Physical characterisation and stability of purified whey proteins in the presence of novel calcium salts

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

Doctor of Philosophy

by

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This thesis is dedicated to my parents Catherine and Eugene Grace. Thank you for your constant support.

Declaration

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

Signed: _____

Date: _____

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To my supervisor Dr. Jennifer McManus: I am thankful for your endless patience and support over the last 6 years. The knowledge you have imparted is priceless and I can't begin to explain how grateful I am that I had the opportunity to work for you. Your drive, scientific expertise and execution of a task is what I strive to emulate. You inspire me to be better.

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Publications arising from this thesis

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Abstract

The demand for highly concentrated whey protein solutions with a high mineral content is significant due to their versatility in providing the entire amino acid and mineral content needed in any stage of the life cycle, from infants to athletes. However, these products require extensive thermal processing to eliminate microbial growth, leading to protein instability during processing. Thermal instability can lead to precipitation, viscosity increases and heat exchanger fouling causing pressure increases, final yield reduction and ultimately leaving the final product to be un-aesthetic to the consumer.

Whey consists of two major proteins, α-lactalbumin and β-lactoglobulin. The main nutrition source for infants is human milk which consists of predominantly the whey proteins α-lac (80%) and β-lac (20 %). Both the protein and mineral content of bovine and human milk differ; hence, the composition of bovine whey must be altered to produce infant milk formulae (IMF). The second commodity product for which whey protein is used is for sports drinks. In these products, the entire protein content is whey based, but the protein content is predominantly composed of β-lactoglobulin. For athletes the aim is to supplement a diet to allow better muscle recovery and maintain an adequate nitrogen balance to facilitate muscle protein synthesis. Therefore, there is a need to understand how supplementation of whey protein solutions with minerals, particularly calcium impacts the stability and processability of the protein, particularly at high protein concentrations, close to those of processing conditions.

α-lac was extensively purified to attain a protein which is > 98% pure by SE-HPLC, free from β-lac contamination. The pure protein had a greater ability to refold after a thermal denaturation, when compared to solutions containing even small quantities of β-lactoglobulin. The effect of increasing calcium concentration ($CaCl_2$) was then investigated using quasi-elastic light scattering (QELS) to assess solution stability and DSC to assess thermal stability. We found that by manipulating the protein: calcium ratio, it was possible to acquire a high concentration (100 mg ml^{-1}) α-lac solution with added salts, beyond calcium concentrations normally employed during processing.

We then probed the effect of adding calcium amino acid salts to whey proteins α-lactalbumin, β-lactoglobulin and their mixtures in Whey Protein Isolate (WPI) and examined the solution and structural stability of these protein in the presence of $Ca(Gly)_2$ and $Ca(Lys)_2$. The calcium amino acid salts alter both the solution and thermal stability of whey. There was also a significant impact on the distribution of protein aggregate sizes produced after heat

treatment. Calcium amino acid salts may offer an alternative strategy for the supplementation of whey protein beverages with more desirable nutrient profiles and processing characteristics.

List of Abbreviations

%T – Percentage transmission

α-lac – α-lactalbumin

β-lac – β-lactoglobulin

ΔH – Enthalpy

ΔH_{me} – Enthalpy from endotherm midpoint to end point.

ΔH_{VH} –Van't Hoff enthalpy

A – Absorbance

B_{22} – Osmotic second virial coefficient

BSA – Bovine serum albumin

Ca^{2+} – Calcium ion

$CaCl_2$ – Calcium chloride

$Ca(Gly)_2$ – Calcium glycinate

$Ca(Lys)_2$ – Calcium lysinate

CH_3COONa – Sodium acetate

CN – Casein

C_p – Heat capacity

D_0 – Diffusion coefficient at concentration 0

D_c – Diffusion coefficient at concentration c.

DLS – Dynamic light scattering

DSC – Differential scanning calorimetry

EDL – Electric double layer

EDTA – Ethydiaminetetaacetic acid

EBT – Erichrome Black T

ES – electrostatic

F.A.A.S – Flame atomic absorbtion spectroscopy

Gly – Glycine

h – Planck's constant

HCl – Hydrochloric acid

HPLC – High performance liquid chromatography

Hz – Hertz

IEX – Ion exchange

k_D – Net protein interaction parameter

LF – Lactoferrin

Lys – Lysine

mM – Millimolar

M – Molar

MWCO – Molecular weight cut off

NaCl – Sodium chloride

NaN_3 – Sodium azide

NH_4OH – Ammonium hydroxide

NaOH – Sodium hydroxide

OD – Optical density

PAGE – Polyacrylamidegel electrophoresis

pI – Isoelectric point

QELS – Quasi elastic light scattering

RFU – Relative fluorescence intensity

R.I. – Refolding Index

SDS – Sodium dodecyl sulphate

SDS-PAGE – Sodium dodecyl sulfate polyacrylamide gel electrophoresis.

SEC – Size exclusion chroamatography

SE-HPLC – Size exclusion high performance liquid chromatography

TEMED – Tetramethylethylenediamine

$\mathbf{T_m}$ – Melt transition temperature

$\mathbf{t_R}$ – Retention time

Tris-HCl – Tris(hydroxymethyl)aminomethane hydrochloride

UV – Ultraviolet

UV-Vis – Ultraviolet – visible

VDW – van Der Waals

v/v – volume per volume

w/v – weight per volume

ϕ – volume fraction

λ – wavelength

λ_{ex} – Excitation wavelength

λ_{em} – Emission wavelength

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Chapter 1: Introduction

1.0 Introduction

1.1 Protein stability and dairy processing

All dairy products require processing during their manufacture. This involves heating/cooling to eliminate microbes, as well as the addition of mineral additives to make the product more nutritionally rich for consumers. As dairy solutions are colloidal systems consisting of a number of proteins and salts, this processing is the cause of both protein self-association and heat-induced aggregation. Salt addition can contribute to charge screening which leads to self-association, while heating causes protein unfolding and subsequently aggregate formation. These factors are problematic in a single protein system (only one protein species present), however this is exacerbated when the system is a multi-protein one, each protein with a different isoelectric point (pI), net surface charge and melt transition temperature (T_m).

