereas the other corresponded to α-lactalbumin dimers (~ 28 kDa). These results showed that, although heterogeneous polymers formed between β-lactoglobulin and α-lactalbumin, homogeneous polymers of each protein were formed during the heating of a mixture of these proteins.

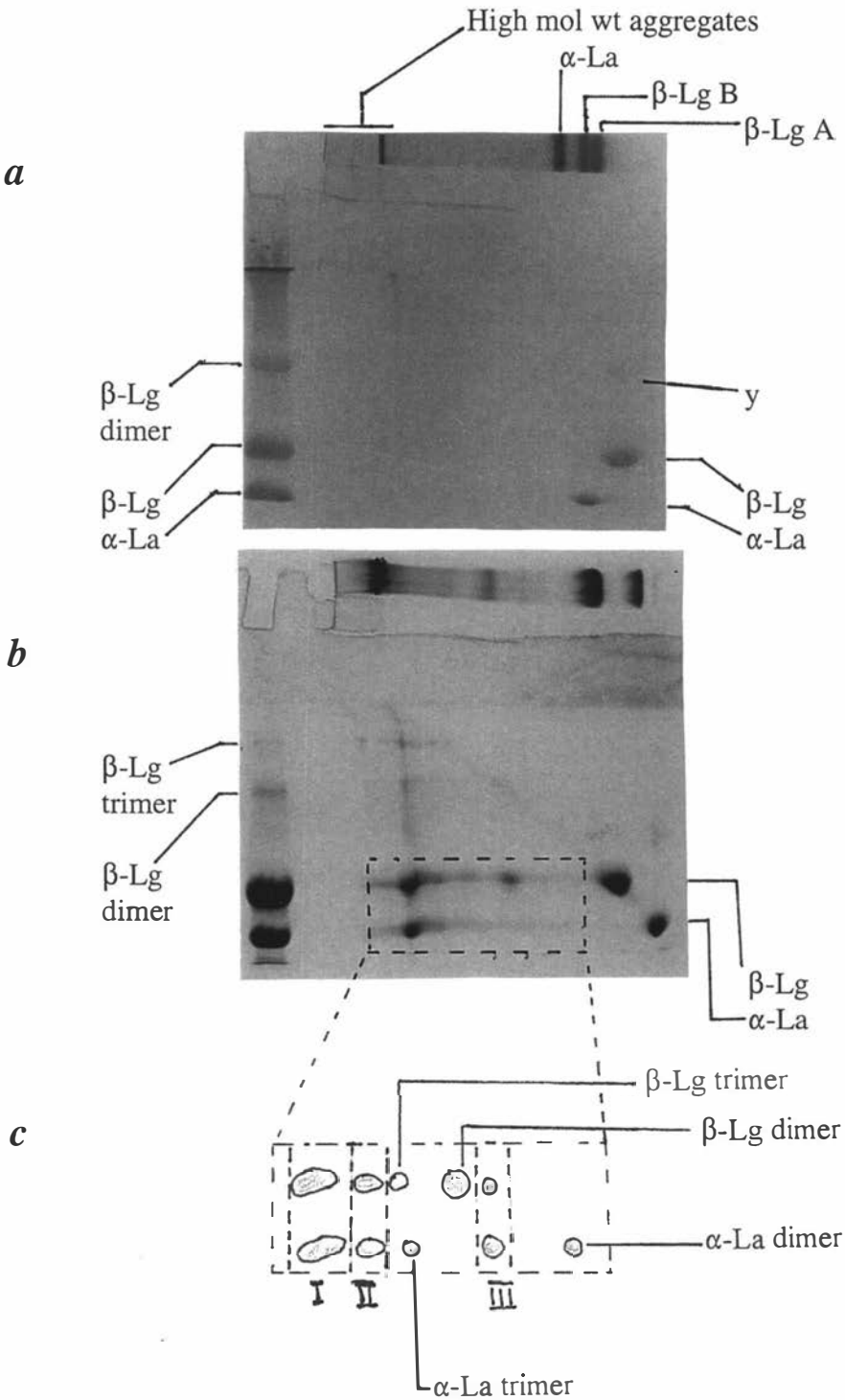


Figure 8.22. Native-SDS (*a*) and SDS (*b*) 2D-PAGE patterns of a 100 g/kg, 2:1 mixture of β-lactoglobulin and α-lactalbumin solution heated at 75 °C for 8 min. © diagrammatic representation of the marked box in (*b*).

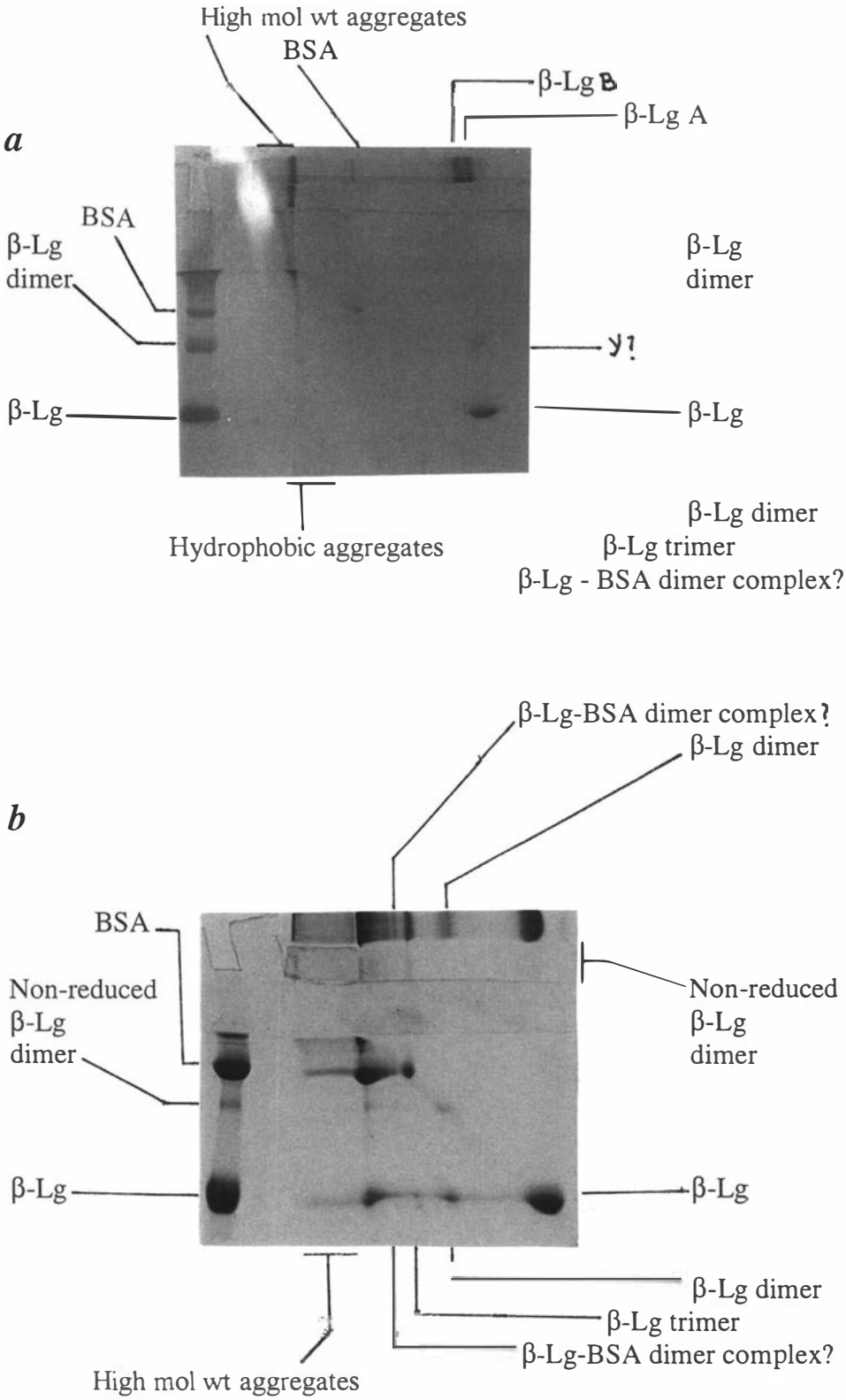


Figure 8.23. Native-SDS (*a*) and SDS (*b*) 2D-PAGE patterns of a 100 g/kg 2:1 mixture of β-lactoglobulin and BSA heated at 75 °C for 6 min.

