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Influence of Protein Concentration on Heat-Induced Aggregation and Subsequent Functionality of Whey Proteins

A thesis submitted to the National University of Ireland in fulfilment of the requirements for the degree of

Doctor of Philosophy

by

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This thesis is dedicated to my parents Anne and P.J. Buggy. Thank you for your never ending support.

Declaration

I hereby certify that this thesis has not been submitted before, in whole or in part, to thesis or any university for any degree and is, except where otherwise stated, the original work of the author.

Signed:_____

Date: _____

Maynooth University

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Abstract

The usage of whey proteins as a source of nutrition in foods and beverages is ever increasing, creating a demand for highly concentrated and functional forms of protein. Their use as an ingredient to increase protein content in foods is growing, and the ability to withstand thermal treatment while concentrated is paramount to reducing process instability, e.g., high viscosity, precipitation and/or fouling of heat exchangers. The objective of this thesis was to investigate the effect of protein concentration on the heat-induced aggregation of whey proteins and subsequent increases in viscosity in dispersed and emulsified systems. Increasing the protein content of whey solutions from 1 to 12% (w/w) was accompanied by a reduction in denaturation temperature and increase in both viscosity and the shear rate at which onset of turbulent flow was observed. On heating to 85 °C for 30 s, the particle size (Dynamic light scattering, DLS) of aggregates was significantly ($P < 0.05$) affected by both protein concentration (1 to 12 % w/w) and pH (6.2, 6.7 and 7.2). Aggregates of ~ 50 nm were formed at higher pH (7.2) and protein concentration (12%), contributing to greater heat stability upon subsequent heating compared to aggregates of ~ 100 nm formed from the heat-treatment of 1% whey at pH 6.2. In an emulsified system, simulating that of a 1st stage infant formula, increasing the ratio of α-lactalbumin to β-lactoglobulin significantly ($P < 0.05$) reduced the level of large soluble aggregates formed on heating, improved emulsion stability and reduced apparent viscosity during concentration. Differences in these physicochemical properties are attributed to underlying molecular changes in the mechanism(s) of aggregation due to a reduction in the total number free thiol groups in the system as β-lactoglobulin is replaced by α-lactalbumin.

To better understand the complex interactions involving β-lactoglobulin, $^{1}H–^{13}C$ HSQC solution NMR (Nuclear Magnetic Resonance [13C,1H]–Heteronuclear Single Quantum Correlated spectroscopy) and Fourier Transform Infrared (FTIR) spectroscopy were used to observe molecular rearrangement at low (1% (w/w)) and high concentration (12% (w/w)) at either 62 or 85 °C. FTIR showed that there was formation of antiparallel inter-bonded β-sheets in both 1 and 12% samples heat-treated at 85 °C for 10 minutes, indicating protein denaturation and subsequent aggregation. Partial reversible denaturation was observed by NMR after heat-treatment at 62 °C; however, at 85 °C, denaturation and the subsequent aggregation of β-lactoglobulin monomers was extensive. Results suggest that although β-lactoglobulin monomers and dimers are denatured, the aggregates formed are comprised of unfolded β-lactoglobulin monomers that behaved "native-like". This thesis demonstrates that by controlling the extent and size of aggregates through reduction in β-lactoglobulin and soluble 'seed' aggregates, it is possible to modulate viscosity in concentrated whey protein dispersions and emulsified systems.

Abbreviations used

β-lg	β-lactoglobulin
α-lac	α-lactalbumin
(w/w)	weight/weight
WPC	Whey protein concentrates
TCA	Trichloroacetic acid
BSA	Bovine serum albumin
NMR	Nuclear magnetic resonance
HSQC	Heteronuclear single quantum correlated spectroscopy
FTIR	Fourier transform infrared
FE-SEM	Field emission – scanning electron microscopy
TEM	Transmission electron microscopy
CCP	Colloidal calcium phosphate
WPI	Whey protein isolate
EDTA	Ethylenediaminetetraacetic acid
SH	Sulfhydryl
DSC	Differential scanning calorimetry
M	Molar
mg	Milligram
g	Gram
mm	Millimetre
DLS	Dynamic light scattering
NTU	Nephelometric turbidity units
AFM	Atomic force microscopy
PEG	Poly-ethylene glycol
HCT	Heat coagulation time
RP-HPLC	Reserved Phase – High Pressure Liquid Chromatography
PdI	Polydispersity index
IMF	Infant milk formula
L	Litre
h	hour
min	Minutes
s	Seconds

Hz	Hertz
SDS-PAGE	Sodium dodecyl sulphate-polyacrylamide gel electrophoresis
Pa.s	Pascal seconds
mPa.s	Millipascal seconds
IMF	Infant milk formula
°C	Degrees Celsius
TS	Total solids
NaOH	Sodium hydroxide
HCL	Hydrochloric acid
mM	Millimolar
CLSM	Confocal laser scanning microscopy
µl	Microliter
ml	Millilitre
V	Volts
Da	Daltons
kDa	Kilodaltons
µm	Micrometre
nm	Nanometres
M_w	Molecular weight
$CaCl_2$	Calcium chloride
NaCl	Sodium chloride
SEC	Size exclusion chromatography
HPLC	High Pressure Liquid Chromatography
RF	Refractive index
GLM	General linear model
Au	Absorbance
kCal	Kilocalories
kJ	Kilojoules

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Research Objectives

The use of whey proteins as ingredients in foods and beverages can lead to challenges during manufacture and ultimately affect finished product quality. For instance, thermal treatment is necessary to reduce microbial load, although in systems where high protein concentrations are used, instability can be exacerbated during processing, manifesting in defects such as excessive protein aggregation, precipitation, sedimentation or gelation. Secondary defects include high viscosity in-process causing 'burn-on' on heated surfaces and/or burnt particle formation during spray drying of powder (Jun & Puri, 2005).

The objective of this thesis was to understand the impact of protein concentration on the heat-induced aggregation and subsequent functionality of whey proteins, ultimately to produce more heat stable concentrated whey protein systems. More specifically, the aim was to elucidate the effect that protein concentration has on the *in situ* generation of aggregates by heat-treatment, and the effect that the morphology of these different aggregates may have on their heat stability when subsequently used in a solution or an emulsion. Protein-protein and protein-fat interactions differ depending on the amount of individual whey protein (e.g., α-lactalbumin or β-lactoglobulin) incorporated. By increasing the quantity of α-lactalbumin (at a fixed total protein content, which subsequently decreases the quantity of β-lactoglobulin) in a model infant milk formula system, the role of individual whey proteins on the stability of formula heat-treated pre- or post-homogenisation was examined. Changes in aggregate structure were investigated at the molecular level, by studying the effect of heat on the formation of β-lactoglobulin aggregates (purified system) at low and high protein concentration. Investigation of protein conformational changes and their effect on aggregate structure, underpins the mechanisms by which

subsequent physicochemical properties ensue. The research sought to understand how heat-treatment influenced the heat stability of whey solutions at different protein concentrations, to gain insights into what the optimal aggregate morphology should be to obtain a whey protein system with greater thermal stability. The study endeavours to deepen the understanding of how heat-treatment at different whey protein concentrations affects the subsequent functionality of aggregates formed, and how this impacts subsequent thermal processing.

CHAPTER 1

Introduction

1.1 Introduction

Whey proteins, and other components of bovine milk, are important ingredients in the sports, medical and infant nutrition sectors, including in infant formula and growing-up milks. Although whey proteins are highly nutritional, their use in products can be challenging, as they are typically heat liable at the high temperatures (> 65 °C) required during processing for the reduction in microbial load of most foods and beverages. Their use in infant formula also provides an added challenge as the composition of bovine milk is different to human milk; manipulation of bovine milk constituents can address this problem and produce milk that is better suited to an infant.

1.2 Composition and Constituents of Bovine Milk

Milk produced by mammals is typically comprised of a blend of protein, fat, carbohydrate, minerals and vitamins, with the ratio and type of each constituent differing, depending on the mammal. Fresh bovine milk has a protein content of approximately 3.3% (w/w); composed of 80% casein protein and 20% whey protein (dependent on lactation period) including immunoglobulins and minor proteins. Both of these protein fractions are highly sought after commodities in the food manufacturing industry. Much research has been carried out investigating the behavioural and functional properties of the whey and casein protein fractions of milk. However, particular attention has been paid to whey proteins, as the isolation of whey proteins from milk has provided the opportunity for use of these components as ingredients in a varying range of products including infant milk formula.

1.2.1 Whey Proteins

Liquid whey can be produced from the manufacture and processing of cheese and can be removed from the casein fraction of milk by isoelectric precipitation at pH 4.6 at 20 °C (Mullvihill & Donavan, 1987). What remains is a soluble fraction of whey protein which was once considered a waste product and fed to animals as feed or pumped as effluent into river systems, but is now regarded as a valuable supply of high quality protein.

The composition of the whey protein fraction of milk is subdivided into α-lactalbumin (α-lac), β-lactoglobulin (β-lg), lactoferrin, bovine serum albumin (BSA), immunoglobulins, some non-protein nitrogen and a proteose-peptone fraction (de Wit & Klarenbeek, 1984b; Morr, 1985; Mullvihill & Donavan, 1987). Whey proteins are globular in form and are heat sensitive, unlike caseins.

1.2.1.1 β-lactoglobulin

As the most abundant whey protein in bovine milk, β-lg comprises 10 - 12% (w/w) of the total protein content and approximately 50% (w/w) of the whey fraction (Sawyer, 2013). It has a molecular weight of ~ 18.3 kDa and is comprised of 162 amino acid residues per monomer (Figure 1.1) (Fox & Mc Sweeney, 1998). The protein can exist in more than twenty different genetic variations with β-lg A and β-lg B being the most abundant. The defining difference between these two native forms relates to their respective amino acid compositions at positions 64 and 118. Asp and Val are at position 64 and 118 respectively for β-lg A with Gly and Ala being substituted in these positions for β-lg B (Croguennec et al., 2004). Each monomer has an approximate diameter of 2 nm, consists of eight anti-parallel β-sheet strands with (+1)8 topology (i.e. each successive β-strand is adjacent to the preceding one) (Brownlow et al., 1997) and

contains two disulfide bonds at amino acid position 66-160 and 106-119 respectively with one free sulfhydryl group, buried inside the native protein (Figure 1.1) (Mc Kenzie et al., 1972; Sawyer, 2003).

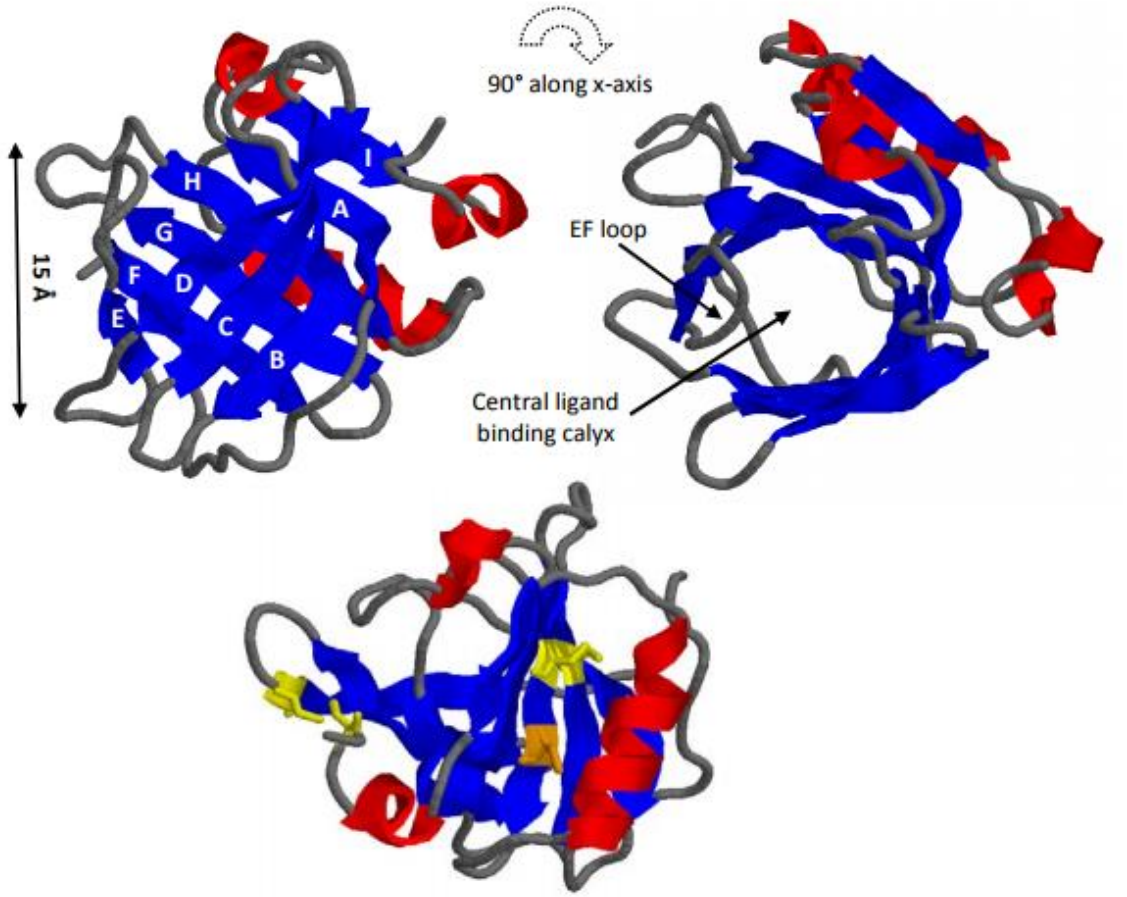


Figure 1.1: Image of a β-lactoglobulin monomer; α–helices are represented in red, β–sheets are represented in blue. Cysteine residues involved in disulfide bond formation are represented in yellow, with the reactive thiol group (Cys 121) represented in orange (from O'Loughlin et al., 2014).

It is this sulfhydryl group that facilitates covalent bond formation with other proteins. When the protein is exposed to denaturing conditions (either physical or chemical), this free thiol becomes exposed, allowing it to form disulfide bonds with free thiols on adjacent proteins (Sawyer, 2013; Swaisgood, 1982). This can then lead to aggregation

of partially and fully denatured protein (see section 1.3.1 for a more comprehensive description). The globular structure of this protein contains 10 - 15% α-helix, 43% β-sheet and 47% random unordered coil structure (Boye et al., 1996). Above the isoelectric point of 5.2 (between pH 5.5 and 7.5), the protein exists as a dimer, formed via non-covalent interactions, while below pH 3.5 and above pH 7.5, the dimer undergoes dissociation and β-lg exists as a monomer. It has been noted that a number of different ligands can be bound to β-lactoglobulin such as free fatty acids, aromatic compounds and vitamins A, D, E, K (Perez & Calvo, 1995).

1.2.1.2 α-lactalbumin

4% (w/w) of the total protein found in bovine milk is comprised of α-lac, which is 15% (w/w) of the total whey fraction (Brew, 2013). α-lac has a molecular weight of ~ 14 kDa and consists of 123 amino acid residues with four tryptophan residues and a high ratio of other essential amino acids (Figure 1.2) (Permyakov & Berliner, 2005; Lisak Jakopovic, et al., 2016). The protein has four disulfide bonds per monomer and has a Ca^{2+} binding elbow. Other monovalent and divalent minerals such as Mg^{2+}, Mn^{2+}, K^{+} and Zn^{2+} can also be bound (Barbana & Perez, 2011; Kronman et al., 1981). This elbow's functional properties relates to its structural role in the folding of the protein and helping to stabilise α-lac. It is thought that the binding of Ca^{2+} or other monovalent and divalent ions results in the stabilisation of the molecule against irreversible denaturation (Prestrelski et al., 1991). Upon removal of Ca^{2+} from the binding elbow, unfolding within the local region of the molecular structure occurs, and a change in the backbone results in the transition of the binding elbow from a rigid complex to a disordered loop (Hosseini-Nia et al., 2002; Prestrelski et al., 1991).

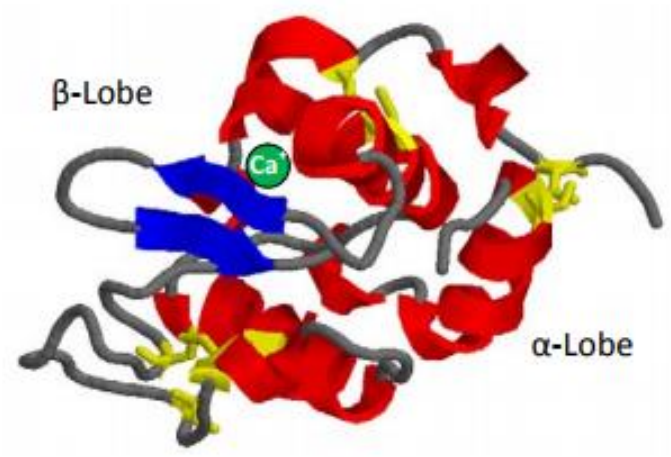


Figure 1.2: Image of an α-lactalbumin monomer; α–helices are represented in red, β–sheets is represented in blue, with the regions in yellow representing the eight cysteine residues (from O'Loughlin et al., 2014).

Chrysina et al. (2000) used x-ray crystallography to study the structure of both forms, with the main differences being within the cleft region of the molecule, which is situated on the opposite side to the Ca^{2+} binding site. It has been suggested that there is an effect from charge repulsion due to the negatively charged aspartic acid residues in the calcium-binding site, and it is this effect which generates a slight structural change between the two forms. The ability of the protein to bind Ca^{2+} is displayed by holo α-lac while the removal of Ca^{2+} from the native structure (apo α-lac) reverts the protein to its molten globule state and this allows for conformational flexibility (Chrysina et al., 2000; Wijesinha-Bettoni et al., 2001).

The synthesis of lactose requires α-lac as it acts as a regulatory protein for the specific substrate glycosyltransferase (Barbana & Perez, 2011). It is noted by Brew (2013) that Ca^{2+} is not essential for the activity of lactose production but it is recognised as a requirement for refolding of denatured protein (Music, 1985).

1.2.1.3 Bovine serum albumin (BSA)

1 - 4% (w/w) of total whey protein in fresh bovine milk is BSA. It is an elliptical shaped protein with a molecular weight of ~ 66 kDa and contains 583 amino acid residues, including 17 intramolecular disulfide bonds and one free sulfhydryl group (Wynn, 2013). It is structured in a series of small loops with three different domains. The concentration of BSA in milk is low (0.1 - 0.4 g/L^{-1}) but it is a major protein in blood (3 - 4 g/L^{-1}). It has no specific functionality in milk and is believed to be a leakage protein from blood which binds metals and fatty acids (Fanali et al., 2012). Levels of BSA within milk vary throughout different stages in lactation, with milk in early lactation periods containing greater amounts (Guidry et al., 1980; Sheldrake et al., 1983).

1.2.1.4 Lactoferrin

Lactoferrin, a red coloured iron binding glycoprotein, is a minor whey protein with multiple biological and physiological functions. This single chain glycosylated protein is composed of 700 amino acids with two globular domains (N and C lobes, connected together by a short α-helix) and 17 intramolecular disulfide bonds; it lacks a free sulfhydryl group (Lönnerdal, 1985; Pierce et al., 1991). The concentration of lactoferrin in human milk varies significantly between species; breast milk is rich in lactoferrin with concentrations ranging from 6 g/L in early milk to 2 g/L in mature milk (Sànchez et al., 1988; Rai et al., 2014), while the quantities present in bovine milk are much less at 0.8 g/L in early milk compared to 0.1 g/L in later milk (Sànchez et al., 1988).

Although this protein is a minor whey protein in bovine milk, it is beneficial to human health as it acts as a nonspecific bacterial inhibitor through its ion (most commonly iron) binding properties (Wakabayashi et al., 2006). The mechanism of this ion binding system has been extensively studied (Aly et al., 2013; Donovan, 2016; Mayeur et al.,

2016). The bacterial inhibiting activity of the protein is due to its tendency to readily and reversibly bind Fe^{3+}, which is needed by several bacteria for bacterial cell growth (Lönnerdal, 2017). In its bound form, lactoferrin is referred to as holo-lactoferrin; in its free form apo-lactoferrin. Holo-lactoferrin has a more closed structure than apo-lactoferrin and therefore it is more resistant to proteolysis (Conesa et al., 2010). Lactoferrin has also been linked with anti-inflammatory and anti-cancer properties. Due to the relatively high isoelectric point of lactoferrin (8.7), its association with other proteins is mostly due to charge interactions (further discussed in section 1.3.2).

1.2.1.5 Immunoglobulins

The main physiological role of immunoglobulin is to provide immune protection for new born infants. Immunoglobulins account for 10 - 15% (w/w) of whey protein in both human and bovine milk (Golinelli et al., 2014). Post-partum, the levels of immunoglobulins found in milk decreases rapidly. There are five classes of immunoglobulin: IgA, IgG (subdivided into IgG1 and IgG2), IgD, IgE and IgM. These immunoglobulin's all have the same basic structure with two heavy chains (50 - 70 kDa) and two light chains (22.4 kDa) connected by a number of disulfide bonds (Hurley & Theil, 2011; Hurley & Theil, 2013).

1.2.2 Non Protein Nitrogen

Components solubilised by 12% trichloroacetic acid (TCA) are regarded as non-protein nitrogen compounds. Within bovine milk, the percentage of compounds solubilised by TCA is 5 ~ 6%. The components which constitute non-protein nitrogen are as follows: urea, peptides, uric acid, amino sugars and alcohols, ammonia, creatine, creatinine,

nucleic acids, low molecular weight peptide hormones, free amino acids, growth factors, carnitine and choline (Atkinson & Lönnerdal, 1995). Human milk has increased levels of up to 25%, when compared to bovine milk, as the free amino acid taurine (which can be converted to cysteine) is in abundance. As the conversion of taurine to cysteine is important in infant development, infant formula often has added taurine to emulate human milk composition, as taurine is not found in bovine milk (O'Mahony & Fox, 2013). Urea is the largest component of non-protein nitrogen in milk comprising of 50% (w/w) of the non-protein nitrogen sources. This component has a substantial effect on the heat stability of milk with lactational and seasonal variances reflecting differences in urea quantity and therefore differences in relative heat stability (Muir & Sweetsur, 1976).

1.2.3 Caseins

The casein protein fraction of bovine milk is approximately 80% (w/w) of the total protein. This fraction can be subdivided into α_{s1}-casein, α_{s2}-casein, β-casein and κ-casein and can be precipitated by isoelectric precipitation at pH 4.6. α_{s1}-casein, α_{s2}-casein and β-casein are calcium sensitive caseins, in that they can precipitate by binding calcium, while κ-casein is calcium insensitive (Huppertz, 2013). When first discovered, it was believed that caseins were composed of random coil structures with little or no order - however they do contain some secondary and tertiary structure (Byler, 1986). The majority of casein found in milk (~ 95% (w/w)) does not exist in the form of individual proteins suspended in solution, but instead are held together in roughly spherical particles called casein micelles, by non-covalent interactions and intermolecular bonds (McMahon & Oommen, 2013). Both the casein micelle, and the

interactions which govern the formation of these micelles, are discussed in more detail in sections 1.2.2.5 and 1.3.2, respectively.

1.2.3.1 α_{s1}-casein

Typically α_{s1}-casein comprises 37% (w/w) of the casein portion of milk which equates to 12 - 15 g/L in bovine milk. The protein has a molecular weight of 24 kDa and is composed of 199 amino acid residues, 8 (sometimes 9) of which are phosphorylated (Huppertz, 2013). It is the serine residue which allows for most of the phosphorylation with α_{s1}-casein containing 16 Ser residues. The phosphorylation of these residues provides stability for the calcium phosphate nanoclusters in the casein micelle structure. Calcium phosphate can bind through interactions between two SerP residues as each SerP has a single negative charge, while calcium has two positive charges (Mercier et al., 1972). The binding of this calcium is important nutritionally, but also improves casein solubility and heat stability, which is a very important factor in the processing and manufacture of milk protein products. α_{s1}-casein does not contain any cystine residues and therefore cannot form any disulphide linked bonds. The isoelectric point of the protein varies from 4.4 to 4.8 depending on the state of the protein and the degree of phosphorylation (Trieucuot, 1981). As previously stated, although originally thought to contain little secondary structure, work by Creamer & Parry, (1981) demonstrated that α_{s1}-casein has a secondary structure comprised of 20% α-helix, with varying reported values of β-sheet structure of 17 - 20% by Byler (1986) and Creamer & Parry (1981), and 34 - 36% by Malin et al. (2005).

1.2.3.2 αs_2-casein

The casein fraction of milk consists of ~ 10% αs_2-casein and contains 207 amino acid residues with different levels of phosphorylation and intermolecular disulfide bonding

(Swaisgood, 1992). There can be up to thirteen phosphorylated residues within the protein with these residues residing within three different sections of the amino acid sequence and also contains two cystine residues in the form of a disulphide linked bond. Like α_{s1}-casein, the molecular weight of αs_2-casein is 24.3 kDa, with an isoelectric point of phosphorylated αs_2-casein of 4.9, considerably lower than the predicted isoelectric point of unphosphorylated αs_2-casein of 8.3 (Huppertz, 2013).

Numerous techniques and studies have estimated the secondary structure of αs_2-casein with one of the most recent suggesting that the protein consists of 46% α-helix, 9% β-sheet, 19% non-continuous α-helix or β-sheet, 12% turns, 7% polyproline II and 7% unspecified secondary structure (Farrell et al., 2009).

Although αs_2-casein contains the greatest number of phosphorylated residues within the caseins, it is the most highly sensitive to calcium precipitation. Precipitation can occur at a calcium concentration as low as 2 mM. It is recognised that precipitates from calcium are readily solubilised in 4 M urea solutions, resulting in the theory that there are no calcium mediated protein-protein interactions and that the protein-protein interactions are as a result of hydrogen bonding and the hydrophobic effect (Walstra & Jenness, 1984).

1.2.3.3 β-casein

β-casein contains 209 amino acid residues and has a molecular weight of 24 kDa (when Ser residues are phosphorylated) and represents up to 35% of the casein fraction in bovine milk (Dickinson, 2006). The isoelectric point of the protein is 4.7 and is estimated to be 5.1 for the non-phosphorylated protein (Trieucuot, 1981). Although bovine β-casein contains 5 phosphorylated serine residues, it does not contain any sulfhydryl groups and therefore cannot form disulfide bonds.

It is estimated that bovine β-casein has a secondary structure containing from 7% - 25% α-helix, 15 - 33% β-sheet and 20 - 30% turns (Creamer & Parry, 1981; Farrell et al., 2001; Graham et al., 1984). β-casein is the most hydrophobic of all the caseins with a highly hydrophobic outer surface. It exists in monomeric form under 4 °C, but forms thread like chains of approximately 20 units in length above 4 °C. If the temperature is raised above 8.5 °C, agglomeration and larger aggregate formation occurs (Dauphas et al., 2005).

Although β-casein is calcium sensitive, it is less so than α_{s1} and αs_2-casein. At temperatures greater than 18 °C, β-casein is precipitated at Ca^{2+} concentrations in the range of 8 - 15 mM, rendering β-casein insoluble under these conditions. Below 18 °C, β-casein is more soluble and is stable at concentrations of Ca^{2+} up to 400 mM. β-casein can bind up to seven Ca^{2+} ions per molecule under physiological conditions, but this can be increased with an increase in temperature. An increase in ionic strength however, typically within the range of 8 - 15 mM Ca^{2+}, leads to reduced binding of calcium (Farrell et al., 1988; Parker & Dalgleish, 1981).

1.2.3.4 κ-casein

κ-casein is the smallest of all the caseins with 169 amino acid residues and comprises roughly 12% (w/w) of casein in bovine milk (Huppertz, 2013). The degree of phosphorylation in this protein is low, with only one phosphorylated group, although di- and tri-phosphorylated κ-caseins can also exist. Two Cys residues (Cys11 and Cys88) give κ-casein the ability to create disulfide bonds (Mercier et al., 1981). κ-casein has a molecular weight of 19 kDa with an isoelectric point of 5.6 in its phosphorylated form (Walstra & Jenness, 1984). κ-casein is the only casein that is calcium insensitive and does not precipitate out of solution if excess calcium is present. Its ability to remain

soluble in the presence of calcium helps to stabilise the other caseins and form the casein micelle.

One of the main properties of κ-casein is that it contains 5% carbohydrate (not found in any other casein). It is usually glycosylated and the carbohydrate exists as a tri- or tetra-saccharide of *N*-acetylneuraminic acid, galactose and *N*-acteylgalactosamine and is positioned towards the end of the C terminus, attached through an *O*-threonyl linkage.

1.2.3.5 Casein Micelle

95% (w/w) of the casein fraction of bovine milk exists as large spherical colloidal assemblies know as casein micelles. These assemblies are comprised of a combination of different casein proteins (94% (w/w)), low molecular weight salts and colloidal calcium phosphate (Dalgleish, 2011). They are highly hydrated, with each micelle containing 2 - 4 g water for each gram of protein (de Kruif, 1999; Morris et al., 2000). The micelles have a large surface area and have a mass between 10^6 and 10^8 Da. They are highly stable under heating and can withstand high temperatures of 140 - 150 °C for 15 - 20 minutes before heat-induced coagulation occurs (McMahon & Oommen, 2013). Even when coagulation of bovine milk does occur, it is not due to the denaturation of the proteins in the casein micelle, but due to other reactions taking place within the bulk milk. Casein micelles are stable at high calcium concentrations (up to 200 mM at 50 °C) but do become unstable at alkaline pH of 9 or above. In bovine milk, approximately 65% (w/w) of the total calcium is bound to casein in the form of colloidal calcium phosphate (Neville et al., 1994). Micellar caseins are roughly spherical in shape and have a size distribution that ranges between 50 - 600 nm, with the average diameter of the casein micelle being 120 - 180 nm (Farrell et al., 1990). Holt (1992) suggested a nanocluster model (Figure 1.3) for the description of the casein micelle. The surface of

the casein micelle can be viewed under Field Emission – Scanning Electron Microscopy (FE-SEM) which provides some structural information to provide some scientific basis to the nanocluster model. In this model, it is suggested that phosphorylation of sub casein proteins (α_{s1}-, α_{s2}- and β-casein) can bind salts, in particular colloidal calcium phosphate (CCP), creating a stabilised protein structure. The surface of this structure is primarily composed of κ-casein, which provides steric stabilisation through κ-casein repulsion and electrostatic stabilisation through a surface ζ-potential of -20 mV.

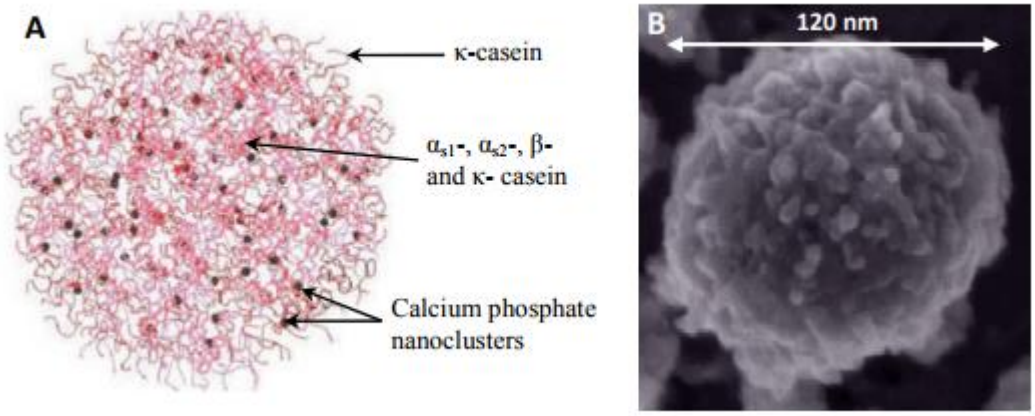


Figure 1.3: (A) Schematic representation of the nanocluster model of the casein micelle as proposed by de Kruif & Holt (2003). (B) An electron micrograph image of a micelle (Dalgleish et al., 2004).

Dalgleish (2011) suggested the use of cryogenic TEM to ascertain properties of the casein micelle. TEM has shown that within the micelle, there are areas of equally distributed electron dense regions, providing supportive evidence to neutron scattering results, that these regions consist of calcium phosphate nanoclusters. It is postulated by Dalgleish, 2011 that the structure of the micelle cannot be solely maintained by the hydrophobic effect, as the micelle would dissociate if exposed to low temperatures, but as this is not the case, other interactions must be taking place.

1.3 Thermal Denaturation of Proteins

The thermal treatment of dairy ingredients is needed to reduce the bacterial load and ensure a product which is safe for human consumption. However proteins, in particular whey proteins, are heat labile, decreasing the stability of dairy proteins during heat-treatment, especially when concentrated, ultimately influencing finished powder functionality. In particular, α-la, β-lg and BSA undergo conformational changes including unfolding, denaturation and aggregation which are generally accepted to be irreversible under particular conditions (Tolkach & Kulozik, 2007). Denaturation can be defined as the unfolding of a protein, which gives rise to a change away from its native structure, and permits the exposure of hydrophobic residues from within the core of the protein molecule (Galani & Apenten, 1999). Subsequent aggregation of protein is caused by both non-covalent interactions and covalent bond formation, as outlined below, with both types of interactions affected by factors such as salt concentration, temperature, length of heating time and pH.

1.3.1 Disulfide Bond Formation in Proteins

The formation of covalent bonds in heat-treated protein systems typically involves inter- (interaction between polypeptide chains) and intra- (interaction within a polypeptide chain) molecular disulfide bonds (Schmid et al., 2017; Surroca et al., 2002; Wijayanti et al., 2014) (Figure 1.4). These bonds are formed through sulfhydryl-disulfide interchange or sulfhydryl oxidation reactions (Havea et al., 2004), with this functional group commonly referred to as either a thiol or sulfhydryl group. The bonds formed are permanent and have strength of ~ 200 - 400 kJ/mol (Dickinson, 1997a; Eissa & Khan, 2006).

The presence of free thiol groups in both β-lg and BSA monomers can induce, when heated above ~ 65 °C, the disruption of inter- and intra-molecular bonds, resulting in the possible formation of covalently bonded protein aggregates (Havea et al., 2004; Morr, 1975; Verheul et al., 1998).

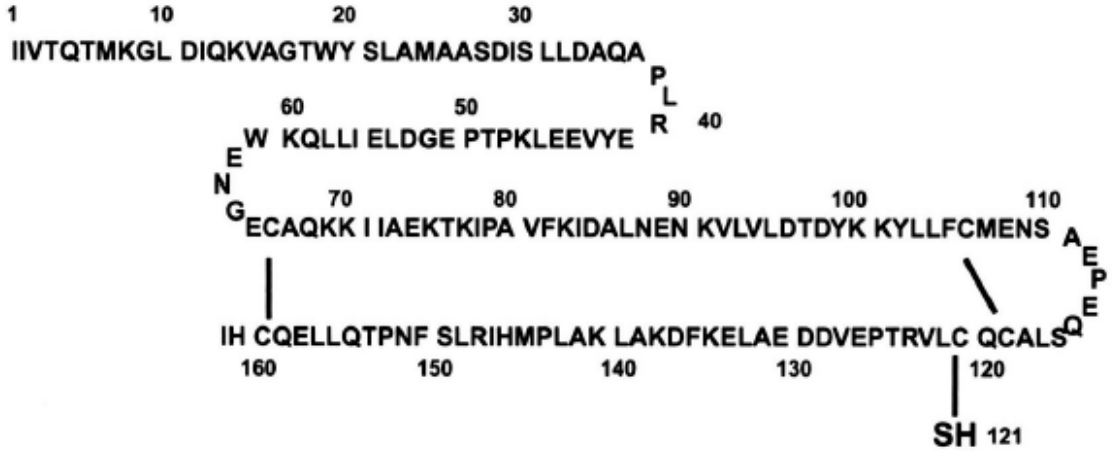


Figure 1.4: The primary structure of bovine β-lactoglobulin. Disulfide linkages are located between Cys-106 and Cys-119, and Cys-66 and Cys-160 residues, with a free sulfhydryl group (free cysteine) located at residue Cys-121 (From Liu et al., 2007).

As the main whey protein in bovine milk, β-lg plays an important role in determining physical characteristics of protein systems containing whey proteins during heating. Heat-treatment results in the denaturation and unfolding of β-lg, permitting intermolecular reactions via thiol-disulfide interchanges, leading to protein aggregation with other β-lg monomers and dimers (Manderson et al., 1998), other whey proteins (Donovan, 1987) and caseins (Guyomarc'h et al., 2009; O'Kennedy & Mounsey, 2006). As α-lac is stabilised by four disulfide bonds, but does not contain a free thiol group, it cannot initiate irreversible aggregation in the same way (Brew, 2013). Consequently, denaturation of pure α-lac can be reversible (Boye et al., 1997a). However, most dairy protein systems contain both β-lg and α-lac, e.g., infant formula, and α-lac can be

incorporated into aggregates as a result of thiol-disulfide interchanges and hydrophobic interactions (Dalgleish et al., 1997; Fitzsimons et al., 2007).

1.3.2 Non-covalent Interactions in Proteins

Non-covalent interactions are weaker interactions, when compared to covalent bonds, and primarily exist between individual protein molecules and/or two different functional groups within the same protein molecule. They play an essential role in protein folding and DNA replication, allowing for both the formation of a proteins three-dimensional structure, and the flexibility needed for unfolding i.e. during DNA replication (Cèrny & Hobza, 2007). The main types of non-covalent interactions are hydrogen bonding, van der Waals, electrostatic interactions and the hydrophobic effect.

1.3.2.1 Hydrogen Bonding

Hydrogen bonding is an attractive interaction which occurs when a hydrogen atom binds to a lone pair of electrons on a neighbouring electronegative atom (Bryant & McClements, 1998). Figure 1.5 shows an example of hydrogen bonding in water, where the hydrogen bond donor is covalently bound to another atom, in this case oxygen, and is freely available to the acceptor atom.

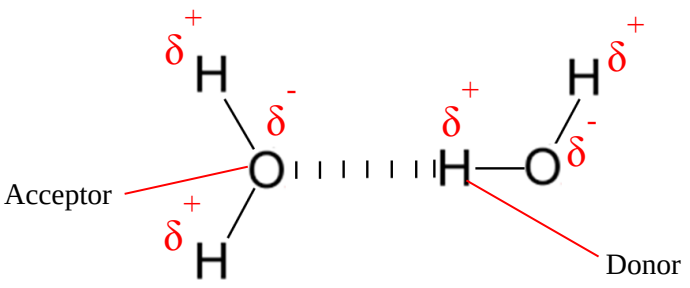


Figure 1.5: Attraction between a positively charged hydrogen (donor) atom and a negatively charged oxygen (acceptor) atom in water.

The hydrogen bond acceptor atom, in this case also oxygen, accepts the free lone pair of electrons given by the hydrogen atom (donor). The strongest hydrogen bonds exist when the hydrogen bond donor atom and acceptor atom align with each other (Berg et al., 2002; Hubbard & Haider, 2010).

1.3.2.2 van der Waals Interactions

van der Waals interactions can only occur when two molecules are separated by a short distance, although not close enough to be repelled by the negative charges from outer electron shells (known as van der Waals contact distance). They form when two atoms approach each other and create an attractive force (Figure 1.6). The interactions are resultant from fluctuations in the distribution of electrons in an atom, resulting in transient unequal electron distribution, and therefore the formation of a transient electric dipole, with an electrical dipole defined as charge separation due to positive and negative charges.

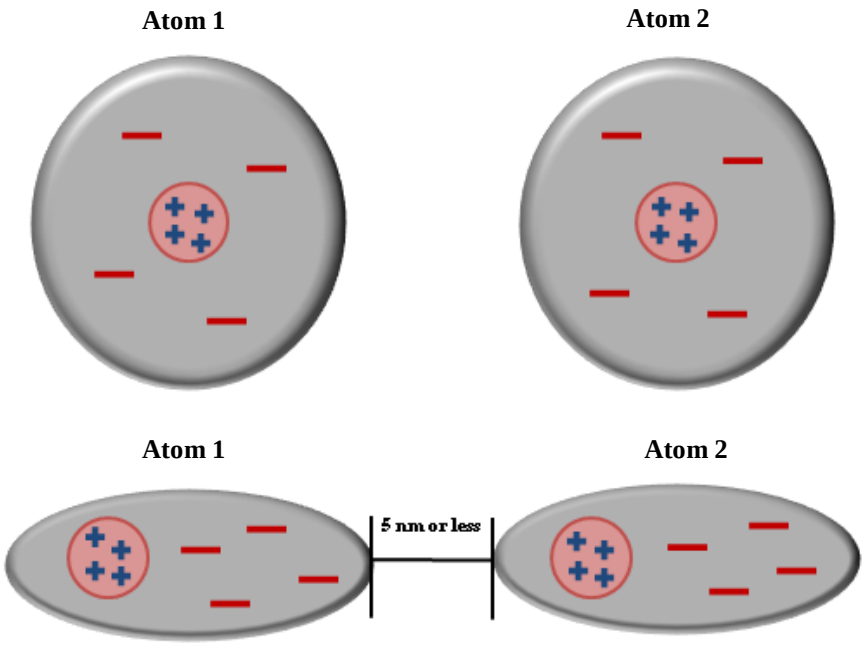


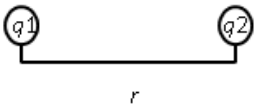
Figure 1.6: Schematic diagram of a van der Waals interaction between two atoms.

If two atoms are distanced close enough to each other, the transient dipole of one atom will perturb the electron cloud of the other atom; this in turn generates a transient dipole in the second atom, causing a weak attraction between the two atoms (Lodish et al., 2002). van der Waals interactions occur at distances of twice the length of the covalent bond between the same atoms.

1.3.2.3 Electrostatic Interactions

Electrostatic interactions exist between two atoms, molecules or surfaces which have permanent electrical charge i.e. ions or dipoles, and bear opposite electrochemical charges; they can be attractive or repulsive (Bryant & McClements, 1998). *Coulomb's* law governs the energy of an electrostatic interaction:

$$E = kq1q2/Dr$$



(Equation 1.1)

where *E* is energy, *k* is a proportionality constant (k = 332 for 1 kCal/mol or 1389 for 1 kJ/mol), *q1* and *q2* are the charges of the two opposing molecules (units for electric charge), *D* is the dielectric constant (dependent on external environment) and *r* is the distance between the two opposing molecules (in angstroms) (Berg et al., 2002). A number of parameters affect the charge of a protein including the charge sign, pH and the solution the protein is residing in; proteins are negatively charged above their isoelectric point, positively charged below, with the net charge at the isoelectric point of the protein being zero (Bryant & McClements, 1998; Majhi et al., 2006).

At a basic level, the DLVO (Derjaguin, Landau, Verwey and Overbeek) theory can be used to describe the interaction potential between two particles, and provides an explanation of the stability of suspended colloids, by determining the sum of

electrostatic and van der Waals interactions that exist between particles as they approach each other (Figure 1.7).

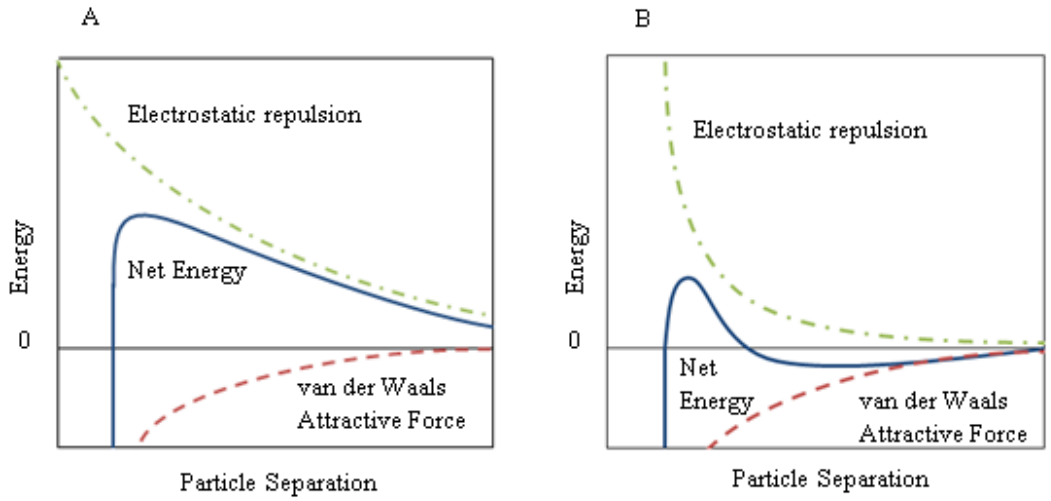


Figure 1.7: Schematic representation of the free energy variation with particle separation (A) and at higher salt concentrations (B).

Particles are initially separated as they approach each other due to electrostatic repulsion. This repulsive force can however be overcome, if the particles are provided with sufficient energy, typically due to a change in temperature, which will allow the attractive force to dominate and cause irreversible flocculation of particles (Figure 1.7, A). In high salt concentrations, the electrostatic charge of particles is lowered, resulting in the likely association of particles through reversible flocculation (Figure 1.7, B).

1.3.2.4 Hydrophobic Effect

The hydrophobic effect is a relatively strong attractive force, which exist between non-polar groups, separated by water. It originates from the ability of water molecules to form strong hydrogen bonds with other water molecules, but with non-polar molecules only able to interact via weaker van der Waals forces with neighbouring molecules.

When these non-polar groups are placed in water, they disrupt the hydrogen bonding network that surround the water molecules, therefore forcing rearrangement of the molecules around the hydrophobic surface. Rearrangement of these molecules is thermodynamically unfavourable, with the system trying to minimise the contact area between the water molecules and the non-polar groups, resulting in the production of attractive forces between these non-polar groups. Typically these interactions are longer in range than other non-covalent interactions (Bryant & McClements, 1998).