1.1.1 Dairy processing

All dairy products require processing to some degree before they are safe to consume. A principle component of milk - whey protein, can be well utilized by humans as it provides the full range of amino acids and minerals optimal for survival and health (Vegarud et al., 2000; Hambraaues, 2003; McGregor & Poppitt, 2013; Layman et al, 2018). The processing of milk however can lead to protein denaturation and proteins may lose functionality as a result. Some of the processing steps of milk involve repeated heating and cooling to increase shelf life and also decrease bacterial growth in the final product. Processing causes the formation of whey protein aggregates which leads to heat exchanger fouling, pressure increases and reduction in run times (Delplace et al, 1994; De Jong, 1997; Visser & Jeurnik, 1997; Bansal, 2006; Sadeghinezhad et al, 2013).

Processing can be divided into two categories, **thermisation** and **pasteurisation**. Thermisation is the mildest of all thermal treatments and pasteurisation encompasses the treatments in order of increasing severity - pasteurisation, extended shelf-life processing (ESL), ultra-high temperature (UHT) processing and in-container sterilisation (Deeth & Lewis; 2017, Ritota et al, 2017). These processes are summarised in table 1.1.

1.1.2 Thermisation

Thermisation involves heating milk from 57 - 68 °C for 5 - 30 s. It is then cooled and held chilled before moving forward to the pasteurisation step. Thermisation is necessary as it reduces the growth of bacteria that can release heat resistant proteases; hence it primes the solution for pasteurisation. Thermisation causes almost no irreversible changes in the milk

except for the inactivation of some enzymes. (Walstra et al, 2005; Sfakianakis et al, 2014; Deeth & Lewis, 2017).

Table 1.1: *Summary of thermal dairy processing methods (adapted from Deeth & Lewis, 2017).*

Heat treatment	Temperature time/range	Bacteria effect	Enzyme effect
Thermisation	57 - 68 °C for 5 - 30 s	Destroys the majority of non-spore forming bacteria.	Inactivates no enzymes
Pasteurisation	63 °C for 30 min 65 °C for 15 min 72 - 82 °C for 15 - 30 s (Continuous HTST)	Destroys non-spore forming pathogens and spoilage bacteria.	Inactivates milk alkaline phosphatase and lipase
ESL(extended shelf-life processing)	123 - 145 °C for 1 - 5 s	Destroys non-spore forming pathogens and spoilage bacteria and **mesophilic spores.**	Inactivates milk alkaline phosphatase, lipase and lactoperoxidase
UHT (ultra-high temperature processing)	138 - 145 °C for 1 - 10 s	Destroys all non-spore forming pathogens spoilage bacteria except high heat resistant spores.	Inactivates milk alkaline phosphatase, lipase and lactoperoxidase and most plasmin
In-container sterilisation	115 - 120 °C for 10 - 30 min	Destroys all bacteria except extremely heat resistant ones	Inactivates virtually all enzymes

1.1.3 Pasteurisation

Initial **pasteurisation** involves a stepwise increase in temperature (63 °C - 82 °C) followed by holding milk at specific temperatures for set amounts of time depending on the step. The two main methods of initial pasteurisation are low temperature, long time (LTLT) and high temperature, short time (HTST) (Escuder-Vieco et al, 2018). LTLT is best for batch processing while HTST is performed as a large continuous process allowing processing efficiency to reach as high as 50,000 L h^{-1} of raw milk. Similar to thermisation it results in the reduction of even more enzymes that can lead to an unpleasant taste; specifically milk alkaline phosphatase and lipase (Sharma & Rajput, 2014; Rankin et al, 2010). This process also has the benefit of removing heat resistant pathogens such as *Coxiella burnetii* (Cerf & Condron, 2006; Eldin et al, 2013).

ESL processing is more aggressive than standard pasteurisation, in that temperatures as high as 145 °C are reached. Its purpose is to destroy nearly all non-spore forming pathogens and bacteria that can lead to milk spoilage. It builds on standard pasteurisation where it also inactivates lactoperoxidase.

UHT processing is more aggressive than ESL, where higher temperature ranges are reached and holding times at these temperatures are even longer. Specifically, these are 138 - 145 °C for up to 10 s, compared to ESL's ranges of 123 - 145 °C for 5 s. UHT carries the advantage also of eliminating most plasmin and proteases, as well as ESL's benefits.

In-container sterilisation is the most severe form of pasteurisation, where all heat resistant bacteria are eliminated completely and virtually all enzymes are inactivated (Juffs & Deeth, 2007; Deeth & Lewis, 2017).

1.1.3.1 Pasteurisation methods

1.1.3.1.1 Low temperature, Long time (LTLT) processing

The LTLT method heats milk solutions to 63 - 65 °C for 15 - 30 min (Myer et al, 2016; Ritola et al, 2017). This method has the advantage of being small scale, allowing flexibility in the treatment of batches. The LTLT method doesn't produce a superior end product compared to the more commonly used HTST.