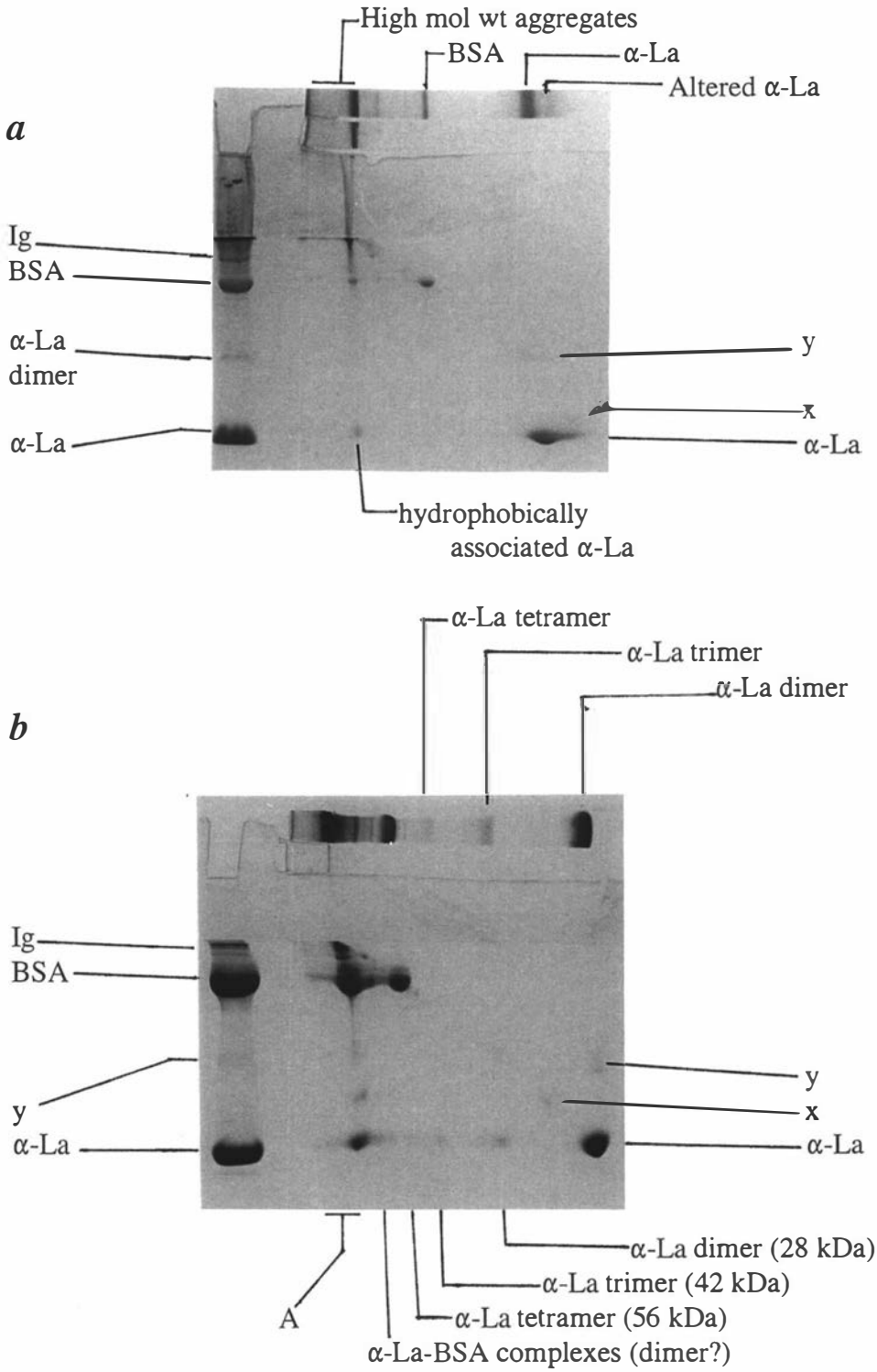


Figure 8.24. Native-SDS (*a*) and SDS (*b*) 2D-PAGE patterns of a 100 g/kg 1:1 mixture of α-lactalbumin and BSA heated at 75 °C for 6 min.

8.8.3. Heated mixture of β-lactoglobulin and BSA

The native-SDS 2D-PAGE pattern of the heated mixture of β-lactoglobulin and BSA (Fig. 23*a*) showed some faint regions corresponding to both proteins dissociated from the material caught on top of the resolving gel and within the stacking gel, indicating the presence of hydrophobically-associated aggregates.

The SDS 2D-PAGE pattern (Fig.8.23*b*) showed that the bands in the sample strip resolved to give spots in the 2D gel corresponded to monomeric β-lactoglobulin and BSA, β-lactoglobulin dimer and trimer, and higher molecular weight aggregates consisting of the two proteins. The fine band following the BSA band in the sample strip resolved to give spots corresponding to both β-lactoglobulin and BSA, indicating that this band was a 1:1 complex dimer of the two proteins. There were also spots corresponding to "non-reduced covalently linked β-lactoglobulin dimers" (similar spots were identified in Fig. 8.19*b*).

8.8.4. Heated mixture of α-lactalbumin and BSA

The native-SDS 2D-PAGE pattern of the heated mixture of α-lactalbumin and BSA (Fig. 8.24*a*) showed spots that corresponded to both monomeric and dimeric α-lactalbumin dissociated from the material caught on top of the resolving gel of the sample strip. This indicates that both proteins were involved in the formation of hydrophobically-associated aggregates. The spot labelled "y" was not identified. The material that travelled ahead of the α-lactalbumin band (sample strip) dissociated to give a faint band that travelled behind the α-lactalbumin monomeric band (labelled "x").

When compared with the SDS 2D-PAGE pattern of α-lactalbumin heated alone (Fig. 8. 20*b*), the heated mixture of α-lactalbumin and BSA (Fig. 8.24*b*) showed some spots that corresponded to α-lactalbumin polymers (dimer ~ 28 kDa, trimer ~ 42 kDa and tetramer ~ 56 kDa). The band that travelled behind BSA (sample strip), on reduction, gave intense bands that corresponded to both α-lactalbumin and BSA. These results confirm that α-lactalbumin polymers were formed during the heat treatment of a mixture of BSA and α-lactalbumin (Fig. 8.6). The same α-lactalbumin spots were also

observed in the 2D-PAGE of a mixture of α-lactalbumin and β-lactoglobulin (Fig. 8.22). The spots that resolved from the material caught on top of the resolving gel of the sample strip, labelled "A", can probably be attributed to a mixture of homogeneous and heterogeneous aggregates of the two proteins.

8.8.5. Heated mixture of β-lactoglobulin, α-lactalbumin and BSA

The native-SDS 2D-PAGE pattern of the heated mixture of the major whey proteins (β-lactoglobulin, α-lactalbumin and BSA) (Fig. 8.25*a*) showed that the material caught on top of the resolving gel (sample strip) partially dissociated to give spots that corresponded to the three proteins. A number of spots corresponding to disulphide-linked intermediate aggregates that dissociated from the same material were also observed. This suggests that disulphide-linked intermediate aggregates consisting of dimers, trimers, and tetramers were incorporated into higher molecular weight aggregates via hydrophobic associations.

The SDS 2D-PAGE pattern of the heated mixture (Fig. 8.25*b*) showed all the features discussed in Figs. 8.22-8.24. It is interesting to note that the α-lactalbumin and β-lactoglobulin spots, as observed in Fig. 8.22, were more apparent in the heated mixture of the three proteins. The material that was resolved from the material on top of the resolving gel (sample strip), labelled "A", probably consisted of different homogeneous and heterogeneous aggregates of the three proteins. These results suggest that, even in a mixture of the three proteins, the protein interactions that lead to the formation of these homogeneous aggregates still took place in addition to the formation of heterogeneous aggregates.