1.3.3 Heat Induced Aggregation of Whey Proteins

A number of mechanisms have been proposed for the denaturation and aggregation of whey proteins, in particular for β-lg, as it is the main whey protein in bovine milk, and unlike α-lac, contains a free thiol group. Earlier whey protein denaturation models describe a two-step mechanism of protein unfolding/denaturation (first step), and protein association (aggregation, second step) which resulted in irreversibly aggregated protein complexes (Mullvihill & Donavan, 1987). Roefs and de Kruif (1994) moved this model forward, and suggested a three step model involving activation, propagation and termination. In this model, aggregation through disulfide bonds occurs as follows:

$$B_2 \rightleftharpoons 2B \rightarrow B^*$$

where B_2 is equal to the non-covalent dimer that exists between β-lg monomers (dependent on physicochemical factors), B is the β-lg monomer, and B^* is the activated monomer. After initial heating, protein unfolding is initiated and exposure of a free sulfhydryl group allows for propagation:

$$B + B^* \rightarrow B^*_{i+1}$$

This step of the reaction allows for B^* to react with native β-lg molecules to form an activated dimer (B^*_2) through disulfide interchange reaction, allowing for the exposure of one of the disulfide groups from the initial disulfide bond, resulting in the availability of a free sulfhydryl group to allow further aggregation. The third step is a termination reaction step, and occurs as follows:

$$B^*_i + B^*_j \rightarrow B_{i+j}$$

When two activated monomers (B^*_I and B^*_j) aggregate through disulfide linkage of their exposed free sulfhydryl groups, they can no longer aggregate through disulfide bonding, as no free sulfhydryl group remains, thus terminating the aggregation reaction (Brodkorb et al., 2016). However, this model has its limitations, as it is only accurate for β-lg heat-treated between ~ 65 and 70 °C, low ionic strength and neutral pH (Wijayanti et al., 2014).

Although the model proposed by Roefs and de Kruif (1994) was comprehensive with regard to the formation of β-lg aggregates through disulfide bonds, it did not take into account non-covalent interactions, and only observed protein aggregation for purified β-lg, at relatively low heating temperatures. Oldfield et al. (1998) however, improved on the model proposed by Roefs and de Kruif (1994). This model outlines not only aggregation by free sulfhydryl groups, but also the hydrophobic effects that occur when hydrophobic groups are exposed upon unfolding of whey protein molecules. It is postulated that at lower heating temperatures (< 75 °C), these exposed hydrophobic groups aggregate through the hydrophobic effect, and upon further heating to temperatures > 75 °C, these aggregates are converted to disulfide bonded aggregates. Similarly, in the presence of α-lac, below 80 °C, β-lg and α-lac form aggregates via the hydrophobic effect, which are converted to disulfide bonded aggregates, at temperatures

above 80 °C. This model is more comprehensive than the previous models discussed, as the model was representative of skim milk over a wider range of temperatures i.e. 70 to 130 °C, with heating times ranging from 3 to 1800 seconds. This model also uses direct steam injection, which is more applicable to large scale food and beverage manufacture than lab scale heating (Wijayanti et al., 2014).

1.3.4 Casein - whey Protein Interaction

Both covalent and non-covalent interactions between whey proteins and other ingredients during the manufacture of foods and beverages are inevitable, and can have a positive or negative impact on the quality of the final food product. These interactions can have a significant effect on both the physical and chemical characteristics of a protein system during processing, e.g. in-process control of heat stability, viscosity and gelation, or in the finished powder, e.g. wettability, dispersibility or formation of 'white flecks' on rehydration. The physical phenomena of precipitation, coagulation and gelation are the resulting effect of whey protein denaturation and subsequent aggregation, which is dependent on environmental conditions such as temperature, pH, protein and mineral concentration, as previously mentioned. The tertiary structure of caseins is relatively low, resulting in increased heat-stability, when compared to whey proteins (Donella-Deana et al., 1985; Swaisgood, 2003); as an example, Fox & McSweeney (1998) studied the heat stability of sodium caseinate and found that solutions can be heat-treated at 140 °C, and held for over 1 hour without visible aggregation. However, the interaction of caseins with other proteins does occur during heat-treatment, with these reactions are central to producing stable dairy protein systems. The thermal stability of skim milk is linked to cysteine residues in κ-casein, located at the surface of the casein micelle, and their interaction with β-lg. Mixtures of

κ-casein and β-lg are able to aggregate through disulfide bonds and the hydrophobic effect (Guyomarc'h et al., 2009). The formation of complexes is dependent on pH, with complex formation either helping to increase or decrease thermal stability in skim milk (Tessier & Rose, 1964). Jeurink & de Kruif (1993) studied the interaction of β-lg and κ-casein in heat-treated skim milk and found that there was an increase in viscosity with the association of unfolded β-lg molecules and casein micelles (heat-treatment of 85 °C). Heat-treatment at pH < 6.9 allows for the formation of β-lg covalent bonds with casein micelles *via* κ-casein, and in turn helps stabilise the micelle against precipitation (Wijayanti et al., 2014). Due to the dissociation of κ-casein into the serum phase at pH > 6.9, interaction of β-lg and κ-casein occurs causing the formation of complexes; this is detrimental to the structure of the casein micelle, and as a result, the protein system is destabilised (Singh & Fox, 1987). Likewise, interactions between casein and whey proteins in infant formula have been studied, with pH being a contributing factor to protein stability. McSweeney et al. (2004) showed that an infant formula made up of skim milk powder (SMP) and whey, heat-treated at 140 °C for 80 s, between pH 6.5 – 6.8, resulted in the formation of whey/casein complexes, which precipitated post heating. However, when the pH was raised to 6.9 – 7.1, the supernatant of the solution, after isoelectric precipitation, contained native β-lg. The effect of pH is important as infant formula manufacturers target a pH of ~ 6.8 during batch make-up by careful selection of mineral salts and their order of addition to the liquid mix (further discussed in section 1.3.4 of this introduction). Understanding casein/whey protein interactions and pH is further complicated by the drop in pH during concentration on evaporation of dairy protein systems (Murphy et al., 2015). It is important to monitor the pH during processing, as a significant drop in pH (e.g., pH < 6.5) can lead to increased viscosity and protein precipitation during evaporation as the pH drops further, thus causing

fouling and contributing to manufacturing downtime. It is thought that pH affects the availability of κ-casein which subsequently has an effect on the type of protein aggregates that can be formed (Creamer et al., 1978). Protein concentration of both κ-casein and whey protein can have an effect on the amount of protein bound to each κ-casein molecule with roughly a 1:3 κ-casein to whey protein ratio (Guyomarc'h et al., 2003). It is suggested that the ratio becomes distorted depending on the concentration of each of the proteins in milk, although, Donato et al. (2007) reported that excess κ-casein or sodium caseinate, in skimmed milk of neutral pH and heated to 90 °C, had a minor influence on the size and number of protein aggregates formed, with excess whey protein having a much more prolific effect, where both the quantity and size of protein aggregates were altered. It is suggested that κ-casein, when added in excess, is structurally different to that of κ-casein found on the surface of the casein micelle as it resides in the serum phase and has insignificant interactions with whey protein and does not affect the casein micelle. It is proposed that κ-casein residing on the surface of the micelle is more accessible than when in the serum phase, as on the micelle, the κ-casein is widely distributed across the surface giving greater access for whey proteins to interact compared to that of the aggregated κ-casein in the serum fraction. A recent study by Goodison et al. (2017) demonstrated that heating whey protein isolate (WPI) and casein proteins together in a model infant formula solution, compared to heating individually and then mixing, resulted in increased stability. The greater stability was attributed in-part to the significantly lower level of exposed free thiol groups compared to those formulations where the skim milk and whey protein were heated separately. Murphy et al. (2015) showed that casein at a concentration similar to that used in infant formula increases the temperature at which β-lg denatures and also the onset of viscosity increase during heating.

1.3.5 Effect of Minerals on Whey/Casein Interaction

Milk and whey are a good source of minerals and minor trace elements, with many of the minerals present in the in the form of salts, e.g., calcium phosphate. In addition to their primary function to provide nutrition in dairy beverages, they impart stability to the colloidal casein micelle, have an impact on protein stability during heat-treatment and provide buffering capacity. Milk salts include but are not limited to citrates, phosphates and chlorides containing H^+, K^+, Na^+, Mg^{2+} and Ca^{2+} ions. Salts can reside within the casein micelle, or in the serum phase in solution, and can be organic (e.g., citrate) or inorganic substances (e.g., phosphate). Bovine milk contains large amounts of calcium and phosphate salts, with over half of inorganic phosphate and two-thirds of calcium present in the colloidal casein micellar complex together with Mg^{2+} and citrate. Within the serum phase of milk are the remaining fractions of calcium and phosphate, which are often found in the form of complexes with citrate, phosphate or chloride as cationic or anionic pairs. Environmental conditions have a significant impact on salt equilibrium and behaviour. The level of monovalent and divalent ions such as Ca^{2+}, Mg^{2+}, Na^+ and K^+ that are present in an infant formula concentrate play a significant role in protein-related destabilisation of an infant formula liquid concentrate.

1. 4 Whey Proteins and In-process Emulsion Stability

1.4.1 Lipids

Milk fat consists of a mixture compounds which include triacylglycerols, diacylglycerols, monoacylglycerols, phospholipids, sterols, fat soluble vitamins (i.e., A, D, E, K) and free fatty acids etc. The principle fatty acids in milk include saturated

(Butyric, Caproic, Caprylic, Capric, Lauric, Myristic, Palmitic, Stearic), monounsaturated (Oleic) and unsaturated (Linoleic, Lineolenic and Arachidonic) acids. Milk fat can be described as a water insoluble course emulsion with a large fat globule size distribution, ranging between 1 to 20 μm. These milk fat globules are stabilised by a membrane, i.e., a lipid bilayer comprising of proteins, phospholipids, lipoproteins, cerebrosides, nucleic acids and enzymes. In bovine milk, >98% of lipids are composed of individual fatty acids attached to glycerol carbon backbones, with the most common type of lipid being triglycerides (three fatty acids attached to a glycerol backbone) (Figure 1.8).

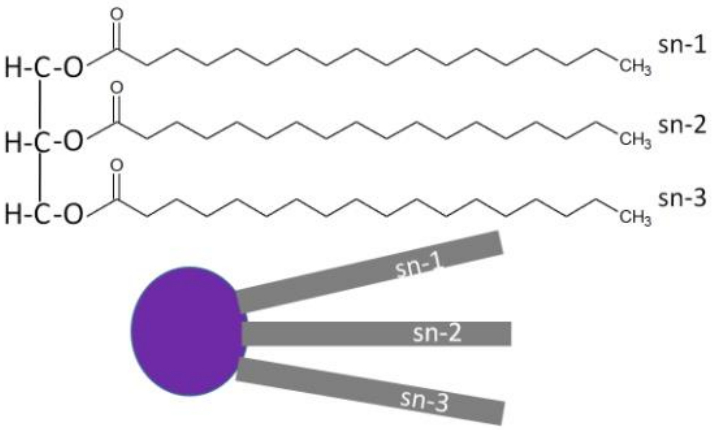


Figure 1.8: Schematic diagram of a triglyceride.

The three fatty acids on the glycerol backbone are designated as *Sn*-1, *Sn*-2 and *Sn*-3, with Sn denoting "stereospecific numbering". Bovine and human milk fats have notably different fatty acid composition and hence different nutritional and functional attributes. One compositional difference which has generated interest amongst infant formulae manufactures is the make-up of triglycerides attached at the Sn positions, with palmitic acid predominately esterified to the glycerol backbone at *Sn*-2 in human milk, whereas it is esterified at position *Sn*-3 in bovine milk (Hunter, 2001). This difference

affects the absorption of fat by infants, as when palmitic acid is found at the *Sn*-2 position, as in human milk, the triglyceride is much more easily absorbed, resulting in higher quantities of overall fat being absorbed (Small, 1991). By preforming transesterification, commercially available vegetable oils can be produced with increased levels of palmitic acid at position *Sn*-2, providing a nutritional replacement fat in infant formula (O'Callaghan, 2001). Because of differences in fatty acid composition, bovine milk fat is not the primary source of fat used during the production of infant formula. Vegetable oils including palm, coconut, sunflower and soybean oil can be used in infant formulations in combinations designed to simulate the fatty acid composition of human milk.

1.4.2 The Homogenisation Process

Many foods and beverages produced using dairy ingredients will use emulsification as a method of incorporating lipids into a product. Typically an oil-in-water emulsion is formed though the homogenisation of an aqueous and oil phase, in the presence of proteins and/or emulsifiers. Coarse emulsions are produced during the batch make-up process, i.e., the shearing of protein ingredients, carbohydrate, minerals and vitamins with a fat blend usually by recirculation through a high shear powder entrainment system. This mix is unstable owing to its non-uniform emulsion droplet size and bimodal fat globule size distribution ranging from small to large droplets, which are likely to cream or flocculate. A two-stage valve-type homogenisation step is typically used to form a homogenous and stable infant formula emulsion (Figure 1.9). The initial stage during homogenisation involves the disruption of existing fat droplets and particles creating a narrower fat globule size distribution (Lam & Nickerson, 2015; McCarthy et al., 2015). For the manufacture of dairy emulsions and powders containing

fat, the oil-in-water mixture is forced through a valve, typically at a pressure between 150 - 180 bar (15 – 18 MPa), inducing, depending on certain factors, a uniform and stable emulsion. A second homogenisation step, generally carried out at ~ 20% of the first stage pressure, to break up flocculates that may have formed immediately after the first homogenisation step insuring that all large fat globules and flocculates have been reduced to the smallest droplet size.

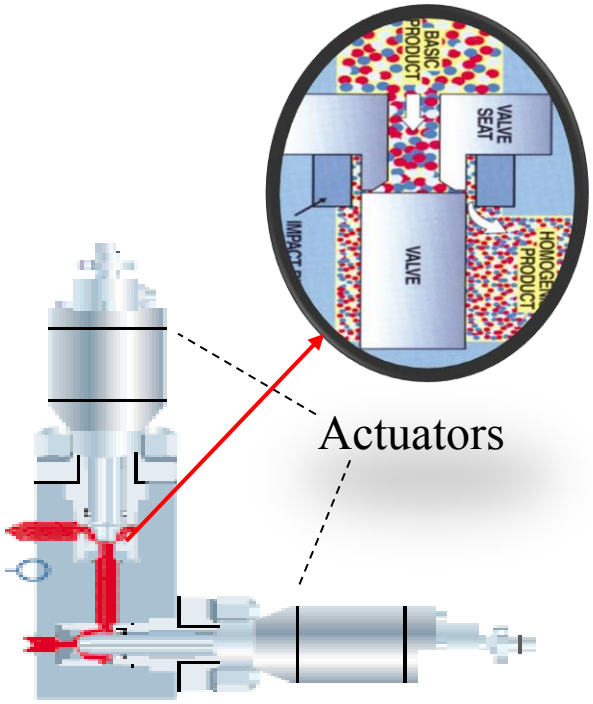


Figure 1.9: A schematic diagram of a two-stage homogeniser, with valve assembly set up.

According to Stoke's law, the smaller the fat globule size, the more stable an emulsified system is to creaming (Dickinson & Golding, 1998):

$$v_s = \frac{(\rho_f - \rho_p)}{18\mu} . g . d_p^2 \qquad \text{(Equation 1.2)}$$

where v_s is the velocity of the separating particles, ρ_f is the particle density (mass) in kg/m^3, ρ_p is the density of the fluid (mass) in kg/m^3, g is gravitational acceleration (9.81 m.s^{-2}), μ is the continuous phase viscosity and d_p is the mean particle diameter.

1.4.3 Behaviour of Dairy Proteins at the Oil-in-water Interface

McCarthy et al. (2015) used a factorial statistical design to determine the optimal total solids (TS), pre-heat-treatment temperature, and first- and second-stage homogenisation pressures for maximum emulsion stability in a model infant formula during processing. The dispersion of fat in the aqueous phase forms an interface where proteins can interact and adsorb, reducing interfacial tension, increasing stability of emulsion droplets through the formation of hydro-dynamically and electrically charged protein layers, providing resistance to flocculation and coalescence of fat globules (McClements, 2004; Ye, 2008). Formation of a stable emulsion with mono-modal fat globule size distribution is a critical step in the manufacture of infant formula and determines subsequent stability throughout evaporation, drying and powder rehydration. Insufficient emulsification due to critically low protein concentration can cause an unstable emulsion to flocculate and/or coalesce resulting in free fat and poor reconstitution of dairy powders. The affinity of a protein to adsorb to the interface relates to the flexibility, linearity and hydrophobicity of its structure (Dalgleish et al., 2002). In mixed protein systems containing both the same ratio of casein to whey proteins, preferential adsorption of caseins occurs as they contain little secondary structure and are more open flexible molecules and contain hydrophobic regions within their amino acid sequence. In particular, β-casein and α_{s1}-casein are highly surface active, containing hydrophobic residues which anchor to the interface (Dickinson, 2001) (Figure 1.10). In comparison, the globular whey proteins are less susceptible to

adsorption, especially in their native globular form (Dickinson, 1998). However, upon unfolding during thermal treatment, these proteins become more flexible, expose hydrophobic amino acid residues and are therefore more likely to adsorb to an emulsion interface in conjunction with casein. For instance, pre-heat-treatment and pH has been shown to alter structural characteristics and emulsifying properties of α-lac (Lam & Nickerson, 2015; Raikos, 2010).

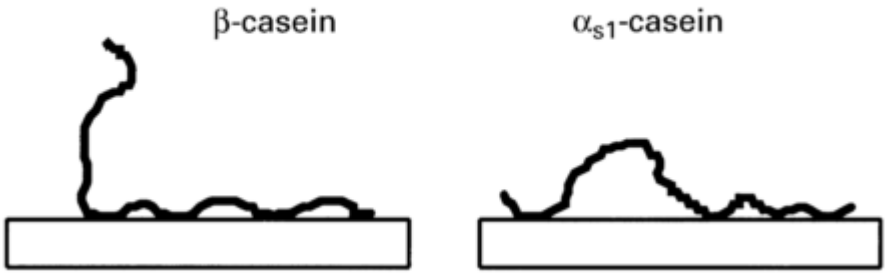


Figure 1.10: Proposed sketch of the adsorption of (i) β-casein and (ii) α_{s1}-casein at a solid hydrophobic surface.

In the processing of dairy based powders, emulsions are primarily stabilised by dairy proteins, however, emulsifiers such as lecithins and monoacylglycerols /diacylglycerols can be added to help increase emulsion stability in certain formulations, e.g. those made using hydrolysed whey protein ingredients. As protein or an emulsifier is needed to stabilise the interfacial surface of each fat droplet formed during homogenisation, the protein to fat ratio has an important role in emulsion stability, both during, and post processing (Creamer & Parry, 1981; McDermott, 1987; Ye, 2008; McCarthy et al., 2013). McCarthy et al. (2012) described how a reduction in the protein (60% (w/w), whey protein) to fat ratio of a model 1st stage IF from 0.43 to 0.21, significantly increased fat globule size from 0.74 to 0.81 μm (Sauter mean diameter (D[3,2]) post homogenisation). Furthermore, the fat globule size for all emulsions significantly

increased on evaporation, with the greatest increase being observed when the protein to fat ratio was lowered to 0.21. The study suggests that the critical protein to fat ratio needed to achieve a stable infant formula is 0.26, and the protein to fat ratio is decreased below this level, emulsion stability is significantly reduced, particularity on evaporation. Removal of water from the continuous phase compresses emulsions and reduces the quantity of water between the interface of neighbouring droplets, thus causing distortion, disruption and short-range interactions to occur leading to coalescence (Aranberri et al., 2004).

1.4.4 Emulsion Instability

While infant formula emulsions are primarily stabilised by the protein found at the fat droplet interface, pH and ionic strength are important; fat globule stability is governed by van der Waals interactions, electrostatic repulsion and short-range interactions all of which are sensitive to changes in pH and ionic strength (Demetriades et al., 1997). As the isoelectric point of the majority of dairy proteins range between 4 and 6, the pH of the continuous phase can affect the strength of electrostatic repulsions provided by proteins embedded on the surface of fat droplets. Lowering the pH within this range reduces the net charge of the protein on the surface of droplets causing flocculation and destabilisation of the emulsion. Depending on the composition of protein on the surface of the emulsion droplet, emulsions may de-flocculate when the pH is either further reduced or increased. Both casein and whey protein-based emulsions are destabilised at pH ~ 5. However, at this pH, casein undergoes irreversible protein precipitation while emulsions stabilised by whey proteins can be deflocculated when the pH is decreased to pH < 3 or increased to pH > 7 (Dickinson, 2010). For example, higher levels of α-lac are found at the interface at pH 3, due to a change in tertiary structure of the protein,

with preferential adsorption of its monomeric structures favoured by the lower pH (Hunt & Dalgleish, 1994). Emulsions containing globular whey proteins are thermodynamically unstable during heat-treatment due to molecular rearrangement of protein molecules on the surface of the oil droplets (Tcholakova et al., 2006). Without sufficient pre-heating, β-lg, exposed on emulsion droplets may destabilise due to partial molecular rearrangement induced by homogenisation and heat-denaturation (Dickinson, 1998), an important consideration for manufacturers of whey-dominant infant formula. This partial rearrangement can result in protein-protein bridging and flocculation of protein molecules on the surface of oil droplets, or cause depletion flocculation where detached surface proteins interact with unfolded protein in the aqueous phase, thus causing coalescence of oil droplets and a subsequent increase in creaming rates. This effect is confirmed by recent research which shows that increasing the α-lac within a whey protein fraction (i.e., reduced in β- Lg) of a model infant formula, significantly ($P < 0.05$) increased stability of the emulsion and reduced viscosity during subsequent evaporation (Crowley et al., 2016; Buggy et al., 2017). Replacing β-lg with α-lac reduced the total number of free thiol groups within the protein system and less aggregation was observed. As a consequence both the surface of emulsion droplets and the continuous phase contain fewer molecules with exposed free thiol groups, thus reducing the occurrence of bridging between emulsion droplets and increases in viscosity on concentration.

1.5 Role of Whey Proteins in Infant Formula

Bovine milk has a different composition to human milk, i.e., in both type and content of fat, protein, carbohydrate, minerals and vitamins (Table 1.1). Particular interest has surrounded the production of whey-protein dominant infant formula, the nutritional

content of which must closely mimic of that of human breast milk. Protein ingredients made from bovine sources, are commonly used in infant formula (non-fat macromolecules) (Nasripour et al., 2006) and are blended together to achieve the same quantity of amino acids (some conditionally indispensable) as is in human milk to attain the protein requirements needed for sufficient growth and development of an infant (European Commission).

Table 1.1 - Compositional differences between, infant formula, human and bovine milk

	Infant Formula	**Human**	**Bovine**
		per 100 mL	
Energy (kcal)	60 - 70	71	69
Total protein (g)	1.2 - 2.0*	0.9	3.3
Casein	N.S	0.3	2.6
Whey protein	N.S	0.6	0.7
Fat (g)	2.9 - 3.9*	3.8	3.7
Carbohydrate (g)	5.9 - 9.1*	7	4.8

Data: Gurr, 1981; Thompkinson & Kharb (2007). From Murphy (2015).

*Calculated from European Commission, (2006) for energy content = 65 kcal 100 mL^{-1}

For example, in the formulation of a 1st stage infant formula, skim milk and demineralised whey powder are mixed with lactose, and minerals, to produce a formula which is safe for infant consumption (contains the right levels of protein, carbohydrate and minerals). By adding the demineralised whey powder to skim, a different whey/casein ratio can be produced, which can better mimic the protein profile in human milk, at a ratio of 60:40, respectively. However, this change in ratio is dependent on the stage of lactation, with protein contents varying throughout the lactation season (Lönnerdal & Lien, 2003). European regulations do not state what the casein to whey

protein ratio should be, although the ratio is generally modified to include larger amounts of whey protein; European manufactures of infant formula who export to countries such as China, do however have to follow regulations, with a minimum label claim for whey protein of 60% (Peoples Republic of China, 2010). In human milk, as the casein content is lower than the whey protein content, the curd formed during digestion is softer, and is therefore is more easily digested and leading to a faster rate of gastric emptying, compared to the curd from bovine milk, which is coarse, flaky and firm, and more difficult to digest (Nakai & Li-Chan, 1987; Thompkinson & Kharb, 2007). The buffering capacity of human milk proteins is also lower than bovine milk proteins and, therefore, reduces gastric acidity to a greater extent (Davis et al., 1994). While altering the casein:whey protein ratio is beneficial for simulating the amino acid profile of human milk, it has also been determined to affect infants' microbiota in a similar manner to breast milk (Rochata et al., 2007).

For infant formula to be nutritionally viable source for infant nutrition, it must match the amino acid profile of human milk, and contain the same amount of essential and non-essential amino acids (Table 1.2). The amino acid composition of human milk provides all 11 essential amino acids necessary for infant growth and development (Dewey et al., 1996).

However, while skim milk has a large abundance of many amino acids, it is deficient in methionine, cystine and tryptophan (Lien, 2003); differences in phenylalanine:tyrosine and cysteine:methionine ratios between bovine and human milk are considered particularly critical (Thompkinson & Kharb, 2007). Therefore, to ensure an adequate supply of all amino acids, the protein content of infant formula (1.3 - 1.5 g/ 100 g) is higher than that of human milk (~ 1.0 g/ 100 g).

Table 1.2 - The minimum levels of indispensable and conditionally indispensable amino acids in infant formulas, based on breast milk, and expressed in mg per 100 kJ and 100 kcal.

Amino acid	mg/ 100 kJ	mg/ 100 kcal
Cystine	6	24
Histidine	11	45
Isoleucine	17	72
Leucine	37	156
Lysine	29	122
Methionine	7	29
Phenylalanine	15	62
Threonine	19	80
Tryptophan	7	30
Tyrosine	14	59
Valine	19	80

Commission Directive 2006/141/EC (European Commission, 2006); amended by Commission Regulation (EC) No 1243/2008 amending Annexes III and VI. From Mc Carthy (2013).

For infant formula produced by non-fat bovine constituents, the crude protein content must be greater than 1.8 g/100 $kcal^{-1}$ (European Commission, 2006; Codex Alimentarius, 2007; Peoples Republic of China, 2010), with formula produced using hydrolysed proteins needing a protein content of greater than 2.25 g/ 100 kcal (European Commission, 2006; Codex Alimentarius, 2007; Peoples Republic of China, 2010).

1.6 Effect of Concentration of Whey in Infant Formula Systems

The relationship between whey protein denaturation, aggregation and in-process viscosity is determined by the concentration, temperature and pH at which the formulation is heat-treated. Formulations containing whey proteins which are heated at low total solids content behave differently than those heated at high solids due to a variety of factors. Murphy et al. (2015) attempted to decouple macronutrient (casein, whey protein, lactose and fat) interactions during heating of model systems, by measuring physical changes in the behaviour of macronutrients, in isolation and combination, over the range of concentrations used during the manufacture of 1st stage infant formula. Addition of casein (as phospho-casein - PCN) to whey protein (as whey protein isolate -WPI) resulted in an increase in β-lg denaturation temperature, with a subsequent increase in the temperature at which viscosity began to significantly increase. Measurement of the increase in viscosity under shear conditions can provide useful data, which is particularly relevant during industrial heat-treatment steps, and can be correlated to the temperature at which the onset of denaturation occurs. Mixtures of whey protein and casein exhibited similar viscosity increase to that of whey protein alone, confirming the important role of whey protein in viscosity development.

1.7 Manufacturing Practices

Infant formulations can be manufactured in either liquid ready-to-feed or powder formats. Both feature a number of key unit operations which are influenced by the physical stability of protein system used. Whey proteins, in particular, have an important role in viscosity development, gelation and ultimately production efficiency during infant formula manufacture, particularly in the case of 1st stage formulae where

the whey to casein ratio is 60:40. The production of powdered infant formula can be carried out by either dry-blending, wet blending, or a combination of both (U.S. Food and Drug Administration, 2003). The wet-blending process is more widely used and is described herein (Figure 1.11). Infant formula manufacturing plants are designed for maximum production efficiency with control measures incorporated to reduce/prevent microbial contamination of the final powder in both the wet and dry processes (Mullane et al., 2007). Most are batch processes which take place in large jacketed mixing vessels and involve entrainment and recirculation with high shear to ensure adequate ingredient mixing before subsequent processing. Once the carbohydrates, fat, protein, and minerals have been dispersed in water, the mix is held under agitation ensuring complete hydration of the powders. Whey ingredients are added after or during lactose addition, at a temperature of ~ 65 °C, so as to buffer with casein prior to mineral addition. The quantity and composition of mineral salts is dependent on the innate levels provided by the protein ingredients, required to achieve the target label claim. Fat soluble vitamins are added to the fat phase while the water-soluble vitamins are incorporated directly into the protein, carbohydrate and fat mix. Blends of vegetable oils (e.g., sunflower, palm, coconut, and soybean) make up the fat proportion of formulations, to best recreate the lipid profile of human milk fat (Bar-Yoseph et al., 2013).

The order of addition of the minerals is carefully carried out to achieve a consistent pH of ~ 6.8 throughout the batch make up and the mix can be adjusted using an alkali or citric acid (Montagne et al., 2009) if required. Control of pH (at ~ 6.6 - 6.9) throughout batch make-up is important as variation in pH can lead to protein aggregation, and fouling during subsequent heat-treatment (McSweeney et al., 2004; Simmons et al., 2007). The infant formula batch, with all ingredients added, can support survival/growth

of microbes, necessitating the use of heat-treatment to ensure safety and shelf life (D'Agostina et al., 2003).

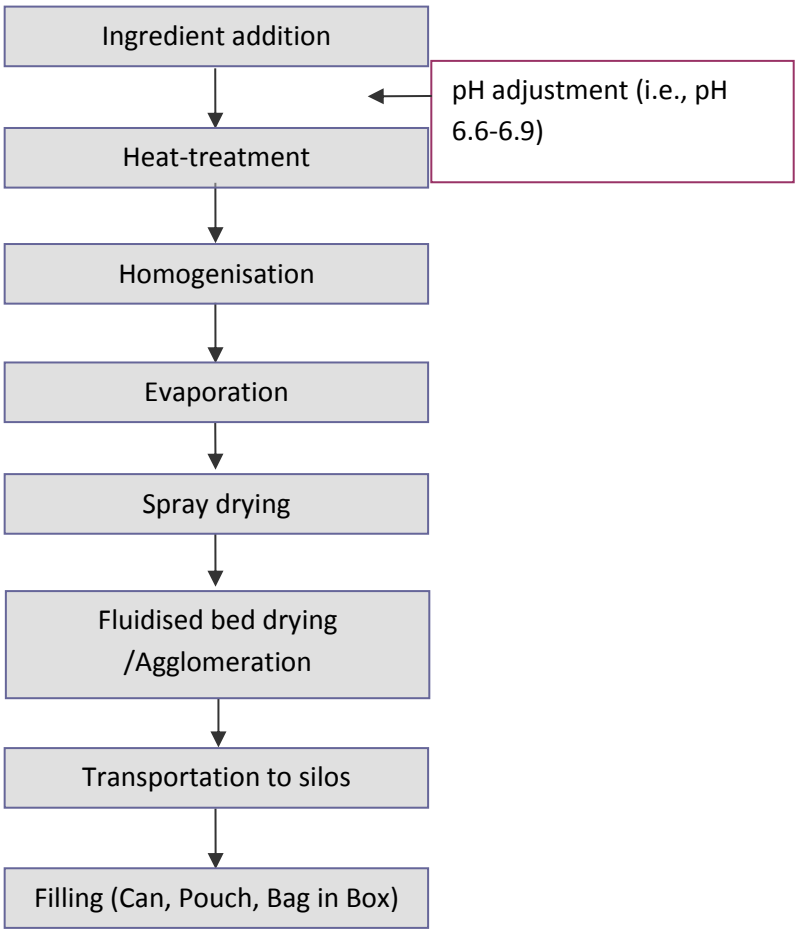


Figure 1.11: A flow diagram of a typical wet-process for infant formula manufacture.

The time/temperature combinations used for processing can vary between manufacturers and often between individual formulae, depending on composition. Traditionally, heat-treatment has been by either ultra-high temperature (UHT) and/or retort sterilization (McSweeney et al., 2004) for liquid products, or high temperature short-time (HTST) treatment prior to spray-drying for powdered formulae (Lönnerdal & Hernell, 1998). Selecting the required heat-treatment is dependent on the balance

between the need to achieve high microbial specification and minimise degradation of nutrients/bioavailability and product quality.

A common method of measuring the nutritional quality of infant formula powders after thermal treatment is the determination of the content of available lysine (D'Agostina et al., 2003). In addition to loss of nutritional value, incorrect heat-treatment of infant formula can have a negative effect on processing efficiency, such as the fouling of heat-exchangers (Fryer et al., 1995) that leads to down-time in processing. Many of these negative effects are related to the thermal history of proteins used in whey dominant formulae. After heat-treatment, the infant formula mix is homogenised and ingredients which are sensitive to heat-treatment i.e. vitamins are added. Fat-soluble vitamins are sometimes pre-encapsulated and, therefore, made to solubilise in water, so that they can be added directly to the mix, before or after homogenisation (Montagne et al., 2009). Typical homogenisation pressures used during manufacture of infant formula are ~ 15 and 5 MPa for first- and second-stage homogenisation, respectively (Liu et al., 2012) at 45 – 66 °C. The stability of the resultant infant formula emulsion is strongly influenced by type of protein system used, and positioning of the homogenisation and heat-treatment steps (Buggy et al., 2017). Homogenisation may take place before or after evaporation, depending on the pre-heat-treatment used, the thermal history of the whey proteins, type of mineral salts added, and the cumulative thermal load of the unit operations during infant formula manufacture. In typical processes, evaporation is used to remove water thus concentrating the solids content of the liquid infant formula mix. Evaporation is a relatively economical and low-energy process compared to spray-drying where water removal is energy-intensive and expensive. The solids content of liquid infant formula concentrates may be increased from ~ 20 - 40% total solids (TS) post-heat-treatment to between 50 and 55% TS after evaporation (Westergaard, 2004).

A number of different evaporators/configurations may be used, from single-effect (stage), to multiple-effect falling-film evaporators. Falling-film evaporators work on the basis that thermal evaporation is carried out under low pressure (i.e., under vacuum), resulting in the boiling off of water at low temperatures, usually 45 - 50 °C (Liu et al., 2012). Evaporation of infant formula mixes causes increases in viscosity and fat globule size, as well as changes to other functional properties (McCarthy et al., 2012). Concentrated product from the evaporator is pumped to the top of a spray dryer, where it is turned into a fine spray, i.e., by atomization. This process converts liquid feed into droplets ~ 10 - 400 µm in size, resulting in an increase in the surface area for evaporation to take place, and improved thermal efficiency of the dryer. A multistage spray-dryer is used for manufacture of infant formula to create powder agglomerates which more readily rehydrate. The evaporated infant formula concentrate is spray-dried to yield a powder with residual moisture content of approximately 2 - 4% (w/w).

Typical air temperatures used for infant formula drying are shown in Table 1.3. At the base of the main drying chamber, the moist particles enter the internal fluidized bed (second-stage drying), where drying air fluidises the product. The powder, with reduced moisture content, then enters an external fluidised vibrating bed for subsequent drying and cooling of the powder (Westergaard, 2004). Key physical attributes of an infant formula concentrate are its viscosity during thermal treatment, emulsion formation and concentration, which are intrinsically linked to functionally of the whey protein ingredients used.

Table 1.3 - Typical air temperatures of plant equipment during infant formula manufacture (Montagne et al., 2009).

	Temperature
Main chamber drying air	180 – 200 °C
Integrated fluid bed air	50 – 60 °C
External fluid bed cooling air	20 – 30 °C
Exhaust air	80 – 100 °C

Data from: Montagne et al. (2009). From: Mc Carthy (2013).

1.8 Conclusions

The physicochemical properties of protein solutions have a significant influence on the structure and stability of individual protein molecules, aggregates and protein adsorbed onto the surface of fat globules during emulsification. However, if protein denaturation and aggregation can be controlled by manipulating these properties, the possibility of creating more concentrated protein systems is viable.

CHAPTER 2

Effect of Mineral Salts on the Physicochemical Properties of Whey Proteins

2.1 Abstract

Whey proteins are widely used in the food industry due to their nutri- and techno-functional properties. Many factors affect the physical stability of whey proteins including pH, ionic strength and temperature. Solutions of whey protein were prepared at different concentrations, i.e., 1, 4, 8, 10, 12 and 16% (w/w). Salts (tri-sodium citrate, calcium citrate and calcium chloride) were added individually to each whey solution at concentrations of 5, 10, 30, 60 and 90 mM. Samples were analysed for changes in pH, turbidity and viscosity. A significant increase ($P < 0.05$) in pH was observed with the addition of tri-sodium citrate, increasing with salt concentration. The opposite effect was noted with addition of calcium chloride. There was no significant difference between control samples containing no mineral salts, and those samples containing calcium citrate, at any protein concentration. Data from rheological analysis showed that there was no significant effect of salt addition on viscosity at fixed protein concentration, although, as expected, an increase in protein concentration resulted in a significant increase ($P < 0.05$) in viscosity. The study demonstrated the effect of pH, protein concentration and ionic strength has on unheated whey protein systems and provides information which is important, pre-processing.

2.2 Introduction

Whey was once considered a waste product, however with the emergence of membrane separation technology, is now recognised as a valuable commodity, primarily sold as a protein concentrate in powder format. Whey protein has a high nutritional value and can provide functionality within foods, such as gel and foam formation, or emulsifying properties (Demetriades et al., 1997; Ramos et al., 2012; Schmitt et al., 2007).

The isoelectric point (pI) of β-lg in its native form is 5.2, with the protein existing as a dimer between pH 5.5 and 7.5, while below pH 3.5 and above pH 7.5, the dimer undergoes dissociation and exists as a monomer. The pI of α-lac is slightly lower at pH 4.8, and as α-lac does not have a free thiol group, it can, in a purified state, undergo partial and sometimes reversible denaturation during heating (Boye et al., 1997a). Although the pI range for whey proteins is between 4.8 - 5.2, the precipitation of denatured whey proteins is usually carried out at pH 4.6 at a temperature of 20 °C (Mullvihill & Donavan, 1987).

Although changes in pH can alter the stability of the soluble phase of whey protein solutions, ionic salts, often introduced to dairy based beverages to increase the nutritional profile i.e., infant formula, and/or help with pH regulation of beverages, can also cause physical changes to occur (Lönnerdal & Hernell, 2016; Ryan & Foegeding, 2015). Many salts added to food formulations contain calcium, sodium and/or citrate ions. In infant formula, a range of mineral salts are added at differing levels to reach specific targets of each individual mineral per 100g of powder [European Union (2006/141/EC)]. Hence, mineral salt addition impacts the pH and ionic strength of the protein solution, which may impact protein surface charge and therefore its colloidal stability. Consequently, the addition of mineral salts to protein systems can cause

precipitation, gelation and fouling of processing surfaces. Calcium in particular, in dairy protein systems, induces aggregation and coagulation. However, it has previously been observed that inclusion of casein in a protein system containing whey protein and a salt (i.e. formation of a casein/whey protein blend can produce a protein system which is more stable against coagulation (Ryan et al., 2012).

The Hofmeister series ranks specific ions on their influence on the physical behaviour of colloidal systems (Zhang & Cremer, 2006). It is based on the classification of ions on their ability of being able to salt in or out proteins, with anions generally accepted as having a more pronounced effect. The Hofmeister series for anionic ions is commonly written as (Bakker, 2008; Xie & Gao, 2013):

$$CO_3^{2-} > SO_4^{2-} > S_2O_3^{2-} > H_2PO_4^{-} > F^{-} > Cl^{-} > Br^{-} > I^{-} \approx NO_3^{-}$$

The classification of these ions was initially based on the ions ability to change the hydrogen bonding network of water, with all species to the left of Cl^- classified as kosmotropes ("water structure makers") and all to the right chaotropes ("water structure breakers") (Collins & Washabaugh, 1985). However, more recent research has challenged this theory suggesting that changes in bulk water with the addition of salts cannot explain specific ion effects through the Hofmeister series (Batchelor et al., 2004; Gurau et al., 2004; Omta et al., 2003). Curtis et al. (2002) measured protein-protein interactions for lysozyme and ovalbumin in concentrated salt solutions, and found that the typical salting-out effect of the Hofmeister series occurred when the binding of ions to the protein surfaces was weak, due to increased hydrophobic attraction. However, the strength of the attraction was dependent on the non-polar fraction of the surface of the protein, and the surface tension of the salt solution. More recent research by Okur et al. (2017) used molecular simulations, in conjunction with experimental work, and

determined that the stronger the attraction between an anion and a protein, the lower the ability of that ion to cause salting-out effects. This phenomenon is dependent on the surface charge of the protein, in particular, the protein backbone and charged side chains, and the strength of their interaction with the specific ion.

Additives such as citrate, EDTA, phosphates etc. can form soluble metal complexes with polyvalent metal ions such as Ca^{2+} and Mg^{2+}, usually forming a ring structure (Lucey & Horne, 2009). In dairy products containing casein micelles, this allows for the movement of insoluble calcium, held within the casein micelle, to be diffused into the serum phase of milk. The addition of large amounts of chelating agents results in a smaller casein micelle diameter with a decrease in casein micelle density (Udabage et al., 2000). The removal of calcium from colloidal calcium phosphate within the casein micelle effectively causes dissociation of the micellar structure (Gaucheron, 2005). In whey based protein systems, addition of these agents can improve heat stability and reduce age gelation and deposition of product on the internal surface of heat exchangers and membrane plants. Different sequestrate agents have different affinities for binding Ca^{2+}. The effectiveness of an agent can be compared by analysing the stability constants for particular metals. The stability constant for a metal complex can be calculated using the following equation:

$$K = \frac{[ML]}{[M][L]} \qquad \text{(Equation 2.1)}$$

Where *M* is the metal ion, *L* is the sequestrant and *ML* is the metal complex (Furia, 1972). If ranked, phosphates and citrates would have the following chelating affinities: log-chain phosphates > tripolyphosphate > pyrophosphate > citrate > orthophosphate (Vanwazer & Callis, 1958). Polyphosphates are anionic in nature, allowing for repulsion of similar charged structures such as proteins above the proteins isoelectric

point. Below the isoelectric point of proteins, precipitation can occur through cation-anion interactions.

This study investigates the effect of ionic salts (calcium, citrate and sodium) on the stability of whey protein systems which have not undergone thermal denaturation giving an insight into whey protein systems pre-processing. The effect of differing salt levels on the physical stability of whey protein at low and high protein concentrations was observed; the effect of both of these parameters on pH, viscosity and turbidity were investigated to better understand the effect of ionic strength and pH on whey protein systems before heat-treatment.

2.3 Materials and Methods

2.3.1 Sample Preparation

Whey protein isolate (WPI; BiPro, Davisco Foods Intl., Inc. Le Sueur, Minnesota, U.S.A.), with a protein content of 93.3% as determined by Kjeldahl, was acquired from Davisco Foods Intl., Inc. Le Sueur, Minnesota, U.S.A. A 32% (w/w) WPI solution, with a native pH of 7.10 ± 0.01 was prepared using distilled water at room temperature (22 °C) with gentle stirring using a magnetic bar for 6 hours. After the powder was rehydrated, the solution was placed at 4 °C overnight and continuously stirred. The following morning, the protein solution was re-equilibrated to room temperature with aliquots used to dilute samples to a protein content of 1, 4, 8, 12 and 16% (w/w). Each sample was adjusted to pH 5.7, 6.2, 6.7, 7.2 or 7.7 using 2 M NaOH or 4 M HCl.

Davisco BiPro alpha and beta powders, with protein concentrations of 89.4% and 86.5% (as determined by Kjeldahl), respectively, were used to prepared samples of α-lac or β-lg. Samples of both proteins were hydrated as outlined above for WPI solutions.

2.3.2 Addition of Salts

Calcium chloride $CaCl_2$, calcium citrate $Ca_3(C_6H_5O_7)_2$ and tri-sodium citrate $Na_3C_6H_5O_7$ were added individually to each of the whey protein solutions (i.e. 1 to 16% (w/w) protein), at the required protein concentration, to formulate samples with salt levels ranging from 0 to 90 mM. A stock solution of 180 mM of each salt was added at the appropriate amount to produce protein/salt solutions with the required levels. The pH of each sample was recorded using a Mettler Toledo S220 SevenCompact pH bench-top meter at room temperature.

2.3.3 Turbidity Measurements

The turbidity of samples was measured using a HACH 2100N turbidity meter by placing 30.0 ml of sample into a 25.0 mm glass turbidity tube and analysed at room temperature. The units of turbidity were expressed as Nephelometric Turbidity Units (NTU).

2.3.4 Rheological Measurements

An AR2000ex controlled-stress Rheometer (TA Instruments, Crawley, UK) fitted with a standard Din and concentric cylinder was used for the analysis of the apparent viscosity of the protein samples. The diameter of the Din and concentric cylinder were 27.5 mm and 30.0 mm respectively with 19 g of sample placed in the cup for analysis. Samples were measured at their respective protein concentrations, 1 to 16% (w/w), using a pre-shear of 200 s^{-1} for 30 s, ramp shear rate from 0 s^{-1} to 1000 s^{-1} over 4 min, holding at 1000s^{-1} for 1 min followed by a shear rate decrease from 1000s^{-1} to 0s^{-1} over 4min. Temperature was kept constant at 22 °C using a Peltier apparatus (± 0.1 °C). All samples were degassed before analysis and a tetradecane solvent trap was used to prevent sample evaporation.

2.3.5 Differential Scanning Calorimetry

Differential scanning calorimetry (DSC) was used to measure the denaturation temperature of β-lg, α-lac and WPI samples. Samples were brought to the required pH before being placed in Tzero Hermetic pans, sealed, and analyzed by a Q2000 DSC (TA Instruments, New Castle, DE, USA) with an approximate sample weight of 20 mg. A ramp rate of 2 °C min^{-1} from 40 °C to 90 °C was used for analysis, with measurements taken under nitrogen purged conditions. The reference used was an empty pan. The

peak minimum of the endotherm post linear integration was taken as the denaturation temperature. Data analysis was performed using Universal Analysis 2000 software (TA Instruments).

2.3.6 Statistical analysis

Minitab® 17 statistical analysis package (Minitab Ltd, Coventry, UK, 2014), was used for analysis of variance (ANOVA; Tukey's HSD). The level of significance was determined at ($P < 0.05$).