1.1.3.1.2 High temperature, short time (HTST) processing

HTST is the most common form of dairy pasteurisation. It allows larger scale, continuous processing and longer run times compared to the LTLT method (Deeth & Lewis, 2017). HTST methods also carry the advantage of a high preservation rate of immunoglobulins (~87 - 100%) (Escuder-Vieco, 2018). The HTST method performs heating and cooling actions using low/high temperature water to regulate pipe temperature (done in step 4, heat exchanger, see figure 1.1). The purpose of the separators (5) is to separate cream/fat (yellow) and skim milk (light blue). These can be remixed later, once homogenisation has occurred (stage 12) (Baro, 2011; Deeth & Lewis, 2017,). Any component density that is greater than the continuous milk phase is placed back into the production and the cycle redone (black) (step 13-15). Alternatively, both the fat components and skim components can be processed separately to yield skim milk and milk containing a desired amount of fat.

1.1.4 Spray drying

Once milk has been pasteurised, the next step before packaging as milk powder is spray drying. Spray drying in the context of foods involves atomisation of a liquid (milk) to produce

a dry powder (powdered milk). This powder can then be reconstituted by the consumer for the intended purpose (Vega & Roos, 2006; Taneja et al, 2015).

The process of spray drying fulfils the aim of changing a fluid into a dry powder. This is done by atomising the fluid in the path of a hot drying gas stream (Rattes & Oliveira, 2007; Fang et al, 2012). The spray drying process involves the steps (in order) spraying, drying of the atomised feed, formation of a powder and separation/collection (Schafroth et al, 2012; Sosnik & Seremeta, 2015). The raw milk fluid is first pumped into the atomiser. The atomiser forms droplets in the path of a high temperature gas stream (Fatnassi et al, 2013) (in most cases the gas is air). The gas stream dries the atomised liquid into a powder. The powder is separated from the gas via either cyclone or bag filter. This process is illustrated in figure 1.2.

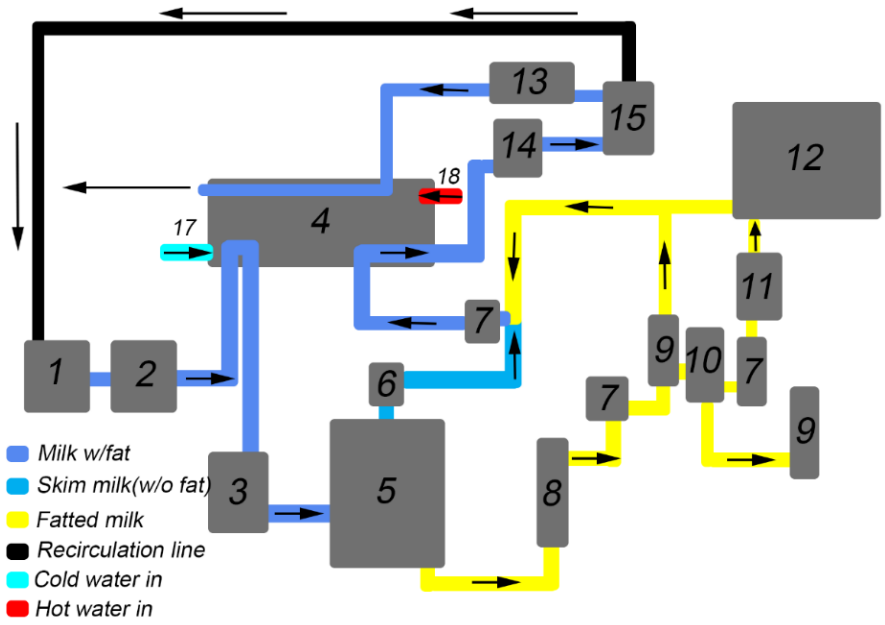


Figure 1.1: *Production line for pasteurised milk with homogenisation.1 Balance tank; 2 Feed pump; 3 flow controller; 4 Plate heat exchanger; 5 Separator; 6 Pressure valve; 7 Flow transmitter; 8 Density transmitter; 9 Regulating valve; 10 Shut-off valve; 11 Check valve; 12 Homogeniser; 13 Booster pump; 14 Holding tube; 15 Flow diversion valve; 16 Process control; 17 Cold water introduction; 18 Hot water introduction (adapted from Deeth and Lewis 2017).*