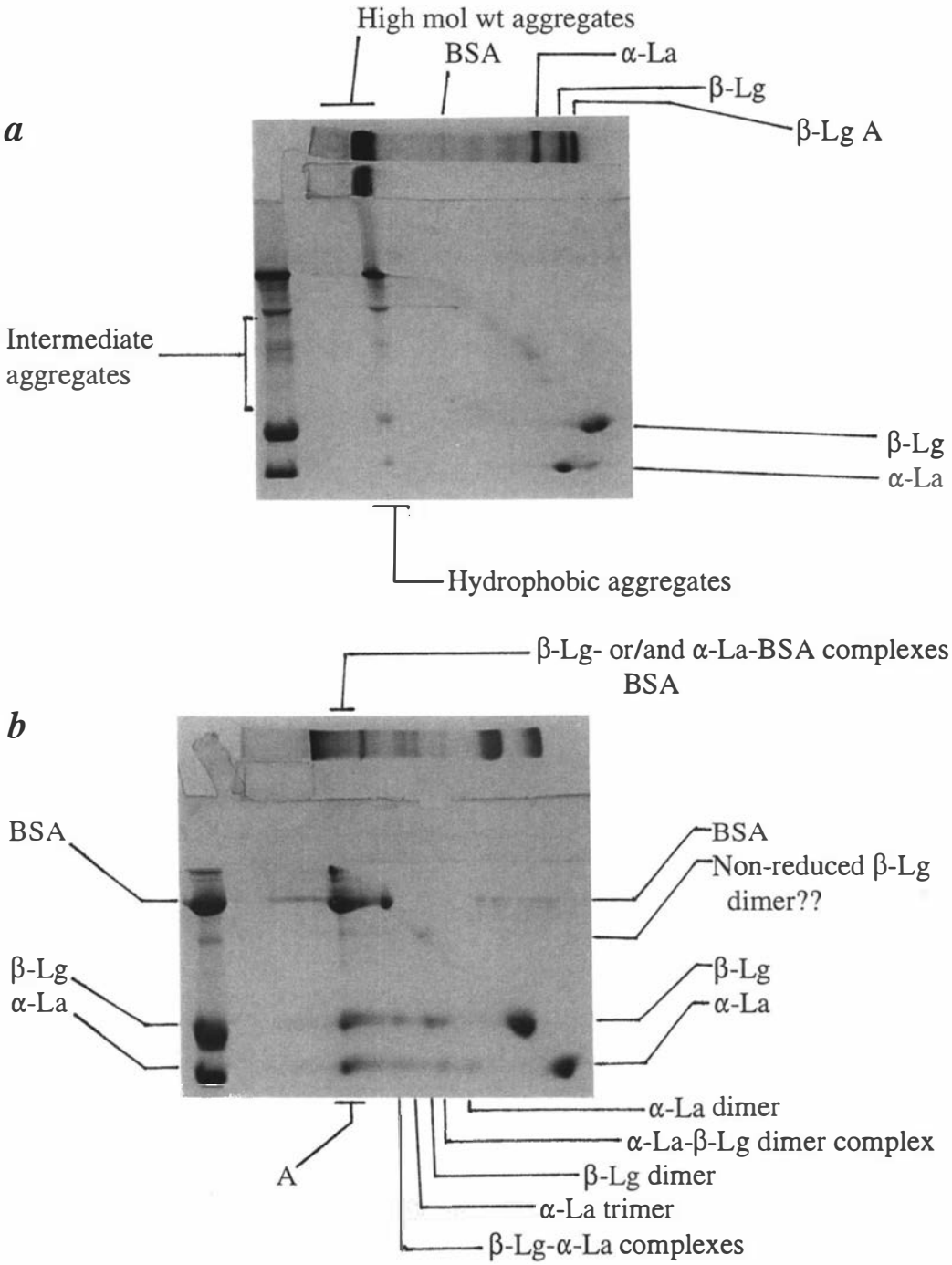


Figure 8.25. Native-SDS (*a*) and SDS (*b*) 2D-PAGE patterns of a 100 g/kg 2:1:1 mixture of β-lactoglobulin, α-lactalbumin and BSA heated at 75 °C for 6 min.

8.9. Development of protein aggregates during heat treatment of protein solutions

The 1D-PAGE results, Figs. 8.1-8.11, showed that during the heating of protein solutions the loss of monomeric proteins resulted in the formation of protein aggregates that increased in size with heating time. During the early stages of heat treatment, the aggregates formed were small enough to migrate into the resolving gel (e.g. see Fig. 8.1). The aggregates continued to increase in size with heating time until they could no longer migrate into the resolving gel, but remained caught on top of the resolving gel, and then within or on top of the stacking gel. With more severe heating, the aggregates may be so large that they become insoluble.

The development of heat-induced aggregates in mixtures of β-lactoglobulin, α-lactalbumin and BSA was followed using SDS 2D-PAGE (Fig. 8.26). Experience in 2D-PAGE analysis showed that, when a sample of an unheated protein mixture that has been separated in 1D-PAGE is run on a second dimension, the monomeric proteins will fall on a diagonal line as illustrated in Fig. 8.26*a*. If the sample strip contains intermediate aggregated proteins, reduction before the second dimension separation will result in the aggregates being resolved to give off-diagonal spots within region "A" (Fig. 8.26*a*). If the sample strip contains larger protein aggregates that are caught within the stacking gel, reduction before the second dimension separation will result in protein spots appearing within the region labelled "B".

The SDS 2D-PAGE pattern of the unheated mixture of β-lactoglobulin, α-lactalbumin and BSA (illustrated in Fig. 8.26*a*) showed monomeric proteins only. After heating at 75 °C for 30 s, a small region of low intensity spots corresponding to BSA at the top end of region A was seen (Fig. 8.26*b*). This indicates that BSA had started to form aggregates during the early stages of heating. After heating for 1 min, the BSA spots became more intense (Fig. 8.26*c*), and an almost continuous horizontal faint line corresponding to β-lactoglobulin and a faint spot corresponding to α-lactalbumin appeared in region A. After heating for 2 min (Fig. 8.26*d*), a similar pattern was observed, except that a faint line corresponding to BSA monomers appeared in region B, and the series of spots corresponding to monomeric β-lactoglobulin and α-lactalbumin became more intense. After heating for 6 min (Fig. 8.26*e*), the spots

corresponding to the proteins in region A and the BSA band in region B were more intense. After heating for 8 min (Fig. 8.26*f*), the intensities of the three protein bands in region B were similar. These results show that, when a mixture of the three major whey protein components (β-lactoglobulin, α-lactalbumin and BSA) is heated at 75 °C, BSA forms the initial aggregates. β-Lactoglobulin and α-lactalbumin are involved in the aggregation process at a later stage.