2.4 Results and discussion

2.4.1 The Effect of Salts on pH and Turbidity

An increase in whey protein concentration from 1 to 16% (w/w) resulted in an increase in the solution pH of samples from 6.6 to 7.0 respectively (Figure 2.1 A, B, C). The addition of calcium citrate at each protein concentration, up to a salt concentration of 90 mM, did not alter the pH, with no significant difference noted between samples with added mineral salts and the control sample where no salt was added. However, the addition of tri-sodium citrate and calcium chloride did change the pH of the whey protein solutions, and was salt concentration dependant. Addition of tri-sodium citrate, even at a concentration of 5 mM, resulted in a significant ($P < 0.05$) increase in the pH of whey protein solutions at 1% (w/w) protein. With addition of 10 mM tri-sodium citrate and above, a significant ($P < 0.05$) increase in pH was observed for samples at each protein concentration. The opposite effect was noted with addition of calcium chloride, with a significant decrease ($P < 0.05$) in pH observed, with an increase of only 5 mM salt; at a salt concentration of 90 mM, the pH decreased from 7.0 (control) to 6.3.

The impact of additives on pH showed that the effect of citrate on the pH of whey concentrates was dependent on the type of citrate salt added. The addition of calcium citrate did not significantly affect pH, most likely due to calcium citrate having very poor solubility in water, even though it is commonly used as a food additive and/or food flavouring due to its high bioavailability (Pak et al., 1987; Vavrusova et al., 2017). However, during the production of beverages, salts are often used as a way to regulate pH, thus the addition of calcium citrate may be less effective in pH regulation of beverages - although this is dependent on the product type, as a synergistic effect could exist.

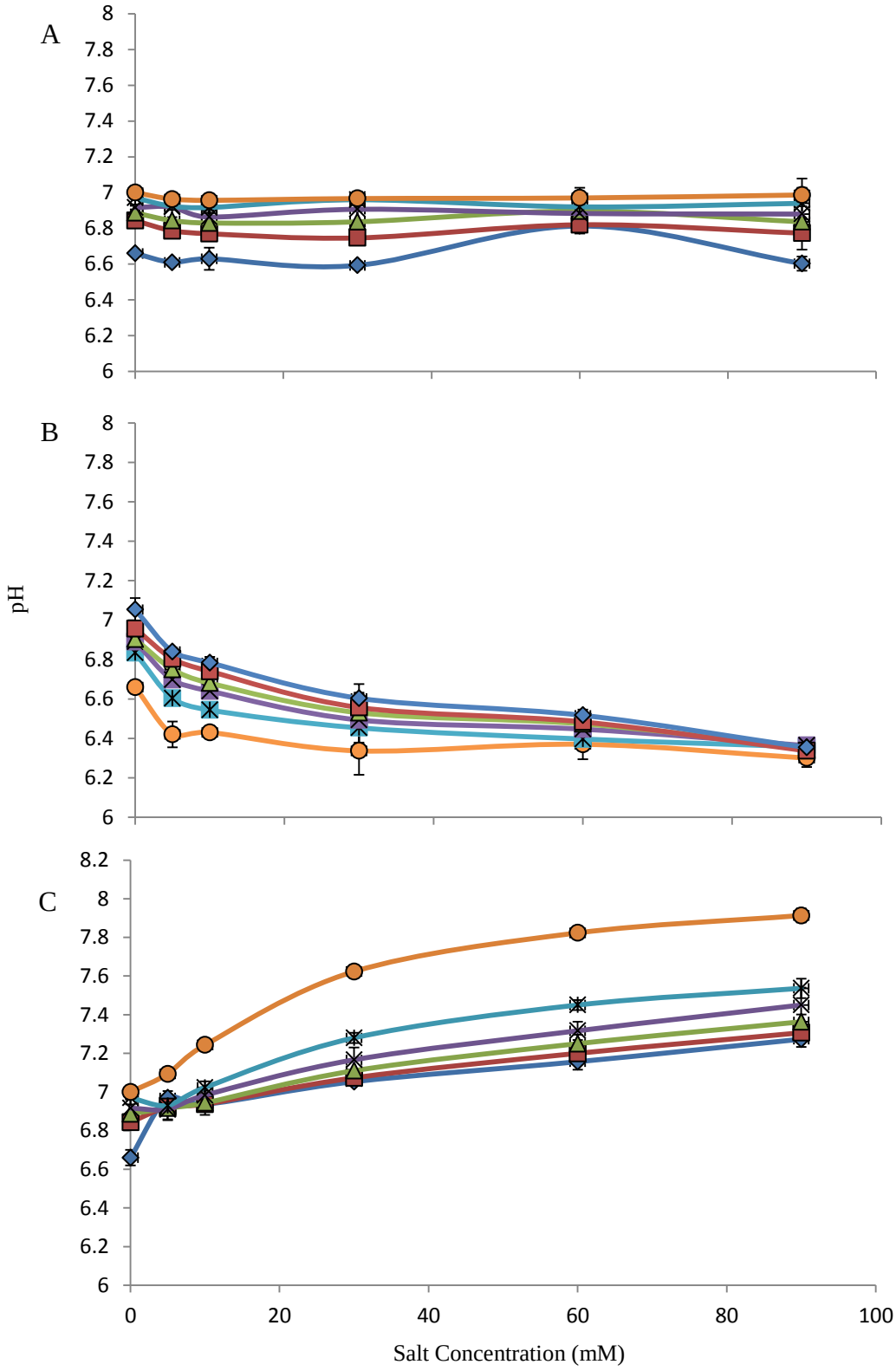


Figure 2.1: The effect of salt concentration, (A) calcium citrate, (B) calcium chloride, (C) tri-sodium citrate and whey protein concentration (1%, ; 4%, ; 8%, ; 10%, ; 12%, ; 16%,) on pH.

Although calcium citrate has poor solubility in water, as polar compounds, tri-sodium citrate and calcium chloride are highly water soluble (Leusbroc et al., 2010; Soleymani et al., 2016). Association of Ca^{2+} ions from calcium chloride, with OH^- ions from water forms calcium hydroxide (medium strength base), allowing for free hydrogen ions to be liberated and associate with the free H^+ ions, forming HCl, causing a decrease in pH. As tri-sodium citrate is a conjugate base of citric acid, when it dissociates in water, sodium hydroxide is formed (causing an increase in pH), allowing for any of the positively charged free hydrogen ions to bind to the negatively charged free citrate molecules. The buffering capacity of whey protein, particularly at lower salt concentrations (10 mM and below), increases with increasing protein concentration.

As expected, an increase in protein concentration resulted in an increase in turbidity at each pH (Figure 2.2). However, at low pH (5.5), the turbidity of samples at 1%, 16% and 32% (w/w) protein was statistically ($P < 0.05$) greater than at pH 6.2, 6.7, 7.2 and 7.7. The same trend is noted for turbidity for samples at pH 6.2, 6.7 and 7.2, with an initial significant ($P < 0.05$) increase in turbidity with increasing protein concentration from 1 to 16%; no further increase was observed from 16 to 32% protein. At pH 7.7, a significant ($P < 0.05$) increase in turbidity was observed at a protein concentration of 32%, when compared against turbidity values obtained at pH 6.2, 6.7 and 7.2. This turbidity increase is most likely due to the unfolding of β-lg due to the Tanford transition of the protein, causing an increase in protein interaction. Above ~pH 7.2, changes in the local confirmation of the EF loop (residues 85-90) results in the opening of the loop which is closed at lower pH values. It is understood that the opening of this loop allows for the binding of ligands (Qin et al., 1998).

As the protein concentration is increased, the proportion of protein molecules which come in contact and/or collision with each other are increased, therefore increasing the potential for protein aggregation (Kessler, 2002). The addition of salts, or change in pH, enhances this potential for aggregation, as either attractive or repulsive interactions can be caused, due to changes in electrostatic potential energy. The strength and range of electrostatic interactions between proteins can be significantly reduced in the presence of electrolytes, due to electrostatic screening by counter-ions (Bryant & McClements, 1998).

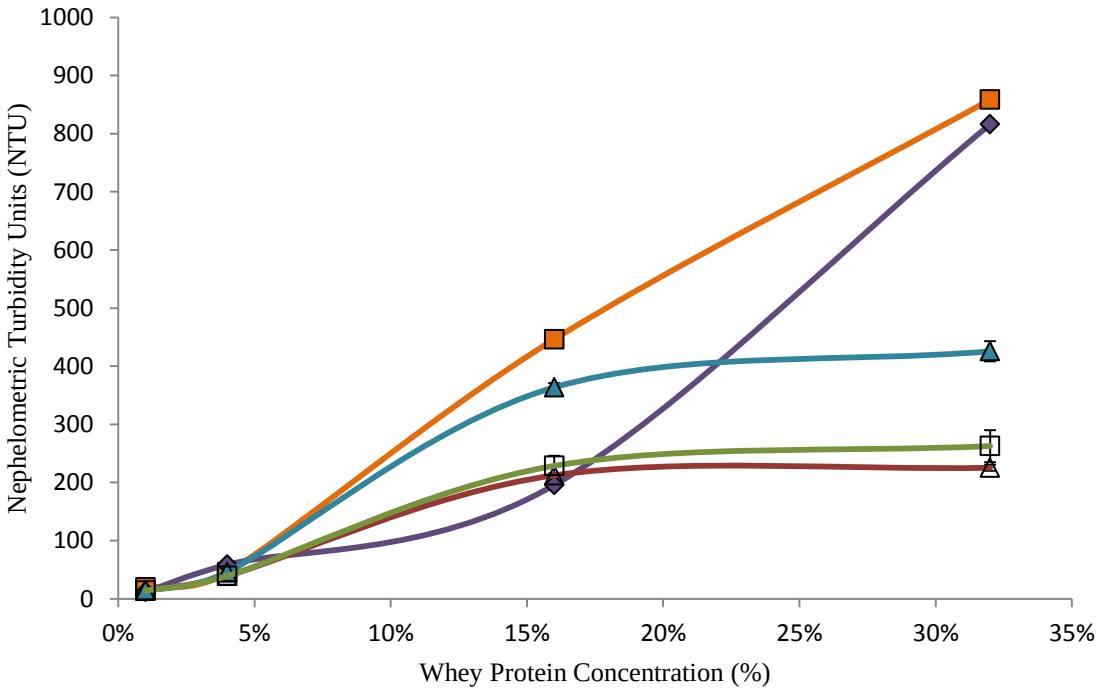


Figure 2.2: The effect of pH (5.7, ■; 6.2, ▲; 6.7, □; 7.2, Δ; 7.7, ♦) and whey protein concentration on turbidity.

2.4.2 Viscosity

The viscosity of α-lac, β-lg (Figure 2.3) and WPI (Figure 2.4) was measured at pH 6.2, 6.7 and 7.2. At 1% protein, no significant difference was observed between the viscosities of α-lac and β-lg. However, at 16% protein, the viscosity of β-lg was marginally greater than for α-lac, although not significantly. For WPI samples, an increase in protein concentration resulted in an increase in viscosity, with pH having no significant effect (Figure 2.4). All samples, including α-lac and β-lg, achieved turbulent flow above a critical shear rate as indicated by the point of inflection shown in Figure 2.3, which increased with increasing protein concentration. Turbulent flow is indicated by the sudden change in viscosity and concomitant increase in shear-thickening, i.e., 'inflection point'. By plotting the critical shear rate at which each protein concentration achieved turbulent flow (Figure 2.4), the relationship between turbulent flow and protein concnetration was observed. Fouling of dairy products within heat exchangers is both costly and problematic, as fouling can cause the formation of burnt particles, burn on and gelation of food proteins (Bansal & Chen, 2006; Guerin et al., 2007). Although heat transfer in heat exchangers is a complex phenomenon, it is understood that a fluid under turbulent flow will produce less fouling than a fluid flowing with laminar flow, depending of the type of heat exchanger used (Kerche et al., 2016). Therefore, knowledge based on the turbulent flow of whey protein solutions before they enter heat exchangers can be of benefit when trying to increase the efficiency of heat transfer within a heat exchanger. Palsson et al. (2013) increased the shear stress within an annular heat exchanger by inducing "swirling flow" of fluid at the beginning of the pipe used for heating. This resulted in the production of shear stress close to the walls of the heat exchanger pipes under laminar flow, although the overall back pressure was reduced.

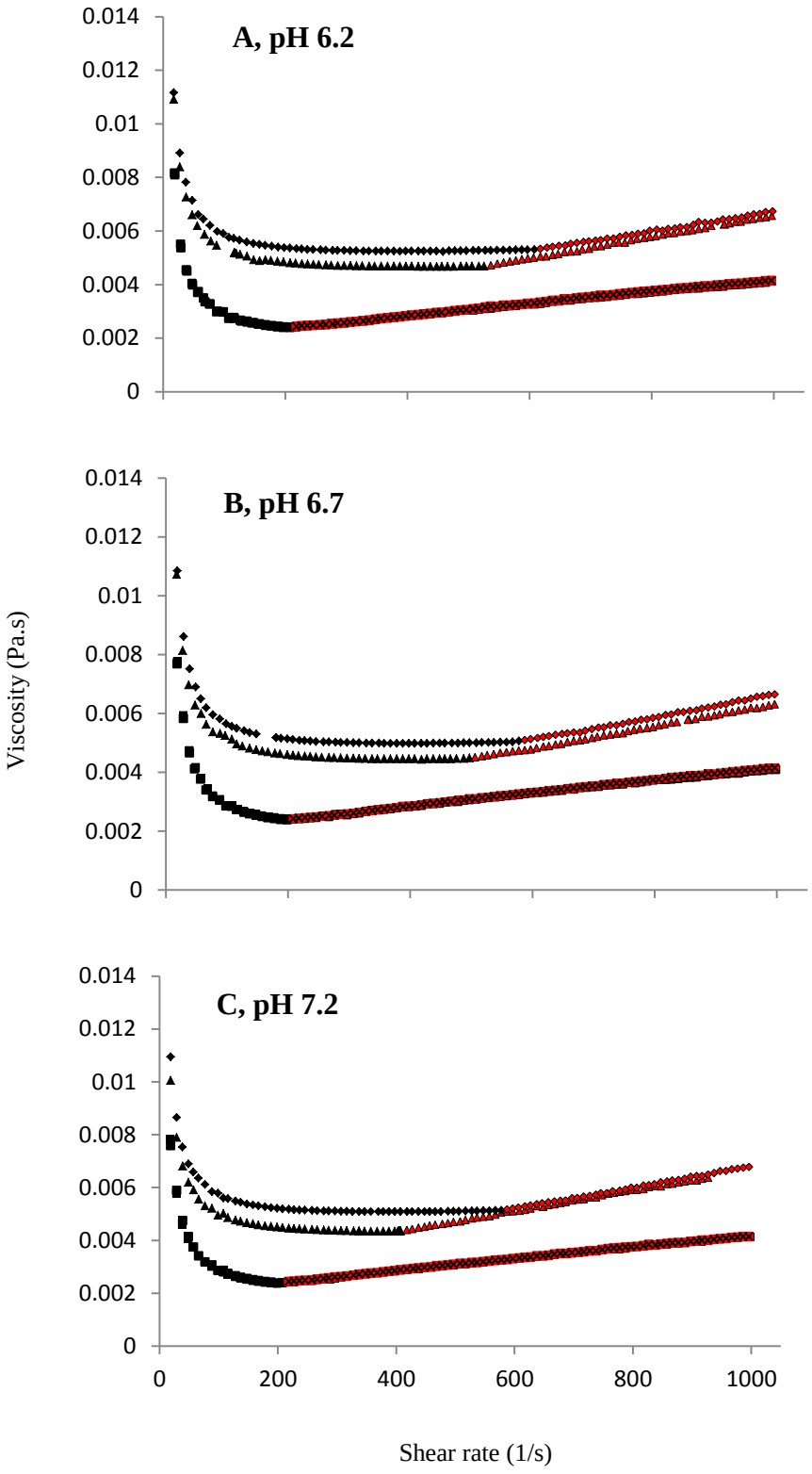


Figure 2.3: Rheology measurements of the viscosity of individual whey proteins (1% β-lactoglobulin (■), 1% α-lactalbumin (×), 16% α-lactalbumin (▲) and 16% β-lactoglobulin (◆) at pH 6.2 (A), 6.7 (B) and 7.2 (C). Symbols in red denounce when each sample has entered into turbulent flow.

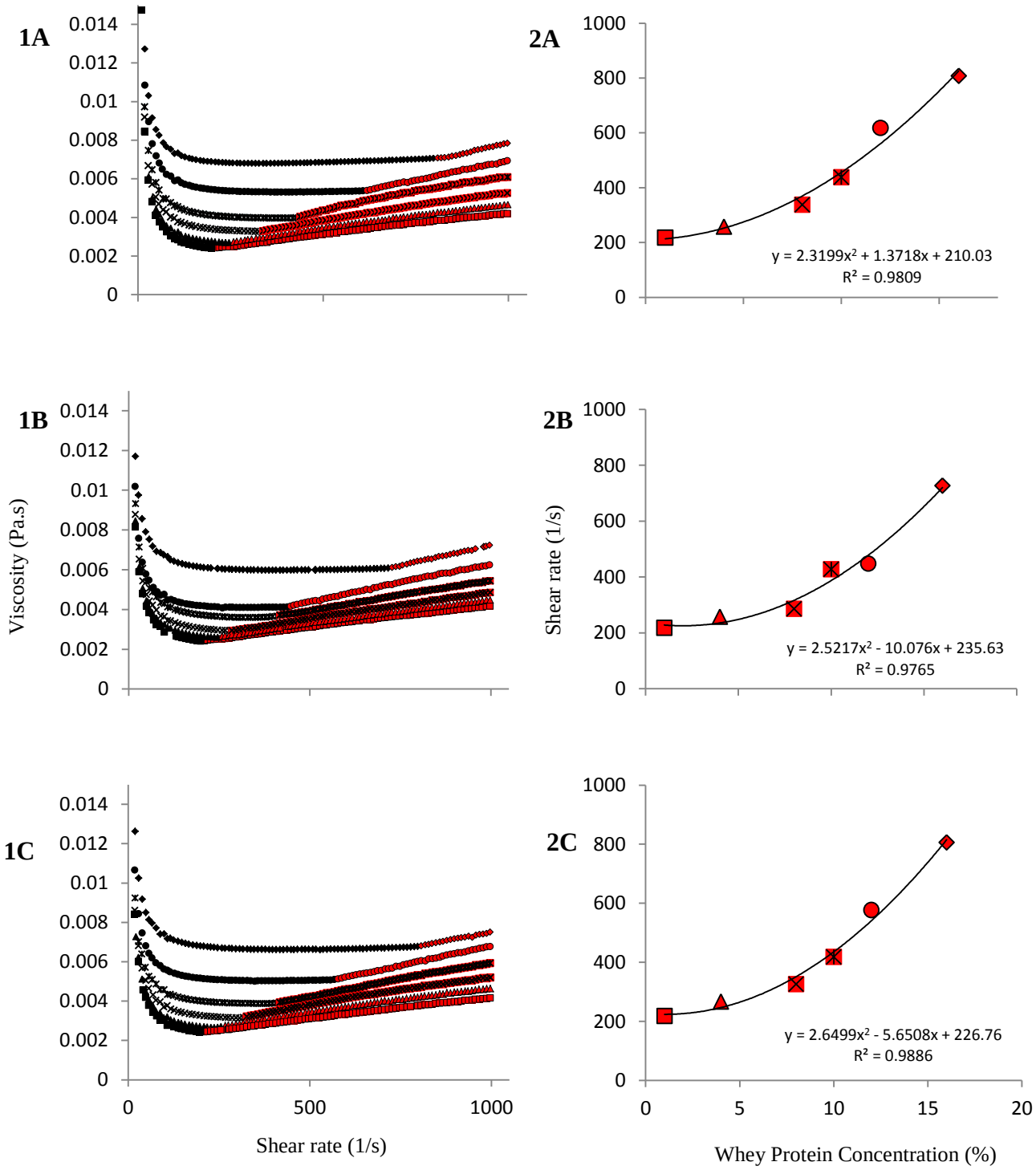


Figure 2.4: Viscosity rheology of WPI concentrations (1) and the polynomial linear plot of the turbulence inflection point (2) of 1 (■), 4 (▲), 8 (×), 10 (∗), 12 (●) and 16 (◆) % at pH 6.2 (A), 6.7 (B) and 7.2 (C). Symbols in red denounce when each sample has entered into turbulent flow.

2.4.3 Denaturation Temperature

A significant ($P < 0.05$) decrease in denaturation temperature was observed with an increase in protein concentration for α-lac samples from 4 to 16% protein (Table 2.1); the same trend was not noted for the denaturation temperature of β-lg samples.

Table 2.1 - The Denaturation temperature of whey proteins at different pH

Concentration & Protein Type	pH	Denaturation Temperature (°C)
4% α-lac	6.7	68.17 ± 0.80^{a}
8% α-lac	6.7	65.72 ± 0.67^{b}
16% α-lac	6.7	65.14 ± 0.17^{ab}
4% β-lg	6.7	77.71 ± 0.93^{a}
8% β-lg	6.7	77.38 ± 0.15^{a}
16% β-lg	6.7	76.55 ± 0.71^{a}
16% WPI - α-lac	6.2	65.94 ± 0.12^{a};
- β-lg		76.59 ± 0.75^{a}
16% WPI - α-lac	6.7	65.34 ± 0.56^{a};
- β-lg		76.27 ± 0.82^{b}
16% WPI - α-lac	7.2	63.43 ± 0.21^{b};
- β-lg		75.84 ± 0.32^{c}
32% WPI - α-lac	6.7	63.12 ± 0.12^{b};
- β-lg		71.92 ± 0.02^{d}
16% α-lac	6.7	62.83 ± 0.20;
:β-lg (1:1)		76.31 ± 0.02^{a}
16% α-lac:β-lg (0.4:1)	6.7	76.14 ± 0.07^{a}

[a–d] Values within a column with different superscripts are statistically different at $P < 0.05$.

However, when both β-g and α-lac denatured in the presence of each other i.e., in WPI, increasing concentration resulted in a significant ($P < 0.05$) decrease in denaturation temperature for both α-lac and β-lg at pH 6.7, when the protein content was increased from 16 to 32%. The pH was also found to have an effect on the denaturation temperature of both whey proteins at 16% WPI, with a decrease in the denaturation temperature observed when pH was increased from 6.2 to 6.7 to 7.2.

Although significant differences were observed for the denaturation temperature of β-lg when α-lac was present, when the two ratios of α-lac:β-lg were analysed ((1:1) and (0.4:1)), no significant difference in the denaturation temperature for β-lg was noted. The denaturation temperature for α-lac could not be compared as the quantity of α-lac in low ratio samples could not be detected within thermographs.

2.5 Conclusions

This study demonstrates the effect of pH, protein concentration and addition of mineral salts on the physiochemical properties of unheated whey protein systems. The turbidity of whey protein solutions increased with increasing protein concentration. Rheological behaviour of the same solutions was characterised by the onset of turbulent flow during viscosity measurements, i.e., critical shear rate above which viscosity increased rapidly, an effect which was found to be dependent on protein concentration. Denaturation temperature of whey protein solutions decreased with increasing protein concentration. The results show the relevant impact that pH, protein concentration and salt has on unheated whey protein systems and provides useful insight into considerations which need to be examined before thermal processing of whey protein systems.

CHAPTER 3

Pilot-scale Formation of Whey Protein Aggregates Determine the Stability of Heat-Treated Whey Protein Solutions – Effect of pH and Protein Concentration

3.1 Abstract

Denaturation and consequent aggregation in whey protein solutions is an important factor that impacts on product functionality during subsequent industrial processing. Solutions of whey protein isolate (WPI) prepared at 1, 4, 8 and 12% (w/w) and pH 6.2, 6.7 or 7.2, were subjected to heat-treatment (85 °C × 30 s) using a pilot-scale heat exchanger. The effects of heat-treatment on whey protein denaturation and subsequent aggregation were determined by chromatography, particle size, turbidity and rheological analyses. The influence of pH and WPI concentration during heat-treatment on subsequent thermal stability was also investigated. WPI solutions heated at pH 6.2 were more extensively denatured, had a greater proportion of insoluble aggregates, higher particle size and turbidity, and were significantly ($P < 0.05$) less heat stable than equivalent samples prepared at pH 6.7 and 7.2. The effects of WPI concentration on denaturation and subsequent aggregation behaviour were more apparent at higher pH, where the stabilising effects of charge repulsion became increasingly influential. Solutions containing 12% (w/w) WPI had significantly ($P < 0.05$) higher apparent viscosities, at each pH, compared to lower protein concentrations, with solutions prepared at pH 6.2 forming a gel. Smaller average particle size and a higher proportion of soluble aggregates in WPI solutions, pre-heated at pH 6.7 and 7.2, resulted in improved thermal stability on subsequent heating. Higher pH during secondary heating also increased thermal stability. This study offers insight into the interactive effects of pH and whey protein concentration during pilot-scale processing and demonstrates how protein functionality can be controlled through manipulation of these factors.

3.2 Introduction

Dairy proteins, and in particular, whey proteins, have been the subject of extensive research due to their excellent nutritional properties, amino acid profile and functional properties; and are used in the production and manufacture of nutritional beverages, including infant formula, sports and lifestyle foods (Holt et al., 2013; Playne et al., 2003). The primary whey proteins in milk are β-lactoglobulin (β-lg) and α-lactalbumin (α-lac) and their ability to undergo structural changes during heating is of particular importance in influencing subsequent aggregation. Thermal treatment is a critical processing step during the manufacture of most foods and beverages for reduction of microbial load. However, the thermal stability of whey proteins is relatively low, with the formation of whey protein aggregates during heat-treatment leading to changes in viscosity, turbidity, particle size, protein precipitation and gel formation (Ryan et al., 2012).

Both α-lac and β-lg are compact globular proteins, with molecular weights of ~ 14 kDa and ~ 18 kDa, respectively; α-lac is a monomer at neutral pH while β-lg exists as a dimer between pH 5.5 and 7.5 (Mullvihill & Donavan, 1987). The mechanism leading to protein aggregation following thermal denaturation is dependent on a number of factors i.e. protein type and concentration, pH, ionic strength, heating temperature and type of heat-treatment (Dissanayake et al., 2013; Kehoe & Foegeding, 2011). A single monomer of β-lg contains two disulfide bonds, with one buried free sulfhydryl (SH) group (Verheul et al., 1998). This free SH group can become exposed during denaturation/unfolding and aggregate with other β-lg monomers and dimers through disulfide interchange reactions, resulting in the exposure of another free SH group; the reaction is terminated when two β-lg monomers or aggregates form a disulfide linkage between their exposed SH groups (Roefs & de Kruif, 1994). However, unlike β-lg, α-lac

does not contain any free SH groups, and therefore, in the absence of β-lg, can only aggregate through non-covalent interactions such as electrostatic and/or hydrophobic and van der Waals interactions (Anema, 2009; Roth, 1996). Previous studies have observed that due to the lack of a free SH group, aggregation of the native α-lac monomer can be reversible, depending on physicochemical and environmental conditions (Boye et al., 1997a). β-lg aggregation, with protein species containing a free SH group and/or disulfide bridges, is not reversible. Previous research into whey protein denaturation and aggregation has focused on the formation of aggregates through heat-treatment of purified whey proteins, often at low concentrations, using lab scale heat-treatments, which can be slow to reach final heating temperatures (Croguennec et al., 2004; Galani & Apenten, 1999; Verheul et al., 1998). However, for industrial applications, scientific research using whey protein concentrates (WPCs) and whey protein isolates (WPIs) at high concentrations are applicable, as these ingredients are similar to the types of ingredients used in the manufacture of infant formula and nutritional beverage products. Previous work has also been carried out, albeit less extensively, using pilot scale heating equipment, typically in the heat-treatment of skim milk (Corredig & Dalgleish, 1996; Oldfield et al., 1998), and to a lesser extent, WPC and WPI (Erabit et al., 2016; Petit, 2013). Studying the behavior of whey proteins under relevant processing conditions is fundamental to the understanding and optimization of the variables required for the production of stable and viable whey protein products.

The objective of this study was to examine the effects of protein concentration and pH on heat-induced whey protein denaturation and subsequent aggregation, and determine whether ensuing soluble aggregate content and size, along with remaining native protein, affect thermal stability of solutions upon subsequent heating. The findings of

this study should give further insights into factors affecting the thermal stability of whey proteins during the processing of dairy-based beverages, including infant formula.

3.3 Materials and Methods

3.3.1 Preperations of Protein Solutions

Whey protein isolate (WPI, (BIPRO^{TM})), with protein content of 93.3%, as determined by Kjeldahl (IDF, 2001), was sourced from Davisco Foods Intl., Inc. (Le Sueur, Minn., U.S.A.) The mineral content of 0.05% P, 0.04% K, 0.08% Ca and 0.66% Na, was analyzed by ion coupling plasma mass spectroscopy (ICPMS). Whey protein solutions (2L) of 1, 4, 8 and 12% (w/w) (total protein) were prepared by hydration of WPI in distilled water and stirred at room temperature (22 °C) for approximately 2 hours, before overnight storage at 4 °C, under gentle agitation, to unsure full rehydration of the powder. Solutions were then re-equilibrated to room temperature and adjusted to the required pH (6.2, 6.7 or 7.2) using 2M NaOH or 4M HCl. Control (unheated) samples were separated from each batch with the remaining solution used for further processing. Heat-treatment of solutions was carried out using a MicroThermics Lab scale tubular heat exchanger (MicroThermics, North Carolina, U.S.A.) with a set flow rate of 1 L/min and holding-tube volume of 0.5 L. Solutions were pre-heated to a temperature of 65 °C, with a final heating temperature of 85 °C for 30 seconds, with subsequent cooling to 15 °C. Same day analysis was carried out for measurements of aggregate morphology and size to minimize any change to aggregates over time. Each sample batch was replicated four times. Throughout this publication, samples will be coded as follows: e.g., H1-6.7 represents a 1% WPI solution heated at pH 6.7.

3.3.2 Quantification of Native Protein

Reversed phase HPLC was used to quantify remaining native protein, in unheated and heat-treated samples, using a Waters 2487 dual wavelength absorbance detector, at wavelengths of 214 nm and 280 nm. A Source™ 5RPC (150 ×4.6 mm) column (Sigma Aldrich, Wicklow, Ireland) was used for separation using solvent A (0.1%

trifluoroacetic acid in MilliQ water) and solvent B (90% Acetonitrile, 0.1% TFA) at a flow rate of 1 mL min^{-1}. Gradient conditions for elution were described by Kehoe et al. (2011). Samples were diluted to a protein content of 0.25% (w/w), in 0.1 M sodium acetate buffer at pH 4.6, before centrifugation at 20,000 x g (25 °C) for 25 mins. Supernatants were filtered through a 0.22 μm (high-velocity filters, Millipore (UK) Ltd., Durham, UK) before injection. β-Lactoglobulin, α-lactalbumin and BSA standards (Sigma Aldrich, Ireland) were used for column calibration. Data was processed using Waters Empower® software.

3.3.3 Differential Scanning Calorimetry

Denaturation temperatures of α-lac and β-lg were determined by differential scanning calorimetry (DSC). Aliquots of control and heat-treated samples (20 mg) were placed in Tzero Hermetic pans, sealed, and analysed by a Q2000 DSC (TA Instruments, New Castle, DE, USA). The temperature was ramped from 20 to 100 °C at a rate of 5 °C min^{-1} under nitrogen purged conditions. An empty pan was used as a reference for heat flow to the sample, and denaturation temperatures expressed as the peak minima of the endotherms following post-linear integration. Data analysis was carried out using Universal Analysis 2000 software (TA Instruments).

3.3.4 Determination of Molecular Weight of Soluble Aggregates

The formation of soluble aggregates was determined by size exclusion chromatography (SEC), using a Waters 2695 separation module HPLC system. Heated and control samples were diluted to 0.25% (w/w) protein in 20 mM sodium phosphate buffer at pH 7.0 (mobile phase). Filtration of samples though 0.45 μm low protein binding filters (Sartorius Stedim Biotech GmbH, Germany) ensured removal of larger, non-soluble aggregates. A TSK Gel $G2000SW_{xL}$ run in series with a $G3000SW_{XL}$, 7.8 x 300 mm column (TosoHaas Bioscience GmbH, Stuttgart, Germany), under isocratic conditions,

at a flow rate of 0.5 ml min^{-1} over 1 hour, was used to elute 20 µl of sample to a Waters 2487 dual wavelength absorbance detector at wavelengths of 214 nm and 280 nm. The following molecular weight standards were used for column calibration: cytochrome c (12 kDa), α-lactalbumin (~ 14 kDa), β-lactoglobulin (~ 18 kDa), carbonic anhydrase (29 kDa) ferritin (44 kDa), bovine serum albumin (~ 66k kDa) and aldolase (158 kDa). Data analysis and integration were carried out using Waters Empower® software. HPLC grade MilliQ water was used in the preparation of all buffers and samples. Buffers were vacuum filtered through 0.45 µm high velocity filters (Millipore (UK) Ltd., Durham, U.K.) before analysis.

3.3.5 Particle Size Analysis

Following heat-treatment, the z-average hydrodynamic diameter was determined by Dynamic Light Scattering (DLS) using a Zetasizer Nano ZS (Malvern Instruments, Malvern, UK) with a HeNe laser, with a wavelength of 633 nm, and measured at a scattering angle of 173 °. Samples of H4, H8 and H12 were diluted to 1% (w/w) using distilled water, before further dilution to 1 in 30 in order to avoid multiple scattering, and measured in a plastic cuvette at 22 °C. The volume distribution was determined using the cumulative method.

3.3.6 Measurement of Turbidity

The absorbance of heated and unheated samples was measured using a HACH 2100N turbidity meter at room temperature (22 °C), using 30.0 mL sample in a 25.0 mm glass turbidity tube. The turbidity of samples was expressed in Nephelometric Turbidity Units (NTU).

3.3.7 Atomic Force Microscopy

Aggregates formed by heat-treatment of 1 and 12% (w/w) WPI at pH 6.7, were imaged using atomic force microscopy (AFM). Samples were prepared at a protein concentration of 0.01% protein, deposited onto a surface of freshly cleaved mica, which was fixed to a glass microscopy slide and dried in an incubator at 38 °C, before storage in a desiccator (relative humidity ~ 19%) prior to analysis. Samples were analysed using an Asylum Research MFP- 3DAFM (Asylum Research UK Ltd., Oxford, UK). Images were recorded in AC mode as described previously by Kehoe et al. (2011). All images were processed using AFM imaging software Igor 6.12A with 3D images compiled using Argyle Light software.

3.3.8 Heat Coagulation Time as a Function of pH

Aliquots of H1-6.7 and H4-6.7 were concentrated to 6% (w/w) by dialysis against a polyethylene glycol (PEG) solution (8% (w/w)) using dialysis tubing with a 5 kDa molecular weight cut-off). PEG was used for removal of water from the protein solutions through concentration by osmotic stressing (Bouchoux et al., 2009). The protein content of H8-6.7 and H12-6.7 was reduced to 6% (w/w) using MilliQ water.

Aliquots (10 mL) of WPI at 6% (w/w) protein were adjusted to create a pH range from 6.4 to 7.2 with 0.1 pH unit increments using 0.1 M HCl and 0.1 M NaOH. Samples were adjusted initially and stirred for 4 hours before final readjustment. 3.4g of each sample was placed into a 4 ml glass tube (120 mm tube length, 10 mm outer radius, 7 mm inner radius; Hettich Benelux BV, Geldermalsen, the Netherlands). Each tube was stoppered using a rubber bung, placed in a steel rack and submerged in a temperature controlled silicone oil bath (Hettich ESP oilbaths; Hettich Benelux BV) at 140 °C and rocked back and forth at a speed of 7 oscillations per min. Heat stability was determined

as the amount of time (min) required for visible flocculation or gelation of proteins to occur. Analysis of heat stability was performed in triplicate.

Aliquots of H4, H8 and H12 WPI at pH 6.2, 6.7 and 7.2 were also analyzed using the same Heat Coagulation Time (HCT) method, with each sample standardized to 4% protein, adjusted to create a pH curve ranging from 6.4 to 7.2, with 0.2 pH unit increments.

3.3.9 Rheological Measurements

An AR2000ex controlled-stress Rheometer (TA Instruments, Crawley, UK) fitted with a standard DIN concentric cylinder and cup was used for the analysis of apparent viscosity. The diameters of the bob and cup were 27.5 mm and 30.0 mm respectively. Heat-treated (85 °C x 30s) whey protein samples of (1, 4, 8 and 12% (w/w)) were measured using a shear rate sweep from 0 to 800 s^{-1} over 4 min, holding at 800 s^{-1} for 1 min, followed by a shear rate decrease from 800 to 0 s^{-1}, over 4 min. Samples were pre-sheared at 200 s^{-1} for 30 s prior to analysis. Temperature was maintained at 22 °C using a Peltier apparatus (± 0.1 °C). All samples were free of air bubbles and surface foam before analysis and a tetradecane solvent trap was used to prevent sample evaporation.

Small-amplitude oscillatory shear measurements were carried out using an ARG2 controlled stress rheometer (TA Instruments, Crawley, UK) for the analysis of thermal gelation. A standard DIN concentric cylinder was used as per viscosity measurements. Frequency measurements and strain were kept constant at 0.1 Hz and 0.5% respectively, with samples pre-heated to 45 °C for 5 min. The temperature was then increased from 45 °C to 80 °C, at a rate of 5 °C/min, and held for 90 min at 80 °C.

3.3.10 Stastical Analysis

Minitab® 17 statistical analysis package (Minitab Ltd, Coventry, UK, 2014), was used for analysis of variance (ANOVA; Tukey's HSD). The level of significance was determined at ($P < 0.05$).

3.4 Results and Discussion

3.4.1 Protein Denaturation

The amount of native protein in heated and unheated solutions, at a protein concentration of 1, 4, 8 and 12%, and pH of 6.2, 6.7 and 7.2, was quantified using RP-HPLC. Samples were diluted in sodium acetate buffer at pH 4.6 and large protein aggregates removed by sedimentation by centrifugation at 20,000 g for 25 min. Samples were subsequently injected onto the HPLC column after filtering. Table 3.1 shows the amount of native protein present in unheated control samples, and samples heat-treated at 85 °C for 30 s. A significant ($P < 0.05$) loss in native protein was observed for all samples after heat-treatment, compared to the unheated control sample. An increase in protein concentration from H1 to H12 resulted in a decrease in the quantity of native protein. Kehoe et al. (2011) showed previously that an increase in protein concentration resulted in more extensive aggregation in mixed whey protein systems. At higher protein concentration, there is an increase in the collision rate between protein molecules, as the rate of collision is proportional to the number of primary particles (Kessler, 2002; Wolz & Kulozik, 2015).

Table 3.1 also shows the amount of native α-lac, β-lac A and β-lac B protein present in samples as a function of both protein concentration and pH. β-lg A and B are the two most common variants of bovine β-lg, although other variants also exist (Sawyer, 2013). Historically, it has been reported that β-lg B is more heat labile compared to β-lg A, and undergoes greater levels of denaturation upon heat-treatment at pH 6.8 (Gough & Jenness, 1962). However, more recent research has shown that the heat stability of both β-lg variants is dependent on a number of factors including protein concentration and pH. Nielsen et al. (1996) observed that β-lg samples, heat-treated at protein

Table 3.1 - RP-HPLC results for individual and total native protein in unheated and heat-treated samples prepared at 1, 4, 8, and 12% (w/w) at pH (6.2, 6.7, 7.2).

Protein (%)		Native α-lac (%)	Native β-lg A (%)	Native β-lg B (%)	Total Native Protein (%)
Unheated		25.83 ± 1.0[a]	43.71 ± 0.4[a]	30.46 ± 1.0[a]	100 ± 1.8[a]
Heated	6.2				
1		12.52 ± 0.9[b]	21.58 ± 1.7[cd]	12.58 ± 4.6[de]	46.48 ± 2.7[cd]
4		9.88 ± 1.7[bc]	23.25 ± 0.1[c]	13.32 ± 3.6[de]	46.45 ± 1.9[cd]
8		11.01 ± 3.3[b]	13.40 ± 2.9[fgh]	7.25 ± 2.6[fg]	31.66 ± 4.9[ef]
12		10.77 ± 1.9[bc]	8.78 ± 4.0[h]	8.77 ± 2.6[fg]	28.03 ± 6.1[fg]
	6.7				
1		7.81 ± 1.8[bc]	28.77 ± 3.6[b]	14.85 ± 0.9[d]	51.44 ± 3.9[bc]
4		1.44 ± 0.8[cd]	12.24 ± 0.5[fgh]	26.33 ± 2.5[b]	39.01 ± 3.4[de]
8		11.24 ± 2.5[b]	15.13 ± 3.7[ef]	6.95 ± 4.0[fg]	33.32 ± 3.1[ef]
12		5.62 ± 1.0[cd]	9.60 ± 2.3[gh]	4.25 ± 2.4[g]	19.48 ± 1.8[g]
	7.2				
1		10.23 ± 2.3[bc]	30.67 ± 0.5[b]	19.47 ± 0.9[c]	60.37 ± 6.2[b]
4		7.88 ± 1.5[bc]	17.02 ± 1.0[def]	20.23 ± 1.4[c]	45.14 ± 2.4[cd]
8		9.54 ± 3.3[bc]	14.17 ± 3.3[fg]	4.95 ± 2.6[fg]	28.65 ± 3.4[fg]
12		8.37 ± 2.8[bc]	19.84 ± 2.3[cde]	9.02 ± 2.6[ef]	37.23 ± 4.3[def]

[a–h] Values within a column with different superscripts are statistically different at $P < 0.05$.

concentrations below 5% (w/w) (pH 7.0), showed more rapid denaturation of β-lg B, whereas β-lg A was more heat sensitive at protein concentrations above 5% (w/w). O'Kennedy & Mounsey (2006) reported preferential denaturation of β-lg A at acidic pH (pH 5.0 – 5.5) and β-lg B at more neutral pH (6.5 - 7.0). In the current study, β-lg A was generally more heat stable, although this effect was less pronounced at pH 6.2. The proportion of native β-lg A remaining was lower at higher protein concentration (H8 and H12), as supported by the findings of by Nielsen et al. (1996), although samples still contained a greater proportion of native protein when compared to β-lg B. Qin et al. (1999) studied the structures of β-lg A and B and found that although the two variants differ only at two amino acid sites, 64 and 118, (variant A has an aspartic acid residue at position 64 and a valine residue at 118, while variant B has a glycine and alanine residue at position 64 and 118, respectively), β-lg B is less thermally stable due to differences in structure on the β-strand (V118A), which alters the dynamic properties of the molecule by disrupting the internal hydrophobic packing. In comparison to β-lg and its variants, the percentage of native α-lac remaining in heat-treated samples decreased with increasing pH. Law & Leaver (2000) studied the effect of pH on whey protein denaturation in skim milk and found that the rates of denaturation and aggregation of α-lac increases with increasing pH.

The denaturation temperatures of α-lac and β-lg were determined from DSC measurements. Although reversibility of protein denaturation has been previously observed using proteins such as lysozyme (Blumlein & McManus, 2013), no evidence of reversibility was observed in DSC thermographs of unheated samples following a second heating cycle (data not shown). The denaturation temperature of α-lac and β-lg corresponded with previous research, with temperatures ranging from 64.5 - 66.5 °C and 75.5 - 78.5 °C, respectively (Figure 3.1). At pH 6.2 and 6.7, there was a significant

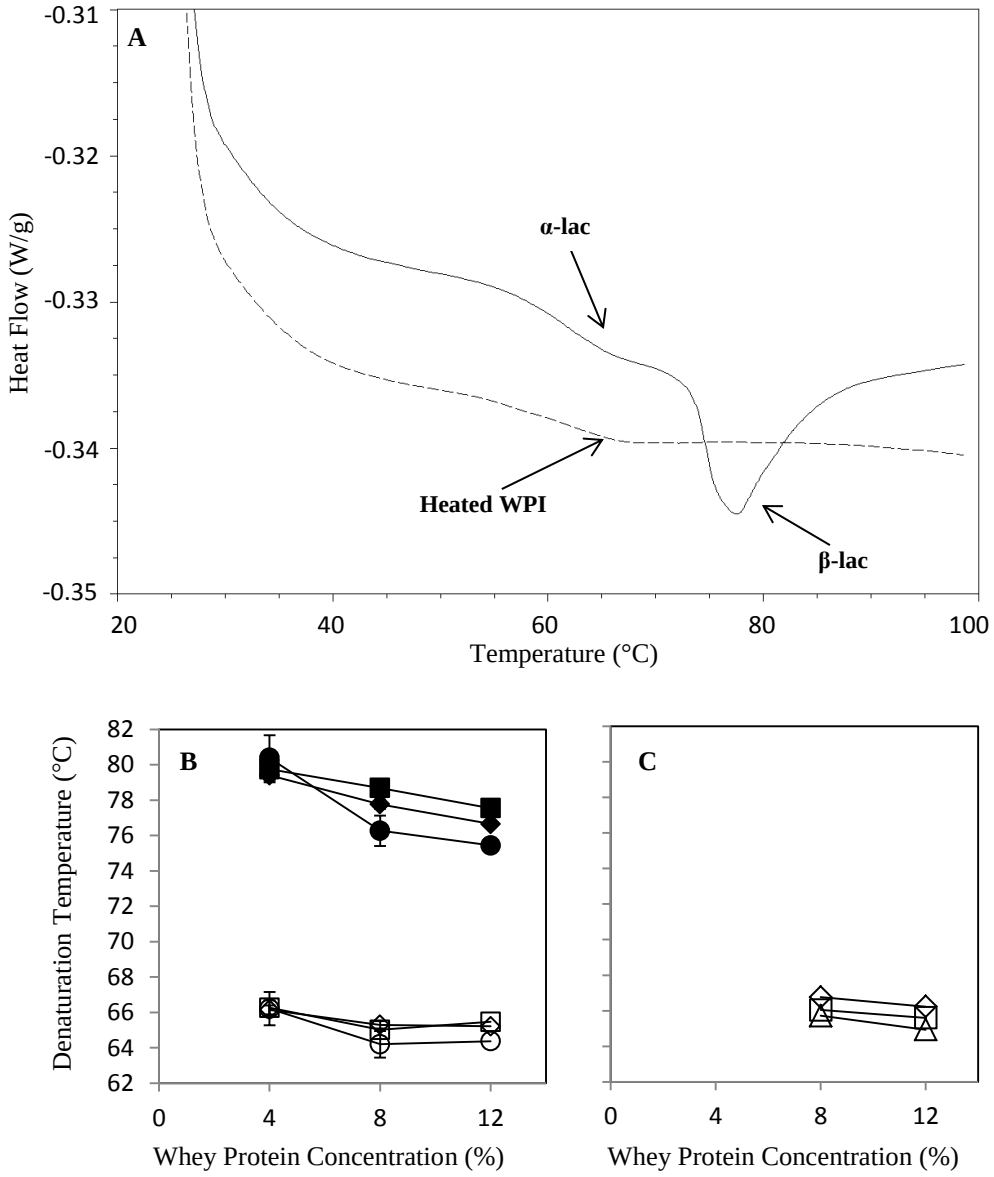


Figure 3.1: Differential Scanning Calorimetry (DSC) for heated (85 °C x 30s) and unheated WPI. Graph A represents an example of heated and unheated 12% WPI solutions at pH 6.7 and displays the endothermic peaks for α-lactalbumin, β-lactoglobulin and heated mixed protein system heated from 20 to 100 °C at a heating rate of 5 °C/min. Graph B represents the denaturation temperature of α-lactalbumin (open symbols) and β-lactoglobulin (closed symbols) at pH 6.2 (□,■), 6.7 (◇,◆), pH 7.2 (○,●) of unheated WPI samples at 4, 8 and 12%. Graph C represents the denaturation temperature of heated WPI samples (8 and 12%) at pH 6.2 (◇), pH 6.7 (□), pH 7.2 (△).