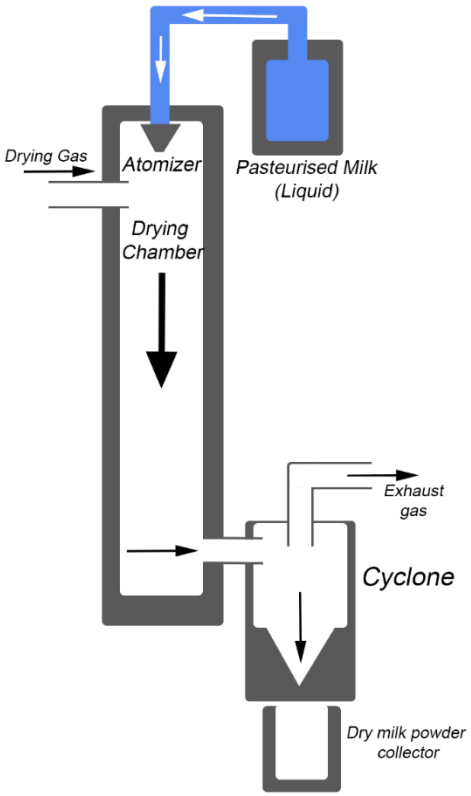


Figure 1.2: *Schematic of a typical spray dryer to produce powdered milk*

1.1.5 Infant milk formula (IMF) production

Infant milk formula production is very similar to powder milk production in that they are both pasteurised and spray dried to produce a powder that can be reconstituted and is safe to consume. However infant formula requires several additional steps throughout production to meet nutritional requirements of infants (Blanchard et al, 2013). Infants require a higher mineral content (specifically calcium) and also a higher concentration of the whey protein α-lactalbumin (α-lac) from cows' milk, to match the protein composition of human milk (Martin et al, 2016). Infants who are formula fed have an increased risk of food-borne infections and as such additional measures are required to eliminate this risk (Penders et al, 2006; Stuebe, 2009). Infant formula production itself is a 10-step process that is outlined in figure 1.3. The main 3 differences between this and powdered milk production are i) at some point minerals (calcium) are added to the mixture, ii) whey protein (α-lac) is added to the mixture at some point to increase the whey: casein ratio of the product (Fenelon et al, 2019) , , iii) additional quality control once the powder is formed to eliminate any risk of foodborne bacteria presence. These steps vary depending on the desired method of formulation, whether it is dry-mixing or wet-mixing.

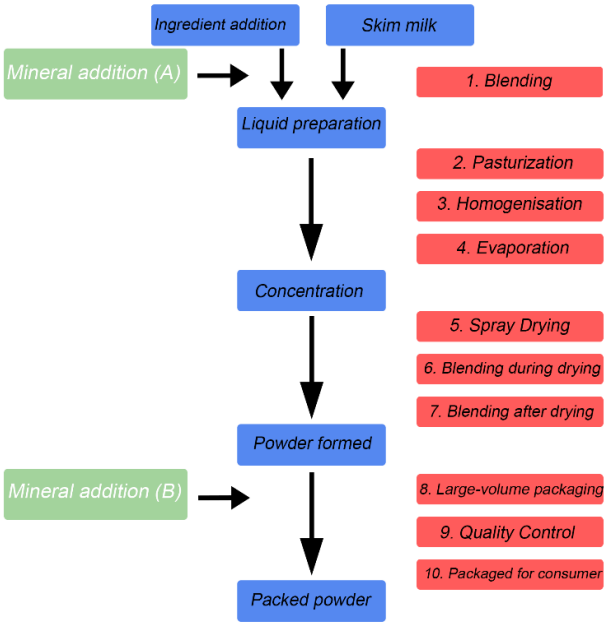


Figure 1.3: *Flow chart demonstrating the 10 steps in producing infant formula (adapted from Blancard et al, 2013).*

Dry mixing involves using powdered starting materials. Whey based powders are mixed with mineral additives in large batches to produce a uniform powdered infant formula (Kent et al, 2015). The main advantage of dry mixing is the reduction in energy cost, however it's imperative that raw materials are free from bacteria as no heating is done to destroy them. The post processing risk of contamination in dry mixing is much higher compared to wet-mixing (Blancard et al, 2013).

Wet-mixing follows the steps outlined in figure 1.3. It produces the most homogenous solution and includes more quality control than dry-mixing. The starting material used is skim milk which is supplemented with whey powder and at this point mineral salts can be added also (mineral addition A). Once pasteurisation and homogenisation have occurred (the same as milk powder) an evaporation step is needed to partially dehydrate the solution, to inactivate enzymes and destroy bacteria. Spray drying occurs to remove the majority of moisture. To ensure an optimal spray, the viscosity of the solution needs to be accurately controlled and must not exceed 200 mPa.s. This corresponds to a solid content of about 50% (w/v) (Blancard et al, 2013; Jiang & Guo, 2014). At this point mineral salts can also be added (mineral addition B). Mineral and other additive additions can technically be done at any time through steps 1-10, however the process outlined here is a generic procedure to produce the fewest issues. The powder is then packaged into large storage containers and screened for bacteria. Post screening - the powder is packaged for consumer use.

1.2 Protein aggregation

Protein aggregation that occurs through the formation of non-native conformation states is irreversible in a broad range of colloidal systems, especially during processing and long-term storage (Roberts, 2006). In food-based systems, food protein aggregation, microstructure and rheological properties must be characterised and controlled in order to ensure optimal sensory experiences for the consumer as well as ensuring issue free processing (Mezzenga & Fischer, 2013; Nicolai, 2013). In beverages such as milk, self-association and aggregation occurs due to thermal processing, but also the presence of a high concentration of additives and acute changes in solution pH (Brodkorb et al, 2016). Milk solutions such as sports drinks and infant formulas have the added complication of requiring a high protein concentration and high mineral content (specifically calcium), both of which can lead to protein aggregation in most cases. A stable milk product is produced when any aggregation that does occur results in soluble aggregates that can maintain colloidal stability over the course of the product shelf life (Ryan & Foegeding, 2015).

1.3 Protein Structure

Proteins are linear polymers built of monomer units called amino acids. The function of a protein is directly dependent on its three-dimensional structure, in most cases. Proteins can provide a wide variety of molecular functions and this is in part due to their ability to fold into many different three-dimensional structural shapes (Andreeva et al, 2008; Sillitoe, 2015). Amino acids are the building blocks of proteins. An α-amino acid consists of a central carbon atom, called the α-carbon, linked to an amino group, a carboxylic acid group, a hydrogen atom, and an R group. The R group is referred to as the side chain. With four groups connected to the tetrahedral α-carbon atom, α-amino acids are chiral; the two mirror-image forms are called the *L* and *D* isomer. A typical amino acid structure is shown in figure

1.4.