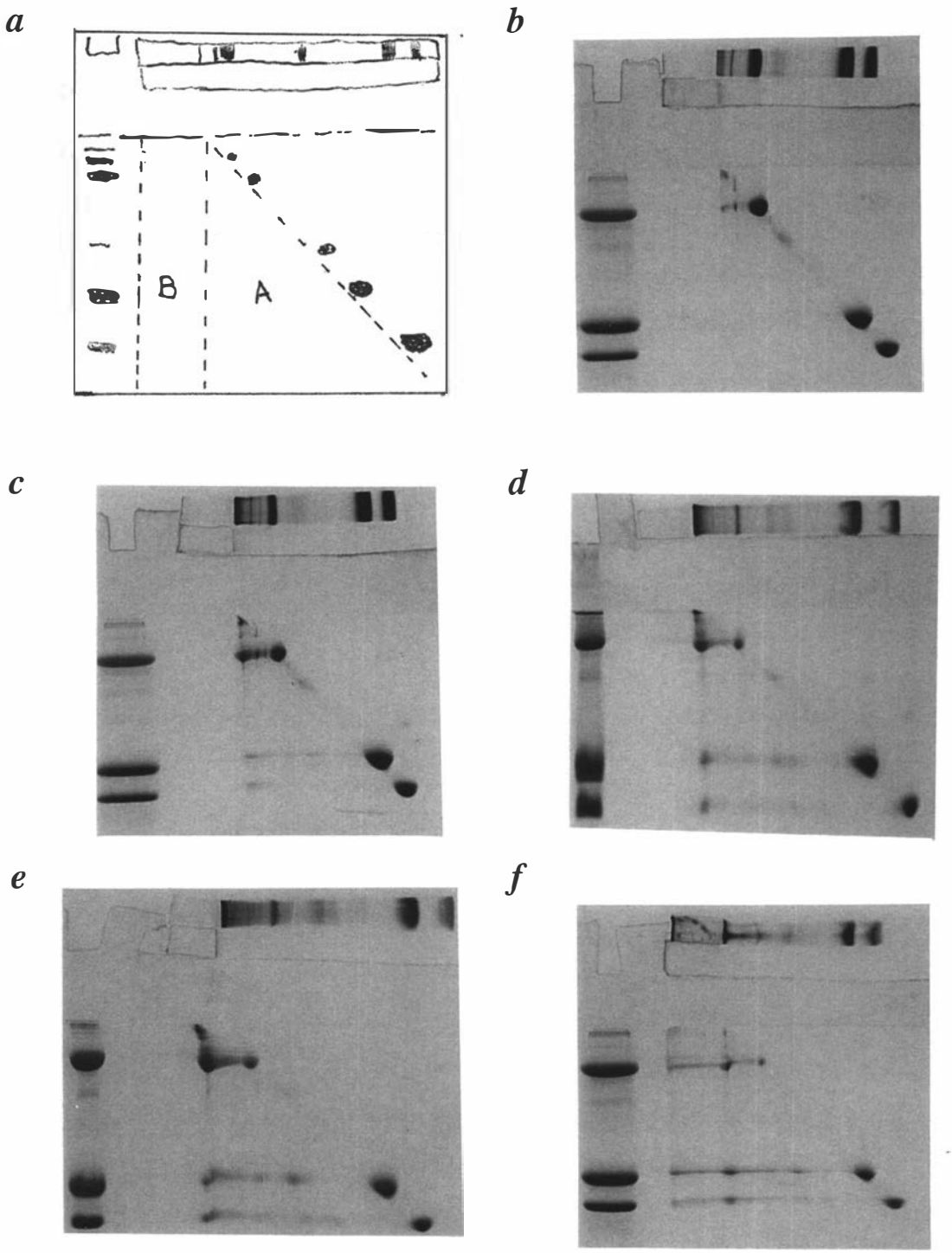


Figure 8.26. SDS 2D-PAGE patterns of a 100 g/kg, 2:1:1 mixture of β-Lactoglobulin, α-Lactalbumin and BSA heated at 75 °C for 0.5 (*b*), 2 (*c*), 4 (*d*), 6 (*e*) and 8 (*f*) min. (*a*) is a diagrammatic representation of the SDS 2D-PAGE patterns of the unheated mixture, indicating the two regions A and B where reduced aggregates would appear.

8.10. Discussion

In this study, electrophoresis was used to characterise the 'protein' products formed during heat treatment of different combinations of β-lactoglobulin, α-lactalbumin and BSA in model systems. In this study, the proteins were dissolved in WPC permeate, derived directly from the WPC solution, was used. This provided the true WPC mineral environment.

8.10.1. Individual protein solutions

The heat-induced denaturation and aggregation of β-lactoglobulin has been well documented (Griffin *et al.*, 1993; Huang *et al.*, 1994; Iametti *et al.*, 1995; Hoffmann *et al.*, 1996; and many others). The β-lactoglobulin molecule has two disulphide bonds and one free thiol group (McKenzie, 1971; McKenzie *et al.*, 1972). The three-dimensional structure of the molecule has been elucidated at neutral pH by X-ray crystallography (Papiz *et al.*, 1986; Monaco *et al.*, 1987; Brownlow *et al.*, 1997). It is basically a β-barrel consisting of eight antiparallel strands with a +1 topology (Belloque & Smith, 1998). The three-turn α-helix and a ninth β-strand lie outside the barrel. Recent studies have added a β_{10}-helix to the picture (Brownlow *et al.*, 1997). Its overall shape is a calyx, with a hydrophobic pocket that is able to bind small hydrophobic molecules, such as retinol (Papiz *et al.*, 1986), see Fig. 2.3. Prabakaran & Damodaran (1997) demonstrated using circular dichroism spectroscopy that, in the heat-induced denaturation and aggregation of β-lactoglobulin, the initiation reaction involves critical conformational changes to form reactive monomers. This conformational change was also reported in a more recent study using ^{1}H nuclear magnetic resonance and deuterium exchange (Belloque & Smith, 1998), and it was reported that the denaturation of β-lactoglobulin occurred in stages. At 55 °C, strand E and the A-B loop became unfolded (Papiz *et al.*, 1986). Strand A became partially flexible at 55 °C and lost the protective action of the α-helix at 75 °C, which became unfolded. At 75 °C, gelation occurred, with no observable opening of the β-barrel, although its internal face was partially exposed.

The reactive β-lactoglobulin species then react with each other via sulphydryl-disulphide exchange reactions to form, predominantly, dimers. Initially, only dimers are formed but, when the dimer concentration reaches a critical value, continued thiol-disulphide interchange reactions lead to the formation of higher molecular weight polymers (Prabakaran & Damodaran, 1997). Manderson *et al.* (1998), using SDS-PAGE, observed that, when solutions of β-lactoglobulin were heated at 80 °C, relatively high concentrations of disulphide-bonded dimers were present in the system. They suggested that these dimers could be important intermediates in the further aggregation of β-lactoglobulin.

The results of the current study (Fig. 8.1*b*) showed that, when relatively concentrated solutions of β-lactoglobulin (100 g/kg) were heated, during the initial stages of heating (up to 2 min at 75 °C) predominantly dimers were formed, and higher molecular weight aggregates appeared at later stages of heating. The formation of higher molecular weight aggregates (trimers, tetramers and other polymers) probably involves interactions between the exposed thiol group and disulphide bonds of two dimers or between those of a dimer and another reactive monomer.

Similar detailed information on the mechanisms of aggregation of BSA and α-lactalbumin is not available as yet. The BSA molecule has 17 disulphide bonds and one free thiol group (Reed *et al.*, 1975). The present study showed that, upon heating at 75 °C, BSA readily undergoes denaturation and aggregation (Figs. 8.3, 8.5, 8.6, 8.8, 8.9 & 8.11), supporting the findings of many other workers (Paulsson *et al.*, 1986; Matsudomi *et al.*, 1992; Gezimati *et al.*, 1996*a, b*). It is tempting to suggest that BSA behaves in a similar fashion to β-lactoglobulin, giving rise to various dimers, trimers and high molecular weight BSA polymers. However, such aggregates were not observed in the current study, partly because the resolving gel pore size in the SDS-PAGE system used was too small for good resolution of these large molecular aggregates and partly because the original protein mixture contained a number of contaminants, including dimeric BSA. When heated (75 °C) in admixture with β-lactoglobulin or α-lactalbumin (or both), BSA formed dimer complexes with either protein (Figs. 8.5, 8.6 & 8.8). These results need further confirmation using a technique

that provides better resolution than PAGE analysis. The formation of these complexes probably took place via sulphydryl disulphide interchange reactions between a reactive BSA and a reactive monomer or dimer of either β-lactoglobulin or α-lactalbumin. This implies that BSA probably undergoes some initial critical conformation changes to render reactivity, similar to β-lactoglobulin.

The α-lactalbumin molecule has four interchain disulphide bonds but has no sulphydryl group (Eigel *et al.*, 1984). Upon heating a 100 g/kg α-lactalbumin solution under mild conditions (75 °C), α-lactalbumin molecules did not form aggregates that continued to exist at low temperature after the solution was cooled (Fig. 8.2), supporting earlier findings (Matsudomi *et al.*, 1992; Hines & Foegeding, 1993; Calvo *et al.*, 1993; Gezimati *et al.*, 1996*a*, *b*). The results, however, showed that heating under these conditions altered the α-lactalbumin molecules which appeared as regions travelling ahead of or behind native-like α-lactalbumin (Figs. 8.6 & 8.8). This alteration probably exposes some of the disulphide bonds, as in the case of heated β-lactoglobulin, but because of the lack of a free thiol group the altered molecules do not proceed to form α-lactalbumin aggregates. It was reported earlier (Rüegg *et al.*, 1977) that α-lactalbumin underwent a thermal transition at ~ 64 °C, observable in differential scanning calorimetry, and, when the heated solution was cooled, the protein appeared to regain 80-90% of the ability to undergo the thermal transition. It is probable that, when α-lactalbumin is heated under these mild conditions (75 °C), the molecules adopt the "molten globule state" (Kuwajima, 1989; Hirose, 1993; Privalov, 1996).