($P < 0.05$) decrease in the denaturation temperature of β-lg at higher whey protein concentrations (8 to 12%).

For pre-heated solutions (85 °C x 30 s), a decrease in denaturation temperature occurred, although the decrease occurred at a lower denaturation temperature, and was not statistically significant. It has previously been observed that increasing the protein concentration of β-lg results in a decrease in its denaturation temperature (Haug et al., 2009; Qi et al., 1995), with comparable results also observed in mixed whey protein systems (Boye & Alli, 2000; Murphy et al., 2014).

3.4.2 Characterisation of Aggregates Formed

3.4.2.1 Size Exclusion Chromatography

Size Exclusion Chromatography HPLC (SEC-HPLC) was used to quantify the molecular weight (M_w) distribution of soluble aggregates produced during heating. Aggregates too large to pass though the SEC columns, i.e., insoluble aggregated material, were excluded by prior filtration through 0.45 μm filters. This resulted in a maximum molecular weight range cut-off for soluble aggregates between ~ 1 kDa and ~ 600 kDa. Figure 3.2, (A) depicts a typical chromatogram of unheated and heated (85 °C x 30 s) WPI. Figure 3.2, (B) shows both the quantity of insoluble protein and the percentage distribution of soluble aggregates detected by SEC-HPLC. Although an effect of protein concentration was evident for samples heat-treated at pH 6.7 and 7.2, this effect was not observed for samples heat-treated at pH 6.2. No significant difference was observed in the quantity of insoluble material or soluble aggregates formed at this pH. It appears that the interactive effects of pH and protein concentration are dominated by diminution of charge repulsion effects at the lower pH examined. In contrast, samples heat-treated at pH 6.7 and 7.2 showed a more pronounced protein

concentration effect, with an increase in soluble aggregate formation in the M_w range of 280 - 600kDa, with increasing protein concentration, and a concomitant decrease in lower M_w species (< 24 kDa).

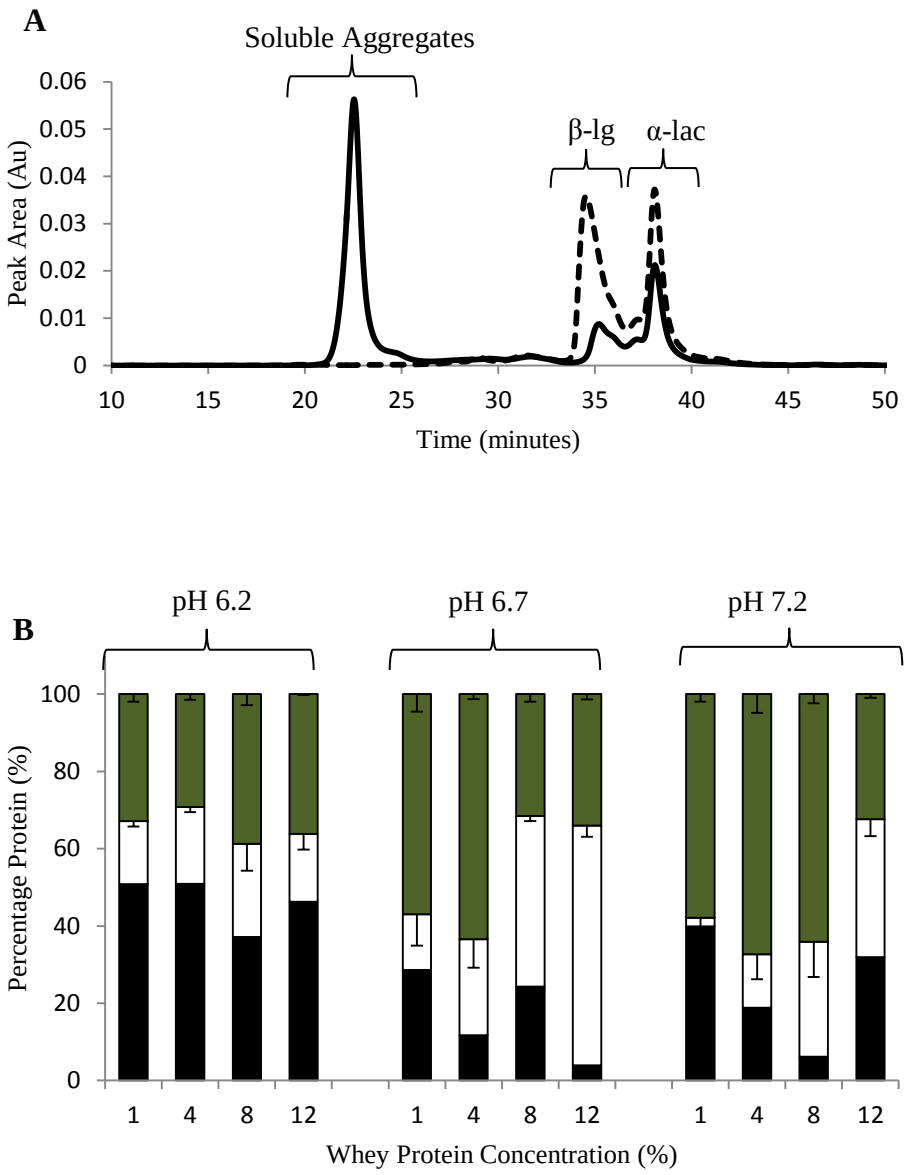


Figure 3.2: (A) A representative SEC-HPLC chromatogram of an unheated (control – broken line) and heat-treated (85 °C x 30 s – unbroken line) whey protein sample at 8% (w/w) at pH 6.7. (B) M_w distribution of soluble protein aggregates and insoluble material as determined by size exclusion chromatography after samples (1, 4, 8 and 12% (w/w)) underwent heat-treatment of 85 °C x 30 s at pH 6.2, 6.7 and 7.2. Distribution represented as: insoluble material ■, ≤280 ▭, 280 to 600 ▬ kDa.

These findings are in agreement with previous measurements of soluble aggregate formation in β-lg solutions. Mehalebi et al. (2008) also observed an increase in the formation of large soluble aggregates with increasing β-lg concentration (pH 8.0), and a decrease at pH 6.0. In contrast, Schmitt et al. (2007) reported that 1% whey protein, heat-treated at 85 °C for 15 min, resulted in fewer soluble aggregates with increasing pH. This was attributed to reduced protein interactions as a result of increased overall negative charge. Ryan et al. (2013) reported that small, compact and highly charged soluble aggregates are resistant to changes in ionic strength and can improve the thermal stability of whey protein-containing beverages during heat-treatment.

3.4.2.2 Dynamic Light Scattering

The particle size of aggregates in unfiltered solutions, as determined by dynamic light scattering (DLS), was significantly ($P < 0.05$) different at each WPI concentration (H1, H4, H8 and H12) heat-treated at pH 6.7 and 7.2. Increasing protein concentration, (from H1 to H8) resulted in a significant decrease ($P < 0.05$) in z-average size (Figure 3.3, A). The z-average diameter, determined from a cumulants analysis, is based on the measurement of the mean by the scattered light intensity, in combination with sample polydispersity. The polydispersity index (PdI) is used as a measure of the breadth of the molecular weight distribution of polymer samples (Rane & Choi, 2005). All particle size distributions measured in the experimental work presented had a single peak distribution and was therefore monomodal in distribution. However, it was noted that the PdI for samples of H1-6.2 and H4-6.2 were lower than the equivalent samples heat-treated at pH 6.7 or 7.2 (Table 3.2). This result suggests that although these samples were had similar distributions, they contained less different size classes of particle in their distribution compared to the equivalent samples heat-treated at pH 6.7 or 7.2.

Samples heat-treated at pH 6.2, overall, had a greater z-average particle size than samples heat-treated at higher pH. The isoelectric point of α-lac and β-lg is in the range of pH 4.6 - 5.2 depending on the conformation of the individual protein (native/non-native) (El-Salam et al., 2009); in comparison, the pH of rehydrated WPI solutions (dependent on protein concentration) is typically in the range of pH 6.6 to 7.0 with fresh raw milk having a pH of 6.8 (Mullvihill & Donavan, 1987).

Table 3.2 - Polydispersity Index of each heat-treated (85 °C x 30 s) whey protein solution, prepared at 1, 4, 8, and 12% (w/w) at pH (6.2, 6.7, 7.2), as determined by Dynamic Light Scattering.

Protein content	pH	Polydispersity
(%)	6.2	
1		0.06 ± 0.01^{a}
4		0.06 ± 0.01^{b}
8		0.17 ± 0.03^{c}
12		0.34 ± 0.05^{c}
	6.7	
1		0.10 ± 0.03^{a}
4		0.08 ± 0.01^{a}
8		0.25 ± 0.02^{b}
12		0.25 ± 0.01^{b}
	7.2	
1		0.23 ± 0.02^{a}
4		0.29 ± 0.06^{a}
8		0.27 ± 0.01^{a}
12		0.26 ± 0.01^{a}

[a–c] Values within a column (each individual pH) with different superscripts are statistically different at $P < 0.05$.

A decrease in pH towards the isoelectric point causes a reduction in overall net negative charge (at pH above the pI) and increases the probability of protein aggregates forming.

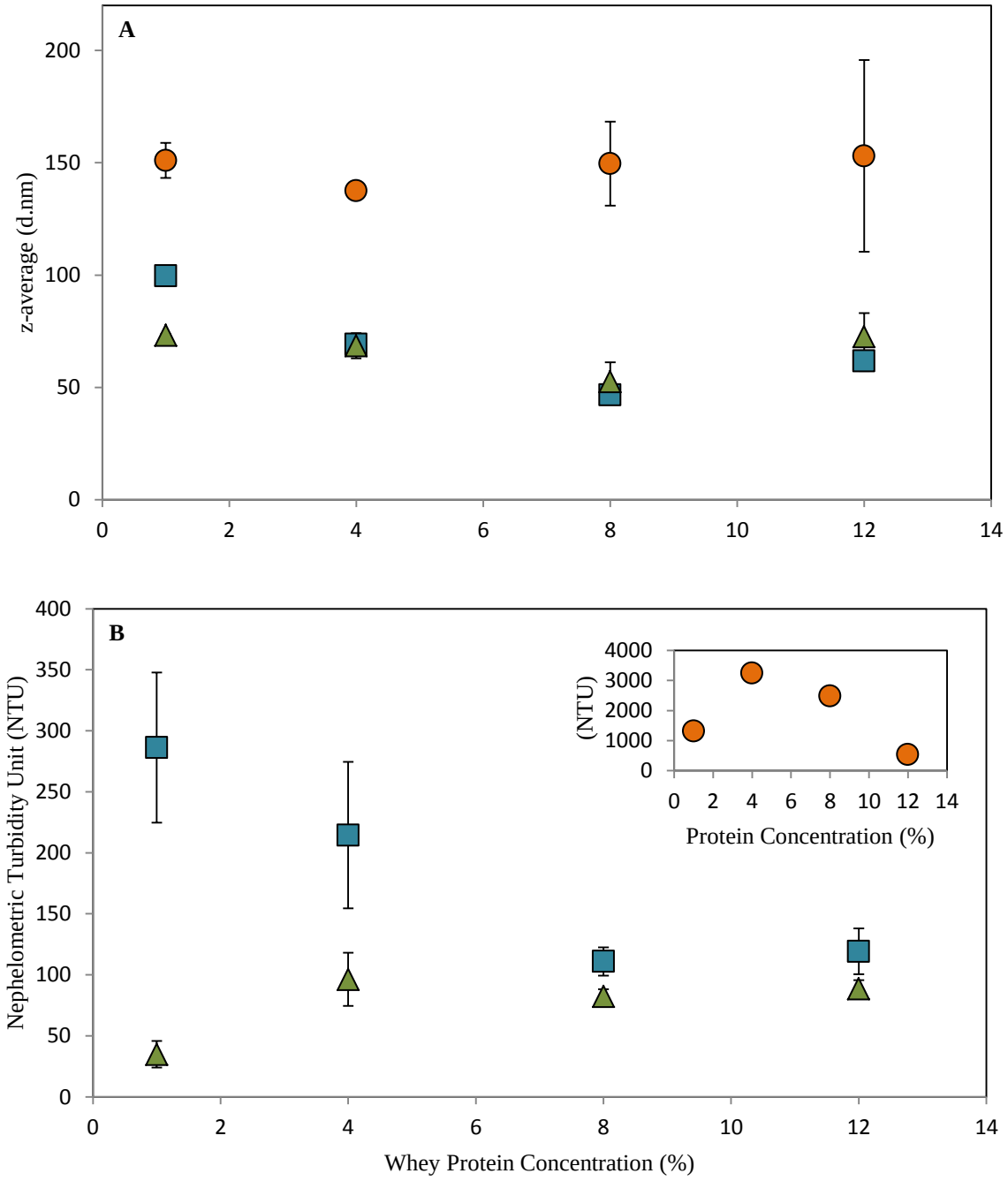


Figure 3.3: Particle size and turbidity measurements of heated whey protein samples (1, 4, 8 and 12% (w/w) protein heat-treated at 85 °C x 30 s). Graph (A) represents the particle size of the whey protein samples at different concentration; pH 6.2 (●), pH 6.7 (■), pH 7.2 (▲). Graph (B) represents the turbidity of whey protein samples at different concentration; pH 6.2 (●), pH 6.7 (■), pH 7.2 (▲). The insert in graph B represents the turbidity of samples heat-treated at pH 6.2.

Increased association of proteins can ultimately lead to the formation of covalently bonded aggregates (Brodkorb et al., 2016), and an increase in the average particle size of aggregates, compared to whey proteins heat-treated at a pH further away from the isoelectric point (Cornacchia et al., 2014).

A probable charge repulsion effect is observed in samples heat-treated at 1% protein, with significant differences ($P < 0.05$) in the z-average of particles formed at pH 6.2, 6.7 and 7.2. These differences may reflect a greater contribution of pH at lower protein concentration towards aggregate formation, due to differences in net negative charge at each pH. As pH 6.2 is the closest pH to the isoelectric point of both α-lac and β-lg, it is not as highly charged as protein molecules at pH 6.7 or 7.2 and therefore is not as resistant to aggregation as whey proteins at higher pH. The probability of interactions between protein molecules occurring in a 1% solution is lower than in an equivalent solution of 12%. In this case, pH dominates interactive forces to a greater extent than protein concentration. SEC-HPLC results support the observation, as it is clear that pH effects dominate at lower pH, and protein concentration dominates at higher pH.

3.4.2.3 Turbidity

The turbidity of heat-treated whey protein solutions varied as a function of both pH and protein concentration (Figure 3.3, B); a positive relationship was evident between the z-average particle size distribution, and turbidity, for samples heat-treated at pH 6.7. A significant decrease ($P < 0.05$) in turbidity at pH 6.7 was observed with an increase in protein concentration from H1 to H8. A significant increase ($P < 0.05$) in turbidity was also observed at each protein concentration, when samples heat-treated at pH 6.7 and 7.2 are compared to samples heat-treated at pH 6.2 (insert of Figure 3.3, B). Again,

when compared against results for DLS, samples heat-treated at 1% (pH 6.7 and 7.2) had significantly higher ($P < 0.05$) turbidity, with a greater effect of pH at this protein concentration due to the electrostatic repulsions previously mentioned. From turbidity and DLS analysis, it is apparent that samples can be categorized into two groups, the first of which contains larger particles and are more turbid samples (samples heat-treated at pH6.2) and the second of which contains smaller particles and are less turbid samples (samples heat-treated at higher protein concentration at pH 6.7 and 7.2).

3.4.2.4 AFM

AFM was used to better visualize the size, shape and polydispersity of aggregates formed in H1-6.7 and H12-6.7 solutions (Figure 3.4). A positive correlation was observed between the size and height (y-axis) of large aggregates in AFM images, and the z-average diameters determined by DLS (Figure 3.4, A, 4). Drying of particles in air during the AFM preparative process is likely to affect both particle size and shape, and means that the aggregate size determined by AFM is somewhat qualitative. Nevertheless, AFM images demonstrated differences in aggregate morphology and size distribution, as a function of protein concentration. The 3D images (Figure 3.4, 3) show the morphology and shape of individual aggregates, with H1-6.7 having a smoother appearance than aggregates formed at H12-6.7. However, this result must be taken with caution as the individual aggregates observed by AFM may not be representative of all aggregates formed during the heating process.

The polydispersity of samples also appeared different within AFM images. PdI values for samples H12-6.7 and H1-6.7 were 0.25 ± 0.01 and 0.10 ± 0.03, respectively (Table 3.2). Samples H1-6.7 appeared to be more uniform (mono-disperse) in appearance, which corresponds to its lower PdI value.

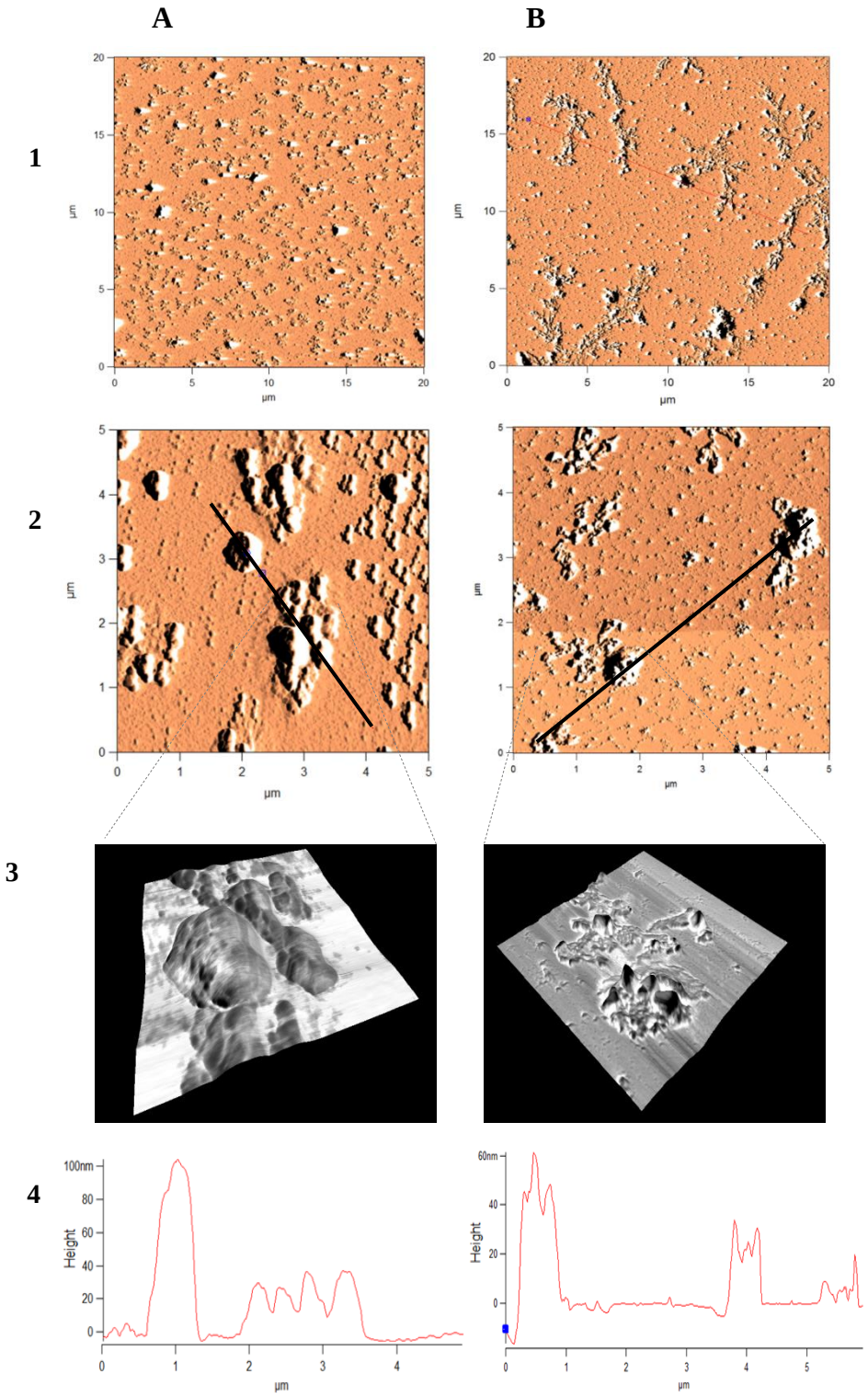


Figure 3.4: AFM images (recorded in air) of aggregates formed after heat-treatment at pH 6.7 (85 °C x 30 s) of (A) 1% whey protein and (B) 12% whey protein. Amplitude images of areas of 20 µm x 20 µm and 5 µm x 5 µm are shown in image 1 and 2, respectively, with 3D images of individual aggregates given in image 3. The bottom graph (4) shows the height profile of the cross section displayed in image 2.

When compared to samples H12-6.7, a more distinctive network structure can be observed, with aggregates clustered in often linear fragments indicating a greater extent of particulate interaction.

3.4.3 Heat Stability as a Function of Aggregate Size and pH

Correlation between aggregates formed from heat-treatment of H4, H8 and H12, at pH 6.2, 6.7 and 7.2 (standardized to 4% (w/w) protein) and the thermal stability of heat-treated solutions, was carried out using the Heat Coagulation Time (HCT) method over a pH range from pH 6.4 – 7.2 (Table 3.3). The HCT method analyses the heat stability of liquid samples by placing glass tubes (rotated) in a silicone oil bath at a set temperature (140 °C) until visual aggregation is observed. Solutions heat-treated at 1% (w/w) protein were very heat stable. No aggregation was apparent after 40 minutes, which was chosen as an arbitrary cut off point for HCT in this experimental work.

It is apparent from Table 3.3 that an increase in pH resulted in an increase in HCT, when samples are compared at the pH at which they were pre-heat-treated at (85 °C x 30 s). This effect is more evident as the pH is increased from 6.4 - 7.2 during the HCT experiment. For samples previously heat-treated at pH 6.2, higher protein concentration resulted in an increase in HCT. In contrast, samples previously heat-treated at pH 6.7 and 7.2 generally tended to show an opposite trend. Samples previously heat-treated at pH 6.7 and 7.2 were deemed thermally stable on subsequent heating at pH 7 and 6.8, respectively. When correlated with DLS results, solutions heat-treated at H8 and H12 at pH 6.7 and 7.2 also had statistically ($P < 0.05$) lower particle size and a higher ($P < 0.05$) proportion of large soluble aggregates (280 to 600 kDa), compared to respective samples with prior heat-treatment at pH 6.2.

Table 3.3 - Heat Coagulation Time data of pre-heated (85 °C x 30 s) whey protein solutions prepared at 4, 8, and 12% (w/w) at pH (6.2, 6.7, 7.2) after being placed in a silicone oil bath at 140 °C.

Protein Content	Heat Coagulation Time (min)		
	pH of Heat-Treatment		
	6.2	6.7	7.2
(%)	pH 6.4 [#]		
4%	13.50 ± 3^{b}	18.24 ± 1^{a}	21.74 ± 2^{ab}
8%	20.44 ± 1^{a}	16.73 ± 0.5^{a}	23.42 ± 1^{a}
12%	17.18 ± 2^{a}	13.22 ± 1^{b}	18.66 ± 2^{b}
	pH 6.6 [#]		
4%	15.05 ± 1^{b}	15.77 ± 1^{a}	21.36 ± 7^{a}
8%	22.86 ± 2^{a}	22.70 ± 1^{b}	15.70 ± 1^{b}
12%	24.34 ± 0^{a}	23.03 ± 1^{c}	23.13 ± 1^{a}
	pH 6.8 [#]		
4%	18.67 ± 0.7^{c}	31.13 ± 6^{a}	32.9 ± 6^{a}
8%	35.74 ± 1^{a}	25.11 ± 1^{b}	$> 40^{*}$
12%	20.58 ± 1^{b}	27.25 ± 1^{ab}	31.17 ± 1^{a}
	pH 7.0 [#]		
4%	22.34 ± 1^{c}	$> 40^{*}$	$> 40^{*}$
8%	29.52 ± 1^{a}	35.08 ± 1^{a}	$> 40^{*}$
12%	28.27 ± 2^{a}	30.67 ± 0.7^{b}	$> 40^{*}$
	pH 7.2 [#]		
4%	22.93 ± 2^{c}	$> 40^{*}$	$> 40^{*}$
8%	38.34 ± 0^{a}	$> 40^{*}$	$> 40^{*}$
12%	31.17 ± 1^{b}	14.45 ± 1	$> 40^{*}$

[a–c] Values within a column (each individual pH) with different superscripts are statistically different at $P < 0.05$.

[*] Samples were stable for up to 40minutes after which the HCT method was interrupted.

[#] pH at which previously heated samples were buffered to before HCT analysis.

Typically, the pH range between 6.8 - 7.2 is a target pH for the manufacture of nutritional beverages containing whey ingredients, including infant formula. The maximum HCT observed in this study also lay within this pH range.

To further understand the influence of whey protein denaturation and subsequent aggregation on the heat stability of a protein solution, HCT analysis was also performed on samples heat-treated at pH 6.7 and protein concentrations of (1, 4, 8 and 12% (w/w)). Samples were then standardized to 6% (w/w) protein, which allowed for the inclusion of pre-heat-treated 1% (w/w) samples, the protein concentration of which was too dilute for visual detection in previous HCT analysis. At pH 6.4, H1-6.7 solutions were significantly ($P < 0.05$) more stable than H8-6.7 samples (Figure 3.5).

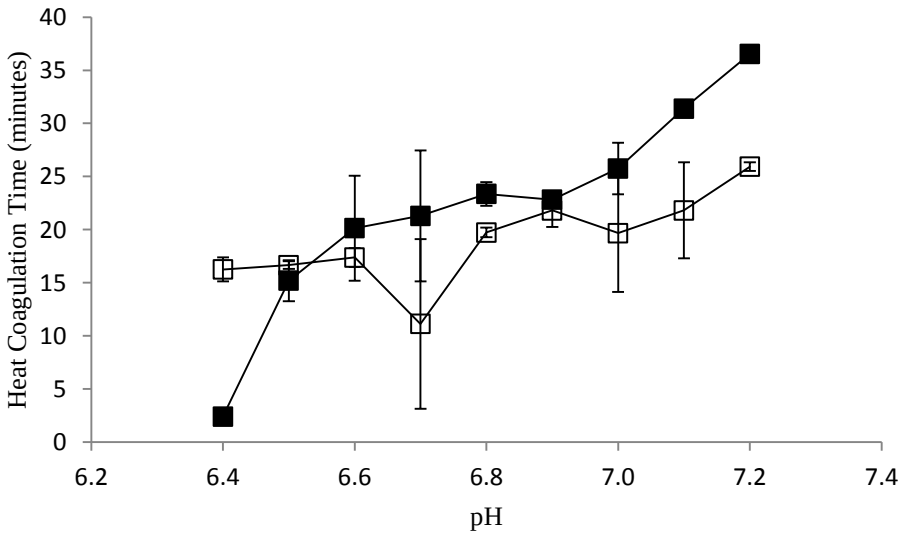


Figure 3.5: Heat coagulation time (HCT) curves for samples of 1% (□) and 8% (■) whey protein solutions, heat-treated at pH 6.7 at 85 °C (x 30 s). Samples used for HCT analysis were standardized to 6% (w/w) protein prior to HCT measurements. HCT was carried out in a silicone oil bath at 140 °C.

However, when the pH of both H1-6.7 and H8-6.7 solutions was increased to 7.0 or above, H8-6.7 solutions had significantly greater ($P < 0.05$) heat stability, with an overall maximum stability of 36.5 minutes. Recent research has focused on the use of

soluble whey protein aggregates to stabilise whey protein systems, in particular model systems, for the production of high concentration whey protein beverages (Ryan and Foegeding, 2015). From SEC-HPLC results it was observed that there were significantly ($P < 0.05$) greater quantities of large-sized soluble aggregates (280 to 600 kDa) for samples H8-6.7 compared to H1-6.7 samples, and when combined with the stabilising effect of pH, helped produce more heat stable whey protein systems at pH values greater than 7.0.

3.4.4 Rheological Measurements

The apparent viscosity of WPI dispersions was analysed immediately post-processing. Measurements demonstrated that an increase in protein concentration resulted in an increase in viscosity, with pH having no significant effect (Figure 3.6). Results obtained were similar to those of previous research carried out on non-Newtonian WPI solutions (Bazinet et al., 2004). H12 samples had significantly higher ($P < 0.05$) apparent viscosity than dispersions prepared at lower protein concentrations. By increasing protein concentration, a greater quantity of protein aggregates are formed upon heating, leading to greater particle interactions, resultant in higher viscosities (Verheul et al., 1998; Kessler, 2002).

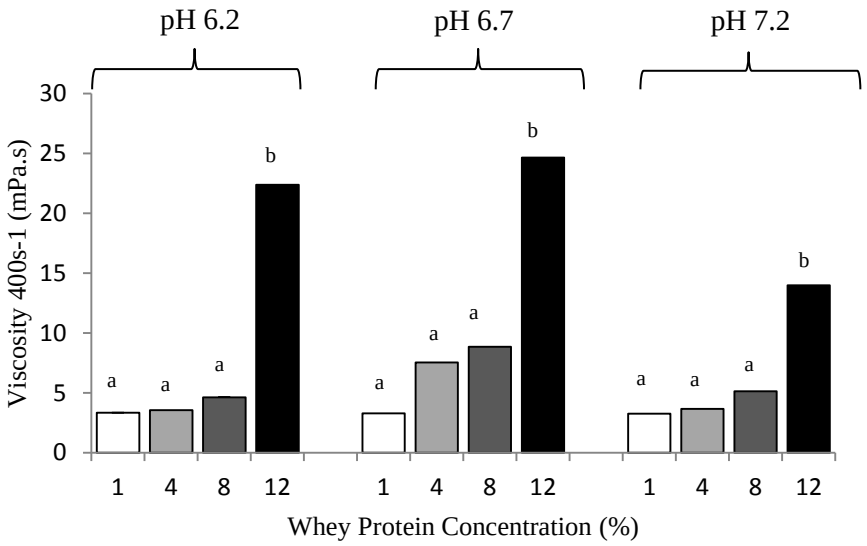


Figure 3.6: Apparent viscosity of heat-treated whey protein samples (85 °C x 30 s) at corresponding pH (6.2, 6.7 and 7.2). Sample points displayed were taken at 400 s^{-1} at 25 °C. Letters differing within each pH differ significantly ($P < 0.05$).

Oscillatory rheology (data not shown), used to measure gel formation and strength (after heat-treatment at 80 °C for 90 min), showed that only H12-6.2 dispersions underwent gelation upon prolonged heating. Whey protein gels formed between pH 4.0 and 6.0 are sometimes referred to as aggregate or particulate gels, as opposed to fine stranded gels, which are formed at higher pH (Stading & Hermansson, 1990). Gelation of this specific sample was most likely due to the quantity and type of aggregates produced following heat-treatment. In order for gel formation to occur, certain parameters must be met in relation to the pH and ionic strength. A critical protein concentration and heating temperature are normally needed, depending on environmental conditions, with both of these parameters tending to increase on moving away from the isoelectric point (Lazidis et al., 2016).

3.5 Conclusions

This study demonstrates that both protein concentration and pH affect the size of whey protein aggregates formed during pilot-scale thermal treatment. A greater proportion of smaller aggregates, formed by heat-treatment of 8 and 12% whey protein solutions (pH 6.7 and 7.2), offered greater heat stability at pH 7.0 upon subsequent heat-treatment (HCT analysis) when compared to larger aggregates formed at lower whey protein concentration (1%). The presence of soluble aggregates, in conjunction with a smaller overall class size, appeared to produce more heat stable whey protein systems. These results demonstrate that is possible to produce more thermally-stable whey protein products when the pH and protein concentration of heat-treated WPI solutions are carefully controlled.

CHAPTER 4

Stabilising Effect of α-lactalbumin on Concentrated Infant Milk Formula Emulsions Heat-treated Pre- or Post- Homogenisation

4.1 Abstract

Protein type and/or heat-treatment pre- or post-homogenisation can affect the physical stability of infant formulations during manufacture. Previous research has described the use of α-lactalbumin addition in infant formulae, but has not demonstrated the effect of heating pre- or post-emulsion formulation during processing. The objective of this study was to evaluate the effect of both of these parameters. Three batches of model 1st stage infant formula, containing differing whey protein ratios, (60:40 whey:casein with α-lactalbumin content 12, 30 or 48% of total protein) were prepared. Each batch was split; one half receiving heat-treatment pre-homogenisation and the second half homogenised and then heat-treated. Emulsion stability was determined using LumiFuge centrifugation with emulsion behaviour investigated through the use of size exclusion chromatography, SDS-PAGE, particle size and viscosity. There was a significant ($P < 0.05$) reduction in the formation of large soluble aggregates upon increasing α-lactalbumin concentration in emulsions heat-treated either before, or after, homogenisation. Heat-treatment of formulations post-homogenisation resulted in a higher ($P < 0.05$) particle size distribution; increasing α-lactalbumin concentration to 30 or 48% significantly ($P < 0.05$) reduced the particle size distribution in these emulsions. The viscosity of concentrates (55% total solids) containing 12% α-lactalbumin, heat-treated post- homogenisation, were significantly greater ($P < 0.05$) than the equivalent emulsions heat-treated pre-homogenisation; increasing the α-lactalbumin concentration to 30 or 48% significantly ($P < 0.05$) reduced viscosity. When the α-lactalbumin content was increased to 48% as a percentage of the total protein, heating before or after emulsion formation had no effect on concentrate viscosity. The findings demonstrate the importance of thermal denaturation and subsequent aggregation of whey proteins

(and in particular, the ratio of α-lactalbumin to β-lactoglobulin) prior to homogenisation of infant formula emulsions.

4.2 Introduction

The physical stability of infant milk formulae (IMF) is dependent on both macronutrient composition and processing conditions during manufacture, with protein-protein and protein-fat interactions essential in determining the stability of this oil-in-water emulsion (McDermott, 1987). Model 1st stage infant formula is based on human breast milk to provide the specific nutrient, mineral and amino acid profile needed for growth and development of an infant. In both human and bovine milk, whey protein and casein are the two dominant fractions of protein present. The relative proportions differ however; bovine milk has a whey protein to casein ratio of 20:80 while in human milk, this ratio is 60:40. Consequently whey proteins, which are small globular proteins, are enriched in 1st stage infant formula to reflect the 60% content found in human milk. These proteins play an important role in determining the stability of IMF and a substantial amount of research has been carried out investigating their interactions with casein. Much of the research previously carried out on individual whey proteins is focused on the heat denaturation of the whey protein β-lactoglobulin (β-lg) (Gough & Jenness, 1962; Morr, 1975; Roefs & de Kruif, 1994; Walstra & Jenness, 1984). Upon heating to 65 °C or above, β-lg begins to unfold from its native globular form, allowing for interactions between the unfolded state and other β-lg monomers, dimers, whey proteins and caseins (Gough & Jenness, 1962; Morr & Ha, 1993; Mounsey & O'Kennedy, 2009; Swaisgood, 1982). Conformational changes in β-lg monomers and dimers disrupts inter- and intra-molecular bonds, resulting in the exposure of one free thiol group and two intra-molecular disulfide bonds per monomer, which can promote further aggregation through thiol/disulfide bridging and bonding (Morr & Ha, 1993; Verheul et al., 1998). The first and second order kinetics of this reaction are dependent on environmental factors i.e. pH, protein type and concentration, and heating

temperature; if not controlled, this reaction can cause rapid aggregation, fouling and in some cases gelling of protein in heat exchangers during processing (Jun & Puri, 2005).

The most abundant whey protein in human milk is α-lactalbumin (α-lac). Similar to β-lg, it is a globular protein in its native state. Under neutral conditions, when heated to temperatures greater than ~ 60 °C, it begins to unfold. Unlike β-lg, the α-lac monomer in its unfolded or non-native state does not contain free thiol groups, but unfolding does expose four intra-chain disulfide bonds (Mullvihill & Donavan, 1987). It therefore is more stable than β-lg against self-association following unfolding; aggregation that does occur between α-lac monomers can be reversible, depending on certain factors i.e. source, purity, environmental conditions of the solution, etc. (Wang et al., 2006). McGuffey et al. (2007) observed that when a commercial source of α-lac was aggregated by heating to 95 °C for 1h, aggregation was up to 70% reversible. Introducing β-lg to an α-lac system however, reduces the ability of α-lac to refold into its native form because of the formation of α-lac/β-lg aggregates through disulfide bridging between free thiol groups and intra-chain disulfide bonds (Dickinson, 1997b; Matsudomi et al., 1992; Parris et al., 1993). This binding can lead to the formation of aggregates containing exposed free thiol groups which subsequently aggregate. Under certain conditions, the free thiol groups exposed on these smaller aggregates can cause

further aggregation due to disulphide bridging, resulting in the creation of larger aggregates.

Earlier work has demonstrated that oil-in-water emulsions can be stabilised by protein (fat: protein ratio dependent), by lowering the surface tension at the interface during emulsification and reducing coalescence through structural stabilisation of the emulsion (McCarthy et al., 2012; Walstra, 1983). The formation of fat droplets during

homogenisation leads to the adsorption of protein on the surface of the droplets, creating a sterically-stabilizing layer, with preferential adsorption of specific individual proteins dependent on the protein concentration, protein structure at time of adsorption, pH and ionic strength (Dickinson, 1997b). Preferential adsorption in casein protein emulsion systems is dominated by α_{s1}-casein and β-casein, as these proteins are in abundance in the casein fraction. They are proteins which are both flexible and linear; these are important factors for surface protein adsorption, with β-casein being more hydrophobic, and hence having greater surface activity (Dickinson, 1988). Globular proteins, in their native form, are less susceptible to adsorption than flexible caseins. However, Matsumura et al. (1994) observed that in the molten globule state, both α-lac and β-lg are readily adsorbed to the emulsion droplet interface, with α-lac being more freely adsorbed in comparison to β-lg.

The objective of this study was to determine the effect of increased α-lac concentration on in-process stability of infant milk formulae, with the effect of heat-treatment before or after homogenisation being of specific interest. This study showed α-lac enriched IMF, heated post-homogenisation, produces aggregates of smaller size and resulted in less viscous concentrated formula, with the most α-lac enriched formulae displaying the best processing characteristics. The findings of the study provide added scientific knowledge which could be beneficial during the concentration and production of spray dried IMF.

4.3 Materials and Methods

4.3.1 Materials

A commercial source of WPI (BiPro) and α-lac were obtained from Davisco Foods Intl., Inc. (Le Sueur, Minnesota, U.S.A.) having protein contents, determined by Kjeldahl, of 92.3% and 91.8% respectively. The α-lac powder was analysed by differential scanning calorimetry (DSC), with 46.2% of the powder in the apo form and 53.8% in the holo form of the protein. Lactose was sourced from Glanbia Nutritionals (Carlow Rd., Kilkenney, Ireland) and sunflower oil was purchased from a local supermarket. Skimmed milk was acquired from the Animal and Grassland Research and Innovation Centre, Moorepark, Fermoy, Co. Cork, Ireland. The composition of the powder ingredients and of the skim milk (each individual batch) used in the experimental design are given in Table 4.1.

The protein, lactose and fat content of the skim milk were determined for each individual replicated trial using Fourier Transform Infrared Spectroscopy (FTIR) analysis (DairySpec FT, Bentley Instruments, Inc. Chaska, Minnesota, USA).

4.3.2 Preparation of Infant Formula Emulsions

Three formulations of 1st stage IMF were prepared with a ratio of protein: fat: lactose of 1.3: 3.5: 7.3, respectively, with each batch (10 L) containing a different level of α-lac; 12, 30 or 48% (percentage of total protein). Whey and casein proportions of total protein were that of a typical infant formula (60:40). Emulsions will be referred to as E12, E30 or E48 in this publication representing emulsions containing 12, 30 and 48% α-lac of total protein, respectively. The formulations were prepared at room temperature by reconstitution of dry ingredients in pre-determined quantities of skimmed milk and

sunflower oil, with mixtures standardised to 30% total solids (TS) using deionised water. Emulsions were adjusted to pH 6.8 using 1 M NaOH, mixed by magnetic stirring for 15 min, and subsequently readjusted and processed. Due to variations in skim milk composition (Table 4.1), varying quantities of protein, oil and lactose were added to each formulation for each batch replication to achieve the target composition. All emulsions were manufactured in triplicate.

4.3.3 Processing Conditions

The three individual batches of emulsion were split equally before processing, with the first half of E12, E30 or E48 heat-treated pre-homogenisation and the second half heat-treated post-homogenisation. These batches were blended using a laboratory scale Silverson mixer prior to homogenisation with an in-line two stage valve homogeniser, Model NS2006H (GEA, Niro Soavi, Parma, Italy). Each of the batches was heat-treated using a MicroThermics® laboratory scale tubular heat exchanger (MicroThermics, North Carolina, U.S.A.) to 100 °C (preheating temperature of 65 °C) at a flow rate of 1 L min^{-1} using a holding time of 30 s. Homogenisation downstream, carried out at 65 °C, had a first stage pressure held at 17.5 MPa and a second-stage pressure of 3.5 MPa. Upstream homogenisation was carried out at the same respective pressures and temperature. Post processing, sodium azide (0.02%) was added to limit microbial growth. Particle size, pH and viscosity analysis were carried out immediately, with the remaining sample held at 4 °C overnight with gentle agitation for analysis [sodium dodecyl sulphate polyacrylamide gel electrophoresis (SDS-PAGE), concentrated viscosity, SEC-HPLC, CLSM] the following day.

4.3.4 Compositional Analysis

Determination of total protein of emulsion samples was carried out using the Kjeldahl method of analysis (IDF 2001), using a conversion factor of 6.38.

The TS of liquid emulsions, pre- and post-evaporation, were determined using a Smart System 5, Smart Trac System (CEM Corporation, Matthews, North Carolina, USA).

4.3.5 Chromatographic Characterization of Molecular Mass Distribution

A Waters 2695 separation module HPLC system, coupled with a Waters 2487 dual wavelength absorbance detector, was used to identify the molecular weight (M_w) of native proteins and soluble protein aggregates. Emulsion samples were diluted to 2.5g L^{-1} protein in 20 mM sodium phosphate buffer before being filtered through 0.45 μm low protein binding filters (Sartorius Stedim Biotech GmbH, Göttingen, Germany). Samples (20 μl) were injected and eluted though TSK Gel G2000SW_{xL} and a G3000SW_{xL}, 7.8 x 300 mm, (TosoHaas Bioscience GmbH, Stuttgart, Germany) columns run in series using a 20 mM sodium phosphate buffer at pH 7.0, (isocratic conditions), with a flow rate of 0.5 mL min^{-1} over 60 min. M_w standards for calibration of columns were as follows: cytochrome c (12 kDa), α-lactalbumin (~ 14 kDa), β-lactoglobulin (~ 18 kDa), carbonic anhydrase (29 kDa) ferritin (44 kDa), bovine serum albumin (~ 66k kDa) and aldolase (158 kDa). All samples and standards were prepared using vacuum filtered (0.45 μm, high velocity filters, Millipore (UK) Ltd., Durham, U.K.) 20 mM sodium phosphate buffer prepared with HPLC grade MilliQ water. Data analysis and integration was carried out using Waters Empower® software with a wavelength of 214 nm and 280 nm used to detect aggregate elution.

4.3.6 Electrophoresis and Individual Protein Identification

Pre-cast sodium dodecyl sulphate-polyacrylamide gel electrophoresis (SDS-PAGE) gels (Novex® by life technologies™, Carlsbad, California, U.S.A) at a concentration of 12% Bis-Tris (1.0 mm x 10 well) were run with a constant voltage of 200 V for 50 min. SDS-PAGE gels of emulsion samples and emulsion sample supernatants (centrifuged at 20,000 *x g* using an eppendorf Centrifuge 5417R, Hamburg, Germany) were carried out under reducing and non-reducing conditions. Gels were stained using method described by McCarthy et al. (2012). Subsequent individual protein quantification was analysed using a method adapted by Ye et al. (2002).

4.3.7 Measurement of Particle Size Distribution

A laser-light diffraction unit fitted with a 300 RF lens (Mastersizer 3000, Malvern Instruments Ltd, Worcestershire, UK) was used for measurement of fat globule size immediately post processing. The average distribution was calculated using 3NHD presentation and optical parameters set with a particle refractive index of 1.46 and a dispersant index of 1.33. The span index of each sample was calculated using:

$$Span = \frac{D_{v0.9} - D_{v0.1}}{D_{v0.5}} \qquad \text{(Equation 4.1)}$$

where $D_{v0.9}$ is the particle diameter that 90% of the population of the distribution lies below, $D_{v0.1}$ is the particle diameter that 10% of the population of the distribution lies below and $D_{v0.5}$ is the median particle diameter.

Post processing, 30mM calcium, in the form of CaCl2, was added to 100mL aliquots of each batch of emulsion and placed in a refrigerator at 4°C with gentle magnetic stirring. On day 5, the average size distribution of fat globules was re-measured with and

without the addition of sodium dodecyl sulphate (SDS), as described by Tomas et al. (1994), in order to disperse flocculated fat globule clusters. All size measurements were carried out in triplicate.