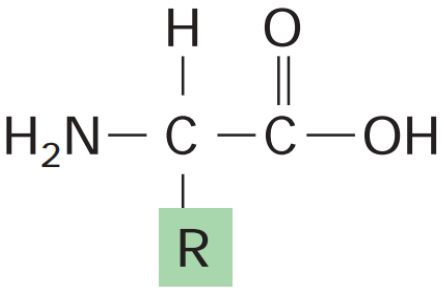


Figure 1.4: *An amino acid with the hydrogen, amino, carboxyl and R group*

Primary structure of a protein arises from the chemical linkage of individual amino acids by amide bonds. These bonds are formed between the N- and C- terminus of amino acids.

Secondary structure is formed by hydrogen bonds between atoms of a polypeptide backbone. The majority of proteins have portions of their respective polypeptide chains that adopt either a coil (α-helix) or sheet structure (β-sheet). The α-helix is a right-handed helical coil that is held together by hydrogen bonding between every fourth amino acid. A diagram showing both can be seen in figure 1.5. There are two types of β-pleated sheets; parallel β-sheets and antiparallel β-sheets. The N-terminus of the polypeptide chain contains the free amino group, and the C-terminus contains the free carboxyl group. If two β-strands run in the same direction, then they form a parallel beta-pleated sheet, and if they run in opposing directions, an antiparallel β-pleated sheet. (Zhang, 2005). These amino acid sequences that form α-helices and β-sheets then fold into a three-dimensional tertiary structure, which is a protein in its native conformation (Dobson, 2004; Parveen, 2017).

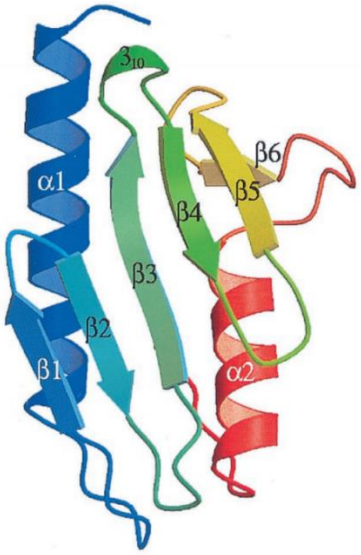


Figure 1.5: *Representation of a typical protein ribbon structure, indicating α-helices and β-sheets. (taken from Cho et al, 2000)*

Tertiary structure is sometimes stabilised by disulfide bonding between sulfur containing amino acids, the hydrophobic effect between non-polar residues, electrostatic interactions and van der Waals forces. Partial or complete unfolding of a protein involves the disruption of protein secondary/tertiary structure and can occur due to changes in temperature, ionic strength, pH or solvent composition (Dill & Shortle, 1991; Wang et al, 2010; Kishore et al, 2012).

Quaternary structure of a protein occurs when proteins that contain more than one polypeptide chain are bonded together by hydrophobic interactions and disulphide bridging.

1.4 Protein-protein interactions

The processing of whey proteins involves changes in pH, temperature and viscosity. The purpose of this to ensure microbial safety and to extend the shelf life of the final product (McKinnon et al, 2009; Sfakianakis & Tzia, 2014; Deeth, 2017). Additives are also introduced to match human milk composition and to supplement the solution mineral profile. During this process the formation of irreversible aggregates occurs, due to the hydrophobic effect and electrostatic interactions in particular, but also due to van der Waals (VDW) interactions and Hydrogen bond formation (Anema & Li, 2003; Chawla et al, 2011; Goyal et al, 2013; Trefalt et al, 2013). Intermolecular protein interactions are summarised in table 1.2. A short-ranged interaction potential implies interactions within the range of the first two hydration layers surrounding a protein and long range implies any range beyond these layers. The strength of these bonds is proportianl to the charge between the two atoms interacting and is inversely proportional to the distance between them.

Table 1.2: *Summary of protein-protein interactions*

Interaction	Attractive (-) / Repulsive (+)	Strength	Range
Van der Waals	-	Weak	Short/Long
Hydration	+	Strong	Short
Hydrophobic	-	Strong	Long
Steric	+	Strong	Short
Hydrogen bonding	-	Weak	Short
Electrostatic	+/-	Weak/strong	Short/Long

1.4.1 Van der Waals (VDW) forces

Van der Waals forces act between all colloidal bodies in a solution, as well as atoms and molecules. Changes in dipole moments between all these are what cause changes in attractive forces as the bodies approach each other (Leckband, 2001; Ozdal et al, 2013; Shaikh et al, 2015). The energy of the VDW interactions decays as the inverse sixth power of separation distance for short range interactions of atoms (Kirsch, 2003), equation 1.1.

$$E = \frac{-C_{VDW}}{D^6} \quad \textbf{1.1}$$

where E indicates energy, c_{VDW} is dependent on the geometry of the acting atoms and D indicates the distance between the centres of them.

1.4.2 Hydrophobic effect

A surface which is hydrophobic usually has no polar or hydrogen bonding sites and this means no affinity for water on the surface in question. Usually bulk water is well structured due to H-bonding between water molecules. The structuring of water molecules in contact with the hydrophobic surface is less entrophically stable relative to well structured hydrogen bonding network mentioned above. For proteins, this causes amino acids with non-polar side chains to be buried in the core of the protein (no contact with water) (Dyson et al, 2006; Liang, 2007).

1.4.3 Hydration forces

Hydration forces are repulsive interactions. They are short-ranged forces that decay exponentially with distance. When excluded volume is increased around the hydrated molecule, it is considered entropy driven as there is a reduction in the amount of space which atoms and molecules can occupy and a decrease in adhesion strength of a flat surface is enthalpy driven, where less energy is required to remove an object from this surface.