Chaplin & Lyster (1986) showed that heating α-lactalbumin (pH 7.0) at 100 °C, resulted in two groups of bands on native-PAGE: one moving faster than native α-lactalbumin and one moving slower, in addition to some denatured protein, which remained at the origin, and some residual native α-lactalbumin. The faster group had unchanged molecular weight but had an increased charge, partly due to hydrolysis of glutamine and asparagine residues. The slower group was shown, using 2D-PAGE, to be oligomers of denatured α-lactalbumin linked by disulphide bonds (Chaplin & Lyster, 1986). It appears that interactions of α-lactalbumin involve a reversible step that precedes one or more irreversible steps. The first step is clearly a conformation change,

in which the compact native structure is lost and the protein is in a disordered and expanded state. This is responsible for the transition reported by Rüegg *et al.* (1977). Following this, there is probably an irreversible step where the molecule is further disrupted and some amino residues are hydrolysed, as reported by Chaplin & Lyster (1986) and shown in the present study as "altered monomeric forms" (Figs. 8.2, 8.6 & 8.8). With further heating, disulphide bonds are lost, leading to aggregation and gelation. The results of Schnack & Klostermeyer (1980) show that a significant loss of disulphide bonds occurs on heating α-lactalbumin at 100 °C for 10-30 min. It was suggested that 15-20% of the disulphides may be lost and that a reactive group similar to a thiol is simultaneously formed. This leads to further aggregation. It appears that, under the mild heating conditions used in the current study, such a reactive group was not formed.

8.10.2. Protein mixtures

The results of the current study clearly demonstrated that, when a mixture of β-lactoglobulin and α-lactalbumin was heated at 75 °C, disulphide-bonded aggregates between the two proteins as well as homogeneous aggregates of each were formed (Fig. 8.22). Other studies have shown that, when α-lactalbumin is added to β-lactoglobulin and the mixtures are heated, the two proteins interact through thiol-disulphide interchange reactions, to form heterogeneous aggregates (Matsudomi *et al.*, 1992, 1993; Hines & Foegeding, 1993; Gezimati *et al.*, 1997). The formation of homogeneous α-lactalbumin aggregates has not been reported before. These results of the current study demonstrated that the formation of homogeneous α-lactalbumin aggregates requires the presence of β-lactoglobulin at these temperatures. These observations suggest that β-lactoglobulin has a catalytic effect on the protein interactions involving α-lactalbumin. The presence of a free thiol group on a reactive β-lactoglobulin molecule probably initiates the reactions that lead to the formation of homogeneous α-lactalbumin polymers.

Analyses of heated mixtures of BSA and β-lactoglobulin showed that aggregates consisting of β-lactoglobulin or BSA (Fig. 8.23) were formed. The fine

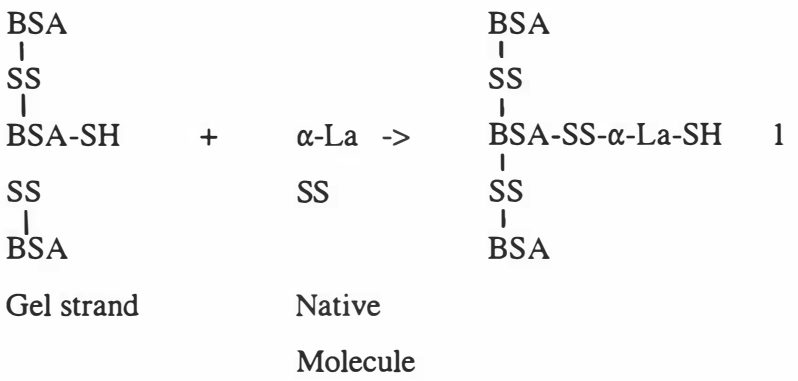
bands following the BSA band in 1D-PAGE, which appeared at the early stage of heat treatment (Fig. 8.5), were probably complex polymers incorporating both β-lactoglobulin and BSA (Fig. 8.23). Gezimati *et al.* (1996*a*) reported that BSA undergoes aggregation at a lower temperature than β-lactoglobulin, which is expected on the basis that the thermal transition temperature for BSA is lower than that for β-lactoglobulin, under a range of conditions at neutral pH (de Wit & Klarenbeek, 1984). What is interesting, however, is the indication that BSA interacts with β-lactoglobulin to form heterogeneous aggregates. The bands corresponding to these aggregates were very fine and lightly stained (Figs. 8.5 & 8.11), indicating that only small quantities of these aggregates were present in the heated protein systems. This would be expected because the majority of the BSA molecules would have been denatured and aggregated during the very early stage of heating (~ 1 min at 75 °C). After the β-lactoglobulin molecules undergo conformation changes (i.e. ~ 2 min), there are only a limited number of reactive monomeric BSA molecules left in the heated mixture that can react with reactive β-lactoglobulin to form β-lactoglobulin-BSA complexes. It is also likely that the homogeneous BSA aggregates form disulphide bonds with either β-lactoglobulin monomers or polymers at a later stage in the heating process.

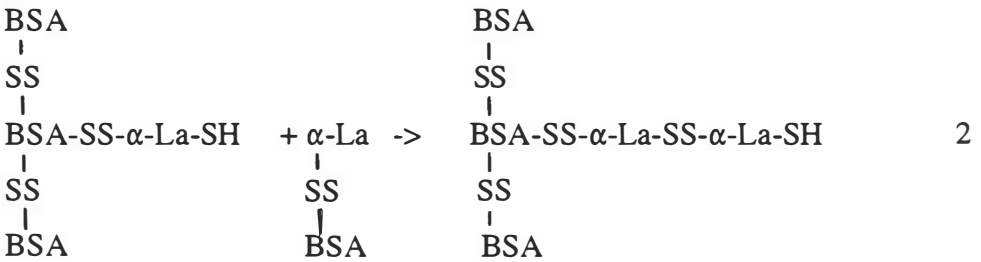
Analyses of a heated mixture of BSA and α-lactalbumin (Figs. 8.6-8.10) showed that intermediate aggregates were formed by α-lactalbumin molecules only (Fig. 8.8 & 8.24). These homogeneous α-lactalbumin aggregates were not formed when a solution of α-lactalbumin was heated alone (Figs 8.2 & 8.20). There was also evidence of an α-lactalbumin-BSA complex being formed in the heated mixtures (Figs 8.6, 8.8, 8.11 & 8.24). The formation of these heterogeneous α-lactalbumin-BSA aggregates probably followed the same mechanism as discussed for the formation of the β-lactoglobulin-α-lactalbumin complex. The free thiol group of a BSA molecule would interact with an exposed disulphide bonds of an α-lactalbumin molecule to form these complexes. Overall, it appears that the aggregation of α-lactalbumin requires the presence of BSA or β-lactoglobulin.

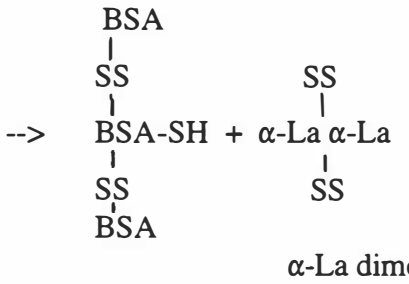
Heat treatment of mixtures of the three proteins (Figs 8.11, 8.25 & 8.26) showed all the aggregation products observed in the heated binary protein mixtures (Figs

8.4-8.10 & 8.22-8.24). In addition, Fig. 8.26 demonstrates that the initial aggregates formed in the heated protein mixtures consisted predominantly of BSA. At a later stage, aggregates involving both β-lactoglobulin and α-lactalbumin were formed.

The mechanism for the formation of α-lactalbumin polymers in these heated protein systems is of interest. It seems possible that the dimers of α-lactalbumin could arise from a reaction that involves adsorption, possibly involving hydrophobic interfaces, of an α-lactalbumin molecule to the thiol-containing strand of BSA, followed by thiol-catalysed disulphide exchange with a second α-lactalbumin molecule and finally thiol exchange back to the BSA strand. The following reactions may explain how the disulphide-linked α-lactalbumin polymers are formed. (Note: When α-lactalbumin is heated in mixture with β-lactoglobulin, there may not be strands of β-lactoglobulin, but reactive monomers. These monomers would have a free thiol group on each molecule which can initiate the following reactions in the same way as the BSA strands).