4.3.8 Confocal Laser Scanning Microscopy

Confocal laser scanning microscopy (CLSM) was performed using a Leica TCS SP5 confocal scanning laser microscope (Leica Microsystems, Wetzler, Germany). Staining of samples was carried out using a dual-labelling fluorescent mixture prepare by pipetting 100 μl of 0.1, %, (w/v), aqueous solution of Fast Green FCF into 100 ml of polyethylene glycol. Nile Red at a concentration of 0.02 % (w/v), was subsequently added. Within imaging analysis, fat globules are stained green with protein aggregates stained red. The diameter of individual fat globules was measured using ImageJ software.

4.3.9 Viscosity Measurements

The viscosity of samples was determined using a control-stress rheometer (AR G2 Rheometer, TA Instruments, Crawley, UK) with a concentric cylinder geometry and tetradecane solvent trap. A Peltier apparatus controlled the sample temperature to (± 0.1 °C) with all samples measured visually free of trapped air and foam. Apparent viscosities of processed emulsions were measured at 25 °C and 10 °C to emulate storage conditions, using a continuous ramp step to a shear rate of $400s^{-1}$ under steady state conditions.

4.3.10 Evaporation and Measurement of Total Solids and Viscosity

Aliquots of individual emulsions were evaporated to > 55% TS using a BUCHI Rotary evaporator (Flawil, Switzerland), Model: Vacuum Controller V-850, Vacuum Pump V-

700, Rotavapor R-212 coupled with a B-491, with the heating Bath set to 52 °C and evaporation occurring over a 2h time period. TS were determined using a CEM 3100 Smart system 5 (CEM Corporation, Matthews, North Carolina, USA). Evaporated samples were diluted to 35 - 55% TS using the distillate of the emulsion samples for dilution to ensure sample integrity. The apparent viscosities of individual evaporated TS samples were measured at 25 °C within 30 min post-evaporation, using the rheometer and methodology previously described.

4.3.11 Analysis of Emulsion Sedimentation and Creaming

The physical stability of emulsions was determined using a LUMiSizer (Lum GMBH, Berlin, Germany) stability analyser and centrifuge, with data analysis carried out using associated SEPVIEW v.4.1 software (Lum, GMBH). Creaming and sedimentation were analysed through the continuous measurement of light passing through the length of a sample during centrifugation. The process is comprehensively described by Tobin et al. (2010). Emulsion samples (400 µl) were syringed into polycarbonate cells (PC 110-131XX; Lum GMBH). Cells containing the emulsion samples were centrifuged at ~ 1500 x *g* at 20 °C for 7.5 hours.

4.3.12 Statistical Analysis

Each treatment was replicated in triplicate using three independent batches of skim milk (composition found in Table 4.1). The Minitab® 17 statistical analysis package (Minitab Ltd, Coventry, UK, 2014), was used for analysis of variance (ANOVA; Tukey's HSD) of protein content, particle size of emulsions and viscosity of emulsions at 10 and 25 °C. The level of significance was determined at ($P < 0.05$).

A split plot design was used to determine the effects of heat-treatment before or after homogenisation, α-lac concentration and their interaction on the viscosity during evaporation, particle size and SEC-HPLC of emulsions. A general linear model (GLM was used for the analysis of variance for the split plot design with statistically significant difference observed at ($P < 0.05$) determined by Fisher's least significant difference test).

Table 4.1 - Composition of dry ingredients and skim milk used in formulations

Composition				
Skim milk	Protein (%)	Fat (%)	Lactose (%)	Total Solids (%)
Trial 1	3.75 ± 0.120	0.27 ± 0.000	4.91 ± 0.020	9.18 ± 0.007
Trial 2	3.56 ± 0.007	0.29 ± 0.000	4.70 ± 0.000	8.82 ± 0.000
Trial 3	3.82 ± 0.007	0.29 ± 0.000	4.97 ± 0.000	9.34 ± 0.007
Powder	Protein (%)	Fat (%)	Ash (%)	Total Moisture (%)
WPI *	97.8	0.2	1.9	5
α-lactalbumin *	93.3	0.3	1.9	4.9
Lactose	-	-	-	5

***** Protein concentrations as determined by Kjeldahl.

4.4 Results and Discussion

4.4.1 Protein Profile and Aggregation Measurements

To ensure consistent preparation of all IMF solutions, compositional analyses confirmed that there was no significant difference between protein concentrations across all batches (Table 4.2). For all IMF preparations described, samples were both homogenised and heated to 100 °C, with the heat step occurring either before or after homogenisation. The molecular weight (M_w) distributions of soluble aggregates (< 0.45 μm in size) present in treated emulsions, were determined by size exclusion chromatography and are displayed in Figure 4.1.

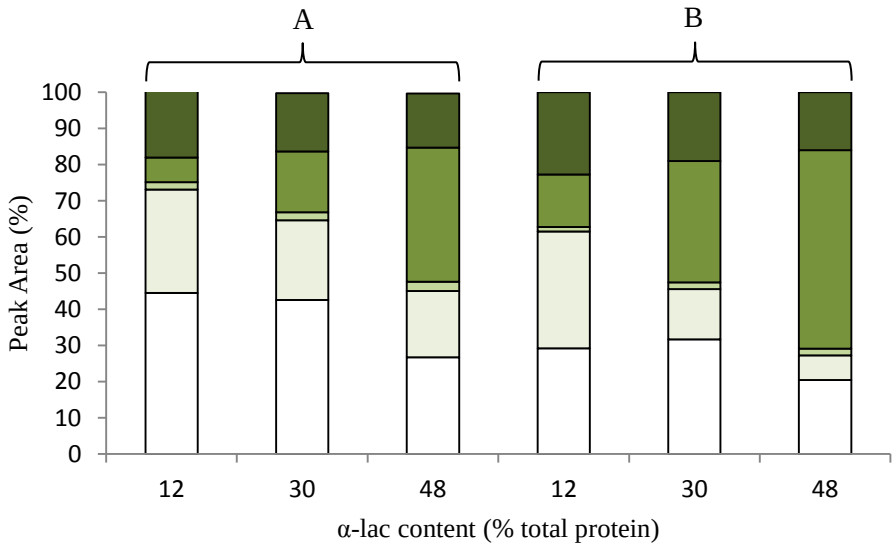


Figure 4.1: M_w distribution of protein aggregates measured by size exclusion high-performance liquid-chromatography. Distribution represented as: < 600 to > 280, < 280 to >28, < 27 to > 20, < 19 to > 13, < 1 kDa. Columns (A) and (B) represent emulsions heated before or after homogenisation, respectively.

In E48 emulsions, a large amount of α-lac was present in its native, monomeric state (M_w ~ 14.2 kDa) for samples heat-treated pre- or post-homogenisation, indicating that a

higher proportion of α-lac did not covalently aggregate with other proteins upon processing. While the α-lac concentration had a significant ($P < 0.05$) effect on the particle size distribution for each individual IMF composition, the processing conditions affected the size distributions in a manner independent of the composition (i.e. no interaction between these two variables was noted). A significant ($P < 0.05$) individual effect for the parameters of α-lac concentration and processing treatment was observed for aggregates found in this M_w range; however no interaction between these two parameters was noted.

With increasing α-lac concentration, the proportion of medium sized soluble aggregates (20 - 280kDa) increased significantly ($P < 0.05$), with a concomitant decrease in large M_w soluble aggregates (660 – 2000 kDa) in all emulsions. Therefore, increasing the α-lac concentration within emulsions produced a greater proportion of smaller and medium sized soluble aggregates and reduced the quantity of larger soluble aggregates in emulsions heat-treated pre- and post-homogenisation. The proportion of soluble protein, i.e., protein material which was able to pass through 0.45 μm filters (Figure 4.2), increased significantly ($P < 0.05$) with an increase in α-lac concentration between emulsions E12 and E30. However, there was no significant difference in the quantity of soluble protein in E48, with no significant effect of heat-treatment pre- or post-homogenisation.

Table 4.2 - Results for protein analysis and viscosity (at 25 and 10 °C)

Protein content (30% TS)	Pre 12%*	Pre 30%*	Pre 48%*	Post 12%*	Post 30%*	Post 48%*
Trial 1	2.52 ± 0.01[a]	2.62 ± 0.13[a]	2.43 ± 0.37[a]	2.43 ± 0.03[a]	2.39 ± 0.77[a]	2.57 ± 0.02[a]
Trial 2	2.46 ±0.01[a]	2.56 ± 0.01[a]	2.57 ± 0.02[a]	2.63 ± 0.18[a]	2.63 ± 0.03[a]	2.65 ± 0.06[a]
Trial 3	2.43 ±0.05[a]	2.59 ± 0.02[a]	2.64 ± 0.01[a]	2.47 ± 0.03[a]	2.53 ± 0.05[a]	2.50 ± 0.16[a]
Viscosity (25 °C)						
	1.48 ± 0.12	1.47 ± 0.05	1.36 ± 0.04	1.33 ± 0.05	1.73 ± 0.02	1.35 ± 0.01
Viscosity (10 °C)						
	2.32 ± 0.33	2.12 ± 0.21	2.03 ± 0.14	2.33 ± 0.07	2.05 ± 0.13	2.08 ± 0.07

* Percentage of α-lactalbumin in total protein.

[a] Values represent the means of triplicate trials ± sample standard deviation; values not sharing a common subscript significantly differ ($P < 0.05$).

Pre – samples heat-treated before homogenisation, Post – samples heat-treated after homogenisation.

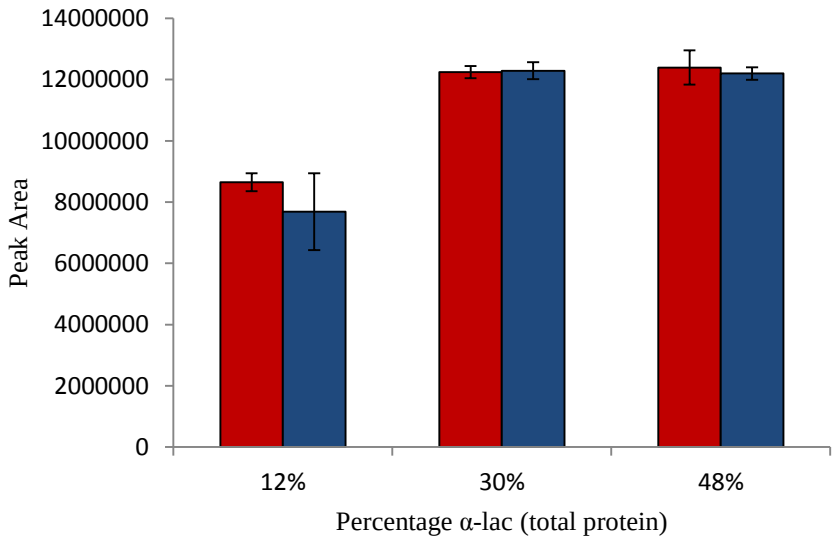


Figure 4.2: Quantity of soluble protein (protein material which was able to pass through 0.45 μm filters) in size exclusion chromatography samples. Blue represents samples heated pre-homogenisation and red samples heat-treated post-homogenisation.

Figure 4.3 shows non-reducing (A) and reducing (B) SDS-PAGE gels of emulsions E12, E30 and E48 heat-treated pre- or post-homogenisation. Non-reducing gels showed an increase in band intensity for α-lac, qualitatively consistent with the size exclusion data, when the α-lac concentration in emulsions was increased. The band intensity for monomeric β-lg does not hold the same trend. As the amount (and hence proportion) of β-lg was decreased in emulsions E12 to E48, an increase in the β-lg monomer band intensity on the non-reducing gel was observed. This is an indication that emulsions containing a higher proportion of β-lg formed a larger number of covalently bonded aggregates, i.e., aggregate complexes formed by the interaction of casein, β-lg and α-lac, consistent with previous observations (Gough & Jenness, 1962; Matsudomi et al., 1992). Comparing the intensities of the β-lg monomer band in the reducing and non-reducing gels, we can speculate that the majority of the aggregated β-lg exists as a covalently bonded moiety, since in the reduced gel, the intensity of the band is

significantly stronger. Furthermore, as the proportion of α-lac is increased, more β-lg monomer is observed (even if the starting concentration is lower) in the non-reducing gel, suggesting that α-lac is reducing the aggregation tendency of the β-lg during processing, consistent with work on processed dairy protein mixtures (Crowley et al., 2016).

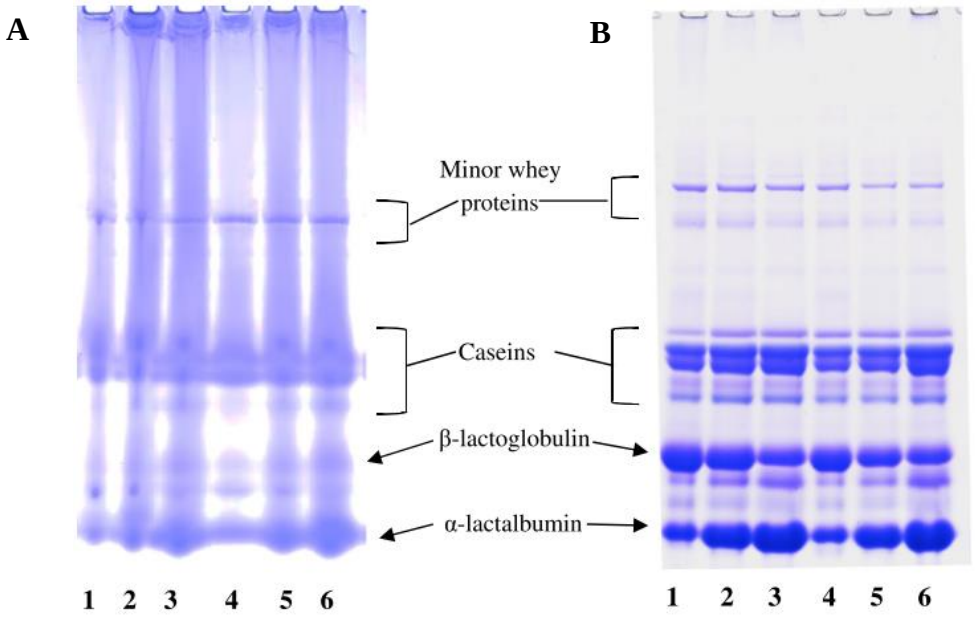


Figure 4.3: Non-reducing (A) and Reducing (B) SDS-PAGE gels of emulsions heat-treated before (1-3) or after (4-6) homogenisation, with emulsions containing 12, 30 and 48% α-lac represented by lanes (1,4), (2,5) and (3,6), respectively.

4.4.2 Particle Size Distribution of Emulsions

To determine if the size distribution of the soluble protein aggregates in the emulsion had a significant effect on the particle size of the emulsion itself, laser diffraction and confocal microscopy were used to characterise the emulsion particle sizes. The size distribution of particles in emulsions E12, E30 and E48 heat-treated pre-homogenisation, measured by laser diffraction were not significantly different, with broad but mono-modal distributions observed for each emulsion (Table 4.3; Figure 4.4 A, B, C).

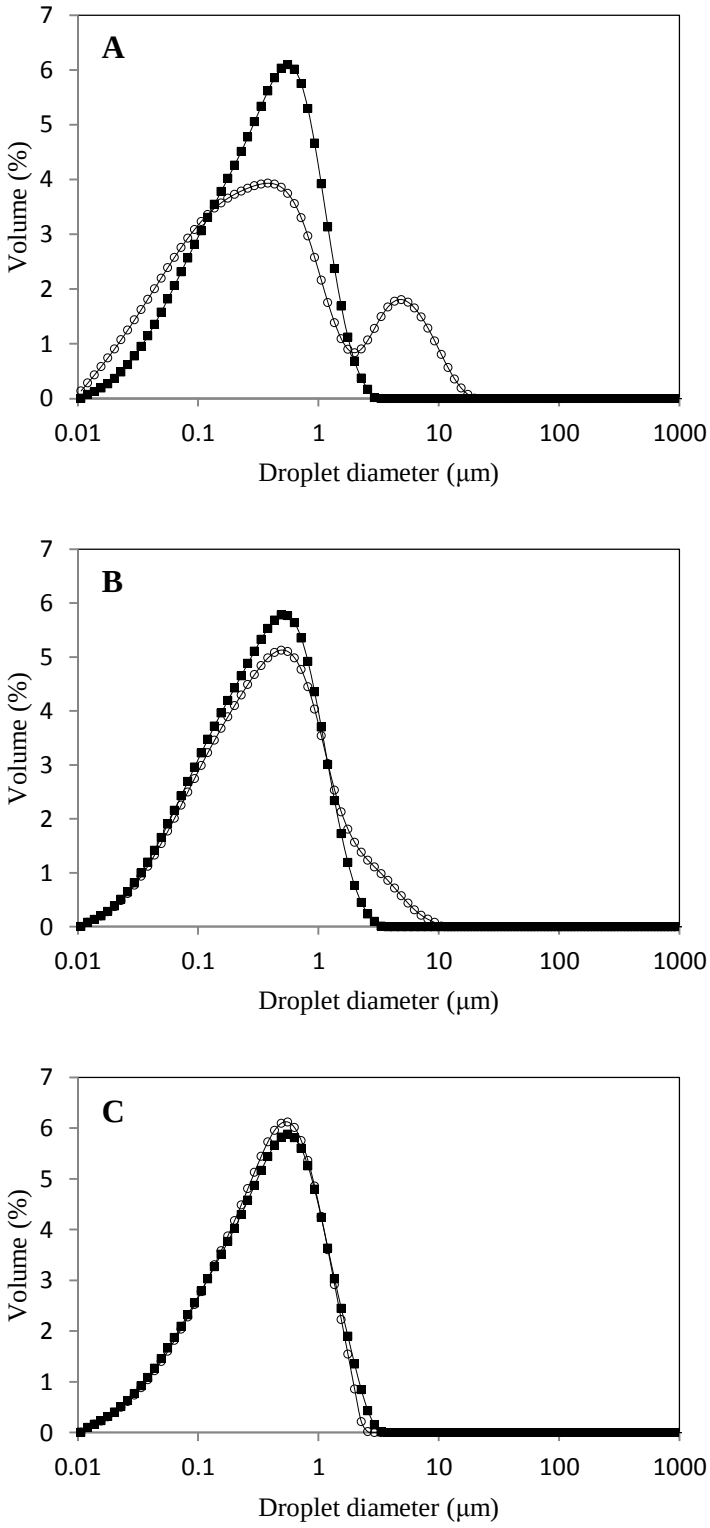


Figure 4.4: Distribution of fat globule size for emulsions containing 12, 30 and 48% α-lac (% of total protein), (a–c respectively), following heat-treatment before homogenisation (filled squares) or after homogenisation (open circles).

For E12 emulsions, there was a difference observed in samples heat-treated post-homogenisation, with an increase in the average particle size distribution, measured as a span index (eqn. 4.1), from 2.4 in the pre-homogenised sample to 8.0 in the post-homogenised sample. Therefore, a significant ($P < 0.05$) interaction between α-lac concentration and heat-treatment of the emulsion pre- or post-homogenisation was observed (Table 4.4). As the α-lac content is increased, the distribution of emulsion particle sizes becomes more uniform in the emulsion heat-treated post-homogenisation. The span index for E30 and E48 samples pre-homogenisation are the same, 2.5 and are only slightly higher than for the E12 sample. For post-homogenised E30 and E48 samples, the span index is slightly higher, 2.8 and 2.7 respectively, but there is little difference between these two α-lac concentrations.

The addition of 30mM CaCl2 to emulsions did not significantly increase particle size (Table 4.5). There was no flocculation of emulsions heat treated pre- or post-homogenisation with / without $CaCl_2$ on day 5, with a non-significant ($P < 0.05$) decrease in particle size noted with addition of SDS (Tomas et al. 1994). This suggests that there was little or no protein aggregation or flocculation between particles during days 0 - 5 with Ca^{2+} having minor effect on the stability of protein at the interface of the oil fat droplets. Droplets that are highly charged and can resist the effects of secondary aggregation associated with the addition of ions (Vardhanabhuti et al. 2001).

Since laser diffraction does not distinguish between protein aggregates and fat globules, confocal microscopy was used for further analysis (Figure 4.5). Confocal laser scanning microscopy images indicate that there are a greater number of larger fat globules present in emulsion E12 heat-treated post-homogenisation (Figure 4.6 and Table 4.6). For E48 emulsions, the size distribution of fat globules is very similar for both pre- and post-

homogenised samples (Figure 4.6). The laser diffraction data and confocal microscopy data are broadly in agreement. It is therefore most likely that the bimodal size distribution in the laser diffraction data observed in the post homogenised E12 sample is due to the formation of larger fat globule sizes in this sample, rather than the formation of protein aggregates. This seems reasonable based on the assumption that if protein aggregates of this size were formed, they would sediment in the sample (and not be measured by laser diffraction) and they would be clearly visible in the confocal images.

Table 4.3 - Particle size and statistical analysis output for the effect of individual parameter and their interactions of emulsions E12 **-** E30 heat-treated pre- and post-homogenisation.

Emulsion	Particle size (μm)					
	D[4,3]	D. v 01	D[3,2]	D. v 05	D. v 09	Span Index
Pre E12	0.49 ± 0.03^{a}	0.04 ± 0.04^{a}	0.22 ± 0.06^{a}	0.42 ± 0.09^{a}	1.06 ± 0.04^{a}	2.4
Pre E30	0.47 ± 0.02^{b}	0.08 ± 0.02^{a}	0.17 ± 0.01^{ab}	0.39 ± 0.08^{a}	1.07 ± 0.02^{a}	2.5
Pre E48	0.48 ± 0.03^{bc}	0.09 ± 0.05^{a}	0.16 ± 0.04^{ab}	0.40 ± 0.12^{a}	1.09 ± 0.02^{a}	2.5
Post E12	1.20 ± 0.06^{c}	0.10 ± 0.07^{a}	0.14 ± 0.03^{ab}	0.43 ± 0.17^{a}	3.56 ± 0.77^{b}	8
Post E30	0.68 ± 0.05^{c}	0.11 ± 0.04^{a}	0.26 ± 0.09^{a}	0.48 ± 0.10^{a}	1.47 ± 0.13^{a}	2.8
Post E48	0.53 ± 0.20^{c}	0.08 ± 0.01^{a}	0.20 ± 0.01^{b}	0.41 ± 0.05^{a}	1.20 ± 0.04^{a}	2.7

[a] Values represent the means of triplicate trials ± sample standard deviation; values not sharing a common subscript significantly differ ($P < 0.05$).

Pre – samples heat-treated before homogenisation, Post – samples heat-treated after homogenisation.

Table 4.4 - Statistical analysis output for the effect of individual parameters and their interactions on particle size[a].

Parameter	Heat-treatment before or after homogenisation (HT)	α-lac concentration	Interaction: HT x α-lac concentration
Particle Size (class)			
D[4,3]	NS	*	NS
D. v 01	NS	NS	*
D[3,2]	NS	NS	*
D. v 05	NS	NS	**
D. v 09	*	**	**

[a] Significant levels: NS - non significant $P > 0.05$, *$P < 0.05$, ** $P < 0.001$.

Table 4.5 - Particle size of emulsions at day 5 with/without $CaCl_2$.

Emulsion	Particle size (μm) Day 5 – Pre 12%*				
with/without	D[4,3]	D. v 01	D[3,2]	D. v 05	D. v 09
NO	1.32 ± 0.10^{A}	0.11 ± 0.06^{A}	0.24 ± 0.11^{A}	0.48 ± 0.07^{A}	1.36 ± 0.10^{A}
CA	1.00 ± 0.07^{A}	0.05 ± 0.01^{A}	0.13 ± 0.01^{A}	0.42 ± 0.09^{A}	1.50 ± 0.04^{A}
SDS	0.76 ± 0.05^{A}	0.05 ± 0.01^{A}	0.14 ± 0.01^{A}	0.37 ± 0.01^{A}	1.48 ± 0.01^{A}
	Pre 30%*				
NO	1.23 ± 1.26^{A}	0.09 ± 0.04^{A}	0.21 ± 0.07^{A}	0.42 ± 0.07^{A}	1.21 ± 0.14^{B}
CA	1.02 ± 0.03^{A}	0.05 ± 0.01^{A}	0.13 ± 0.01^{A}	0.34 ± 0.01^{A}	1.71 ± 0.01^{A}
SDS	1.02 ± 0.03^{A}	0.05 ± 0.01^{A}	0.13 ± 0.01^{A}	0.34 ± 0.01^{A}	1.71 ± 0.02^{A}
	Pre 48%*				
NO	0.84 ± 0.05^{A}	0.08 ± 0.04^{A}	0.19 ± 0.07^{A}	0.42 ± 0.09^{A}	1.27 ± 0.14^{B}
CA	0.81 ± 0.05^{A}	0.09 ± 0.04^{A}	0.21 ± 0.08^{A}	0.44 ± 0.07^{A}	1.41 ± 0.09^{AB}
SDS	0.83 ± 0.01^{A}	0.06 ± 0.01^{A}	0.17 ± 0.01^{A}	0.43 ± 0.01^{A}	1.60 ± 0.01^{A}
	Post 12%*				
NO	1.16 ± 0.30^{B}	0.09 ± 0.05^{A}	0.21 ± 0.09^{A}	0.44 ± 0.08^{A}	3.00 ± 0.20^{B}
CA	1.86 ± 0.18^{A}	0.04 ± 0.01^{A}	0.22 ± 0.12^{A}	0.43 ± 0.01^{A}	4.16 ± 2.50^{A}
SDS	2.47 ± 0.09^{A}	0.04 ± 0.01^{A}	0.12 ± 0.01^{A}	0.43 ± 0.01^{A}	7.45 ± 0.20^{A}
	Post 30%*				
NO	1.31 ± 0.70^{A}	0.09 ± 0.05^{A}	0.21 ± 0.11^{A}	0.42 ± 0.12^{A}	1.59 ± 0.07^{B}
CA	0.96 ± 0.62^{A}	0.09 ± 0.04^{A}	0.19 ± 0.07^{A}	0.41 ± 0.09^{A}	1.58 ± 0.21^{B}
SDS	0.77 ± 0.02^{A}	0.04 ± 0.01^{A}	0.12 ± 0.01^{A}	0.33 ± 0.01^{A}	1.96 ± 0.01^{A}
	Post 48%*				
NO	1.60 ± 2.40^{A}	0.10 ± 0.05^{A}	0.23 ± 0.10^{A}	0.45 ± 0.09^{A}	1.36 ± 0.15^{A}
CA	1.53 ± 1.25^{A}	0.09 ± 0.04^{A}	0.22 ± 0.08^{A}	0.43 ± 0.07^{A}	1.33 ± 0.07^{A}
SDS	0.55 ± 0.01^{A}	0.06 ± 0.01^{A}	0.15 ± 0.01^{A}	0.37 ± 0.01^{A}	1.27 ± 0.01^{A}

*Percentage of α-lactalbumin in total protein; [a] Values represent the means of triplicate trials ± sample standard deviation; values not sharing a common subscript significantly differ ($P < 0.05$); NO – Samples without $CaCl_2$, CA – Samples containing $CaCl_2$, SDS – Samples containing $CaCl_2$ and sodium dodecyl sulfate (SDS).

Table 4.6 - Fat globule size results determined by confocal laser scanning microscopy.

Emulsion	Fat Globule size (μm)	
	Average globule size	Median globule size
Pre E12	0.83 ± 0.31	0.90
Post E12	1.41 ± 0.52	1.27
Pre E48	1.06 ± 0.37	1.00
Post E48	1.18 ± 0.42	1.09

Pre – samples heat-treated before homogenisation, Post – samples heat-treated after homogenisation.

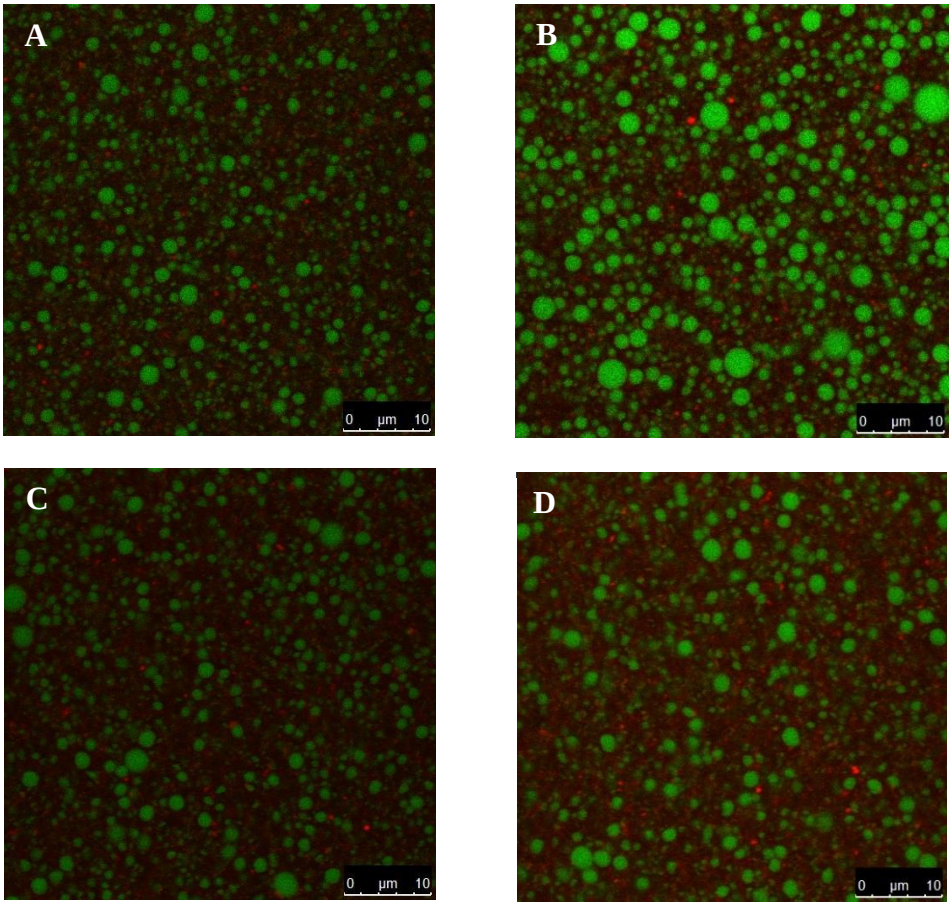


Figure 4.5: Confocal laser scanning microscopy images of emulsions containing 12% α-lac (A and B) and 48% α-lac (C and D). Images A and C depict emulsions heat-treated before homogenisation, with B and D representing emulsions heat-treated after homogenisation. Fat is represented in green, with protein represented in red.

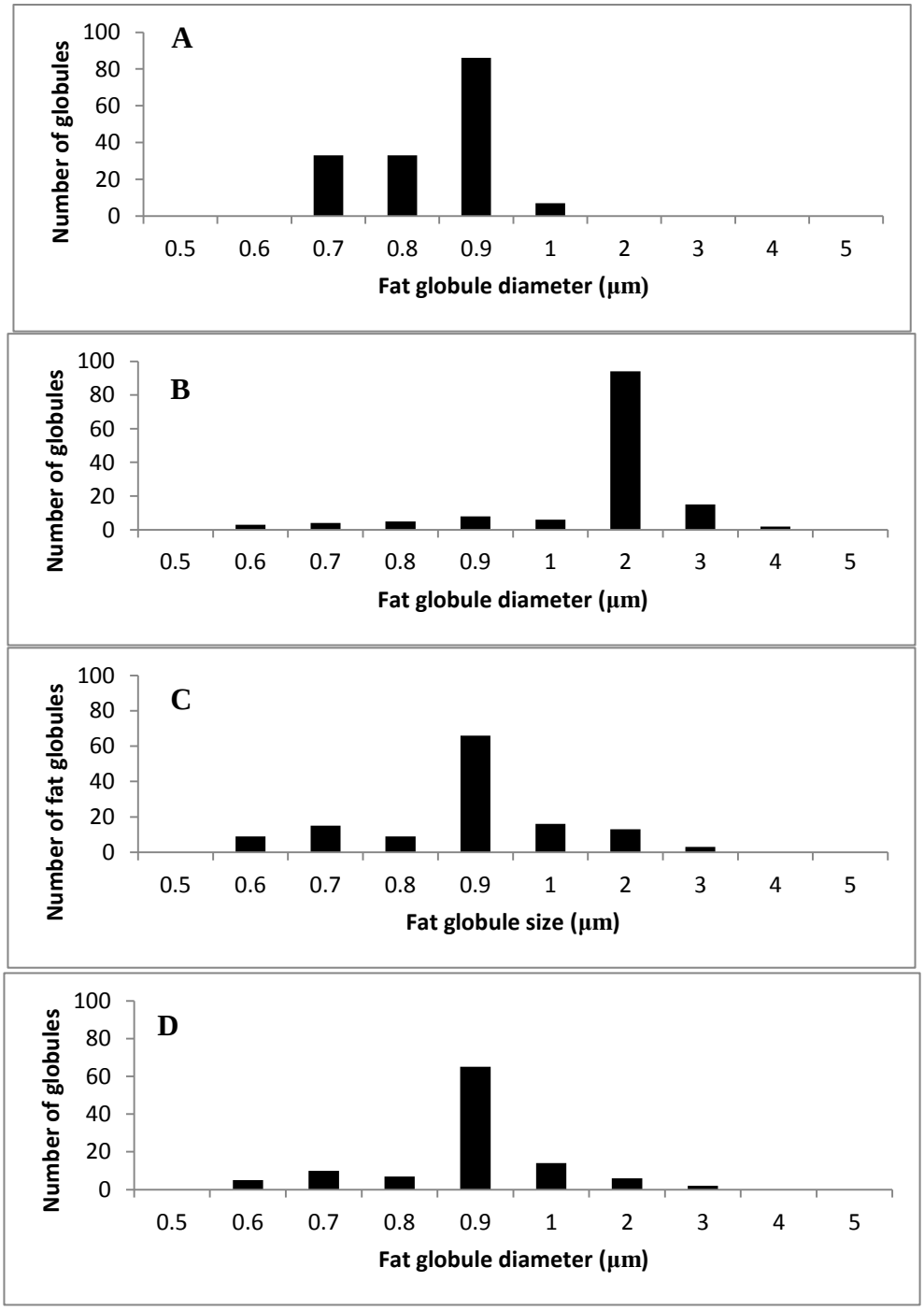


Figure 4.6: Histogram of globule distribution of confocal image (A) E12 heat-treated pre-homogenisation, (B) E12 heat-treated post-homogenisation, (C) E48 heat-treated pre- homogenisation, and (D) E48 heat-treated post-homogenisation, as determined from confocal laser scanning microscopy images (Figure 4.5) using Image J software.

4.4.3 Viscosity

4.4.3.1 Emulsion viscosity

Viscosity of the E12, E30 and E48 emulsions heat-treated pre- or post-homogenisation were analysed immediately post processing at 30% total solids (TS) (Table 4.2). At a shear rate of $400s^{-1}$, there was a significant ($P < 0.05$) effect of temperature on the viscosity as expected; emulsions measured at 25 °C had a significantly ($P < 0.05$) lower viscosity than the equivalent emulsions measured at 10 °C, with the effect of heat-treatment pre- or post-homogenisation having no significant effect (Gonzalez-Tello et al., 2009).

4.4.3.2 Viscosity of concentrated emulsions

Figure 4.7 displays the viscosity profiles (at $400s^{-1}$) for liquid emulsion concentrates as a function of TS. A significant interaction ($P < 0.05$) between the α-lac concentration and processing method is evident. Emulsion E12, heat-treated post-homogenisation, showed the greatest viscosity at a maximum of 54 mPa.s. Moreover, the viscosity of emulsion E12, heat-treated post-homogenisation, was significantly ($P < 0.05$) higher than the corresponding emulsion heat-treated pre-homogenisation. The effect of the homogeniser positioning in-line was less evident when the α-lac concentration was increased in emulsions E30 and E48, with emulsions heat-treated pre- and post-homogenisation observing no significant ($P > 0.05$) difference in viscosity. To identify the reason for the increase in viscosity in the E12 emulsions heated post-homogenisation, we examined the soluble protein aggregate size distributions and the emulsion fat globule size distributions. As previously mentioned, emulsion E12, heat-treated after homogenisation (Figure 4.4, A; Table 4.3), had a significantly ($P < 0.05$) greater span index than the pre-homogenised emulsion.

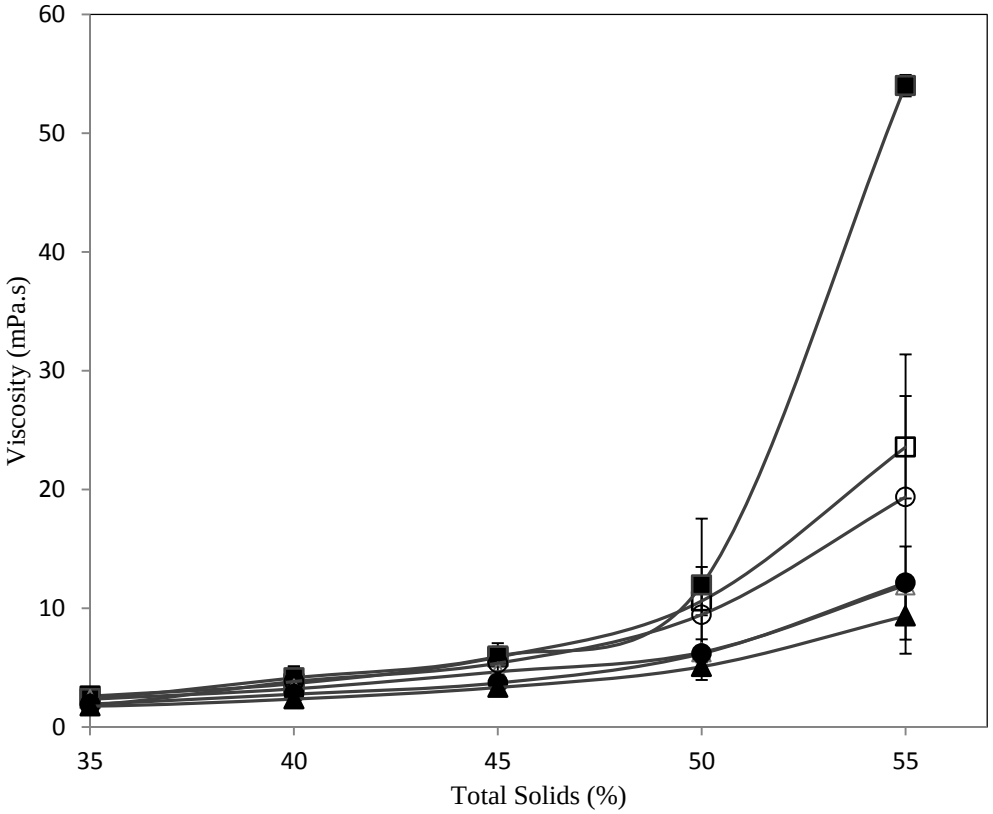


Figure 4.7: Viscosity (shear rate of 400s $^{-1}$) measured for emulsions at solids content ranging from 35 to 55%: 12% (□,■);32% (o,●); and 48% a-lac (Δ,▲). Open symbols represent samples heat-treated before homogenisation and closed symbols samples heat-treated after homogenisation.

However, the relative number of large soluble protein aggregates was lower in the post-homogenised sample (compared with the pre-homogenised sample), but there was a significant (P < 0.05) increase in the fat globule size. Heat-treatment and homogenisation affect the macromolecular arrangement of protein, and facilitates adsorption of protein to the surface of emulsion droplets. Different process conditions can allow for different interactions to take place between proteins that are adsorbed on the droplets and/or in the continuous phase. It is known that homogenisation partially denatures β-lg resulting in exposure of disulfide bonds (Dickinson, 1998). Upon

heating, interaction between unfolded β-lg and other proteins allows for the formation of aggregates which can interact with the fat globule surface. Protein aggregates which do not interact with the globule surface are suspended in the continuous phase. Upon the removal of water through evaporation, enhanced interactions occur between proteins bound to the surface of fat globules and non-adsorbed protein in the continuous phase. This interaction can cause destabilisation of the emulsion, and hence the formation of larger fat globule sizes, and as a result, an increase in the viscosity of the emulsion is observed. By increasing the α-lac concentration within the emulsion, this affect can be mitigated. This is most probably because of the larger number of smaller aggregates formed, which can more effectively absorb to the fat globules in the emulsion, providing the steric stabilisation observed in other emulsion systems (Dickinson, 1997b).

4.4.4 Sedimentation and Creaming Rates

Emulsion stability was analysed using LUMiFuge light transmission profiles (Figure 4.8, A and B). Instability within the emulsions was shown to be dominated by creaming, with emulsions E12 heat-treated post-homogenisation (Figure 4.8, B) displaying increased creaming compared to the respective E12 emulsions, heat-treated pre-homogenisation (Figure 4.8, A). It is suggested that this is due to the increased particle size of samples heat-treated post-homogenisation. Stability decreased with increasing α-lac content for emulsions heat-treated pre- or post-homogenisation; a finding that is most likely due to the corresponding reduction in viscosity in these samples, which would have contributed to an increased rate of creaming.

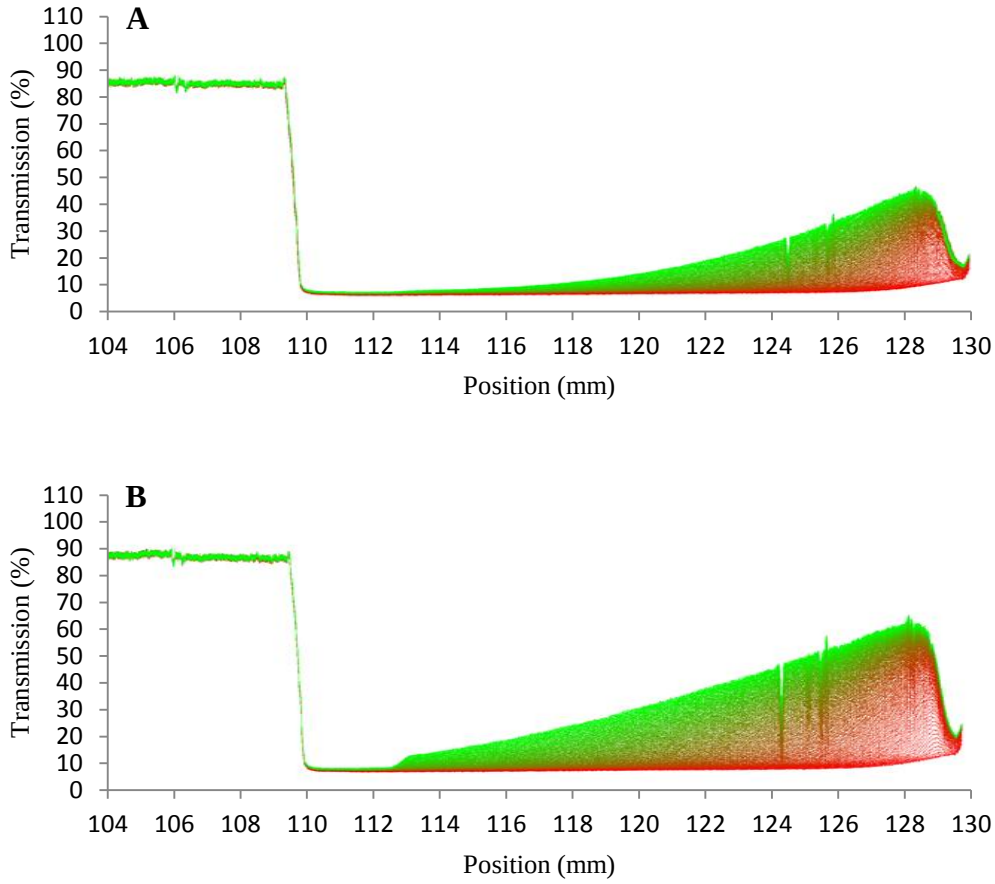


Figure 4.8: Transmission profile of emulsion E12 heat-treated before homogenisation (A) or heat-treated after homogenisation (B).

4.5 Conclusions

We have demonstrated that increased proportions of α-lac can alter the physicochemical properties of IMF emulsions. Increasing α-lac content (% of total protein; as per typical 1st stage IMF) reduced viscosity upon concentration, particularly where emulsions are formed prior to heat-treatment. Since lower quantities of β-lg are present, there are fewer covalently bonded aggregates formed by heat-treatment, which results in a more stable emulsion. Knowledge of the interaction of protein systems with unit operations, i.e., heat-treatment up or downstream of homogenisation, provides insight into how in-process stability of infant formula emulsions can be achieved during new formulation and/or process design.

CHAPTER 5

The Effect of β-lactoglobulin Concentration on Denaturation and Aggregation Studied by NMR

5.1 Abstract

The heat-induced denaturation of the globular protein β-lactoglobulin can cause structural rearrangements and formation of soluble, covalently bonded aggregates. This study determined the effect of β-lactoglobulin concentration (1 and 12% (w/w)) on structural rearrangements, pre- and post-heat-treatment, using chromatography, SDS-PAGE, FTIR and $^{1}H–^{13}C$ HSQC solution NMR (Nuclear Magnetic Resonance [13C,1H]–Heteronuclear Single Quantum Correlated spectroscopy) to determine the denaturation, aggregation and overall conformational changes in molecular structure. Samples were heat-treatment at 62 °C (representing un-aggregated samples) and 85 °C. Results showed that samples heated at 62 °C, even for long periods of time (20 hours), did not contain covalently bonded aggregates, although some reversible conformational changes were observed by NMR analysis. However, samples heat-treated at 85 °C showed a significant ($P < 0.05$) loss in native β-lactoglobulin monomer (determined by reversed phase-HPLC) after 5 minutes of heating, with a further loss after a heating time of 10 minutes. Changes in the secondary structure of 1% β-lg were evident after 10 minutes of heat-treatment at 85 °C; at protein concentration of 12%, changes were observed after only 5 minutes of heating. The findings suggest that while increasing temperature resulted in increased protein denaturation and aggregate formation, along with changes to the secondary structure of the protein, changes in the molecular structure as determined by NMR, were not as extensive as expected, when compared to the native globular structure. This would suggest that although the β-lactoglobulin molecules were heat-modified, the resulting aggregates contained "native-like" molecules.