This is indicated by the equation 1.2:

$$F_{SOL}(D) = Ke^{-D/l} \quad \textbf{1.2}$$

where D is distance, K relates to hydrophilic repulsion forces and l is the correlation length of the ordering of water molecules (Churev & Derjaguin, 1985; Leikin, 1993; Valle-Delgado et al, 2011).

1.4.4 Hydrogen bonding

Hydrogen bonds are formed by the bonds of the positively charged H atoms and electronegative atoms (Leckband, 2001). This type of bonding occurs between amino acids to help stabilise protein secondary structure. This manifests by the bonding of an oxygen atom on a carboxyl group of one amino acid and the hydrogen atom from an amide group on another (Kim, 2002).

1.4.5 Electrostatics

Electrostatic interactions can be both attractive and repulsive. An electric double layer forms around a charged particle (figure 1.6). The Stern layer is located in the innermost section and

is made up of the oppositely charged counterions, which are closly attached by the subsequent electrostatic force. Outside of the Stern layer, it consists of of ions that are both positive and negative (the diffuse and bulk layer); where ions generally move at greater speeds, the further away from the central negatively charged particle they are. The diffuse layer contains a greater concentration of counterions relative to the bulk solution and the electrostatic force from the charged surface have a greater effect here on counterions than the next outer layer (bulk). The charge sign of the ions and surface amino acid side group will dictate whether they will adhere to each other or not in the diffuse and bulk layer. The outermost layer is the bulk solution, which is the figure 1.6 (Bhagavan, 2002; An, 2017).

1.5 Parameters affecting aggregation

1.5.1 Ionic strength

Adding salts to a milk protein solution is common practice, to increase calcium or other mineral concentrations or to induce aggregation for the formation of cheese (Santos, 2013). Salts have a complex effect on the stability of protein solutions by changing both the physical stability and rate of formation of non-native aggregates. They modulate the strength of electrostatic interactions between proteins by specific and non-specific direct interactions with the protein itself and also by charge screening. High salt concentrations can cause charge screening that reduces the effect of electrostatic interactions and can lead to protein self-association. (Arakawa, 1984; Chen et al, 1994; Maclean, 2002). This is particularly relevant in the processing of whey, by being more conducive to self-association and subsequently thermal induced aggregation. (Ako, 2010).

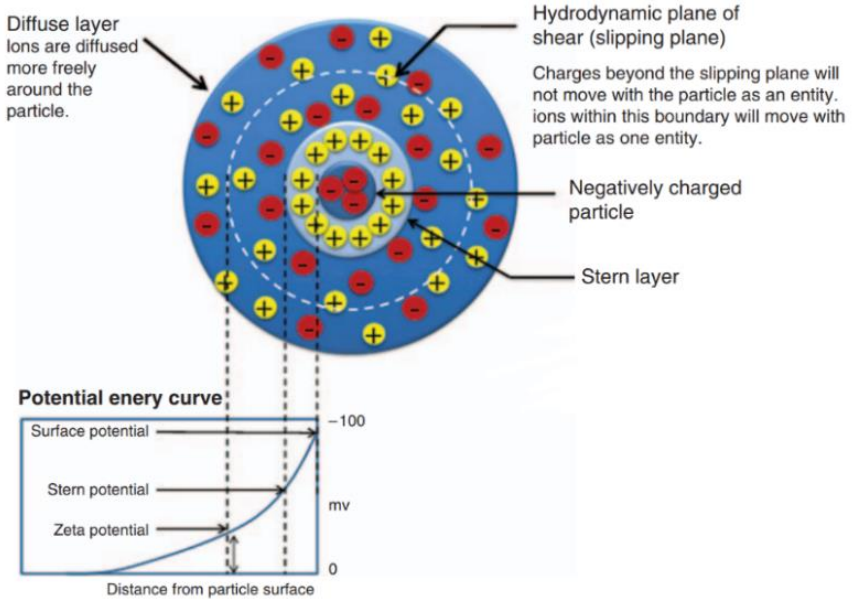


Figure 1.6: *Visual representation of the electric double layer (Taken from Bhagavan, 2012)*

1.5.2 pH

pH has a very strong influence over protein net surface charge. The pH at which the protein net charge is zero is called the isoelectric point (pI). In whey solutions; pHs close to the pI contribute to reduced solubility of protein by reducing the electrostatic repulsion. In the instance when heat is also applied, it results in aggregate formation. (Mleko & Foegeding, 2000; Havea et al, 2009). Figure 1.7 illustrates the change in the effective surface charge (measured here as zeta potential) with varying pH. The isoelectric point in this case is approximately pH 4.5. At the isoelectric point, the net electrostatic charge is zero and does not contribute a repulsive component to the interaction potential, and in many cases, the protein is more likely to self-associate at this pH. Whey protein processing is generally done at pH 6.5-6.7.