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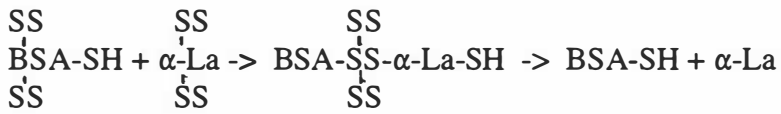
No doubt trimers, tetramers and so on follow from an extension of reaction 2. Similarly altered monomers could arise from a different second step in reaction 1; for example,



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where the newly formed α-lactalbumin has a different disulphide bonding pattern from that of the native protein. It has been shown that the bond between Cys6 and Cys120 is more readily reduced than the other three bonds and that the bond between Cys28 and Cys111 is also readily reduced. This suggests that bond 6-120 is more likely to be involved than the others in these disulphide interchange reactions (Monahan *et al.*, 1995).

The results presented in this study clearly show that when different combinations of β-lactoglobulin, α-lactalbumin and BSA are heated at 75 °C, three types of protein are present: native, disulphide-bonded and a third type that is monomeric in SDS but is not native. McSwiney *et al.* (1994*a, b*) and Gezimati *et al.* (1996*a*) speculated that this third type of protein is formed by aggregation of proteins via hydrophobic associations and that the protein molecules within these aggregates may have structures that are comparable in some fashion with the "molten globule" state (Kuwajima, 1989). These structures may not be folded into native-like conformations. Hirose (1993) speculated that the intermediates formed during the

formation of gels, foams and emulsions may be in the molten globule conformation, as did McSwiney *et al.* (1994*b*) for the hydrophobic aggregates formed in heated β-lactoglobulin solutions. Tani *et al.* (1995) observed that some characteristics of the heat-denatured aggregates of BSA, hen egg white lysozyme and ovalbumin were consistent with these proteins being in the molten globule state, using criteria of Kuwajima (1989) and Ptitsyn (1995*a*). Characteristics of this state are the loosened tertiary structure but largely retained secondary structure, giving greater access to regions of the protein molecules that are inaccessible in the native state. The present results for each protein can be considered in terms of the formation, at the heating temperatures, of hydrophobic aggregates that have some characteristics of the molten globule state. If two or more protein molecules can form an aggregate with hydrophobic region that spans the protein boundaries, then within this low-polarity environment certain reactions, such as the thiol-disulphide interchange might take place more readily. In such an aggregate, it would be expected that the peptide segments of one protein molecule would be able to be co-mmingle with segments of other protein molecules within the putative hydrophobic core of the aggregate. This would allow the occasional close juxtaposition of thiol and disulphide groupings with the possibility of the interchange reaction occurring. Similarly, the formation of the stable hydrophobic aggregates, detected by different electrophoretic behaviour, may also result from the close juxtaposition of appropriate peptide segments (Gezimati *et al.*, 1996*a*).

The kinetics parameters calculated for the loss of both native-like and SDS-monomeric proteins from the heated systems were consistent with a reaction order of $n = 1.5$. This reaction order is in agreement with the results for the thermal loss of β-lactoglobulin from various systems reported by Dannenberg & Kessler (1988), Oldfield *et al.* (1996) and Prabakaran & Damodaran (1997). The 1.5-order of the thermal aggregation implies that the reactions involving these proteins are more complicated than a simple first or second order process.

8.10.3. Involvement of protein interactions in gelation

The results clearly show that, when the mixture of the three proteins (β-lactoglobulin, α-lactalbumin and BSA) is heated at 75 °C, the composition of the aggregates and various complexes changes with heating time. The initial aggregates are formed predominantly by polymerisation of BSA with itself, whereas the aggregates involving β-lactoglobulin and α-lactalbumin are generated at a later stage. Based on the results of this study, the heat-induced gels formed in mixed whey protein solutions can be visualised as a heterogeneous network, consisting of gel strands made up of predominantly disulphide-linked co-polymers of β-lactoglobulin and α-lactoglobulin with BSA aggregates being embedded in the spaces between the gel strands, as depicted in Fig. 8.27. However, the possibility of BSA aggregates interacting with β-lactoglobulin and α-lactalbumin aggregates or strands cannot be ruled out completely.

In conclusion, when α-lactalbumin solution (100 g/kg, pH 6.8) was heated at 75 °C, no aggregates were formed even after heating for 10 min. When α-lactalbumin was heated in 1:2 mixture with β-lactoglobulin or 1:1 mixture with BSA (100 g/kg, pH 6.8), α-lactalbumin was involved in disulphide-linked aggregates with either protein as well as in disulphide-linked aggregates of its own. Under the mild heating conditions used, α-lactalbumin required a free thiol group from either β-lactoglobulin or BSA in order to form disulphide-linked aggregates. The three proteins (β-lactoglobulin, α-lactalbumin and BSA) involve in formation of both hydrophobically-associated and disulphide bonded aggregates when they were heated in different mixtures. When a mixture of the three proteins was heated, BSA formed the initial aggregates and β-lactoglobulin and α-lactalbumin were involved in the aggregation process at some later stage. It is proposed that a gel network formed when heating a mixture of these proteins consists of strands formed predominantly by β-lactoglobulin and α-lactalbumin with "clusters" or "strands" of BSA aggregates embedded in the network.

The losses of both native-like and SDS-monomeric proteins from the heated protein mixtures were consistent with a reaction order of $n = 1.5$. The thermal losses of each protein were different from different protein combinations, suggesting that the proteins may interact with each other via different reaction mechanisms. This area needs further investigation.

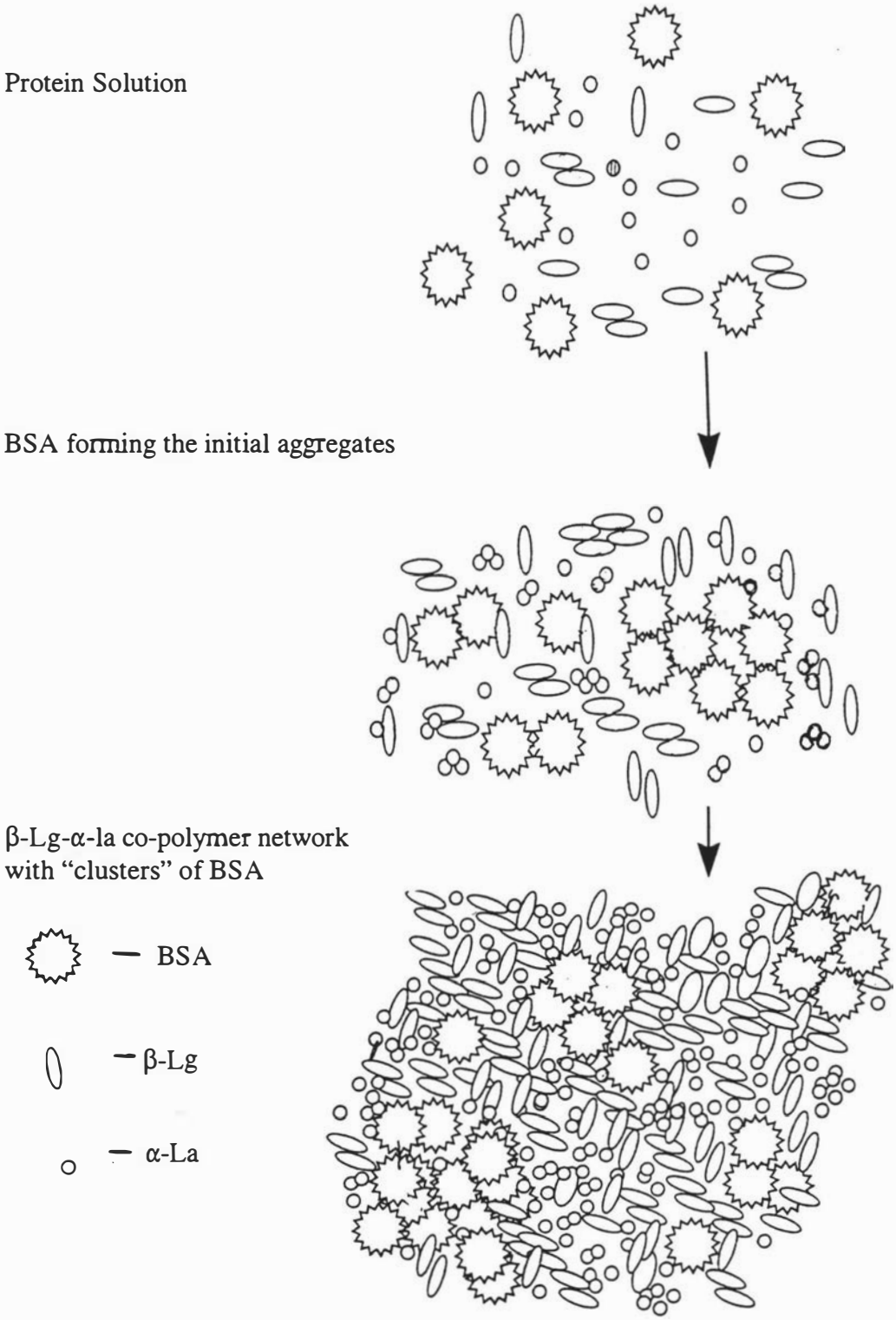

Fig. 8.27. Diagrammatic representation of the formation of β-lactoglubulin and α-lactalbumin gel network embedded with "clusters" or "strands" of BSA.