5.2 Introduction

Bovine β-lactoglobulin (β-lg) is a globular whey protein which is a major component of bovine milk comprising between 7 - 12% (w/w) of the total protein (Euston et al., 1999). It is a widely utilised protein within the food industry, as it can offer functional properties, e.g., gel and foam formation, when heated to a sufficient temperatures to induce protein unfolding and denaturation (Bansal & Bhandari, 2016). Its globular structure is comprised of 162 amino acids, with a molecular weight of ~ 18.3 kDa (depending on the variant of the protein), and when unfolded, can undergo aggregation (Papiz et al., 1986; O'Mahony & Fox, 2013) with continuous and prolonged heating often resulting in gel formation (at critical protein concentration), as aggregates formed through thermal treatment form a gel network (Twomey et al., 1997). The predominant genetic variants of β-lg are A and B, and differ in amino acid composition at residue positions 64 and 118; variant A has an aspartic acid residue at position 64 and a valine residue at 118, while variant B has a glycine and alanine residue at position 64 and 118, respectively. The structural and behavioural differences and interactions of both of these variants has been extensively studied (Cho et al., 2003; Croguennec et al., 2004; Euston et al., 1999; Manderson et al., 1998; Surroca et al., 2002). Although at neutral pH, β-lg predominately resides in dimeric form, each monomer is characterised by the presence of two disulfide bridges (Cys-66/Cys-160 and Cys-106/Cys-119) with one free sulfhydryl group, namely Cys-121 (de Wit & Klarenbeek, 1984a). This free sulfhydryl group is a reactive site, which can bind to other free reactive sites and result in the formation of covalently bonded aggregates, therefore eliminating the possibility of reversing of the denaturation process. Under physiological conditions however, this free sulfhydryl is buried in the interior of the protein.

The effect of heat-treatment on β-lg induces changes within its native globular structure and as a result can often cause fouling within industrial heat exchangers, caused by burnt particles, uncontrolled gelation due to uncontrolled aggregation and formation of covalently bonded protein aggregates (Jun & Puri, 2005; Zuniga et al., 2010). Consequently, understanding β-lg denaturation, aggregation and the morphology of aggregates formed, post heat-treatment, can provide important information about β-lg during thermal processing. Previous studies have examined the secondary, tertiary and quaternary structures of β-lg to varying degrees under differing conditions (Bhattacharjee et al., 2005; Boye et al., 1996; Surroca et al., 2002). It is widely accepted that β-lg monomers contain a β-barrel comprised of eight antiparallel β strands with $(+1)_8$ topology, with each successive strand proceeding one after another (Brownlow et al., 1997; Kuwata et al., 1999). The secondary structure of β-lg consists of 45% β-sheet, 10% α-helix and 45% unordered structure (Whitney, 1988). It belongs to the lipocalin family, with the β-barrel able to bind a range of compounds including small hydrophobic or amphipathic molecules, such as fatty acids and triglycerides, as well as a range of vitamins such as A, D, E and K (Sawyer, 2003; Perez & Calvo, 1995; Le Maux et al., 2013). It is believed that the binding of these molecules may be the function or part of the function of bovine β-lg, as no clear biological function has previously been defined.

Although NMR spectroscopy has been routinely used to study the three-dimensional structure and folding/unfolding/aggregation of globular proteins such as sperm whale myoglobin (Schlichting & Chu, 2000) and hen egg white lysozyme (Radford et al., 1992), the use of NMR as a tool for analysis of β-lg structure has been more challenging, due to monomer-dimer partitioning of β-lg at neutral pH. Unlike some other globular proteins e.g., α-lactalbumin, β-lg does not show any reversibly upon

denaturation, with the covalently bonded protein aggregates formed after heat denaturation resulting in peak broadening within NMR spectra (Sakurai et al., 2009). Numerous studies have used NMR to observe the molecular structure of β-lg at pH 3 (Sakurai & Goto, 2007; Forge et al., 2000; Jara et al., 2014), as β-lg exists as a stable, monomeric structure at this pH. More recently however, work has been carried out using heteronuclear solution NMR at pH 7.0 on the engineered β-lg mutant (A34C), which exists exclusively as a dimer at neutral pH (Sakurai & Goto, 2006), predominantly in relation to the intermediate folding's of the protein during the Tanford transition (Tanford & Nozaki, 1959) of the protein at ~ pH 7.0 (Qin et al., 1998).

As β-lg is found in bovine milk at its natural pH of ~ 6.7, the current research aimed to add to the body of work already present, by providing an insight into the effect of protein concentration on the structure of purified β-lg at 62 and 85 °C, using different analytical techniques including heteronuclear NMR, before and after heat-treatment.

5.3 Material and Methods

5.3.1 Materials

β-lg was isolated by ion exchange chromatography (Fast Protein Liquid Chromatography (FPLC)) using an adapted method previously described by (Pearce, 1995). A commercial source of whey protein isolate (WPI), BiPro from Davisco Foods Intl., Minnesota, U.S.A, was used as the starting material for purification. The β-lg fraction obtained by ion exchange chromatography was dialysed by membrane filtration using an 8 kDa membrane (Sigma-Aldrich, Ireland) to remove the salt added during isolation. Post dialysis, the protein fraction was freeze dried in order to maintain protein integrity. The protein content of the resulting powder was 88% as determined by Kjeldahl.

The lyophilised powder was reconstituted in MilliQ water (Millipore (UK) Ltd., Durham, UK), adjusted to pH 4.6 using 1 M Hydrochloric acid and centrifuged at 32,000 x *g* for 30 min to remove all denatured β-lg present. The supernatant of the centrifuged liquid was decanted and re-adjusted to pH 7.0 using 2 M Sodium Hydroxide. The ionic strength of the fraction was standardised through repeated dialysis against MilliQ water using the membrane previously mentioned. The fraction was again freeze dried post dialysis. The content of purity was determined by reversed-phase HPLC (Kehoe et al., 2011) and found to be > 98% β-lg.

1,4-dioxane used for NMR internal reference was purchased from Sigma-Aldrich, Ireland.

5.3.2 Sample Preparation

Rehydrated (overnight at 4 °C) protein solutions of 1 and 12% (w/w) β-lg were placed in sealed glass tubes and heat-treated separately at two different temperatures, 62 °C and 85 °C, using a temperature controlled water bath. The water bath temperature was measured using an external thermometer. The heating time differed for particular samples; 1% samples heat-treated at 62 °C were heated for up to a total of 20 hours, while 12% samples at this respective temperature were heated for up to 3 hours. Both 1 and 12% samples heat-treated at 85 °C were heated for up to 10 min. Aliquots of 500 µl were removed at 0, 15, 30, 45, 60, 120, 240, 480 and 1,200 min for samples heat-treated at 62 °C and 0, 5 and 10 min for samples heat-treated at 85 °C. All aliquots removed were placed in eppendorf tubes and cooled in an ice bath until sufficiently cooled.

5.3.3 Protein Denaturation

Quantification of protein denaturation was determined by reversed-phase HPLC (RP-HPLC). A Waters 2487 dual wavelength absorbance detector (214 nm – 280 nm) (specific extinction coefficient of 0.96 L g1- cm-1 at 280 nm) in conjunction with a Waters 2695 separation module was used for analysis. The column used for separation was a Source™ 5RPC (150×4.6 mm) column (Amersham Biosciences) run using two solvents, solvent A (0.1% TFA in MilliQ water) and solvent B (90% Acetonitrile, 0.1% TFA). Protein elution was carried out under gradient conditions at a flow rate of 1 mL min^{-1} (Kehoe et al., 2011). Sodium acetate buffer (0.1 M) at a pH of 4.6 was used to dilute samples to a protein content of 0.25% before centrifugation at 20,000g, 25 °C for 25 mins. The supernatant of each sample was filtered through a 0.22 µm before injection. β-Lactoglobulin was used for column calibration. Data was processed using Waters Empower® software.

5.3.4 Size Exclusion Chromatography

Determination of soluble aggregate formation was carried out using size exclusion chromatography (SEC) HPLC. The previously mentioned module and HPLC system were used for aggregate separation. Samples were diluted to 2.5 gL^{-1} protein in sodium phosphate buffer (20 mM) at pH 7.0 with the removal of larger, insoluble aggregates, by filtering samples through 0.45 μm low protein binding syringe filters (Sartorius Stedim Biotech GmbH, Germany). Samples were run on a TSK Gel $G2000SW_{xL}$ run in series with a $G3000SW_{xL}$, (7.8 x 300 mm, TosoHaas Bioscience GmbH, Stuttgart, Germany) with a flow rate of 0.5 mL min^{-1} under isocratic conditions for 1 hour. The following molecular weight standards were used for column calibration; cytochrome c (12 kDa), α-lactalbumin (~ 14 kDa), β-lactoglobulin (~ 18 kDa), carbonic anhydrase (29 kDa), ferritin (44 kDa), bovine serum albumin (~ 66 kDa), aldolase (158 kDa) and thyroglobulin (669 kDa). HPLC grade MilliQ water was used in the preparation all of buffers and samples with buffer being vacuum filtered through 0.45 μm, high velocity filters (Millipore (UK) Ltd., Durham, U.K.). Waters Empower® software was used in aggregate integration and data analysis.

5.3.5 Sodium Dodecyl Sulfate Polyacrylamide Gel Electrophoresis

Samples were loaded onto 10 well pre-cast Sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) gels (Novex® by Life Technologies™, Carlsbad, CA, USA). The gels had a concentration of 12% Bis-Tris with a 1.0 mm well width, consisting of 10 wells, and were run with a constant voltage of 200 V for 50 min. All samples were run under both reducing and non-reducing conditions and stained using a method previously described by McCarthy et al. (2012). A pre-stained protein standard was used as a molecular weight marker for the determination of each protein band

(SeeBlue® Plus 2 Pre-Stained Protein Standard Protocol, Novex® by Life Technologies™, Carlsbad, CA, USA).

5.3.6 Secondary Structure Analysis

Fourier Transform Infrared (FTIR) Spectroscopy (Bruker Tensor 27, Bruker Optik GmbH, Germany) was utilised in the quantitative analysis of secondary structure at each time point. The use of a thermally controlled (25 °C) AquaSpec™ cell allowed for the transmission through a 7 μm path length. Approximately 10 μg of protein was injected into the cell for each measurement with samples heat-treated at 12% diluted to 1% protein with MilliQ water. Background readings were taken against water and subtracted from sample spectra. Qualitative analysis of α-helix and β-sheet structure encompassed atmospheric compensation (aqueous and CO_2) and vector normalisation. All data analysis was carried out on OPUS 5.5 software.

5.3.7 $^{1}H–^{13}C$ HSQC NMR Spectroscopy

Lyophilised β-lg power was dissolved in dH_2O/D_2O (90%/10%) overnight (+10 hours) to allow for complete hydration of the protein. The hydrated β-lg solution was centrifuged at 3900 rpm for 10 min at 4 °C using a Centrifuge 5810R (Eppendorf), and the supernatant was filtered with 0.22 μm syringe filter to remove any insoluble particles. The filtered β-lg solution was amended to yield a protein concentration of 1% (w/w) (10 mg/ml) or 12% (w/w) (extinction coefficient of 1% β-Lg = 9.63cm^{-1}). Once the concentration of the protein solution was confirmed, the samples were heat-treated, depending on the experiment requirement, at 62 °C (for up to 20 hours during real time NMR analysis within the instrument) or 85 °C (for up to 10 minutes in a water bath as previously described). Dioxane was added as internal chemical shift standard (found not

to interact with proteins or partially-folded proteins) and was assigned at 3.75 ppm/67.19 ppm ($^{1}H/^{13}C$ chemical shift)(Fulmer et al. 2010) as a benchmark peak during NMR spectra analysis (De Marco, 1977; Pettersson-Kastberg et al., 2009; Shimizu et al., 1994).

All ^{1}H-^{13}C HSQC solution NMR experiments were performed at 20 °C on Agilent NMR 800 MHz with a triple resonance cold probe. Spectra analysis was carried out with VnmrJ (Agilent Technologies, Santa Clara, CA, USA). All ^{1}H-^{13}C HSQC experiments were acquired with 2s relaxation delay (D1) and acquired data sizes were 2048 × 4096 points in the F_1 and F_2 dimensions.

Further NMR spectra were acquired after insoluble protein precipitation of 1 and 12% (w/w) protein samples, heat-treated at 85 °C using the same NMR parameters. Using 1 M HCl, samples were brought to pH 4.6 before undergoing centrifugation at 20,000 x *g*, 25 °C for 25 mins. The pH was then readjusted to pH 6.7 using 1 M NaOH and the samples were dialyzed extensively (8 kDa ultrafiltration membrane, Sigma-Aldrich, Ireland) to remove all additional salt. The protein concentration of the monomeric and dimeric protein fractions, isolated using the SEC-HPLC method previously mentioned, were measured using the Qubit® Protein Assay Kit (Cambio Ltd, Cambridge, UK) following the method as suggest by the supplier.

5.3.8 Nuclear Magnetic Resonance Temperature Calibration Using 1, 2-ehanediol

This calibration is required when acquiring spectra at high temperature, when there are differences between the temperature value displayed on the control panel and the reading on the temperature probe. Calibration of the temperature of the NMR was

carried out using a 1, 2-ethanediol sample provided in an NMR tube from Agilent, the NMR manufacturer.

Once the 1, 2-ethanediol sample, placed in the NMR probe, was set to 25 °C, the NMR was tuned and matched and 1D Proton spectra were acquired. Within the acquired spectra, 1, 2-ethanediol showed as two peaks with the delta ppm between these two peaks measured and used to calculate the real temperature of the NMR probe using below. The temperature was increased using 5 °C intervals from 25 °C to 60 °C and further increased from 60 °C to 70 °C using intervals of 1 °C. The temperature was equilibrated for a minimum of 10 min for each sample at each temperature (25 °C and 62 °C), with the sample re-shimmed after each temperature change and the same equilibration steps repeated to acquire an accurate temperature.

$$T[K] = -108\Delta\delta + 460.41 \qquad \text{(Equation 5.1)}$$

where $T[K]$ = Real temperature of NMR probe in the Kelvin scale and $\Delta\delta$ = ppm difference between two peaks obtained from 1,2-ethanediol.

5.3.9 Statistical Analysis

The statistical analysis was carried out using the Minitab® 17 statistical analysis package (Minitab Ltd, Coventry, UK, 2014) with the analysis of variance measured by ANOVA (Tukey's HSD). The level of significance was determined at ($P < 0.05$).

5.4 Results and Discussion

The 800 MHz NMR spectrometer employed in these studies incorporates a variable temperature unit, allowing heating (up to 70 °C if necessary) and cooling of the sample *in situ*. Furthermore, the timescale of the NMR experiments are faster than the transitions involved in dimerisation/aggregation. As a result, it was possible to perform real-time (or pseudo real-time experiments), and this has been exploited to distinguish the structural changes between the 1% and 12% samples.

5.4.1 Quantification of β-lactoglobulin Denaturation and Aggregate Formation

Previous studies have quantified β-lg denaturation using a number of different analytical techniques and consequently, have defined β-lg denaturation using a number of different definitions (Briggs, 1945; Lyster, 1970; Roefs & de Kruif, 1994). However, for the purpose of the present study, denatured β-lg is defined as all protein precipitated during isoelectric precipitation (centrifugation at pH 4.6); therefore, native β-lg can be defined as all protein remaining suspended in solution after isoelectric precipitation. The amount of native protein in each β-lg sample was determined using RP-HPLC after isoelectric precipitation, with both 1 and 12% β-lg samples heat-treated at 62 °C displaying no significant difference in the quantity of native protein found in each sample, compared to unheated control samples (Figure 5.1). Neither 1% β-lg samples, heated for a period of 20 hours, nor 12% β-lg samples, heat-treated for 3 hours, contained any detectable denatured protein. Although no protein denaturation was noted at a temperature of 62 °C, samples heat-treated at 85 °C observed significant ($P < 0.05$) protein denaturation after only 5 minutes of heating (Figure 5.1). Samples heat-treated at 1% protein underwent a significant ($P < 0.05$) degree of denaturation (DD), with samples 61% denatured after 5 minutes of heating.

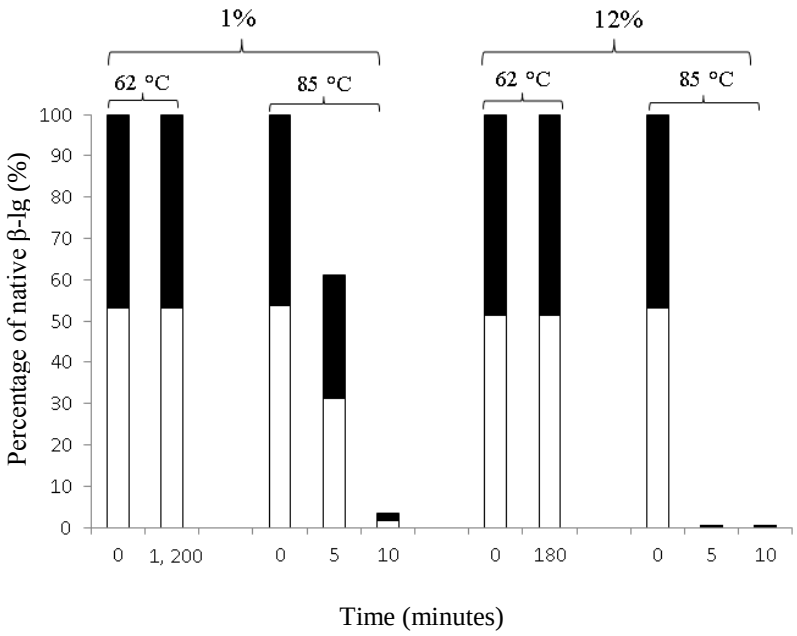


Figure 5.1: Distribution of native β-lactoglobulin A (black section) and B (white section) of 1 and 12% (w/w) samples measured by RP-HPLC after isoelectric precipitation. Samples analysed underwent heat-treatment at 62 °C (0 - 1,200 and 0 -180 min) and 85 °C (0, 5 and 10 min) at pH 6.7.

Furthermore, after an additional 5 minutes of heating, the DD of this sample increased to 98%, after a total heating time of 10 minutes. Although not calculated, it is clear that the overall rate of denaturation for the equivalent 12% sample, heated at 85 °C, was much greater, with a DD of > 99% after 5 minutes of heating; no significant difference in the amount of native protein was noted between samples heated for 5 or 10 minutes at this protein concentration (12%). The β-lg denaturation and aggregation model proposed by Roefs & de Kruif (1994) states that upon unfolding of β-lg monomers, a free sulfhydryl group can become exposed, and aggregate through disulfide interchange reactions; aggregation continues to occur until a disulfide linkage is formed between two exposed free sulfhydryl groups, thus terminating the reaction. Consequently, 12% samples heat-treated at 85 °C for 5 minutes underwent the termination reaction, resulting in no further protein denaturation after an additional 5 minutes of heating. It

has previously been demonstrated that β-lg concentration has an effect on the rate of protein denaturation; an increase in β-lg concentration results in a concomitant increase in the rate of denaturation upon thermal heating (Kehoe et al., 2011; Kessler, 2002; Oldfield et al., 2005), with the current results in agreement.

As previously outlined, samples heat-treated at 62 °C did not observe any detectable protein denaturation. This result was expected, as it is known that the denaturation temperature (T_D) of β-lg is greater than 62 °C. de Wit (1984b) measured the T_D of β-lg by differential scanning calorimetry (DSC) and found that β-lg denaturation was initiated at a temperature of 78 °C, with DSC peak maximum at a temperature of 83 °C. However, more recent studies have acknowledged that the T_D of β-lg is dependent on protein purity, pH, ionic strength and protein concentration (Fitzsimons et al., 2007; Gotham et al., 1992; Qi et al., 1995). Boye & Alli (2000) studied the T_D of β-lg and α-lactalbumin individually (40% (w/v)), and at a 1:1 ratio, and found that the T_D of β-lg decreased from 71.9 to 69.1 °C when α-lactalbumin was added to the protein system. In the current study, the justification for choosing a temperature below the T_D reported by de Wit (1984b) and Boye and Alli (2000) was based, not only in relation to the denaturation temperature of the protein, but also in relation to the temperature at which protein aggregation was absent. A temperature of 62 °C was deemed to be too low to induce the formation of soluble protein aggregates. From the size exclusion chromatograms in Figure 5.2, it is evident that soluble protein aggregates were present in samples, which were heat-treated at a temperature of 64 °C and above (1% β-lg). The necessity of selecting a temperature at which protein aggregation did not occur related to the use of $^{1}H–^{13}C$ HSQC solution NMR to study β-lg molecular structure. The performance of $^{1}H–^{13}C$ HSQC solution NMR under real time heating requires that the

denaturation of protein be slow and in a controlled manner with reduced protein aggregation.

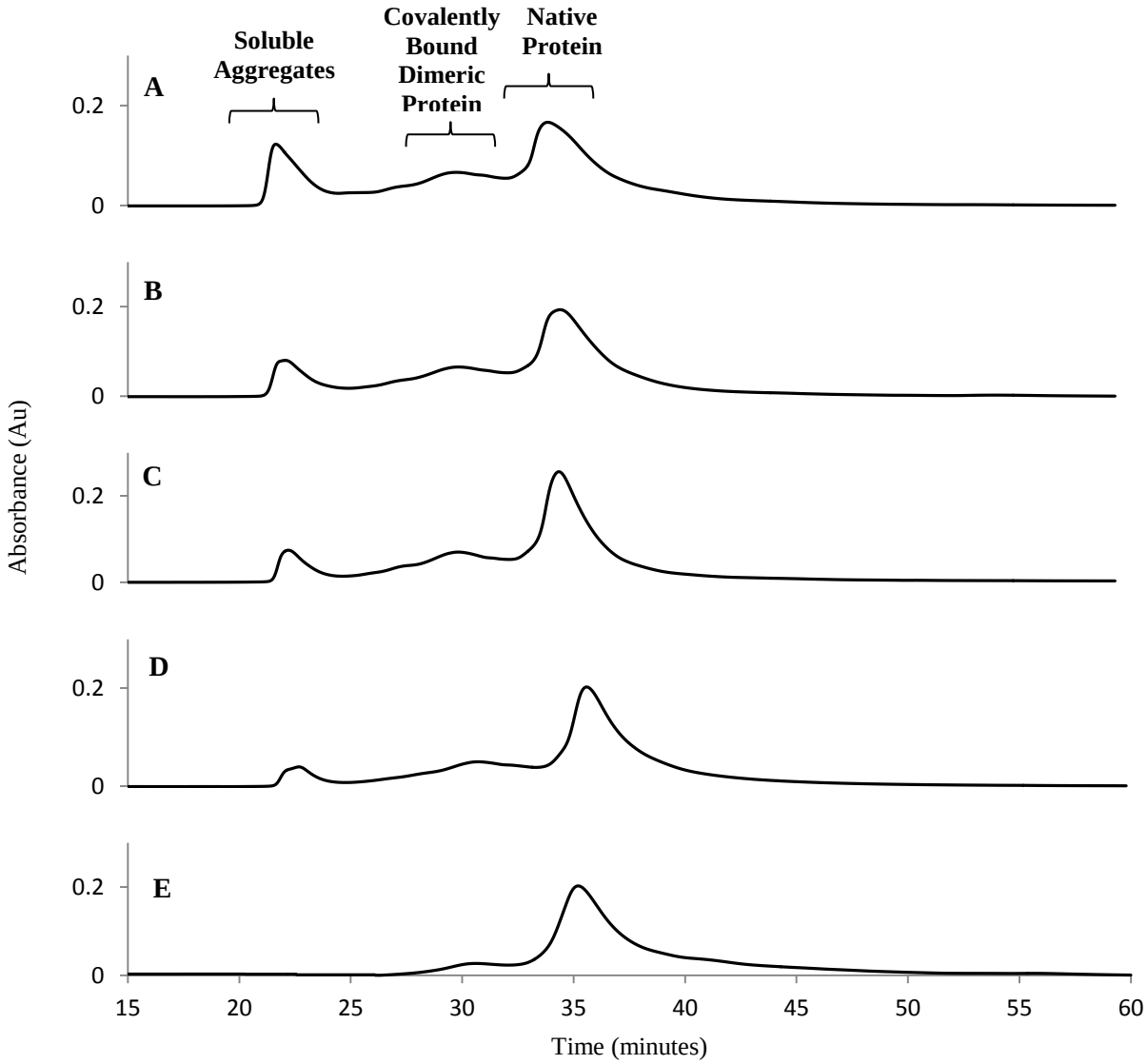


Figure 5.2: Size exclusion chromatograms of 1% β-lg at pH 6.7, heat-treated at 70 (A), 68 (B), 66 (C), 64 (D) and 62 °C (E) for 20 hours.

Although NMR has been routinely used to study the structure of other proteins including sperm whale myglobin (Schlichting and Chu, 2000) and egg lysozyme (Radford et al., 1992), the use of NMR as a tool for analysis of β-lg structure has been

more challenging, particularly as β-lg resides as a dimer at neutral pH. Unlike some other globular proteins (α-lactalbumin), β-lactoglobulin does not show any reversibly upon denaturation, with the covalently bonded protein aggregates formed after heat denaturation resulting in peak broadening within NMR spectra (Sakurai et al., 2009). Numerous studies have used NMR to observe the molecular structure of β-lg at pH 3 (Sakurai and Goto, 2007, Forge et al., 2000, Jara et al., 2014), as β-lg exists as a stable, monomeric structure at this pH. More recently however, work has been carried out on β-lg using heteronuclear NMR, including experiments carried out at pH 7 (neutral pH), predominantly in relation to the intermediate folding's of the protein during the Tanford transition (Tanford, 1959) of the protein at ~ pH 7 (Qin et al., 1998, Sakurai and Goto, 2006).

Each peak in the ^{1}H- ^{13}C HSQC NMR spectrum represents the specific link between a carbon atom and a hydrogen atom, with the resulting chemical shifts in both carbon and proton dimensions extremely sensitive to the surrounding chemical environment of two nuclei, as well as physical conditions such as temperature and pH. It is this sensitivity that allows for the determination of changes in tertiary structure of a protein during HSQC NMR experiments. The ^{1}H- ^{13}C HSQC measurements measured are one-bond correlations which directly correlate to ^{1}H- ^{13}C cross-peaks. This allows for greater sensitivity when compared to direct ^{13}C observation experiments because of ^{1}H detection. Figure 5.3 shows the ^{1}H- ^{13}C HSQC NMR spectrum of 1 % native β-lg (Figure 5.3 (A): control, non-heat-treated sample), 1 % β-lg heat-treated and measured at 62 °C for 20 hours (Figure 5.3 (B): The VT unit was set at 62 °C for the duration of the ^{1}H- ^{13}C HSQC measurement), and the 1% β-lg solution heat-treated to 62 °C for 20 hours and subsequently cooled to 20 °C (Figure 2 (C)).

There are only minor changes after a 20 hour of period sustained heat-treatment at 62 °C (B).

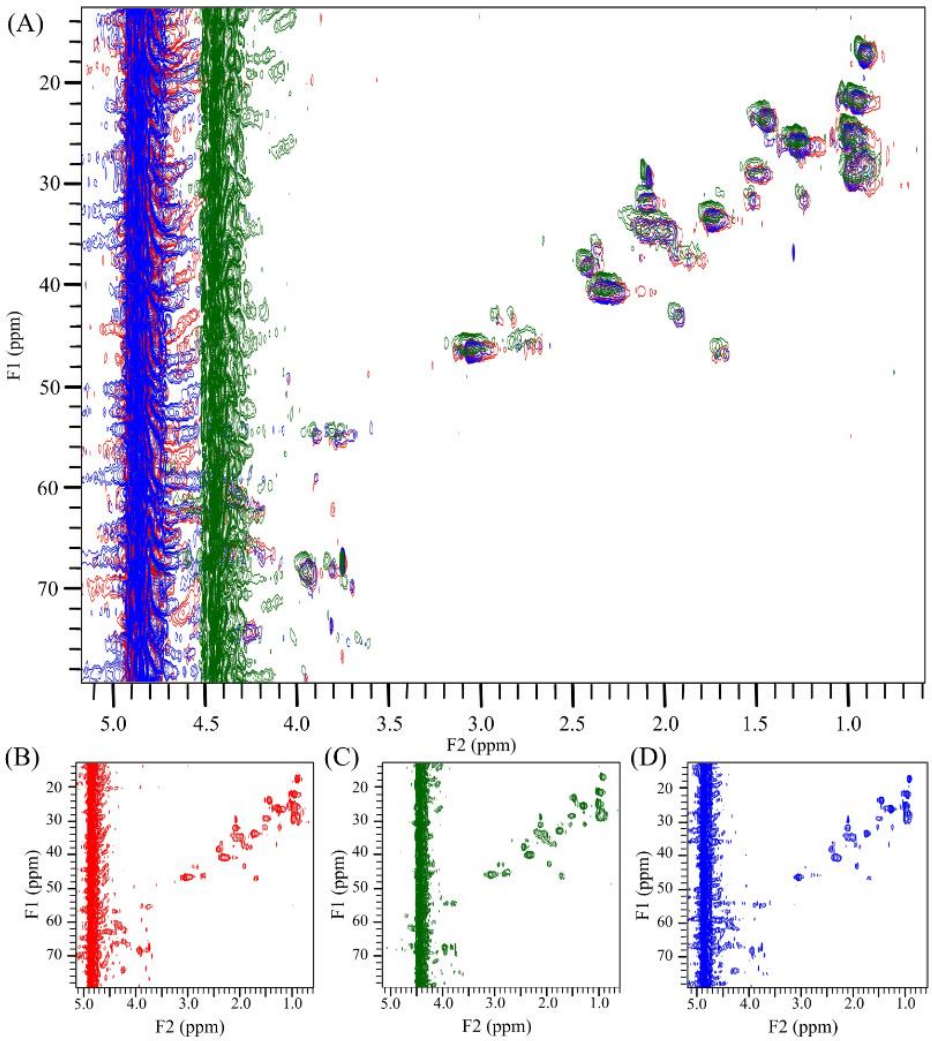


Figure 5.3: ^{1}H-^{13}C HSQC NMR spectra of 1% native β-lg (A), Overlay of spectra (B), (C) and (D), (B), 1% β-lg heat-treated at 62 °C for 20 hours (NMR spectrum acquired at 62 °C) (C), and 1% β-lg heat-treated at 62 °C for 20 hours and subsequently cooled to 20 °C (D). X-axis (F2) represents ^{1}H chemical shift and y-axis (F1) represents ^{13}C chemical shift – Worked carried out by J. Y, Kim & K. H., Mok, Trinity College Dublin.

However, even after the heating process, there were no significant chemical shift changes monitored between the control sample (A) and the cooled sample (C). This suggests that heat-treatment at 62 °C did not induce protein denaturation and only minor

chemical shift changes can be observed in (B), as the unfolded proteins appeared to reversibly renature to the native form (A and C).

There are only minor changes after a 20 hour of period sustained heat-treatment at 62 °C (B). However, even after the heating process, there were no significant chemical shift changes monitored between the control sample (A) and the cooled sample (C). This suggests that heat-treatment at 62 °C did not induce protein denaturation and only minor chemical shift changes can be observed in (B), as the unfolded proteins appeared to reversibly renature to the native form (A and C). The vertical line which appears between 4 – 5 ppm in all three spectra of Figure 5.3 correlates to the observed water signal after solvent suppression. Protein samples are usually prepared in an aqueous solutions containing relatively high water concentrations, of which 55,000 mM is the protein itself (typical protein NMR sample conc. = 1 – 3 mM) with the suppressed water signal a constant artefact of aqueous phase in the solution NMR.

Size exclusion chromatography HPLC (SEC-HPLC) was used to analyse soluble aggregate formation in heat-treated β-lg samples; a direct correlation was found between the length of time, and temperature of heating for the formation of soluble aggregates (Figure 5.4). Although β-lg usually exits as a non-covalent dimer between pH 5.5 - 7.5, unheated control samples only contained ~ 12 % dimeric protein. When this dimer fraction is correlated with SDS-PAGE results (Figure 5.5), it is observed that this dimer is a non-native dimer and most likely originates from the preparation of β-lg. Soluble aggregates were present in samples heat-treated at 85 °C, with 1% β-lg samples containing ~ 11% aggregated material after 5 minutes of heat-treatment. After 10 minutes, the percentage of aggregated protein had increased significantly ($P < 0.05$) to

82%. Both 12% β-lg samples, heated for 5 and 10 minutes, were extensively aggregated (97 and 98%, respectively).

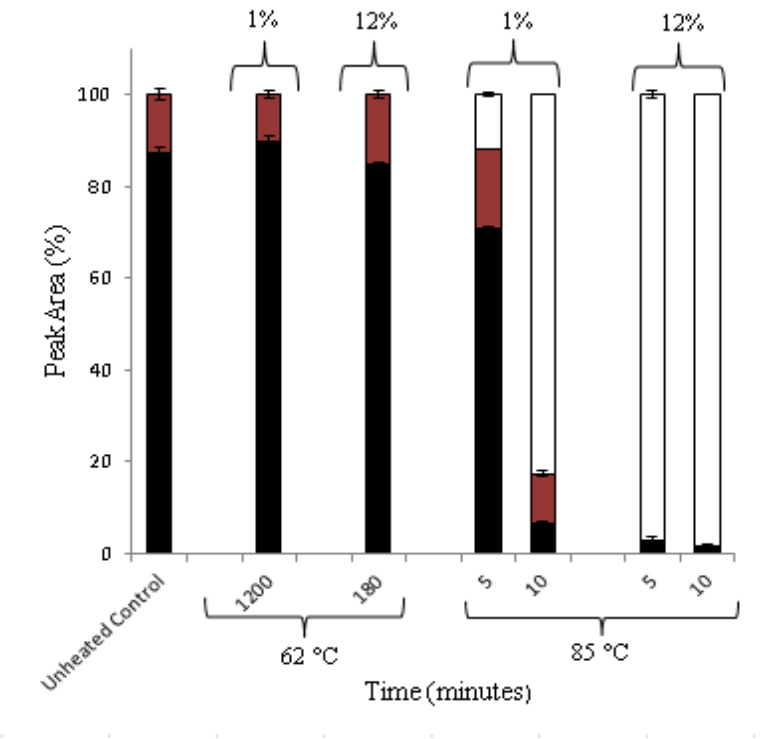


Figure 5.4: Distribution of proteins in the soluble fraction of 1 and 12% (w/w) β-lactoglobulin at pH 6.7, post thermal treatment under different conditions (62 °C for 1200 and 180 min) and 85 °C (5 and 10 min). The quantity of monomeric, dimeric and aggregated (soluble) protein in each sample is represented by the black, red and white regions respectively, as determined by size exclusion chromatography.

Non-native monomers have previously been described as monomeric species which are formed during the initial step of the β-lg denaturation and subsequent aggregation pathway (Croguennec et al., 2003; Kehoe et al., 2011; Surroca et al., 2002). As discussed in the introduction, when protein unfolding occurs, free sulfhydryl groups are exposed, causing the first stages of covalent aggregation via sulfhydryl/disulfide inter-change to occur. By using a thiol blocking agent (N-ethylmaleimide), Croguennec et al. (2003) showed that heat-treatment of β-lg A (7% protein, pH 6.6) heat-treated at 85 °C,

rearranged the disulfide bonds within β-lg monomers resulting in the exposure of Cys119, and the formation of a disulfide bond between Cys106-Cys121. Kehoe et al. (2008) studied the effect of irradiation on β-lg (1%, pH 7, 295 nm for 24 hours) and discovered intermediate monomeric species, where the disulfide bond of Cys66-Cys-160 was cleaved to leave an exposed sulfhydryl group. Some non-native monomers are hypothesised to be less reactive under the conditions they were formed at (Kehoe et al., 2011) and therefore more stable, as they are usually have higher surface charge when compared to the native monomer (Ryan et al., 2013).

At 62 °C, all native protein was found to be intact after heat-treatment, with no soluble aggregate formation, and therefore no non-native monomers present. The quantity of non-native monomer present in both 1 and 12% samples, heat-treated at 85 °C for 5 minutes, was 9.74% and 2.03%, respectively. Previous research has quantified the amount of non-native monomer present in β-lg samples ranging from 2 - 9% (pH 7.0) protein after heat-treatment at 78 °C (Kehoe et al., 2011); an initial increase in the quantity of non-native monomeric species present in samples was observed as a function of time, although a subsequent decrease occurred once secondary aggregation began. Similarly, in this experimental work, a decrease in non-native monomer was observed after denaturation and aggregation became extensive between 5 and 10 minutes of heating at 1% protein (3.0% non-native monomer present), with less non-native monomers present in samples heat-treated at 12% protein at 1.70%, respectively.

Results of non-reducing SDS-PAGE (Figure 5.5), confirmed the extent of covalently linked aggregation detected in samples heat-treated at 85 °C. The sample preparation used for non-reducing conditions involved the use of SDS (in the absence of any reducing agent such as mercaptoethanol), which dissociates non-covalent bonds, leaving

all aggregates linked through disulfide linkages intact. It is apparent that for both 1 and 12% samples, heat-treated at this temperature, extensive covalent aggregation occurred. Streaking of the non-reduced gel on both lanes 6 and 8 was observed, with little to no monomeric protein visible, when compared to unheated samples (lanes 5 and 7, respectively). Aggregates which are too large to enter the separation gel remained on the stacking gel of the wells of lanes 6 and 8. Although faint dimeric protein bands were visible for all unheated samples, there was no clear presence of trimers or oligomers for samples heat-treated at 62 °C.

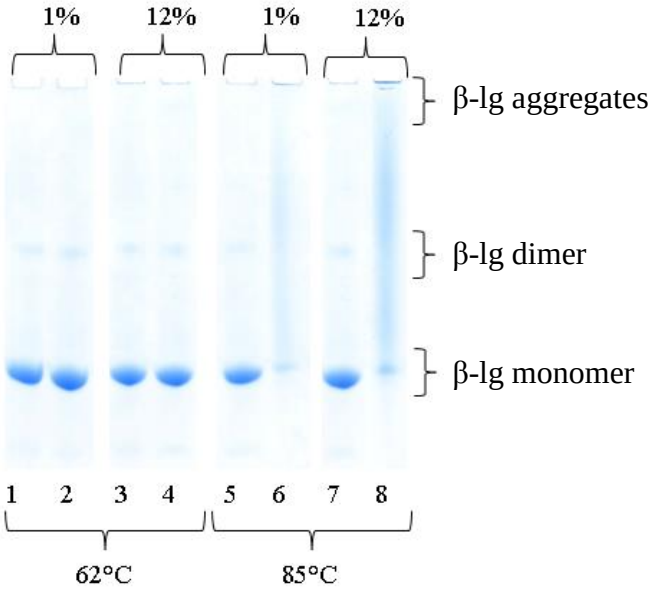


Figure 5.5: Non-reducing SDS-PAGE (12% Bis-Tris) for 1 and 12% β-lactoglobulin samples (pH 6.7) heat-treated at 62 and 85 °C. Lanes (1, 3, 5 and 7) represent unheated control samples; lanes (2 and 4) represent 1 and 12% β-lactoglobulin samples heat-treated for 1200 and 180 min (62 °C), respectively; lanes (6 and 8) represent 1 and 12% β-lactoglobulin samples heat-treated for 10 minutes.

The presence of a dimeric protein band would lead to the suggestion that there were some covalently bound dimeric β-lg molecules present in the original powder. It is most likely that not all covalent dimers were removed during isoelectric precipitation.

Surroca et al. (2002) studied β-lg at pH 6.7, heat-treated at 68.5 °C, for 0 to 6 hours, and determined that a faint dimeric band was present for unheated samples, with trimers beginning to form after 0.25 hours and oligomers forming after 0.5 hours of heat-treatment. However, Zuniga et al. (2010) also studied β-lg aggregation using SDS-PAGE and found that 5% (w/v) samples (pH 6.8), heat-treated at 80 °C, began to form trimers after 120 s of heating, with extensive aggregation and formation of tetramers and oligomers, as well as larger protein aggregates, after 600 s (10 minutes). It is likely that in the current study, the heating temperature of 85 °C is too great a temperature to determine bands for the specific formation of trimeric, tetrameric and oligomeric protein aggregates, as protein denaturation is rapid and the formation of larger disulfide linked protein aggregates prevails.

5.4.2 Analysis of Secondary Structure by Fourier-transform Infrared (FTIR) Spectroscopy

From RP-HPLC and SEC-HPLC results, it is apparent that protein denaturation and aggregation was extensive for samples heat-treated at 85 °C. The determination of secondary structure by FTIR confirmed the extent of changes in secondary structure (Figure 5.6). FTIR analyses the molecular level of proteins by observing the amide I region of the protein, which is present at wavenumbers (1600 - 1700cm^{-1}) within an infrared spectra; analysis focuses on the vibrationional energy, which originates from the amide vibrations, typically of the C=O peptide bonds (stretching vibration) (Bandekar, 1992; Lefèvre & Subirade, 1999). The secondary structure of β-lg has specific thermodynamic stability and when altered, mainly due to external environmental factors i.e., temperature, pH and ionic strength, differences in the stretching vibrations can be detected. These vibrations correlate to specific

wavenumbers within the infrared spectra, which correlate to α-helix and β-sheet structure (Bandekar, 1992).

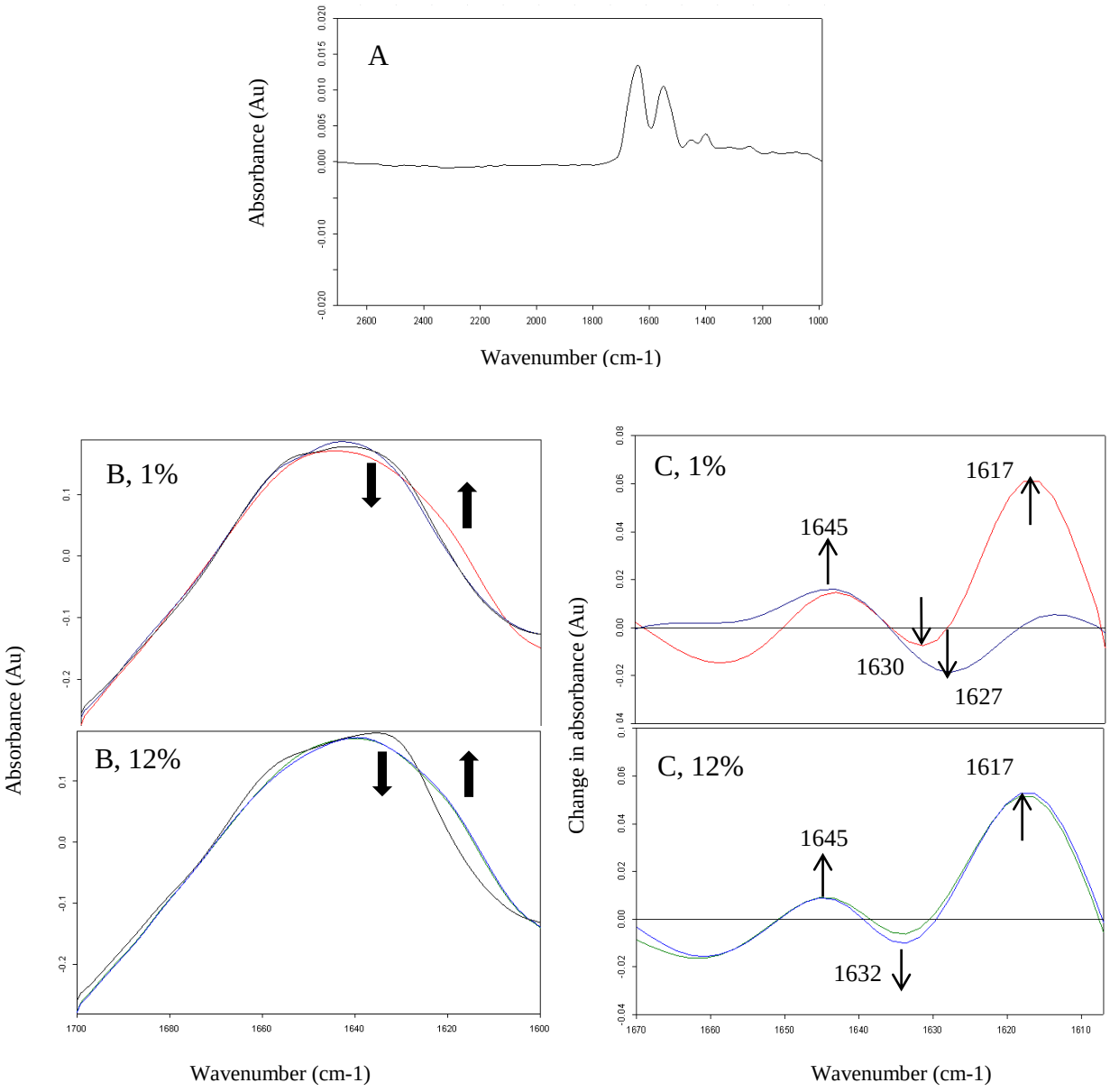


Figure 5.6: (A) Representative FTIR spectra from 3000 – 1000 Wavenumber (cm-1) (B) FTIR spectra of the amide I band, for 1 and 12% (w/w) β-lg samples heat-treated at 85 °C. (C) represents samples heat-treated for 5 and 10 minutes with native spectrum subtracted. Blue and red lines for A and B (top two graphs) represent 1% protein heat-treated for 5 and 10 minutes, respectively. The blue and green line for A and B (bottom two graphs) represent 12% samples heat-treated for 5 and 10 minutes respectively. Black lines in (A) represent native 1 and 12% samples in each respective graph.

No changes in spectra were observed for samples heat-treated at 62 °C, even after 20 hours of heat-treatment (data not shown). However, the spectra in Figure 5.6 (A and B) showed that there was a difference in secondary structure for samples heat-treated at 85 °C, as a function of the length of heat-treatment that samples received. Previous studies have assigned the bands 1617 cm-1, 1627 to 1632 cm-1 and 1645 cm-1 in FTIR spectra to antiparallel inter-molecular β-sheets, strongly bonded β-sheets and α-helix/unordered structure, respectively (Boye et al., 1997b; Fabian et al., 1993; Lefèvre & Subirade, 2000). Samples heat-treated at 1% (w/w) protein (Figure 5.6, A) showed a difference in the spectra between samples heat-treated for 5 or 10 minutes.