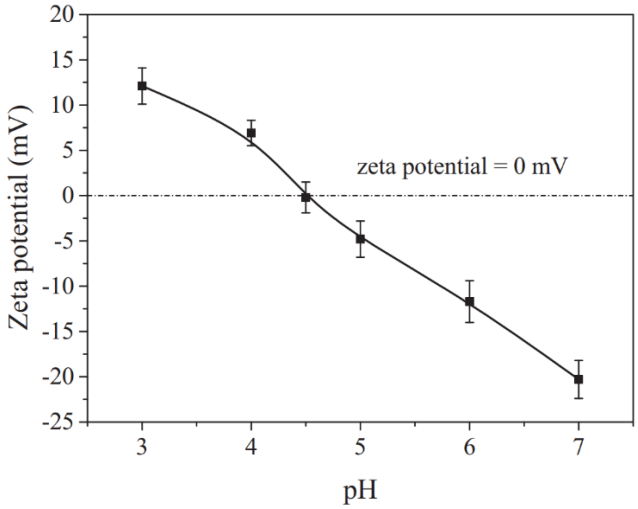


Figure 1.7: *The relationship between zeta potential and pH for BSA (15 g/L). Taken from Li et al, 2016*

1.5.3 Temperature

A native, folded globular protein structure has high stability and low free energy, where on average the folded structure is roughly 5-20 kcal mol^{-1} more stable than the unfolded structure. (Dill, 1990; Pace, 1995). One of the mechanisms leading to protein aggregation occurs via the association of unfolded proteins, by which they unfold when exposed to temperatures higher than their melt transition temperature (T_m). T_m is the midpoint of unfolding transition; the temperature at which half the protein quantity is partially unfolded. Exposure to high temperatures is common practice in dairy processing, to destroy bacteria and inactivate enzymes (Gayà & Calvo, 2018), hence it is an inevitable stress on whey protein solutions. One way to quantify the thermodynamic stability of a protein is using the free energy of unfolding

(ΔG_{unf}). The relationship between free energy and aggregation reaction coordinate can be seen in figure 1.8.

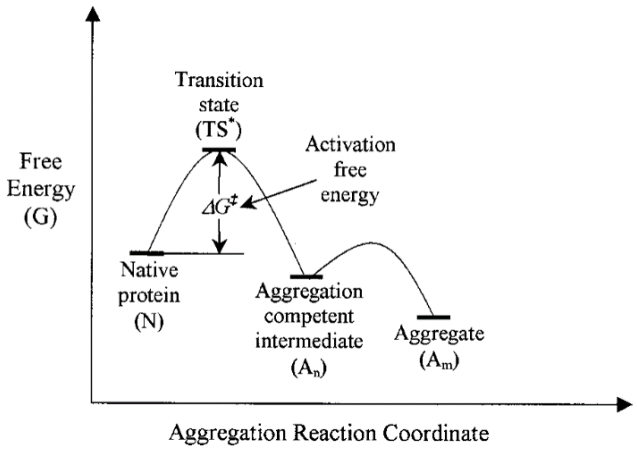


Figure 1.8: *Graph demonstrating the relationship between free energy and protein state (taken from Chi, 2003).*

The aggregation of whey proteins occurs through a multistage process involving first the unfolding of native protein and subsequently its irreversible aggregation. The two main whey proteins of highest concentration in cows and human milk are β-lac and α-lac. β-lac is a native dimer at pH 7.0 and dissociates to monomers at roughly 30 °C. When temperature is increased to a processing relevant temperature of 60 °C, the equilibrium shifts to more irreversible aggregate than oligomer/monomer. This is because β-lac unfolds, exposing its free thiol and the burial of this thiol with another unfolded β-lac free thiol results in a more stable structure (aggregate) than an unfolded monomer.

The aggregation of α-lac however is less complicated than for β-lac. At room temperature and physiological pH, α-lac exists as a monomer and only oligomerises when exposed to higher temperatures (90 °C). α-lac unfolds by a 2-step mechanism where its state is either folded or unfolded, with no intermediates. α-lac can still form disulphide bridges after unfolding, but is less susceptible to this as it contains no free thiols.

1.5.4 Protein concentration

Elevated protein concentrations are desirable in the production of a superior sports drink or baby formula due to them containing both a greater nitrogen content and essential amino acids to ensure efficient muscle recovery and infant health. High protein concentration is also necessary from a processing perspective as its necessary for the final step of spray drying. This can lead to aggregation by macromolecular crowding due to a reduction in excluded volume which decreases the distance between proteins in a colloidal solution. A high protein

concentration is not a causal factor in all aggregation mechanisms, but contributary when combined with thermal treatment, high salt ionic strength and pH in close proximity to its pI (Amin et al, 2014; Perusko et al, 2015; Hall et al, 2016).

1.6 Milk protein environment

Milk (bovine) is a complex colloidal solution. The whey : casein protein ratio is roughly 80 : 20 (Buggy et al, 2016). The whey content is made up of the proteins α-lac (1.2 g/L) β-lac (3.2 g/L), BSA (0.4 g/L) immunoglobulins (0.7 g/L), proteose peptone (0.8 g/L) (Raikos, 2010) and minor amounts of lactoferrin. The 80% casein portion (25.6 g/L) is composed of mainly of κ-casein (Belloque, 2002). The exact contents in bovine milk are summarised in table 1.3.

Table 1.3: *Summary of the proteins present in bovine milk (adapted from Raikos, 2010)*

Protein	g/L	% of total protein
Total protein	**33**	**100**
Total casein proteins	26	79.5
α_{s1}	10	30.6
α_{s2}	2.6	8.0
β	9.3	28.4
κ	3.3	10.1
Total whey proteins	**6.3**	**19.3**
α-lac	1.2	3.7
β-lac	3.2	9.8
BSA	0.4	1.2
Immunoglobulins	0.7	2.1
Proteose peptone	0.8	2.4

Human milk is also composed of a similar complex mixture of whey and casein proteins and depending on lactation stage, the whey : casein ratio shifts can shift from 90:10 to 50:50 from early to late lactation (Ballard & Morrow, 2013; Haschke et al, 2016; Martin et al, 2016). pH changes at different stages also, with human milk starting at pH 7.0 and becoming slightly more alkaline to pH 7.4 at later lactation stages. Bovine milk also becomes more alkaline at later stages of lactation, beginning at pH 6.2 and reaching 6.8 (Tsioulpas, 2007). Regarding the mineral composition, human milk generally has a third the amount of

calcium bovine milk does (~6 and ~30 mM), due to less calcium phosphate bound by casein in human milk. The main salt concentrations of both human and bovine milk are detailed in table 1.4.