CHAPTER 9
OVERALL CONCLUSIONS AND RECOMMENDATIONS

9.1. Study approach

The purpose of this research was to gain greater insights into the interactions of whey proteins during heat-induced gelation of WPC solutions. Previous information in this area has been generated using two approaches. The first approach includes those studies that heat-treat WPC or WPI solutions, and determine the changes in some functional property (e.g. gel strength) as a function of some parameters, such as protein concentration, pH, ionic strength or salt concentration. The interactions of whey proteins are then used as a basis to explain the changes in the macroscopic properties of the heated WPC systems. This approach is very useful for product development work but does not provide insights into the mechanisms of the protein interactions at a molecular level.

The second approach includes those heat-treatment studies that use pure proteins (mainly β-lactoglobulin, α-lactalbumin and BSA) in well-defined model systems. These studies attempt to understand the interactions of each component at a molecular level and often the experiments are done at very low protein concentrations. The choice of the experimental conditions are influenced more by the optimum operating conditions of the equipment used than the requirement to understand the WPC gelation under industrial conditions. For example, to study the rates of denaturation, β-lactoglobulin concentration of 0.1% may be used. The results of these studies, however, may be applicable only to low protein concentration systems. The use of WPC for gelation purpose in the food industry is usually at higher protein concentrations (> 80 g/kg) and the interactions of whey proteins at such high protein concentrations may be different. In a WPC system, there are other proteins (e.g. immunoglobulin, lactoferrin, proteose peptone) which also denature on heating and affect the macroscopic properties of the gels that form. The contributions of these proteins in gel formation are eliminated when a pure protein model system is used. Furthermore, the buffer systems do not usually accurately represent of the mineral

environment of a WPC system.

Recent work in our laboratory involved studies which used mixtures of pure proteins at relatively high concentrations (10% w/w). In addition, proteins were dissolved in a buffer system with pH and mineral environment that was similar to that of a "true" WPC solution. The interactions of whey proteins in heated WPC solutions were then elucidated based on the observations made on pure protein model systems.

In the present study, a combination of both approaches was used, and three different WPCs and model systems of proteins were examined. This involved the examination of the macroscopic properties of the heated WPC solutions (gel development and gel strength) and, more importantly, the heat-induced interactions and aggregation involving individual whey protein were studied. These were carried out by heating the WPC solutions under relatively mild conditions, and interactions and aggregation of whey proteins were determined as the heat treatment progressed. This combination of approaches made it possible to relate the heat-induced changes in whey proteins to the gel forming properties of the WPC and to gain a greater understanding of the factors, including the mechanisms of whey protein interactions, that affect WPC gelation.

9.2. Experimental methodology

A series of experiments, discussed in Chapter 4, were carried out using 1D-PAGE to evaluate the loss of proteins from WPC solution during heat treatment. These results provided information on the rates of loss of individual whey proteins, quantitative estimation of the proportions of proteins that were linked by hydrophobic or disulphide bonds and an indication of some of the intermediate protein aggregates formed during heating. In order to understand the nature of these intermediate aggregates, 2D-PAGE techniques were developed. The development of this technique allowed us to identify the composition of various intermediate protein aggregate species formed during heating of WPC solutions and to differentiate between the aggregates that were disulphide-linked and the non-covalently-linked aggregates. This technique proved to be very useful, as the composition and nature of the intermediate protein aggregate species were clearly identified. Other techniques (size exclusion chromatography,

rheometry, ultracentrifugation, TEM, CSLM, and light scattering) were also used to provide more evidence. Overall, the 2D-PAGE, in combination with the other techniques used in this investigation, generated some new information that increased our understanding of the interactions of whey proteins prior to gel formation in heated WPC solutions.

9.3. Factors affecting WPC gelation

9.3.1. WPC components

The results confirm some of the findings of previous studies in this field and also reveal some new information. The three WPCs used in this study had different protein and mineral compositions. The most significant differences were: 1) the high GMP contents of cheese and rennet WPCs, 2) the higher casein contents of the acid WPC and 3) the different mineral profiles of cheese and acid WPCs. These differences were largely due to the different manufacturing processes used in the production of the WPCs.

GMP

GMP is formed during the early phase of the enzymatic coagulation of milk (as a result of the hydrolysis of κ-casein) for making rennet casein or cheese. The GMP is soluble in milk serum and is concentrated by the membrane processes used to manufacture WPC. It was not possible to follow the heat-induced changes in GMP using the techniques of this study. However, it was clear that gel strength of the cheese WPC solutions (which contained GMP), could be increased significantly by dialysis against an acid WPC solution (i.e. by altering the soluble minerals) (Chapter 6). Therefore, it appears that GMP does not present a significant problem when gel strength is the major functional consideration for WPC. However, the effect of GMP on gelation should be considered in a future study in order to clearly define its role in WPC gelation. From commercial point of view, GMP is a major consideration as it represents up to ~ 20% of the yield. If gel strength can be improved, as shown by the results of this study, then the presence of GMP in WPC represents a significant market return for the cheese WPC manufacturer.

Minerals

The differences in the mineral contents of acid and cheese WPCs were probably largely due to the difference in the pH of the raw whey used for WPC manufacture. The process flow chart (Figure 2.2) shows that the manufacture of cheese WPC starts with the pH of whey the range 6.2 to 6.6. As discussed in Chapter 6, the whey proteins are negatively charged at this pH, hence the cations (e.g. Ca and Mg) will be bound to the protein molecules. During UF, these bound cations are not removed and consequently end up in the WPC product. This explains the high mineral contents of the cheese WPC (Chapter 5). The acid WPC manufacture, on the other hand, starts with whey at ~ pH 4.3. At this pH, the proteins are positively charged and the cations are not strongly bound to proteins. Therefore, they are removed by the UF process, hence the lower mineral content of the acid WPC.

The results of the dialysis experiments (Chapter 6) showed that the differences between the gelation behaviour of acid and cheese WPCs could be explained largely by the differences in their mineral profiles. The salt type and concentration play an important role in determining the gelation behaviour of the WPC solutions. The presence of relatively high concentration of divalent cations significantly increased the proportions of hydrophobic aggregates in the heated WPC solutions, leading to formation of particulated gels. These results are significant because this information could be used to produce high gelling cheese WPC, possibly by reducing the pH of the starting raw whey before the UF stage.

It is evident from the literature (Chapter 2) that there is an optimum ionic strength or mineral concentration for achieving high gel strength. This optimum concentration has not been defined for either acid or cheese WPC, although the results suggested that the mineral content of acid WPC is probably somewhere near optimum, while that of the cheese WPC is higher than the optimum. Clearly, this area requires further study.

Insoluble aggregates

Another aspect that was considered in this research is the "insoluble" aggregates present in the WPCs and their effects on WPC functionality (Chapter 7). The results indicated

that there were some interesting differences between the compositions of the insoluble aggregates in acid and cheese WPCs. The insoluble aggregates present in cheese WPC contained aggregated β-lactoglobulin and α-lactalbumin in addition to the aggregates of BSA and other proteins. By contrast, the insoluble aggregates in acid WPC consisted largely of aggregated BSA, minor proteins and caseins. Preliminary work on the effects of insoluble aggregates on the gelling properties of the WPC solutions indicated that these aggregates had positive effects on the gelling properties of both acid WPCs, but had no effect on cheese WPC gelation.