After 5 minutes of heating, the formation of a band at 1617 cm-1 began, with a significant increase in band strength after an additional 5 minutes of heating. This increase represents an increase in aggregation, with the formation of antiparallel inter-molecular β-sheets. When correlated to RP-HPLC and SEC-HPLC results, it is noted that after 5 minutes of heating at this protein concentration, only ~ 35% of the protein had been denatured, with a small proportion of this denatured material forming soluble aggregates (~ 11%). However, after an additional 5 minutes of heating, extensive denaturation and aggregation was observed. As expected, for 12% samples (Figure 5.6, B), FTIR spectra show no significant difference between samples heated for 5 or 10 minutes, with the formation of antiparallel inter-molecular β-sheets after only 5 minutes of heat-treatment.

These results are both conflicting and supportive of previous studies which analysed β-lg by different IR methods. Lefèvre & Subirade (1999) found (using FTIR) that the heat-treatment (85 °C for 5 minutes) of β-lg at lower protein concentrations (0.25 – 0.5%) resulted in the unfolding of β-lg with no detectable aggregation; however,

increasing protein concentration (1 - 10%) resulted in protein unfolding and aggregation, with the secondary structure of β-lg becoming disordered at higher protein concentrations. This was confirmed by Matheus et al. (2006), who observed that heat-treatment of 10% (w/w) (pH 7.2) β-lg, over a temperature range of 60 - 90 °C, resulted in formation of β-sheet with increasing temperature, with very little change in α-helix structure. Using RAMAN spectroscopy, Seo et al. (2010) determined that heat-treatment of β-lg at native pH from 20 - 100 °C resulted in a two stage denaturation step; the first step was associated with the dissociation of dimeric protein (at ~ 65 °C) and the second step involved a loss in the native α-helix structure with a concomitant formation of β-sheet. In the current study, a change in β-sheet formation is noted, although a change in α-helix structure is not particularly evident.

5.4.3 ^{1}H-^{13}C HSQC Nuclear Magnetic Resonance

As secondary structural changes in heat-treated samples were observed, ^{1}H-^{13}C HSQC NMR was applied to the post heat-treated samples at 85 °C (1 and 12% protein for 10 mins) to determine whether the overall structure of the protein was considerably altered. As described above, the NMR spectra for samples heat-treated at 62 °C did not exhibit significant peak signal changes, suggesting that major changes in the protein structure did not take place (Figure 5.3). As the changes hydrodynamic volume for 1% samples were not deemed to be as significant as those of 12% samples, this results and discussion section will focus on (A) unheated 12% β-lg, (B) heat-treated (85°C for 10 minutes) 12% β-lg and (C) the soluble protein fraction of 12% β-lg heat-treated (85°C for 10 minutes) samples, prepared through isoelectric precipitation (Figure 5.7). Once ^{1}H-^{13}C HSQC spectra were obtained for all samples, the 1D peak traces at the same

chemical peaks from the three spectra (A, B, C) were compared, and from this, three different trends/groups were identified (Figure 5.7).

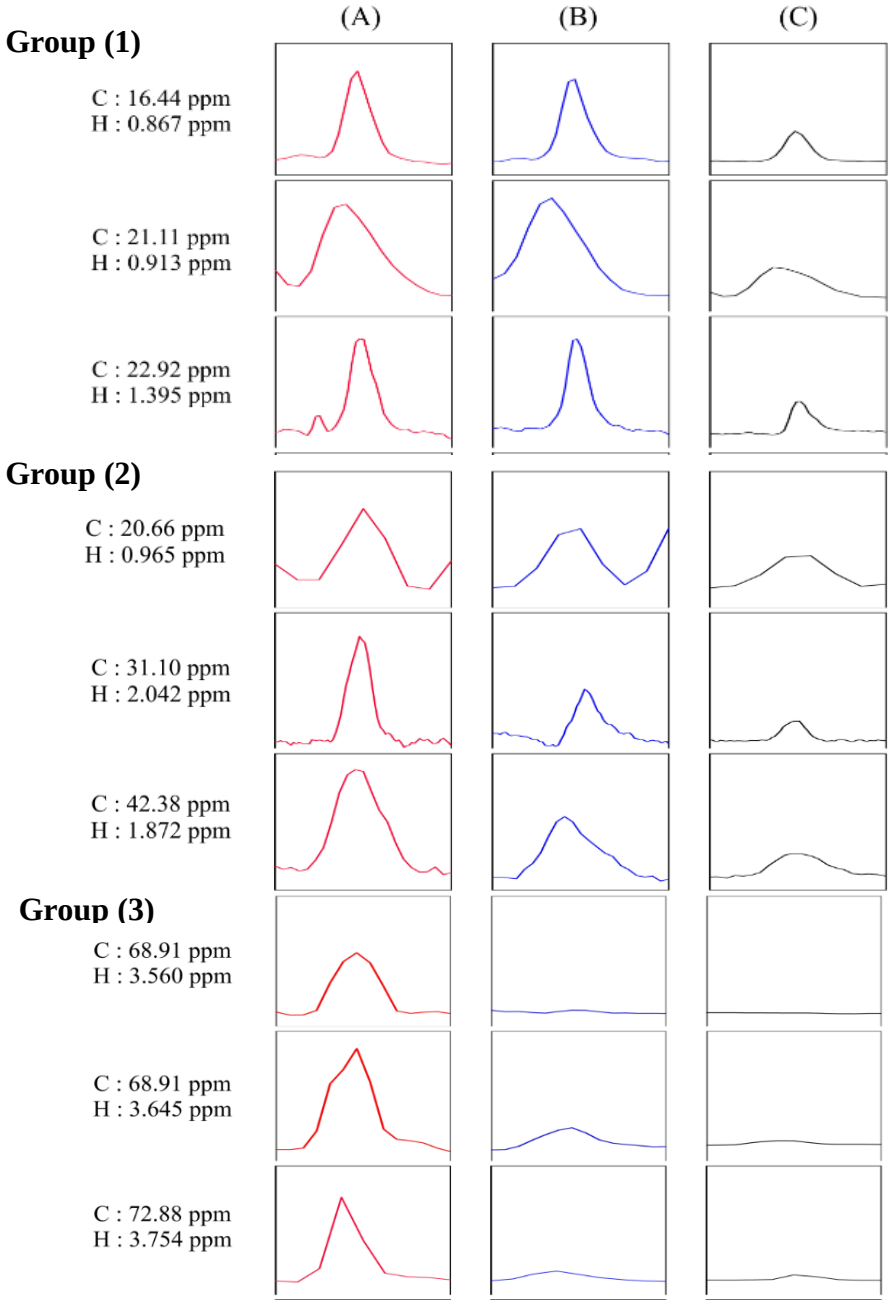


Figure 5.7: Volume comparison of 1D peak obtained by ^{1}H-^{13}C HSQC. Samples are represented by the following: (A) β-lactoglobulin control (native protein at pH 6.7 without heating process), (B) β-lactoglobulin heat-treated at 85°C for 10 mins (pH 6.7), and (C) β-lactoglobulin heated at 85 °C for 10 mins after removal of insoluble material removed through precipitation at pH 4.6 and readjustment to pH 6.7. Group (1) is representative of peaks not altered after heating, group (2) is representative of peaks

which were moderately altered after heating and group (3) is representative of peaks which were entirely altered after heating. (C) and (H) represent the ^{13}C and ^{1}H isotopes – worked carried out by J. Y, Kim & K. H., Mok, Trinity College Dublin.

The three trends observed were Group (1), which was representative of peaks not altered after heating, Group (2), which was representative of peaks which were moderately altered after heating and Group (3), which was representative of peaks which were entirely altered after heating (Figure 5.7). The peaks provided in Figure 5.7 represent specific, unassigned, regions of the β-lg protein, with peak height representing the preservation of structure within the peaks region (if there is no change in peak height) or a change in structure within the region of the peak (diminishing peak height) i.e., the structure of the protein at these particular regions are no longer equivalent to the native structure of unheated β-lg.

Group (1) showed that the peak volume of the specific peak areas analysed did not decrease after heating at 85 °C for 10 mins. However, after the removal of precipitated material (insoluble protein, column C), there was a decrease in peak volume. This result would suggest that the spin populations in which these peaks reside, are not drastically altered after heat-treatment. However, when insoluble material was removed through precipitation, there was a significant decrease in peak volume, although a peak was still detectable. Using SEC-HPLC, the soluble fraction of dimeric and monomeric protein was collected and the protein percentage determined, with protein concentrations of 0.027 and 0.108% respectively representing the peak volume remaining in group (C) of Figure 5.7.

Group (2), as seen in Figure 5.7, showed a gradual decrease in peak volume when the protein was heated at 85 °C for 10 mins, with a further decreased in peak volume when

precipitations were removed from the sample. This appears to reflect that the population of spins that were found in these particular environments have decreased to about 50% – in other words, a proportion of the protein molecules with three-dimensional structures (post-heat-treatment at 85 °C for 10 mins, pH 6.7, 20 °C) identical to the native state have undergone perturbations.

Group (3) in Figure 5.7, showed a significant decrease in peak volume after the heating process, with the remaining peak undetectable after removal of the insoluble material from the sample. This showed that heat-treatment altered the structure of the protein in these regions, while peaks in Group (2) were less altered. The peaks in Group (3) represent regions of the molecular structure where very definite structural changes have occurred after heat-treatment.

Overall, most peaks followed Group 1 and Group 2 trends, suggesting that the protein conformation is not extensively altered upon heat-treatment at 85 °C for 10 minutes. Delahaije et al. (2016) studied the unfolding, refolding and aggregation process of 2% (w/w) β-lg at pH 7.0 by circular dichroism and observed that soluble β-lg, precipitated after heat-treatment, had very similar structure to the native β-lg monomer, with insoluble material present in one "native-like" and two different "non-native like" conformations. The ^{1}H-^{13}C HSQC NMR results, along with the chromatography data, would suggest that in β-lg samples heat-treated at 85 °C, both native-like and non-native like aggregates co-exist. However, further work is necessary in order to be able to assign NMR peaks to individual amino acids, allowing for the quantification of conformational changes in β-lg structure on heating.

5.5 Conclusions

In this study, ^{1}H-^{13}C HSQC NMR was combined with FTIR, and chromatography techniques, to determine the effect of protein concentration on denaturation and protein aggregate formation. It confirms that although protein denaturation of β-lg at a heat-treatment of 85 °C is extensive, the structural rearrangement of protein monomers produced both “native-like” and “non-native like” protein aggregates, with a proportion of soluble material existing as non-native monomers.

CHAPTER 6

General Discussion

6.1 Overall conclusions

The thermal history of a protein influences its behaviour during subsequent heating and concentration, particularly in the presence of other ionic species. This thesis set out to investigate the effect that changing protein concentration has on the structure – function relationship in heated whey protein solutions. To achieve this objective, experiments were carried out on: 1) Chapter 2 - unheated whey protein systems and mineral salts; 2) Chapter 3 - heated whey protein systems and pH; 3) Chapter 4 - emulsified dairy protein systems and protein type; and 4) Chapter 5 - purified β-lactoglobulin systems and structural change after heating. The underlying theme throughout was the effect that a change in protein concentration has on the chemical and physical properties of the protein system. A key conclusion of the research centres on the morphology of the aggregates formed during heating, which was shown to be protein concentration dependant. The findings showed that increasing the protein concentration (in the range 1 to 12% (w/w)) at which whey proteins (primarily α-lactalbumin and β-lactoglobulin) are heated, resulted in significantly ($P < 0.05$) increased proportion of denatured protein and hence, increased levels of soluble aggregates formed (in filtrate, 45 μm pore size), reduced aggregate size (z-average diameter) and greater heat stability at 6% (w/w) protein, pH > 6.9 (Chapter 3).

Physicochemical parameters such as pH, ionic strength and protein concentration have an effect on the physical behaviour of whey proteins, pre- and post-processing. The research in Chapters 2 and 3 provides data to support new processing strategies for whey protein based foods and beverages, by investigating the physical characteristics of whey proteins under different environmental conditions, pre- and post-heat-treatment. Chapter 2 demonstrated that depending on pH, protein concentration and ionic strength

of a whey protein solution, solution turbidity and viscosity differed. Heat treatment of 1 and 4% whey protein solutions at pH 6.7 and 7.2 caused the formation of aggregates with larger z-average sizes, when compared to the z-average of aggregates formed at 8 and 12% protein. At pH 6.2, there was no effect of protein concentration; as pH 6.2 is closer to the isoelectric point of both whey proteins, at this pH whey protein molecules are not as highly charged as at pH 6.7 and 7.2, and therefore are more prone to aggregation upon heat-treatment, when compared to the equivalent samples heat-treated at pH 6.7 and 7.2. Upon subsequent heating, using the heat coagulation time method (HCT), greater heat stability was observed for samples of higher protein concentration, heat-treated at higher pH (8 and 12% protein, pH 6.7 and 7.2). These samples, heated at the higher protein concentration, contained greater quantities of soluble aggregates, had smaller overall aggregate size when compared to samples heat-treated at lower protein concentration (1 and 4%) and were more thermally stable in solution.

Understanding β-lactoglobulin denaturation and subsequent aggregation at a molecular level can improve our understanding of the behaviour of β- lactoglobulin in purified and mixed protein systems (Chapters 2 - 5). This study demonstrated the structural changes of β-lactoglobulin at higher concentration resulted in the formation of aggregates with monomer like species, depending on heating time and temperature. The production of protein intermediates can be beneficial in whey protein systems, as these intermediates are highly charged and can be more resistant to aggregation than the native monomer. Interestingly, under controlled conditions (85 °C x 30 s), while the globular protein β-lactoglobulin underwent structural conformation change, it is hypothesised that the resulting aggregates containing “native-like” and “non-native like” protein aggregates (Chapter 5).

Chapter 4 demonstrated that the reactivity of the protein system used to stabilise the fat globules is key determinant of viscosity during concentration of emulsion-based beverages, such as infant formula. This increase in viscosity was attributed to coalescence of fat globules brought about by emulsion instability. Studying the interaction between the heating and homogenisation steps and the ratio of α-lactalbumin to β-lactoglobulin, showed that emulsion destabilisation was due to increased reaction of the free thiol group associated with denatured β-lactoglobulin.

6.2 Conclusions and future work

This study highlighted the importance of understanding protein denaturation and subsequent aggregation in relation to system viscosity, ranging from pure protein systems to a model infant formula. The results showed that the concentration at which whey proteins are heated affects molecular structure, aggregate morphology and subsequent heat stability in solution and emulsified systems. The findings of the thesis are an important consideration for manufactures of whey protein ingredients and end users such as infant formula manufacturers alike. Future work should concentrate on:

- Investigation of protein-protein interactions in heat-treated mixtures containing pre-aggregated whey protein particles and casein.
- Investigation of the impact of minerals salts on aggregate formation, size and subsequent heat stability using the current work as a basis.
- Assessing the impact of mineral salt addition on protein-protein/protein-fat interactions in an emulsified system (IMF), and the subsequent effect on apparent viscosity.

Bibliography

Aly, E., Ros, G., & Frontela, C. (2013). Structure and Functions of Lactoferrin as Ingredient in Infant Formulas. *Journal of Food Research, 2*(4), 25-36. DOI: 10.5539/jfr.v2n4p25

Anema, S. (2009). The whey proteins in milk: Thermal denturation, physical interactions and effects on the functional properties of milk. In: A. B. Thompson and H. Singh, (Eds.) *Milk proteins: From expression to food*, pp. 274 – 276. Elsevier, Amsterdam.

Aranberri, I., Binks, B. P., Clint, J. H., & Fletcher, P. D. I. (2004). Evaporation rates of water from concentrated oil-in-water emulsions. *Langmuir, 20*(6), 2069-2074. DOI: 10.1021/la035031x

Atkinson, S. A., & Lönnerdal, B. (1995). Nitrogenous components of milk B. Non protein fractions of human milk. In: Jensen, R. G. (Ed.), *Handbook of milk composition*, pp. 369-387. London: Academic Press.

Bakker, H. J. (2008). Structural dynamics of aqueous salt solutions. *Chemical Reviews, 108*(4), 1456-1473. DOI: 10.1021/cr0206622

Bandekar, J. (1992). Amide Modes and Protein Conformation. *Biochimica Et Biophysica Acta, 1120***,** 123-143. DOI: 10.1016/0167-4838(92)90261-B

Bansal, B., & Chen, X. D. (2006). A critical review of milk fouling in heat exchangers. *Comprehensive Reviews in Food Science and Food Safety, 5*(2), 27-33. DOI: 10.1111/j.1541-4337.2006.tb00080.x

Bansal, N. B., & Bhandari. (2016). Functional milk proteins: production and utilization-whey-based ingredients. In: P. L. H. McSweeney, J. A. O'Mahony (Eds.) *Advanced dairy chemistry: Proteins 1B: Applied aspects*, 4th ed, pp. 67-99. New York: Springer.

Barbana, C., & Perez, M. D. (2011). Interaction of alpha-lactalbumin with lipids and possible implications for its emulsifying properties - A review. *International Dairy Journal, 21*(10), 727-741. DOI: 10.1016/j.idairyj.2011.04.009

Bar-Yoseph, F., Lifshitz, Y., & Cohen, T. (2013). Review of sn-2 palmitate oil implications for infant health. *Prostaglandins Leukotrienes and Essential Fatty Acids, 89*(4), 139-143. DOI: 10.1016/j.plefa.2013.03.002

Batchelor, J. D., Olteanu, A., Tripathy, A., & Pielak, G. J. (2004). Impact of protein denaturants and stabilizers on water structure. *Journal of the American Chemical Society, 126*(7), 1958-1961. DOI: 10.1021/ja039335h

Bazinet, L., Trigui, M., & Ippersie, D. (2004). Rheological behavior of WPI dispersion as a function of pH and protein concentration. *Journal of Agriculture and Food Chemistry, 52***,** 5366-5371. DOI: 10.1021/jf049893v

Berg, J. M., Tymoczko, J. L., & Stryer, L. (2002). *Biochemistry* (5th ed.). New York: W.H. Freeman.

Bhattacharjee, C., Saha, S., Biswas, A., Kundu, M., Ghosh, L., & Das, K. P. (2005). Structural changes of beta-lactoglobulin during thermal unfolding and refolding - An FT-IR and circular dichroism study. *Protein Journal, 24***,** 27-35. DOI: 10.1007/s10930-004-0603-z

Bitounis, D., Fanciullion, R., Athanassios, F., Iliadis, A., & Ciccolini, J. (2012). Optimizing Druggability through Liposomal Formulations: New Approaches to an Old Concept. *Interanational Scholarly Research Network, 2012*, 11. DOI: 10.5402/2012/73848432

Blumlein, A., & McManus, J. J. (2013). Reversible and non-reversible thermal denaturation of lysozyme with varying pH at low ionic strength. *Biochim Biophysica Acta, 1834***,** 2064-70. DOI: 10.1016/j.bbapap.2013.06.001

Bouchoux, A., Cayemitte, P. E., Jardin, J., Gesan-Guiziou, G., & Cabane, B. (2009). Casein micelle dispersions under osmotic stress. *Biophysical Journal, 96***,** 693-706. DOI: 10.1016/j.bpj.2008.10.006

Boye, J. I., & Alli, I. (2000). Thermal denaturation of mixtures of alpha-lactalbumin and beta-lactoglobulin: a differential scanning calorimetric study. *Food Research International, 33***,** 673-682. DOI: 10.1016/S0963-9969(00)00112-5

Boye, J. I., Alli, I., & Ismail, A. A. (1997a). Use of differential scanning calorimetry and infrared spectroscopy in the study of thermal and structural stability of alpha-lactalbumin. *Journal of Agriculture and Food Chemistry, 45*(4), 1116-1125. DOI: 10.1021/jf960360z

Boye, J. I., Ismail, A. A., & Alli, I. (1996a). Effects of physicochemical factors on the secondary structure of beta-lactoglobulin. *Journal of Dairy Research, 63*(1), 97-109. DOI: 10.1021/Jf950529t

Boye, J. I., Ma, C. Y., Ismail, A., Harwalkar, V. R., & Kalab, M.(1997b). Molecular and microstructural studies of thermal denaturation and gelation of beta-lactoglobulins A and B. *Journal of Agricultural and Food Chemistry, 45***,** 1608-1618.

Brew, K. (2013). α-lactalbumin. In: P. L. H. McSweeney, P. F. Fox (Eds.) *Advanced Dairy Chemistry, Proteins: Basic Aspects*, vol 1A, pp. 261-275. New York: Springer.

Briggs, D. R., & Hull, R. (1945). Studies on Protein Denaturation. I. Electrophoretic Study Kinetics at Neutrality of Heat Denaturation of β-Lactoglobulin. *Journal of American Chemical Society, 67***,** 2007-2014.

Brodkorb, A., Croguennec, T., Bouhallab, S., & Kehoe, J. J. (2016). Heat-Induced Denaturation, Aggergation and Gelation of Whey Proteins. In: P. L. H. McSweeney, J. A. O'Mahony (Eds.) *Advanced dairy chemistry: Proteins: Applied aspects*, vol. 1B, pp. 155-179. New York: Springer.

Brownlow, S., Cabral, J. H. M., Cooper, R., Flower, D. R., Yewdall, S. J., Polikarpov, I., & Sawyer, L. (1997). Bovine beta-lactoglobulin at 1.8 angstrom resolution - Still an enigmatic lipocalin. *Structure, 5*(4), 481-495. DOI: 10.1016/S0969-2126(97)00205-0

Bryant, C. M., & McClements, D. J. (1998). Molecular basis of protein functionality with special consideration of cold-set gels derived from heat-denatured whey. *Trends in Food Science & Technology, 9*(4), 143-151. DOI: 10.1016/S0924-2244(98)00031-4

Buggy, A. K., McManus, J. J., Brodkorb, A., Mc Carthy, N., & Fenelon, M. A. (2017). Stabilising effect of alpha-lactalbumin on concentrated infant milk formula emulsions heat-treated pre- or post-homogenisation. *Dairy Science & Technology, 96*(6), 845-859. DOI: 10.1007/s13594-016-0306-1

Byler, D. M. S. (1986). Examination of the secondary structure of proteins by deconvoluted FTIR spectra. *Biopolymers, 25*, 469-487. DOI: 10.1002/bip.360250307

Cèrny, J., & Hobza, P. (2007). Non-covalent interactions in biomacromolecules. *Physical Chemistry Chemical Physics, 9*(39), 5291-5303. DOI: 10.1039/b704781a

Cho, Y. H., Singh, H., & Creamer, L. K. (2003). Heat-induced interactions of beta-lactoglobulin A and kappa-casein B in a model system. *Journal of Dairy Research, 70,* 61-71. DOI: 10.1017/S0022029902005642

Chrysina, E. D., Brew, K., & Acharya, K. R. (2000). Crystal structures of apo- and holo-bovine alpha-lactalbumin at 2.2-A resolution reveal an effect of calcium on inter-lobe interactions. *Journal of Biological Chemistry, 275*(47), 37021-37029. DOI: 10.1074/jbc.M004752200

Codex Alimentarius (2007) Standard for infant formula and formulas for special medical purposes intended for infants.

Collins, K. D., & Washabaugh., M. W. (1985). The Hofmeister effect and the behaviour of water at interfaces. *Quarterly reviews of biophysics, 18*(4), 323-422. DOI: 10.1017/S0033583500005369

Conesa, C., Calvo, M., & Sánchez, L. (2010). Recombinant human lactoferrin: A valuable protein for pharmaceutical products and functional foods. *Biotechnology Advances, 28,* 831-838. DOI: 10.1016/j.biotechadv.2010.07.002

Cornacchia, L., De La Fortelle, C. F., & Venema, P. (2014). Heat-induced aggregation of whey proteins in aqueous solutions below their isoelectric point. *Journal of Agriculture and Food Chemistry, 62***,** 733-741. DOI: 10.1021/jf404456q

Corredig, M., & Dalgleish, D. G. (1996). Effect of different heat treatments on the strong binding interactions between whey proteins and milk fat globules in whole milk. *Journal of Dairy Research, 63*(3**),** 441-449. DOI: 10.1017/S0022029900031940

Creamer, L. K., & Parry, D.A. (1981). Secondary structure of bovine alpha-s1 and beta-casein in solution. *Archives of Biochemistry and Biophysics, 211,* 689-696.

Creamer, L. K., Berry, G. P., & Matheson, A. R. (1978). Effect of Ph on Protein Aggregation in Heated Skim Milk. *New Zealand Journal of Dairy Science and Technology, 13*(1), 9-15.

Croguennec, T., Bouhallab, S., Molle, D., O'Kennedy, B. T., & Mehra, R. (2003). Stable monomeric intermediate with exposed Cys-119 is formed during heat denaturation of beta-lactoglobulin. *Biochemical and Biophysical Research Communications, 301***,** 465-471. DOI: 10.1016/S0006-291X(02)02997-2

Croguennec, T., O'Kennedy, B. T., & Mehra, R. (2004). Heat-induced denaturation/aggregation of β-lactoglobulin A and B: kinetics of the first intermediates formed. *International Dairy Journal*, *14*(5), 399-409. DOI: 10.1016/j.idairyj.2003.09.005

Crowley, S. V., Dowling, A. P., Caldeo, V., Kelly, A. L., & O'Mahony, J. A. (2016). Impact of alpha-lactalbumin:beta-lactoglobulin ratio on the heat stability of model infant milk formula protein systems. *Food Chemistry, 194,* 184-190. DOI: 10.1016/j.foodchem.2015.07.077

Curtis, R. A., Ulrich, J., Montaser, A., Prausnitz, J. M., & Blanch, H. W. (2002). Protein-protein interactions in concentrated electrolyte solutions - Hofmeister-series effects. *Biotechnology and Bioengineering, 79*(4), 367-380. DOI: 10.1002/bit.10342

D'Agostina, A., Boschin, G., Rinaldi, A., & Arnoldi, A. (2003). Updating on the lysinoalanine content of commercial infant formulae and beicost products. *Food Chemistry, 80*(4), 483-488. DOI: 10.1016/S0308-8146(02)00316-3

Dalgleish, D. G. (2011). On the structural models of bovine casein micelles-review and possible improvements. *Soft Matter, 7*(6), 2265-2272. DOI: 10.1039/C0SM00806K

Dalgleish, D. G., Goff, H. D., Brun, J. M., & Luan, B. B. (2002). Exchange reactions between whey proteins and caseins in heated soya oil-in-water emulsion systems - overall aspects of the reaction. *Food Hydrocolloids, 16*(4), 303-311. DOI: 10.1016/S0268-005X(01)00103-5

Dalgleish, D. G., Senaratne, V., & Francois, S. (1997). Interactions between alpha-lactalbumin and beta-lactoglobulin in the early stages of heat denaturation. *Journal of Agriculture and Food Chemistry, 45*(9), 3459-3464. DOI: 10.1021/jf970113a

Dalgleish, D. G., Spagnuolo, P. A., & Goff, H. D. (2004). A possible structure of the casein micelle based on high-resolution field-emission scanning electron microscopy. *International Dairy Journal, 14*(12), 1025-1031. DOI 10.1016/j.idairyj.2004.04.008

Dauphas, S., Mouhous-Riou, N., Metro, B., Mackie, A. R., Wilde, P. J., Anton, M., & Riaublanc, A. (2005). The supramolecular organisation of beta-casein: effect on interfacial properties. *Food Hydrocolloids, 19*(3), 387-393. DOI: 10.1016/j.foodhyd.2004.10.005

Davis, T. A., Nguyen, H. V., Garcia-Bravo, R., Fiorotto, M. L., Jackson, E. M., Lewis, D. S. Lee, D. R., & Reeds, P. J. (1994). Amino acid composition of human milk is not unique. *Journal of Nutrition, 124,* 26-32.

de Kruif, C. G. (1999). Casein micelle interactions. *International Dairy Journal 9,* 183-188. DOI: 10.1016/S0958-6946(99)00058-8

de Kruif, C. G., & Holt, C. (2003). Casein Micelle Structure, Functions and Interactions. In P. F. M. Fox, P. L. H. McSweeney (Ed.), *Advanced Dairy Chemistry,* vol 3, pp. 233-254. New York: Kluwer Academic/Plenum Publishers.

De Marco, A. A. (1977). pH dependence of internal references. *Journal of Magnetic Resonance, 26***,** 527-528.

de Wit, J. N. (1984b). Functional properties of whey proteins in food systems. *Netherlands Milk and Dairy Journal, 38***,** 71-89.

de Wit, J. N., & Klarenbeek, G. (1984a). Effects of Various Heat-Treatments on Structure and Solubility of Whey Proteins. *Journal of Dairy Science, 67*(11), 2701-2710. DOI: 10.3168/jds.S0022-0302(84)81628-8

Delahaije, R. J. B. M., Gruppen, H., Van Eijk-Van Boxtel, E. L., Cornacchia, L., & Wierenga, P. A. (2016). Controlling the Ratio between Native-Like, Non-Native-Like, and Aggregated beta-Lactoglobulin after Heat Treatment. *Journal of Agricultural and Food Chemistry, 64***,** 4362-4370. DOI: 10.1021/acs.jafc.6b00816

Demetriades, K., Coupland, J.N., & McClements, D. J. (1997). Physical properties of whey protein stabilized emulsions as related to pH and NaCl. *Journal of Food Science, 62,* 342-347. DOI: 10.1111/j.1365-2621.1997.tb03997.x

Dewey, K. G., Beaton, G., Fjeld, C., Lönnerdal, B., Reeds, P., Brown, K. H, Heinig, M. J., Ziegler, E., Räihä., N. C. R., & Axelsson, I. E. M. (1996). Protein requirements of infants and children. *European Journal of Clinical Nutrition, 50*(Suppl. 1). DOI: 10.1159/000095009

Dickinson, E. (1988). Monte-Carlo Model of Competitive Adsorption between Interacting Macromolecules and Surfactants. *Molecular Physics, 65*(4), 895-908. DOI: 10.1080/00268978800101481

Dickinson, E. (1997a). Enzymic crosslinking as a tool for food colloid rheology control and interfacial stabilization. *Trends in Food Science & Technology, 8*(10), 334-339. DOI: 10.1016/S0924-2244(97)01067-4

Dickinson, E. (1997b). Properties of Emulsions Stabilized with Milk Proteins: Overview of Some Recent Developments. *Journal of Dairy Science, 80*, 2607-2619. DOI: 10.3168/jds.S0022-0302(97)76218-0

Dickinson, E. (1998). Proteins at interfaces and in emulsions - Stability, rheology and interactions. *Journal of the Chemical Society-Faraday Transactions, 94*(12), 1657-1669. DOI:10.1039/A801167B

Dickinson, E. (2001). Milk protein interfacial layers and the relationship to emulsion stability and rheology. *Colloids and Surfaces B-Biointerfaces, 20*(3), 197-210. DOI: 10.1016/S0927-7765(00)00204-6

Dickinson, E. (2006). Structure formation in casein-based gels, foams, and emulsions. *Colloids and Surfaces A: Physicochemical and Engineering Aspects, 288*(1-3), 3-11. DOI: 10.1016/j.colsurfa.2006.01.012

Dickinson, E. (2010). Flocculation of protein-stabilized oil-in-water emulsions. *Colloids and Surfaces B-Biointerfaces, 81*(1), 130-140. DOI: 10.1016/j.colsurfb.2010.06.033

Dickinson, E., & Golding, M. (1998). Influence of alcohol on stability of oil-in-water emulsions containing sodium caseinate. *Journal of Colloid and Interface Science, 197*(1), 133-141. DOI: 10.1006/jcis.1997.5221

Dissanayake, I. M., Ramchandran, L., Donkor, O. N., & Vasiljevis, T. (2013). Denaturation of whey proteins as a function of heat, pH and protein concentration. *International Dairy Journal, 31***,** 93-99. DOI: 10.1016/j.idairyj.2013.02.002

Donato, L., Guyomarc'h, F., Amiot, S., & Dalgleish, D. G. (2007). Formation of whey protein/kappa-casein complexes in heated milk: Preferential reaction of whey protein with kappa-casein in the casein micelles. *International Dairy Journal, 17*(10), 1161-1167. DOI: 10.1051/dst:2008033

Donella-Deana, A., Grankowski, N., Kudlicki, N., Szyszka, R., Gasior, E., & Pinna, L.A. (1985). A type-1 casein kinase from yeast phosphorylates both serine and threonine residues of casein. Identification of phosphorylation sites. *Biochimica et Biophysica Acta (BBA) - Protein Structure and Molecular Enzymology, 829,* 180-187. DOI: 10.1016/0167-4838(85)90187-6

Donovan, S. M. (1987). Thermal-Denaturation and Aggregation of Whey Proteins. *Irish Journal of Food Science and Technology, 11*(1), 87-100.

Donovan, S. M. (2016). The Role of Lactoferrin in Gastrointestinal and Immune Development and Function: A Preclinical Perspective. *Journal of Paediatrics, 173,* (Suppl. 16-28). DOI: 10.1016/j.jpeds.2016.02.072

Eissa, A. S., & Khan, S. A. (2006). Modulation of hydrophobic interactions in denatured whey proteins by transglutaminase enzyme. *Food Hydrocolloids, 20*(4), 543-547. DOI: 10.1016/j.foodhyd.2005.07.005

El-Salam, M. H. A., El-Shibiny, S., & Salem, A. (2009). Factors Affecting the Functional Properties of Whey Protein Products: A Review. *Food Reviews International, 25*(3), 251-270. DOI: 10.1080/87559120902956224

Erabit, N., Ndoye, F. T., Alvarez, G., & Flick, D. (2016). Coupling population balance model and residence time distribution for pilot-scale modelling of beta-lactoglobulin aggregation process. *Journal of Food Engineering, 177***,** 31-41. DOI: 10.1016/j.jfoodeng.2015.12.013

European Commission. (2006). Commission Directive 2006/141/EC of 26 December 2006 on infant formulae and follow-on formulae. *Offical Journal of the European Union.*

Euston, S. R., Hirst, R. L., & Hill, J. P. (1999). The emulsifying properties of beta-lactoglobulin genetic variants A, B and C. *Colloids and Surfaces B-Biointerfaces, 12***,** 193-202. DOI: 10.1016/S0927-7765(98)00074-5

Fabian, H., Schultz, C., Naumann, D., Landt, O., Hahn, U., & Saenger, W. (1993). Secondary Structure and Temperature-Induced Unfolding and Refolding of Ribonuclease-T(1) in Aqueous-Solution - a Fourier-Transform Infrared Spectroscopic Study. *Journal of Molecular Biology, 232***,** 967-981.

Fanali, G., di Masi, A., Trezza, V., Marino, M., Fasano, M., & Ascenzi, P. (2012). Human serum albumin: From bench to bedside. *Molecular Aspects of Medicine, 33*(3), 209-290. DOI: 10.1016/j.mam.2011.12.002

Farrell, H. M., Kumosinski, T. F., Cooke, P. H., King, G., Hoagland, P. D., Wickham, E. D., & Groves, M. L. (1996). Particle sizes of purified kappa-casein: Metal effect and correspondence with predicted three-dimensional molecular models. *Journal of Protein Chemistry, 15*(5), 435-445. DOI: 10.1007/BF01886850

Farrell, H. M., Kumosinski, T. F., Pulaski, P., & Thompson, M. P. (1988). Calcium-Induced Associations of the Caseins - A Thermodynamic Linkage Approach to Precipitation and Resolubilization. *Archives of Biochemistry and Biophysics, 265*(1), 146-158. DOI: 10.1016/0003-9861(88)90380-3

Farrell, H. M., Malin, E. L., Brown, E. M., & Mora-Gutierrez, A. (2009). Review of the chemistry of alpha(S2)-casein and the generation of a homologous molecular model to explain its properties. *Journal of Dairy Science, 92*(4), 1338-1353. DOI: 10.3168/jds.2008-1711

Farrell, H. M., Malin, E. L., Brown, E. M., & Mora-Gutierrez, A. (2009). Review of the chemistry of alpha(S2)-casein and the generation of a homologous molecular model to explain its properties. *Journal of Dairy Science, 92*(4), 1338-1353. DOI: 10.3168/jds.2008-1711

Farrell, H. M., Pessen, H., Brown, E. M., & Kumosinski, T. F. (1990). Structural Insights into the Bovine Casein Micelle - Small-Angle X-Ray-Scattering Studies

and Correlations with Spectroscopy. *Journal of Dairy Science, 73*(12), 3592-3601. DOI: 10.3168/jds.S0022-0302(90)79062-5

Farrell, H. M., Wickham, E. D., Unruh, J. J., Qi, P. X., & Hoagland, P. D. (2001). Secondary structural studies of bovine caseins: temperature dependence of beta-casein structure as analyzed by circular dichroism and FTIR spectroscopy and correlation with micellization. *Food Hydrocolloids, 15*(4-6), 341-354. DOI: 10.1023/A:1019992900455

Fitzsimons, S. A., Mulvihill, D. M., & Morris, E. R. (2007). Denaturation and aggregation processes in thermal gelation of whey proteins resolved by differential scanning calorimetry. *Food Hydrocolloids, 21*(4), 638-644. DOI: 10.1016/j.foodhyd.2006.07.007

Forge, V., Hoshino, M., Kuwata, K., Arai, M., Kuwajima, K., Batt, C. A., & Goto, Y. (2000). Is folding of beta-Lactoglobulin non-hierarchic? Intermediate with native-like beta-sheet and non-native alpha-helix. *Journal of Molecular Biology, 296***,** 1039-1051. DOI: 10.1006/jmbi.1999.3515

Fox, P. F., & McSweeney, P. L. H. (1998). Heat induced changes of milk. In P. F. Fox & P. L. H. McSweeney (Eds.) *Dairy chemistry and biochemistry*, vol. 1, pp. 347-378. UK: Kluwer Academic.

Fryer, P. J., Belmar-Beiny, M. T., & Schreier, P. J. R. (1995). Fouling and cleaning in milk processing. In P. F. Fox (Ed.), *Heat-induced Changes in Milk* vol 1, pp. 391-410. UK: Kluwer Academic.

Fulmer, G. R., Miller, A. J. M., Sherden, N. H., Gottlieb, H. E., Nudelman, A., Stoltz, B. M., Bercaw, J.E., & Goldberg, K. I. (2010). NMR Chemical Shifts of Trace Impurities: Common Laboratory Solvents, Organics, and Gases in Deuterated Solvents Relevant to the Organometallic Chemist. *Organometallics, 29*(9), 2176-2179. doi: 10.1021/om100106e

Furia, T. E. (1972). Sequestrants in food. In T. E. Furia (Ed.), *CRC Handbook of Food Additives*, vol 1, pp. 271-294. Boca Raton, Florida, USA: CRC Press.

Galani, D., & Apenten, R. K. O. (1999a). Beta-lactoglobulin denaturation by dissociation-coupled unfolding. *Food Research International, 32***,** 93-100. DOI: 10.1016/S0963-9969(99)00067-8

Galani, D., & Apenten, R. K. O. (1999b). Heat-induced denaturation and aggregation of beta-Lactoglobulin: kinetics of formation of hydrophobic and disulphide-linked aggregates. *International Journal of Food Science and Technology, 34*(5-6), 467-476. DOI: 10.1046/j.1365-2621.1999.00314.x

Gaucheron, F. (2005). The minerals of milk. *Reproduction Nutrition Development, 45*(4), 473-483. DOI : 10.1051/rnd:2005030

Gelbart, W. M., & BenShaul, A. (1996). The "new" science of "complex fluids". *Journal of Physical Chemistry, 100*(31), 13169-13189. DOI: 10.1021/Jp9606570

Golinelli, L. P., Del Aguila, E. M., Flosi Paschoalin, V. M., Silva, J. T., & Conte-Junior, C. A. (2014). Functional Aspect of Colostrum and Whey Proteins in Human Milk. *Journal of Human Nutrition and Food Science 2*(3), 1035. DOI: 10.1016/j.pcl.2012.10.002

Gonzalez-Tello, P., Camacho, F., Guadix, E. M., Luzon, G., & Gonzalez, P. A. (2009). Density, Viscosity and Surface Tension of Whey Protein Concentrate Solutions. *Journal of Food Process Engineering, 32*(2), 235-247. DOI: 10.1111/j.1745-4530.2007.00213.x

Goodison, A. (2017). Modulating Interactions between Whey and Casein Proteins during Thermal Processing of Infant Formula to Improve Heat Stability. Msc Thesis, University College Cork. Ireland.

Gotham, S. M., Fryer, P. J., & Pritchard, A. M. (1992). Beta-Lactoglobulin Denaturation and Aggregation Reactions and Fouling Deposit Formation - a Dsc Study. *International Journal of Food Science and Technology, 27***,** 313-327. DOI: 10.1111/j.1365-2621.1992.tb02033.x

Gough, P., & Jenness, R. (1962). Heat denaturation of beta-lactoglobulins A and B. *Journal of Dairy Science, 45*(9), 1033-1038.

Graham, E. R. B., Malcolm, G. N., & Mckenzie, H. A. (1984). On the Isolation and Conformation of Bovine Beta-Casein-a-1. *International Journal of Biological Macromolecules, 6*(3), 155-161. DOI: 10.1016/0141-8130(84)90058-8

Guerin, R., Ronse, G., Bouvier, L., Debreyne, P., & Delaplace, G. (2007). Structure and rate of growth of whey protein deposit from in situ electrical conductivity during fouling in a plate heat exchanger. *Chemical Engineering Science, 62*(7), 1948-1957. DOI: 10.1016/j.ces.2006.12.038

Guidry, A. J., Butler, J. E., & Pearson, R.E. (1980). IgA, IgG1, IgG2, IgM, and BSA in serum and mammary secretion throughout lactation. *Veterinary Immunology and Immunopathology, 1*(4), 329-341. DOI: 10.1016/0165-2427(80)90012-4

Gurau, M. C., Lim, S. M., Castellana, E. T., Albertorio, F., Kataoka, S., & Cremer, P. S. (2004). On the mechanism of the Hofmeister effect. *Journal of the American Chemical Society, 126*(34), 10522-10523. DOI: 10.1021/ja047715c

Gurr, M. I. (1981). Review of the progress of dairy science - human and artificial milks for infant-feeding. *Journal of Dairy Research, 48*(3), 519-554.

Guyomarc'h, F., Law, A. J. R., & Dalgleish, D. G. (2003). Formation of soluble and micelle-bound protein aggregates in heated milk. *Journal of Agriculture and Food Chemistry, 51*(16), 4652-4660. DOI: 10.1021/jf0211783

Guyomarc'h, F., Nono, M., Nicolai, T., & Durand, D. (2009). Heat-induced aggregation of whey proteins in the presence of κ-casein or sodium caseinate. *Food Hydrocolloids, 23*(4), 1103-1110. DOI: 10.1016/j.foodhyd.2008.07.001

Haug, I. J., Skar, H. M., Vegarud, G. E., Langsrud, T., & Draget, K. I. (2009). Electrostatic effects on beta-lactoglobulin transitions during heat denaturation as studied by differential scanning calorimetry. *Food Hydrocolloids, 23***,** 2287-2293. DOI: 10.1016/j.foodhyd.2009.06.006

Havea, P., Carr, A. J., & Creamer, L. K. (2004). The roles of disulphide and non-covalent bonding in the functional properties of heat-induced whey protein gels. *Journal of Dairy Research, 71*(3), 330-339. DOI: 10.1017/S02202990400024x

Holt, C. (1992). Structure and stability of bovine casein micelles. *Advances in Protein Chemistry 43,* 63-151. DOI: 10.1016/S0065-3233(08)60554-9

Holt, C., Carver, J. A., Ecroyd, H., & Thorn, D. C. (2013). Invited review: Caseins and the casein micelle: their biological functions, structures, and behavior in foods. *Jouranl of Dairy Science, 96*(10), 6127-6146. DOI: 10.3168/jds.2013-6831

Hosseini-Nia, T., Ismail, A. A., & Kubow, S. (2002). Effect of high hydrostatic pressure on the secondary structures of BSA and apo- and holo-alpha-lactalbumin employing Fourier transform infrared spectroscopy. *Journal of Food Science, 67*(4), 1341-1347. DOI: 10.1111/j.1365-2621.2002.tb10285.x

Hubbard, R. E., & Haider, M. K. (2010). Hydrogen bonds in proteins: role and strength *Encyclopedia of life sciences*. Chichester, UK: John Wiley & Sons, Ltd.

Hunt, J. A., & Dalgleish, D. G. (1994). Effect of Ph on the Stability and Surface-Composition of Emulsions Made with Whey-Protein Isolate. *Journal of Agriculture and Food Chemistry, 42*(10), 2131-2135. DOI: 10.1021/jf00046a011

Hunter, J. E. (2001). Studies on effects of dietary fatty acids as related to their position on triglycerides. *Lipids, 36*(7), 655-668. DOI: 10.1007/s11745-001-0770-0

Huppertz, T. (2013). Chemistry of the Caseins. In P. F. Fox, McSweeney, P.H. (Ed.), *Advance Dairy Chemistry*, vol. 1A, pp. 135-161. New York, USA: Springer.

Hurley, W. L., & Theil, P. K (2013). Immunoglobulins in Mammary Secretions. In P. F. Fox, P. L. McSweeney (Eds.), *Advanced Dairy Chemistry, Protein: Basic Aspects*, vol. 1A, pp. 275-294. New York: Springer.

Hurley, W. L., & Theil, P. K. (2011). Perspectives on Immunoglobulins in Colostrum and Milk. *Nutrients, 3*, 442-474. DOI: 10.3390/nu3040442

IDF 2001. Milk Determination of nitrogen content. *ISO 8969-2.* Brussels International Dairy Federation.

Israelachvili, J. N. (2001). *Intermolecular and Surface Forces*. USA: Elsevier.

Jara, F. L., Sanchez, C. C., Patino, J. M. R., & Pilosof, A. M. R. (2014). Competitive adsorption behavior of beta-lactoglobulin, alpha-lactalbumin, bovin serum albumin in presence of hydroxypropylmethylcellulose. Influence of pH. *Food Hydrocolloids, 35***,** 189-197. DOI: 10.1016/j.foodhyd.2013.05.013

Jeurink, T. J. M., & de Kruif, C. G. (1993). Changes in milk on heating: Viscosity measurements. *Journal of Dairy Research, 60,* 139-150. DOI: 10.1017/S0022029900027461

Jun, S., & Puri, V. M. (2005). Fouling models for heat exchangers in dairy processing: A review. *Journal of Food Process Engineering, 28*(1), 1-34. DOI: 10.1111/j.1745-4530.2005.00473.x

Karshikoff, A. (2006). *Non-Covalent Interactions in Proteins*. UK: Imerial College Press.