9.3.2. Interactions of whey proteins during heat treatment

The concentration of WPC prior to heating had major effect on the behaviour of different proteins during heating (Chapter 4). At low WPC concentrations, higher proportions of low molecular weight intermediate aggregate species (dimers, trimers etc.) were formed, whereas at high WPC concentrations, large aggregates are formed. The rates of loss of monomeric proteins were shown to be different for different WPCs and were related to the aggregate sizes and the types of linkages (disulphide vs hydrophobic linkages) that form the aggregates (Chapter 4, 5, 6). Heating acid WPC solutions resulted in lower rates of loss of monomeric proteins and formation of smaller aggregates, linked largely by disulphide bonds. Heating cheese WPC solutions, on the other hand, resulted in the faster loss of monomeric proteins and the formation of large aggregates that included a high proportion of non-covalently linked material. It was also interesting to note that the properties of the WPC gels were related to the types of protein-linkages involved in aggregate formation. When the aggregates were formed predominantly by intermolecular disulphide-linkages, the gel had high strength. By contrast, when the aggregates consisted of considerable proportions of non-covalently-linked (hydrophobic) material, the gel formed had low gel strength and poor functional properties.

The possibility that there were preferential interactions among some of the whey proteins during heating (Chapter 8) was shown using model protein systems. For example, the loss of monomeric BSA was faster when heated in admixtures with β-lactoglobulin than when it was heated with α-lactalbumin. It has been shown by

some workers (e.g. Paulsson *et al.*, 1986; Matsudomi *et al.*, 1993) that the heat-induced gel formed by BSA becomes stronger when heated in mixture α-lactalbumin. It would be very interesting to define the mechanisms that lead to this preferential interaction and how this phenomenon relates to the macroscopic-properties of the WPC gels. The importance of this area lies in the possibility that the ratios of proteins (e.g. α-lactalbumin:β-lactoglobulin or BSA) in WPC can be manipulated to optimize the desired functionality. This can be done either by genetic engineering, some fractionation techniques or prior heat treatments of either the milk or the whey.

9.3.3. Intermediate protein aggregate species

PAGE analyses of the heated WPC solutions (Chapters 4 - 7) revealed that there were various types of intermediate aggregate species formed during heating. The use of model systems of pure protein (Chapter 8) was very useful as it confirmed and clarified some of the observations made for the heated WPC solutions. The results generally showed that when a WPC solution is heated, the denatured proteins interact to form homogenous aggregate species of each protein as well as heterogeneous aggregates of all possible combinations of the proteins present. The presence of disulphide-linked α-lactalbumin polymers in heated protein mixtures or WPC solutions had not been previously reported and this protein has been considered to reversibly denatured. It was suggested that the formation of these aggregates were catalyzed by a free thiol group of another protein molecule possibly BSA or β-lactoglobulin.

The 2D-PAGE analyses of the heated pure protein mixtures (Chapter 8) clearly showed that when a mixture of β-lactoglobulin, α-lactalbumin and BSA is heated, BSA forms the initial aggregates, and then β-lactoglobulin and α-lactalbumin from co-aggregates at some later stage. Therefore, in these heated solutions, BSA (which is most heat-labile) denatures and forms aggregates, these aggregates are largely homogeneous in nature, i.e. BSA dimers, trimers etc. At a later stage, β-lactoglobulin and α-lactalbumin starts to denature and form co-aggregates. Depending on the heating conditions, heterogeneous aggregates between BSA and the other proteins can also be formed. The concentration of BSA and other heat labile minor whey proteins in WPC is low and they could not form a gel network. So the structure of WPC gels can be

viewed as being a heterogeneous network formed largely by co-polymers of β-lactoglobulin and α-lactalbumin with embedded 'clusters' or "strands" of aggregates of heat labile proteins (Chapter 8).

Because this study only examined the heat-induced changes involving β-lactoglobulin, α-lactalbumin and BSA in detail, the involvement of other proteins, such as immunoglobulin, proteose peptone, GMP etc. should be investigated in the future to determine the role these proteins play in WPC gel formation.

9.4. Further studies

Based on the results presented in this thesis, the following subjects are recommended for further study. These investigations are divided into two categories: the first consists of recommendations for industrial actions while the second consists of recommendations for fundamental studies.

9.4.1. Further investigations by the dairy industry

The following studies are recommended for the dairy industry. Because of the strong emphasis on resources for research and development in industry, these studies can easily be done using the pilot plant facilities that are available at the manufacturing sites.

Functional cheese WPC

Attempts should be made to manufacture a gelling WPC from cheese whey, based on the results of this study. The best start would be to run a pilot plant trial where cheese whey pH is lowered to ~ 4, before UF. The powder produced should be used for functional tests in comparison with the functional properties of the standard cheese WPC, paying particular attention to the gel structure.

Insoluble aggregates

The insoluble aggregates in the WPC, particularly in cheese WPC, should be investigated further. It is recommended that further characterization of the nature and composition of the insoluble aggregates should be carried out, and

the effect of these aggregates on the gelling properties should be further investigated. Investigations should also be conducted to identify the unit operations in the manufacturing processes that form these aggregates, and recommendations could be made on the necessary changes to the processing operations that could optimise the quantities and functionalities of these aggregates in the final product.

Effect of GMP on WPC functionality

Removal of GMP from WPC would be a costly operation although GMP is a valuable products in its own right. The results of this thesis indicate that it does not affect gel strength. This area should be further investigated because the GMP is present at such a high concentration (up to 20 %) in cheese WPC. A good start would be to include GMP in acid WPC and then determine how it affects the acid WPC gel strength.

9.4.2. Fundamental studies

The following subjects are recommended for further fundamental protein chemistry studies. These studies may best be carried out in the academic institutions in conjunction with the dairy industry.

Quantitation of the intermediate aggregate species

Although various species of aggregates were identified and characterised in this thesis, the quantity of each aggregate species formed in heated protein systems was not determined. It is, therefore, recommended that the quantities and proportions of each aggregate species be determined. The need to quantify these aggregate species rests on the possibility that specific aggregate species may favour the formation of a particular type of gels. It was clear from the results in this thesis that polymeric forms of α-lactalbumin were present at relatively low quantities in heated WPC solutions. It would be interesting to quantify each species and then determine if the variations in the quantities of these species are related to the macroscopic properties of the WPC gels. This would help in

developing quantitative models for protein aggregation and gelation.

Quantitation of the proportions of non-covalently linked aggregates

The results of this thesis suggest that the types of linkages involved in forming the protein aggregates play an important role in the functional properties of the WPC gels. It was shown that the higher the proportions of hydrophobically associated aggregates, the 'poorer' the gel strength and structure. This phenomenon should be taken up in a future study, with the specific objective of quantifying the proportions of disulphide-bonded and hydrophobically-linked aggregates, and determining the relationship between these aggregates and gel properties.

Preferential aggregation among the whey proteins

The results indicated that there were preferential interactions among some of the whey proteins. Investigations should be carried out to define the mechanisms that may lead to individual whey proteins interacting preferentially with other proteins.

Role of other proteins

This thesis followed the changes involving β-lactalbumin, α-lactalbumin and BSA. The involvement of other proteins such as immunoglobulin, casein fines, GMP etc. were not considered. This was due largely to the limitation of the techniques used and partly because of the complexity of the topic. It is recommended that the findings of this thesis be used as a foundation for further investigations to consider the interactions of other whey proteins in the formation of WPC gels. Because immunoglobulins represent a considerable proportions (~ 10%) of the total whey proteins, they probably play an important role in gel formation. It was suggested in this thesis (Chapters 5 and 6) that casein fines may also play a role in WPC gel formation by acting as nuclei for aggregation. The effect of casein fines on whey protein gel formation should be further investigated. The behaviour of GMP during heating and its possible

involvement in the aggregation and gelation of cheese WPC should be further investigated.

Quantitation and characterization of bound minerals

The effect of minerals on the aggregation and gelation of whey proteins is a complex area of research. This is because of the many different types of minerals present in the WPC, and because of the unknown amounts that are bound to proteins. In order to clearly understand the mechanisms of mineral action on protein aggregation and gelation in WPC, it would be desirable to determine the state of minerals (i.e. the degree to which minerals are bound) in the WPC, and how this state changes during heat treatments.

Effect of anions

The effect of anions on protein interactions should also be investigated. This thesis and many other studies on the effect of minerals on protein interactions have only considered the cations, but there is very little information on the effect of anions on protein interactions.

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