Kehoe, J. J., & Foegeding, E. A. (2011). Interaction between beta-casein and whey proteins as a function of pH and salt concentration. *Journal of Agriculture and Food Chemistry, 59***,** 349-55. DOI: 10.1021/jf103371g

Kehoe, J. J., Remondetto, G. E., Subirade, M., Morries, E. R., & Brodkrob, A. (2008). Tryptophan-mediated denaturation of beta-lactoglobulin A by UV irradiation. *Journal of Agricultural and Food Chemistry, 56***,** 4720-4725. DOI: 10.1021/jf0733158

Kehoe, J. J., Wang, L., Morris, E. R., & Brodkrob, A. (2011). Formation of Non-Native β-Lactoglobulin during Heat-Induced Denaturation. *Food Biophysics, 6***,** 487-496. DOI: 10.1007/s11483-011-9230-3

Kerche, F., Weterings, M., & Beyrer, M. (2016). The effect of temperature and shear upon technological properties of whey protein concentrate: Aggregation in a tubular heat exchanger. *International Dairy Journal, 60,* 32-38. DOI: 10.1016/j.idairyj.2016.02.032

Kessler, H. G. (2002). Heat Treatment, Processes and Effects - Microorganisms and Conditions of Inactivation. In H. G. Kessler (Ed.), *Food ans Bio Process Engineering*, 86-147. München: Publishing House A. Kessler.

Kronman, M. J. S., & Brew, K. (1981). Characteristics of the binding Ca^{2+} and other divalent metal ions to bovine alpha-lactalbumin. *Journal of Biological Chemistry, 256*, 8582-8587.

Kuwata, K., Hoshino, M., Forge, V., Era, S., Batt, C. A., & Goto, Y. (1999). Solution structure and dynamics of bovine beta-lactoglobulin A. *Protein Science, 8***,** 2541-2545. DOI: 10.1110/ps.8.11.2541

Lam, R. S.H., & Nickerson, M. T. (2015). The effect of pH and temperature pre-treatments on the structure, surface characteristics and emulsifying properties of alpha-lactalbumin. *Food Chemistry*, *173*, 163–170. DOI: 10.1016/j.foodchem.2014.09.078

Law, A. J. R., & Leaver, J. (2000). Effect of pH on the thermal denaturation of whey proteins in milk. *Journal of Agriculture and Food Chemistry, 48***,** 672-679. DOI: 10.1021/jf981302b

Lazidis, A., Hancocks, R. D., Spyropoulos, F., Kreuss, M., Berrocal, R., & Norton, I. T. (2016). Whey protein fluid gels for the stabilisation of foams. *Food Hydrocolloids, 53***,** 209-217. DOI: 10.1016/j.foodhyd.2015.02.022

Le Maux, S., Bouhallab, S., Giblin, L., Brodkorb, A., & Croguennec, T. (2013). Complexes between linoleate and native or aggregated beta-lactoglobulin: Interaction parameters and in vitro cytotoxic effect. *Food Chemistry, 141***,** 2305-2313. DOI: 10.1016/j.foodchem.2013.05.031

Lefèvre, T., & Subirade, M. (1999). Structural and interaction properties of beta-Lactoglobulin as studied by FTIR spectroscopy. *International Journal of Food Science and Technology, 34***,** 419-428. DOI: 10.1046/j.1365-2621.1999.00311.x

Lefèvre, T., & Subirade, M.(2000). Molecular differences in the formation and structure of fine-stranded and particulate beta-lactoglobulin gels. *Biopolymers, 54***,** 578-586.

Leusbrock, I., Metz, S. J., Rexwinkel, G., & Versteeg, G. F. (2010). The solubility of magnesium chloride and calcium chloride in near-critical and supercritical water. *Journal of Supercritical Fluids, 53*(1-3), 17-24. DOI: 10.1016/j.supflu.2009.12.015

Lien, E. L. (2003). Infant formulas with increased concentrations of alpha-lactalbumin. *American Journal of Clinical Nutrition, 77*(6), 1555s-1558s.

Lisak Jakopović, K., Barukčić, I., & Božanić, R. (2016). Physiological significance, structure and isolation of α-lactalbumin. *Mljekarstvo, 66*(1), 3-11. DOI: 10.15567/mljekarstvo.2016.0101

Liu, D. Z., Dunstan, D. E., & Martin, G. J. O. (2012). Evaporative concentration of skimmed milk: Effect on casein micelle hydration, composition, and size. *Food Chemistry, 134,* 1446-1452. DOI: 10.1016/j.foodchem.2012.03.053

Liu, H. C., Chen, W. L., & Mao, S. J. T. (2007). Antioxidant nature of bovine milk beta-lactoglobulin. *Journal of Dairy Science, 90*(2), 547-555. DOI: 10.3168/jds.S0022-0302(07)71538-2

Lodish, H. B., Berk, A., & Zipursky, S. L. (2002). Noncovalent Bonds. In: *Molecular Cell Biology* (4th ed.). New York: W. H. Freeman.Berg

Lönnerdal, B. (1985). Biochemistry and physiological function of human milk proteins. *American Journal of Clinical Nutrition, 42*(6), 1299–317.

Lönnerdal, B. (2017). Excess iron intake as a factor in growth, infections, and development of infants and young children. *American Society for Nutrition, Suppl 6,* 1681-S-1687S. DOI: 10.3945/ajcn.117.156042

Lönnerdal, B., & Hernell, O. (1998). Effects of feeding ultrahigh-temperature (UHT)-treated infant formula with different protein concentrations or powdered

formula, as compared with breast-feeding, on plasma amino acids, hematology, and trace element status. *American Journal of Clinical Nutrition, 68*(2), 350-356. DOI: 10.1093/ajcn/68.2.350

Lönnerdal, B., & Lien, E. L. (2003). Nutritional and physiologic significance of alpha-lactalbumin in infants. *Nutrition Reviews, 61,* 295–305. DOI: 10.1031/nr.2003.sept.295-305

Lucey, J. A., & Horne, D.S. (2009). Milk Salts: Technological Significance In P. F. Fox, P. L. H. Mc Sweeney (Eds.), *Advance Dairy Chemistry: Lactose, Water Salts and Minor Constitutents,* vol. 3, pp. 351-389. New York: Springer.

Lyster, R. L. J. (1970). The denaturation of α-lactalbumin and β-lactoglobulin in heated milk. *Journal of Dairy Research, 37***,** 233-243.

Majhi, P. R., Ganta, R. R., Vanam, R. P., Seyrek, E., Giger, K., & Dubin, P. L. (2006). Electrostatically driven protein aggregation: beta-lactoglobulin at low ionic strength. *Langmuir, 22***,** 9150-9159. DOI: 10.1021/la053528w

Malin, E. L., Browen, E. M., Wichkham, E. D., & Farrell, H. M. Jr. (2005). Contributions of terminal peptides to associative behavior of alpha s1-casein. *Journal of Dairy Science, 88*, 2318-2328.

Manderson, G. A., Hardman, M. J., & Creamer, L. K. (1998). Effect of heat treatment on the conformation and aggregation of beta-lactoglobulin A, B, and C. *Journal of Agriculture and Food Chemistry, 46*(12), 5052-5061. DOI: 10.1021/Jf980515y

Matheus, S., Friess, W., & Mahler, H. C. (2006). FTIR and nDSC as analytical tools for high-concentration protein formulations. *Pharmaceutical Research, 23***,** 1350-1363. DOI: 10.1007/s11095-006-0142-8

Matsudomi, N., Oshita, T., Sasaki, E., & Kobayashi, K. (1992). Enhanced Heat-Induced Gelation of Beta-Lactoglobulin by Alpha-Lactalbumin. *Bioscience Biotechnology and Biochemistry, 56*(11), 1697-1700. DOI: 10.1271/bbb.56.1697

Matsumura, Y., Mitsui, S., Dickinson, E., & Mori, T. (1994). Competitive Adsorption of Alpha-Lactalbumin in the Molten Globule State. *Food Hydrocolloids, 8*(6), 555-566.

Mayeur, S., Spahis, S., Pouliot, Y., & Levy, E. (2016). Lactoferrin, a Pleiotropic Protein in Health and Disease. *Antioxid Redox Signal, 24,* 813–836. DOI: 10.1089/ars.2015.6458

Mc Carthy, N. A. (2013).The Impact of Protein Profile on the Physical Stability of Infant Formulae. PhD Thesis, University College Cork. Ireland.

Mc Kenzie, H. A., Shaw, D. C., & Ralston, G.B. (1972). Location of sulfhydryl and disulfide groups in bovine beta-lactoglobulins and effects of urea. *Biochemistry, 11*(24), 4539 - 4544. DOI: 10.1021/bi00774a017

McCarthy, N. A., Gee, V. L., Hickey, D. K., Kelly, A. L., O'Mahony, J. A., & Fenelon, M. A. (2013). Effect of protein content on the physical stability and microstructure of a model infant formula. *International Dairy Journal, 29,* 53-59. DOI: 10.1016/j.idairyj.2012.10.004

McCarthy, N. A., Gee, V. L., O'Mahony J. A., Kelly, A. L., & Fenelon M. A. (2015). Optimising emulsion stability during processing of model infant formulae using factorial statistical design. *International Journal of Dairy Technology, 68*(3), 334–341. DOI: 10.1111/1471-0307.12240

McCarthy, N. A., Kelly, A. L., O'Mahony, J. A., Hickey, D. K., Chaurin, V., & Fenelon, M. A. (2012). Effect of protein content on emulsion stability of a model infant formula. *International Dairy Journal 25,* 80-86. DOI: 10.1016/j.idairyj.2012.03.003

McClements, D. J. (2004). Protein-stabilized emulsions. *Current Opinion in Colloid & Interface Science, 9*(5), 305-313. DOI: 10.1016/j.cocis.2004.09.003

McDermott, R. (1987). Functionality of Dairy Ingredients in Infant Formula and Nutritional Specialty Products. *Food Technology, 41*(10), 91-103. DOI: 10.1111/j.1541-4337.2012.00199.x

McGuffey, M. K., Otter, D. E., van Zanten, J. H., & Foegeding, E. A. (2007). Solubility and aggregation of commercial alpha-lactalbumin at neutral pH. *International Dairy Journal, 17*(10), 1168-1178. DOI: 10.1016/j.idairyj.2007.04.003

McMahon, D. J. O., & Oommen, B. S. (2013). Casein Micelle Structure, Functions, and Interactions. In P. F. M. Fox, P. L. H. McSweeney (Eds.), *Advanced Dairy Chemistry*, vol. 1A, pp. 185-210. New York: Springer.

McSweeney, S. L., Mulvihill, D. M., & O'Callaghan, D. M. (2004). The influence of pH on the heat-induced aggregation of model milk protein ingredient systems and model infant formula emulsions stabilized by milk protein ingredients. *Food Hydrocolloids, 18*(1), 109-125. DOI: 10.1016/S0268-005x(03)00049-3

Mehalebi, S., Nicolai, T., & Durand, D. (2008). Light scattering study of heat-denatured globular protein aggregates. *International Journal of Biological Macromolecules, 43*(2**),** 129-135. DOI: 10.1016/j.ijbiomac.2008.04.002

Mercier, U., Ribadeau-Dumas, & Grosculaude. (1972). Structure primaire du caskinomacropeptide de la caskine K B, bovine. *European Journal of Biochemistry, 27*, 535-547. DOI: 10.1111/j.1432-1033.1971.tb01238.x

Meza-Nieto, M. A., Vallejo-Cordoba, B., Gonzalez-Cordova, A. F., Felix, L., & Goycoolea, F. M. (2007). Effect of beta-lactoglobulin A and B whey protein variants on the Rennet-induced gelation of skim milk gels in a model reconstituted skim milk system. *Journal of Dairy Science,* 90**,** 582-593. DOI: 10.3168/jds.S0022-0302(07)71541-2

Montagne, D. H., Van Dael, P., Skanderby, M., & Hugelshofer, W. (2009). Infant Formulae-Powders and Liquids. In A. Y. Tamime (Ed.), *Dairy Powders and Concentrated Products*, pp. 294-331. London: Blackwell Publishing Ltd.

Morr, C. V. (1975). Chemistry of Milk Proteins in Food-Processing. *Journal of Dairy Science, 58*(7), 977-984. DOI: 10.3168/jds.S0022-0302(75)84669-8

Morr, C. V. (1985). Functionality of Heated Milk-Proteins in Dairy and Related Foods. *Journal of Dairy Science, 68*(10), 2773-2781.

Morr, C., & Ha, E. (1993). Whey-Protein Concentrates and Isolates - Processing and Functional-Properties. *Critical Reviews in Food Science and Nutrition, 33*(6), 431-476. DOI: 10.1080/10408399309527643

Morris, G. A., Foster, T. J., & Harding, S. E. (2000). Further observations on the size, shape, and hydration of casein micelles from novel analytical ultracentrifuge and capillary viscometry approaches. *Biomacromolecules, 1*(4), 764-767. DOI: 10.1021/bm0055807

Mounsey, J. S., & O'Kennedy, B. T. (2009). Stability of beta-lactoglobulin/micellar casein mixtures on heating in simulated milk ultrafiltrate at pH 6.0. *International Journal of Dairy Technology, 62*(4), 493-499. DOI: 10.1111/j.1471-0307.2009.00522.x

Muir, D. D., & Sweetsur, A. W. M. (1976). The influence of naturally occurring levels of urea on the heat stability of bulk milk. *Journal of Dairy Research, 43*, 495-499. DOI: 10.1017/S0022029900016083

Mullane, N. R., Whyte, P., Wall, P. G., Quinn, T., & Fanning, S. (2007). Application of pulsed-field gel electrophoresis to characterise and trace the prevalence of Enterobacter sakazakii in an infant formula processing facility. *International Journal of Food Microbiology, 116*(1), 73-81. DOI: 10.1016/j.ijfoodmicro.2006.12.036

Mullvihill, M. D., & Donavan, M. (1987). Whey Proteins and their Thermal Denaturation - A Review. *Irish Journal of Food Science and Technology, 11*, 43-75.

Murphy, E. G. (2015). Infant Milk Formula Manufacture: Process and Compositional Interactions in High Dry Matter Wet-mixes. PhD Thesis, University College Cork. Ireland.

Murphy, E. G., Fenelon, M. A., Roos, Y. H., & Hogan, S. A. (2014). Decoupling Macronutrient Interactions during Heating of Model Infant Milk Formulas.

*Journal of Agricultur and Food Chemistry, 62***,** 10585-10593. DOI: 10.1021/jf503620r

Murphy, E. G., Roos, Y. H., Hogan, S. A., Maher, P. G., Flynn, C. G., & Fenelon, M. A. (2015). Physical stability of infant milk formula made with selectively hydrolysed whey proteins. *International Dairy Journal, 40,* 39-46. DOI: 10.1016/j.idairyj.2014.08.012

Music, G. B. (1985). Physiological roles of zinc and calcium binding to alpha-lactalbumin in lactose biosynthesis. *Biochemistry, 24,* 6945-6948. DOI: 10.1021/bi00345a029

Nakai, S., & Li-Chan, E. (1982). Chemical and enzymatic modification of milk proteins. In P. F. Fox (Ed.), *Developments in dairy chemistry: Functional milk proteins*, vol. 4, London: Elsevier Applied Science.

Nasripour, A., Scher, J., & Desorby, S. (2006). Baby foods: Formulations and interactions (a review). *Critical Reviews in Food Science and Nutrition, 46,* 665-681. DOI: 10.1080/10408390500511896

Neville, M. C., Keller, R. P., Casey, C., & Allen, J. C. (1994). Calcium Partitioning in Human and Bovine-Milk. *Journal of Dairy Science, 77*(7), 1964-1975. DOI: 10.3168/jds.S0022-0302(94)77142-3

Nielsen, B. T., Singh, H., & Latham, J. M. (1996). Aggregation of bovine beta-lactoglobulins A and B on heating at 75 degrees C. *International Dairy Journal, 6***,** 519-527.

O'Loughlin, I. B. (2014). Enzymatic hydrolysis of heat-denatured whey proteins. PhD Thesis, University of Limerick. Limerick. Ireland.

O'Callaghan, D. M., O'Mahony, J. A, Ramanujam, K. S., & Burgher, A. M. (2001). Dehydrated Dairy Products - Infant Formula. In: *Encyclopedia of Dairy Sciences*, vol. 3, pp. 1384-1392. London: Academic Press, Elsevier Science.

O'Kennedy, B. T., & Mounsey, J. S. (2006). Control of heat-induced aggregation of whey proteins using casein. *Journal of Agriculture and Food Chemistry, 54*(15), 5637-5642. DOI: 10.1021/jf0607866

O'Kennedy, B. T., & Mounsey, J. S. (2006). Control of heat-induced aggregation of whey proteins using casein. *Journal of Agriculture and Food Chemistry, 54*(15), 5637-5642. DOIo: 10.1021/jf0607866

Okur, H. I., Hladilkova, J., Rembert, K. B., Cho, Y., Heyda, J., Dzubiella, J., Creamer, P. S., & Jungwirth, P. (2017). Beyond the Hofmeister Series: Ion-Specific Effects on Proteins and Their Biological Functions. *Journal of Physical Chemistry B, 121*(9), 1997-2014. doi: 10.1021/acs.jpcb.6b10797

Oldfield, D. J., Singh, H., & Taylor, M. W. (2005). Kinetics of heat-induced whey protein denaturation and aggregation in skim milks with adjusted whey protein concentration. *Journal of Dairy Research, 72***,** 369-378. DOI: 10.1017/S002202990500107X

Oldfield, D. J., Singh, H., Taylor, M. W., & Pearce, K. N. (1998). Kinetics of denaturation and aggregation of whey proteins in skim milk heated in an ultra-high temperature (UHT) pilot plant. *International Dairy Journal, 8*(4), 311-318. DOI: 10.1016/S0958-6946(98)00089-2

O'Mahony, J. A., & Fox, P. F. (2013). Milk Proteins: Introduction and Historical Aspects. In: P. F. Fox, P. L. H. McSweeney (Eds.), *Advanced Dairy Chemistry - Protein: Basic Aspects,* vol. 1, pp. 43-87. New York: Springer.

Omta, A. W., Kropman, M. F., Woutersen, S., & Bakker, H. J. (2003). Negligible effect of ions on the hydrogen-bond structure in liquid water. *Science, 301*(5631), 347-349. DOI: 10.1126/science.1084801

Pak, C. Y. C., Harvey, J. A., & Hsu, M. C. (1987). Enhanced Calcium Bioavailability from a Solubilized Form of Calcium Citrate. *Journal of Clinical Endocrinology & Metabolism, 65*(4), 801-805. DOI: 10.1210/jcem-65-4-801

Palsson, H., Beaubert, F., & Lalot, S. (2013). Inducing Swirling Flow in Heat Exchanger Pipes for Reduced Fouling Rate. *Heat Transfer Engineering, 34*(8-9), 761-768.

Papiz, M. Z., Sawyer, L., Eliopoulos, E. E., North, A. C. T., Findlay, J. B. C., Sivaprasadarao, R., Jones, T. A., Newcomer, M. E., & Kraulis, P. J. (1986). The structure of β-lactoglobulin and its similarity to plasma retinol-binding protein. *Nature, 324*(6095), 383-385. DOI: 10.1038/324383a0

Parker, T. G., & Dalgleish, D. G. (1981). Binding of Calcium-Ions to Bovine Beta-Casein. *Journal of Dairy Research, 48*(1), 71-76.

Parris, N., Anema, S., Singh, H., & Creamer, L. (1993). Aggregation of Whey Proteins in Heated Sweet Whey. *Journal of Agriculture and Food Chemistry, 41*(3), 460-464. DOI: 10.1021/Jf00027a021

Pearce, R. J. (1995). U.S Patent Application.

People's Republic of China, Ministry of Health (2010) National food safety standard: Infant formula.

Perez, M. D., & Calvo, M. (1995). Interaction of Beta-Lactoglobulin with Retinol and Fatty-Acids and Its Role as a Possible Biological Function for This Protein - a Review. *Journal of Dairy Science, 78*(5), 978-988. DOI: 10.3168/jds.S0022-0302(95)76713-3

Permyakov, E. A., & Berliner, L. J. (2005). How to improve nature: study of the electrostatic properties of the surface of alpha-lactalbumin. *Protein Engineering & Design Selection, 18*(9), 425-433. DOI: 10.1093/protein/gzi051

Petit, J., Six, T., Moreau, A., Ronse, G., & Delaplace, G. (2013). beta-lactoglobulin denaturation, aggregation, and fouling in a plate heat exchanger: Pilot-scale experiments and dimensional analysis. *Chemical Engineering Science, 101,* 432-450. DOI: 10.1016/j.ces.2013.06.045

Pettersson-Kastberg, J., Mossberg, A. K., Trulsson, M., Yong, T. J., Min, S., Lim, Y., O'Brien, J. E., Svanborg, C., & Mok, K. H. (2009). alpha-Lactalbumin, Engineered to be Nonnative and Inactive, Kills Tumor Cells when in Complex with Oleic Acid: A New Biological Function Resulting from Partial Unfolding. *Journal of Molecular Biology, 394***,** 994-1010. DOI: 10.1016/j.jmb.2009.09.026

Pierce, A., Colavizza, D., Benaissa, M., Maes, P., Tartar, A., Montreuil, J., & Spik, G. (1991). Molecular-Cloning and Sequence-Analysis of Bovine Lactotransferrin. *European Journal of Biochemistry, 196*(1), 177-184. DOI: 10.1111/j.1432-1033.1991.tb15801.x

Playne, M. J., Bennett, L. E., & Smithers, G. W. (2003). Functional dairy foods and ingredients. *Australian Journal of Dairy Technology, 58*(3), 242-264.

Prestrelski, S. J., Byler, D. M., & Thompson, M. P. (1991). Effect of Metal-Ion Binding on the Secondary Structure of Bovine Alpha-Lactalbumin as Examined by Infrared-Spectroscopy. *Biochemistry, 30*(36), 8797-8804. DOI: 10.1021/Bi00100a010

Qi, X. L., Brownlow, S., Holt, C., & Sellers, P. (1995). Thermal-Denaturation of Beta-Lactoglobulin - Effect of Protein-Concentration at Ph-6.75 and Ph-8.05. *Biochimica Et Biophysica Acta-Protein Structure and Molecular Enzymology, 1248***,** 43-49. DOI: 10.1016/0167-4838(94)00225-6

Qin, B. Y., Bewley, M. C., Creamer, L. K., Baker, E. N., & Jameson, G. B. (1999). Functional implications of structural differences between variants A and B of bovine beta-lactoglobulin. *Protein Science, 8***,** 75-83. DOI: 10.1110/ps.8.1.75

Qin, B. Y., Bewley, M. C., Creamer, L. K., Baker, H. M., Baker, E. N., & Jameson, G. B. (1998). Structural basis of the tanford transition of bovine beta-lactoglobulin. *Biochemistry, 37***,** 14014-14023. DOI: 10.1021/bi981016t

Radford, S. E., Dobson, C. M., & Evans, P. A. (1992). The Folding of Hen Lysozyme Involves Partially Structured Intermediates and Multiple Pathways. *Nature, 358***,** 302-307. DOI: 10.1038/358302a0

Rai, D., Adelman, A. S., Zhuang, W., Rai, G. P., Boettcher, J., & Lönnerdal, B. (2014). Longitudinal changes in lactoferrin concentrations in human milk: a global systematic review. *Critical Reviews in Food Science and Nutrition, 54,* 1539-1547. DOI: 10.1080/10408398.2011.642422

Raikos, V. (2010). Effect of heat treatment on milk protein functionality at emulsion interfaces. A review. *Food Hydrocolloids, 24*(4), 259-265. DOI: 10.1016/j.foodhyd.2009.10.014

Ramos, Ó. L., Pereira, J. O., Silva, S. I., Amorim, M. M., Fernandes, J. C., Lopes-da-Silva, J. A., Pintado, M. E., & Malcata, F. X. (2012). Effect of composition of commercial whey protein preparations upon gelation at various pH values. *Food Research International, 48*(2), 681-689. DOI: 10.1016/j.foodres.2012.06.004

Rane, S. S., & Choi, P. (2005). Polydispersity index: How accurately does it measure the breadth of the molecular weight distribution? *Chemistery of Materials, 17***,** 926-926. DOI: 10.1021/cm048594i

Rochata, F., Cherbuta, C., Barclayb, D., Puccioc, G., Fazzolari-Nescic, A., Grathwohla, D., & Haschkeb, F. (2007). A whey-predominant formula induces fecal microbiota similar to that found in breast-fed infants. *Nutrition Research, 27*(12), 735–740. DOI: 10.1016/j.nutres.2007.09.008

Roefs, S. P., & de Kruif, K. G. (1994). A Model for the Denaturation and Aggregation of Beta-Lactoglobulin. *European Journal of Biochemistry, 226*(3), 883-889. DOI: 10.1111/j.1432-1033.1994.00883.x

Roth, C. M., Neal, B. L., & Lenhoff, A. M. (1996). Van der Waals interactions involving proteins. *Biophysical Journal, 70*(2), 977-987. DOI : 10.1016/S0006-3495(96)79641-8

Ryan, K. N., & Foegeding, E. A. (2015). Formation of soluble whey protein aggregates and their stability in beverages. *Food Hydrocolloids, 43*, 265-274. DOI: 10.1016/j.foodhyd.2014.05.025

Ryan, K. N., Vardhanabhuti, B., Jaramillo, D. P., van Zanten, J. H., Coupland, J. N., & Foegeding, E. A. (2012). Stability and mechanism of whey protein soluble aggregates thermally treated with salts. *Food Hydrocolloids, 27*(2), 411-420. DOI: 10.1016/j.foodhyd.2011.11.006

Ryan, K. N., Zhong, Q. X., & Foegeding, E. A. (2013). Use of whey protein soluble aggregates for thermal stability. A hypothesis paper. *Journal of Food Science, 78***,** R1105-R1115. DOI: 10.1111/1750-3841.12207

Sakurai, K., & Goto, Y. (2006). Dynamics and mechanism of the Tanford transition of bovine beta-lactoglobulin studied using Heteronuclear NMR Spectroscopy. *Journal of Molecular Biology, 356***,** 483-496. DOI: 10.1016/j.jmb.2005.11.038

Sakurai, K., & Goto, Y. (2007). Principal component analysis of the pH-dependent conformational transitions of bovine beta-lactoglobulin monitored by heteronuclear NMR. *Proceedings of the National Academy of Sciences of the United States of America, 104***,** 15346-15351. DOI: 10.1073/pnas.0702112104

Sakurai, K., Konuma, T., Yagi, M., & Goto, Y. (2009). Structural dynamics and folding of beta-lactoglobulin probed by heteronuclear NMR. *Biochimica Et Biophysica Acta-General Subjects, 1790***,** 527-537. DOI: 10.1016/j.bbagen.2009.04.003

Sanchez, L., Aranda, P., Perez, M. D., & Calvo, M. (1988). Concentration of lactoferrin and transferrin throughout lactation in cow's colostrum and milk. *Biological Chemistry Hoppe-Seyler, 369,* 1005-1008. DOI: 10.1515/bchm3.1988.369.2.1005

Sawyer, L. (2003). β-lactoglobulin. In P. F. Fox, P.L.H. McSweeney (Eds.), *Advanced Dairy Chemistry: Proteins*, pp. 319-363. New York, USA: Academic/Plenum Publishers.

Sawyer, L. (2013). β-Lactoglobulin. In P. F. Fox, P.L.H. McSweeney (Eds.), *Advanced Dairy Chemistry, Protein: Basic Aspects*, vol. 1A, pp. 211-261. New York: Springer.

Sawyer, L., & Holt, C. (1993). The Secondary Structure of Milk-Proteins and Their Biological Function. *Journal of Dairy Science, 76*(10), 3062-3078. DOI: 10.3168/jds.S0022-0302(93)77646-8

Schlichting, I., & Chu, K. (2000). Trapping intermediates in the crystal: ligand binding to myoglobin. *Current Opinion in Structural Biology, 10***,** 744-752.

Schmid, M., Prols, S., Kainz, D. M., Hammann, F., & Grupa, U. (2017). Effect of thermally induced denaturation on molecular interaction-response relationships of whey protein isolate based films and coatings. *Progress in Organic Coatings, 104*, 161-172. DOI: 10.1016/j.porgcoat.2016.11.032

Schmitt, C., Bovay, C., Rouvet, M., Shojaei-Rami, S., & Kolodziejczye, E. (2007). Whey protein soluble aggregates from heating with NaCl: Physicochemical, interfacial, and foaming properties. *Langmuir, 23***,** 4155-4166. DOI: 10.1021/la0632575

Seo, J. A., Hedoux, A., Guinet, Y., Paccou, L., Affouard, F., Lerbret, A., & Descamps, M. (2010). Thermal Denaturation of Beta-Lactoglobulin and Stabilization Mechanism by Trehalose Analyzed from Raman Spectroscopy Investigations. *Journal of Physical Chemistry B, 114***,** 6675-6684. DOI: 10.1021/jp1006022

Sheldrake, R. F., Hoare, R. J. T., & McGregor, G.D. (1983). Lactation stage, parity, and infection affecting somatic cells, electrical conductivity, and serum albumin in milk. *Journal of Dairy Science, 66*(3), 542-547. DOI: 10.3168/jds.S0022-0302(83)81823-2

Shimizu, A., Ikeguchi, M., & Sugai, S. (1994). Appropriateness of Dss and Tsp as Internal References for H-1-Nmr Studies of Molten Globule Proteins in Aqueous-Media. *Journal of Biomolecular Nmr, 4***,** 859-862. DOI: 10.1007/BF00398414

Simmons, M. J. H., Jayaraman, P., & Fryer, P. J. (2007). The effect of temperature and shear rate upon the aggeragtion of whey protein and its implications for milk

fouling. *Journal of Food Engineering, 79*, 413-434. DOI: 10.1016/j.jfoodeng.2006.02.013

Singh, H., & Fox, P. F. (1987). Heat stability of milk: role of β-lactoglobulin in the pH-dependent dissociation of micellar κ-casein. *Journal of Dairy Research, 54,* 509-521. DOI: 10.1017/S0022029900025711

Small, D. M. (1991). The Effects of Glyceride Structure on Absorption and Metabolism. *Annual Review of Nutrition, 11*, 413-434. DOI: 10.1146/annurev.nu.11.070191.002213

Soleymani, J., Jouyban-Gharamaleki, V., Jouyban-Gharamaleki, K., Acree, W. E., Kenndler, E., & Jouyban, A. (2016). Solubility of trisodium citrate in water plus methanol mixtures at various temperatures. *Journal of Molecular Liquids, 221*, 166-170.

Stading, M., & Hermansson, A. M. (1990). Viscoelastic behaviour of beta-lactoglobulin gel structures. *Food Hydrocolloids, 4***,** 121-135. DOI: 10.1016/S0268-005X(09)80013-1

Surroca, Y., Haverkamp, J., & Heck, A. J. R. (2002). Towards the understanding of molecular mechanisms in the early stages of heat-induced aggregation of beta-lactoglobulin AB. *Journal of Chromatography A, 970*(1-2), 275-285. DOI: 10.1016/S0021-9673(02)00884-1

Swaisgood, H. E. (1982). Chemistry of Milk Protein. In P. F. Fox (Ed.), *Developments in Dairy Chemistry I*, pp. 1-60. London and New York: Applied Science Publishers Ltd.

Swaisgood, H. E. (1992). Chemistry of Caseins. In P. F. Fox (Ed.), *Advanced Dairy Chemistry 1: Proteins,* vol. 1, pp. 63-110. New York: Elsevier Applied Science.

Swaisgood, H. E. (2003). Chemistry of the caseins. In P. L. H. McSweeney, P. F. Fox (Eds.) *Advanced dairy chemistry: Proteins*, vol. 1, pp. 139-201. New York: Kluwer Academic.

Tanford, C., & Nozaki, Y. (1959). Physico-chemical comparison of beta-lactoglobulins-A and lactoglobulin-B. *Journal of Biological Chemistry, 234***,** 2874-2877. DOI: 10.1021/bi00882a002

Tcholakova, S., Denkov, N. D., Ivanov, I. B., & Campbell, B. (2006). Coalescence stability of emulsions containing globular milk proteins. *Advances in Colloid and Interface Science, 123,* 259-293. DOI: 10.1016/j.cis.2006.05.021

Tessier, H., & Rose, D. (1964) Influence of κ-casein and β-lactoglobulin on the heat stability of skim milk. *Journal of Dairy Science, 47,* 1047-1051. DOI: 10.3168/jds.S0022-0302(64)88843-3

Thompkinson, D. K., & Kharb, S. (2007). Aspects of infant food formulation. *Comprehensive Reviews in Food Science and Food Safety, 6,* 79-102. DOI: 10.1111/j.1541-4337.2007.00020.x

Tobin, J. T., Fitzsimons, S. M., Kelly, A. L., Kelly, P. M., Auty, M. A. E., & Fenelon, M. A. (2010). Microparticulation of mixtures of whey protein and inulin. *International Journal of Dairy Technology, 63*(1), 32-40. DOI: 10.1111/j.1471-0307.2009.00550.x

Tolkach, A., & Kulozik, U. (2007). Reaction kinetic pathway of reversible and irreversible thermal denaturation of beta-lactoglobulin. *Lait, 87*(4-5), 301-315. DOI: 10.1051/lait:2007012

Tresset, G. (2009). The multiple faces of self-assembled lipidic systems. *PMC Biophysics, 2*(3), 1-12. DOI: 10.1186/1757-5036-2-3

Trieucuot, P. G. (1981). Electrofocusing and two-dimensional electrophoresis of bovine caseins. *Journal of Dairy Research, 48,* 303-310. DOI: 10.1017/S0022029900021725

Twomey, M., Keogh, M. K., Mehra, R., & O'Kennedy, B. T. (1997). Gel characteristics of beta-lactoglobulin, whey protein concentrate and whey protein isolate. *Journal of Texture Studies, 28*(4), 387-403.

U.S. Food and Drug Administration (2003). Powdered infant formula. https://www.fda.gov/ohrms/dockets/ac/03/briefing/3939b1_tab4b.htm

Udabage, P., McKinnon, I. R., & Augustin, M. A. (2000). Mineral and casein equilibria in milk: effects of added salts and calcium-chelating agents. *Journal of Dairy Research, 67*(3), 361-370. DOI: 10.1017/S0022029900004271

Vanwazer, J. R., & Callis, C.F. (1958). Metal Complexing by Phosphates. *Chemical Reviews, 58*(6), 1011-1046. DOI: 10.1021/cr50024a001

Vavrusova, M., Garcia, A. C., Danielsen, B. P., & Skibsted, L. H. (2017). Spontaneous supersaturation of calcium citrate from simultaneous isothermal dissolution of sodium citrate and sparingly soluble calcium hydroxycarboxylates in water. *Rsc Advances, 7*(6), 3078-3088. DOI: 10.1039/C6RA25807G

Verheul, M., Pedersen, J. S., Roefs, S. P., & de Kruif, K. G. (1999). Association behavior of native beta-lactoglobulin. *Biopolymers, 49*, 11-20. DOI: 10.1002/(SICI)1097-0282

Verheul, M., Roefs, S. P., & de Kruif, K. G. (1998). Kinetics of heat-induced aggregation of beta-lactoglobulin. *Journal of Agriculture and Food Chemistry, 46*(3), 896-903. DOI: 10.1021/jf970751t

Wakabayashi, H., Yamauchi, K., & Takase, M. (2006). Lactoferrin research, technology and applications. *International Dairy Journal, 16,* 1241-1251. DOI: 10.1016/j.idairyj.2006.06.013

Walstra, P. (1983). Formation of emulsions. In P. Becher (Ed.), *Encyclopedia of emulsion technology*. New York: Marcel Dekker.

Walstra, P., & Jenness, R. (1984). Proteins. In Walstra, P., Jenness, R., Badings (Eds.) *Dairy Chemistry and Physics* (2nd ed.). New York: John Wiley and Sons.

Wang, Q., Tolkach, A., & Kulozik, U. (2006). Quantitative assessment of thermal denaturation of bovine alpha-lactalbumin via low-intensity ultrasound, HPLC,

and DSC. *Journal of Agricultural and Food Chemistry, 54***,** 6501-6506. DOI: 10.1021/jf060895k

Westergaard, V. (2004). Milk powder technology. GEA Niro, Copenhagen, Denmark. https://www.gea.com/en/binaries/Milk%20Powder%20Technology%20-%20Evaporation%20and%20Spray%20Drying_tcm11-33784.pdf

Whitney, R. M. (1988). Proteins of milk. In: *Fundamentals of Dairy Chemistry.* New York: AVI Books.

Wijayanti, H. B., Bansal, N., & Deeth, H. C. (2014). Stability of Whey Proteins during Thermal Processing: A Review. *Comprehensive Reviews in Food Science and Food Safety, 13*(6), 1235-1251. DOI: 10.1111/1541-4337.12105

Wijesinha-Bettoni, R., Dobson, C. M., & Redfield, C. (2001). Comparison of the structural and dynamical properties of holo and apo bovine alpha-lactalbumin by NMR spectroscopy. *Journal of Molecular Biology, 307*(3), 885-898. DOI: 10.1006/jmbi.2001.4530

Wolz, M., & Kulozik, U. (2015). Thermal denaturation kinetics of whey proteins at high protein concentrations. *International Dairy Journal, 49***,** 95-101. DOI: 10.1016/j.idairyj.2015.05.008

Wynn, P. C. (2013). Minor Proteins, Including Growth Factors. In P. F. M. Fox, P.L.H. McSweeney (Eds.), *Advanced Dairy Chemistry, Protein: Basic Aspects,* vol. 1A, pp. 317-329. New York: Springer.

Xie, W. J., & Gao, Y. Q. (2013). A Simple Theory for the Hofmeister Series. *Journal of Physical Chemistry Letters, 4*(24), 4247-4252. DOI: 10.1021/jz402072g

Ye, A. Q. (2008). Interfacial composition and stability of emulsions made with mixtures of commercial sodium caseinate and whey protein concentrate. *Food Chemistry, 110*(4), 946-952. DOI: 10.1016/j.foodchem.2008.02.091

Ye, A., Singh, H., Taylor, M. W., & Anema, S. (2002). Characterization of protein components of natural and heat-treated milk fat globule membranes.

International Dairy Journal, 12(4), 393-402. DOI: 10.1016/S0958-6946(02)00034-1

Zhang, Y. J., & Cremer, P. S. (2006). Interactions between macromolecules and ions: the Hofmeister series. *Current Opinion in Chemical Biology, 10*(6), 658-663. DOI: 10.1016/j.cbpa.2006.09.020

Zuniga, R., Tolkach, A., Kulozik, U., & Aguilera, J. (2010). Kinetics of Formation and Physicochemical Characterization of Thermally-Induced beta-Lactoglobulin Aggregates. *Journal of Food Science, 75*(5), 261-268. DOI: 10.1111/j.1750-3841.2010.01617.x

Appendix I

In this appendix, extra data is presented on the heat stability and viscosity formation of unheated whey protein isolate (WPI) samples investigated in Chapter 2.

The heat stability of samples (Figure 1) was determined at protein concentrations of 4, 8 and 12% protein using the heat coagulation time method described in Chapter 3 – Section 3.3.8 (first paragraph).

The viscosity of unheated samples (Figure 2), at a peak hold of 200 s^{-1} (mPa.s), was determined using the viscosity method described in Chapter 2 – Section 2.3.4.

Small-amplitude oscillatory measurements (Figure 3) were carried out using the method described in Chapter 3 – Section 3.3.9.

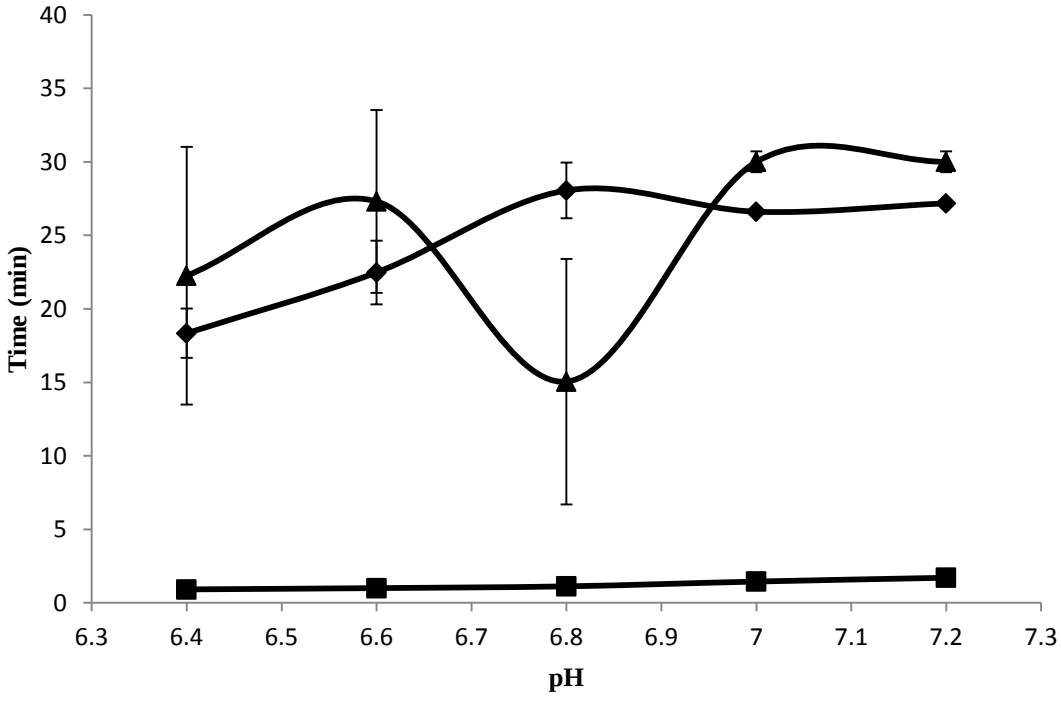


Figure 1: Heat coagulation time curves for 4 (◆) , 8(▲) and 12(■) % WPI samples ranging from pH 6.4 to 7.2.

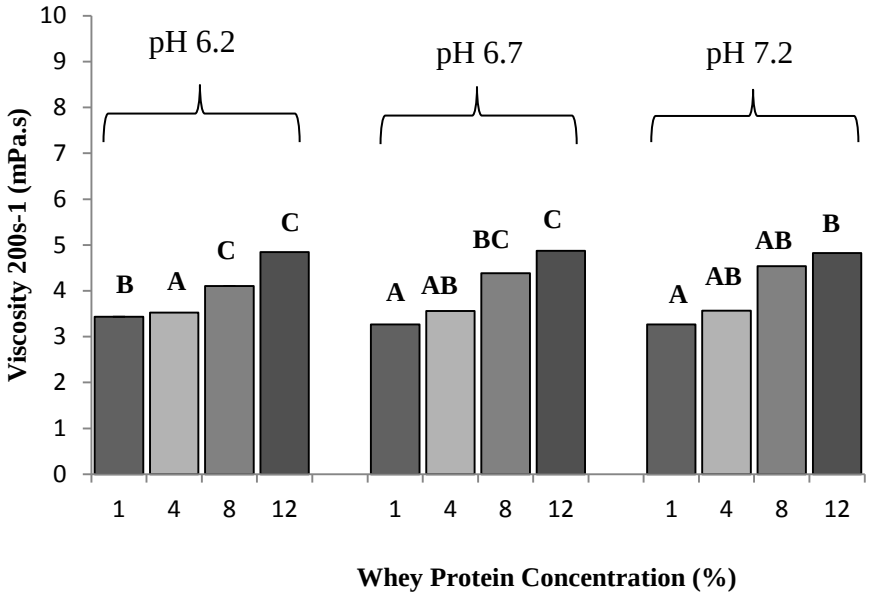


Figure 2: Viscosity of WPI samples (1, 4, 8 and 12% (w/w)) at pH 6.2, 6.7 and 7.2.

[A-C] Values represent the statistical different at $P < 0.05$ the respective pH.

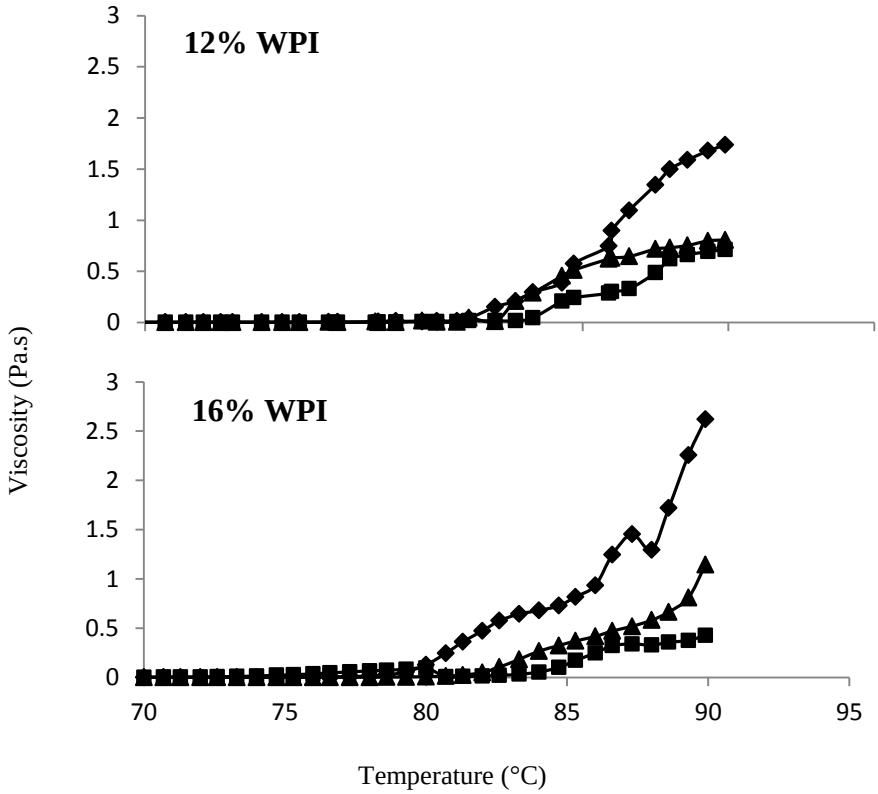


Figure 3: The effect of temperture on the viscosity of both 16% (A) and 12% (B) whey protein isolate at pH 6.2 (▲), 6.7 (■) and 7.2 (◆).