Table 1.4: *Summary of the main ion concentrations in bovine milk (adapted from Gaucheron, 2005) and human milk (from Li et al, 2016)*

Mineral salt	Concentration (mg kg^{-1}) (Bovine)	Concentration (mmol kg^{-1}) (Bovine)	Concentration (mmol kg^{-1}) (Human)
Calcium	1043-1283	26-32	5.53-7.21
Magnesium	97-146	4-6	1.23-1.67
Sodium	391-644	17-28	3.21-5.13
Potassium	1212-1681	31-43	9.6-5.13
Chloride	772-1207	22-34	n/a

1.6.1 α-lactalbumin (α-lac)

α-lac is a 123 amino acid chain metallo-protein found in the milk of most mammals. Its function is the synthesis of lactose (Nicoleta & Rapeanu, 2010). The protein has a relatively low molecular weight of 14,070 Da in human milk and 14,187 Da in bovine milk. The pI can vary from 4.2-4.6, depending on the specific amino acid sequence (Lönnerdal & Lien, 2003; Kamau, 2010; Layman et al, 2018), it has no free thiols and its tertiary structure is stabilised by 4 disulphide bonds. The structure of native α-lac comprises two distinct domains; the first is the large α-helical domain and the second a smaller β-sheet domain. These two separate domains are then linked together by a loop which binds calcium ions specifically (Permyakov, 2000). α-lac exists in two distinct forms; apo (non-calcium bound) and holo (calcium bound) (figure 1.9 and 1.10 respectively). The calcium binding loop is made possible by the carboxylic oxygens of amino acids 82, 87 and 88 with the carbonyl oxygens of amino acids 79 and 84 and two water molecules. This forms a distorted pentagonal bipyramidal structure, with a calcium ion at its centre (Chrysina et al, 2000 Permyakov, 2001; Yarramala et al, 2019). The loop described here is termed the primary calcium binding loop and has an association constant for calcium at roughly 1.7 - 2.0 × 10^7 M^{-1} (Permyakov & Berliner, 2000). The primary calcium binding loop also binds other divalent metals, but their association constant is several orders of magnitude less than calcium. The association constants for these metals are summarised in table 1.5. α-lac also contains several zinc binding sites, the site with the

strongest association constant being 5×10^5 M^{-1} (Permyakov et al, 1991). This site is located roughly 14 Å from the primary calcium binding site (Permyakov et al, 2000).

Table 1.5: *Summary of the association contants of α-lac for the primary cations present in bovine milk at the* ***primary binding site*** *(Adapted from Permyakov & Berliner, 2000)*

Cation	Association constant (M^{-1}) (20 °C)	Association constant (M^{-1}) (37 °C)
Ca^{2+}	2×10^7	3×10^8
Mn^{2+}	n/a	3×10^8
Mg^{2+}	211 ± 20; 46 ± 10	2000 ± 100; 200 ± 20
Na^{+}	36 ± 10	100 ± 10
K^{+}	6 ± 3	8 ± 3

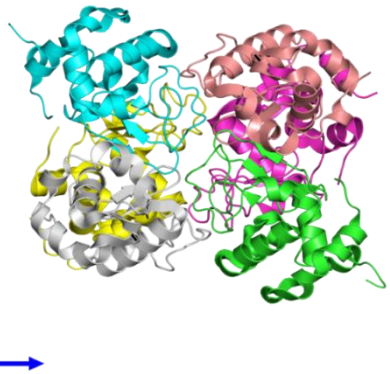


Figure 1.9: *apo α-lac from Bovine Taurus (cow) (taken from protein data bank (europe) PDBe database: ID 1f6r.*

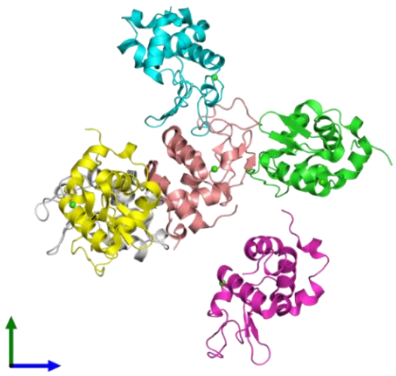


Figure 1.10: *holo-α-lac from Bovine Taurus (cow) (taken from protein data bank (europe) PDBe database: ID 1f6s.*

1.6.2 β-lactoglobulin (β-lac)

β-lactoglobulin is a whey protein present in the milk of most mammals (figure 1.11). It is the most abundant whey protein in bovine milk, making up 50% of total whey protein (Marshall, 2004; Anuradha, 2009 Korhonen, 2011). Its primary structure folds into an 8-stranded antiparallel β-barrel, with a 3-turn α-helix and a 9th (labelled I in figure 1.12) strand that flanks the first (Kontopolis et al, 2004; Mercadante et al, 2012,). It's a lipocalin protein, with a molecular weight (M_w) of ~18,400 kDa, a pI of 5.3 and made of 162 amino acid residues (Brownlow et al, 1997; Simons et al, 2002; Mercadante et al, 2012). β-lac has one free thiol, located at CYS121, which is accessible at pH 7.0 and above (Papiz, 1986, Brownlow et al,1997). β-lac can bind vitamins A, D, ligands, small peptides, palmitic acid, as well as several other hydrophobic compounds, that bind via the calyx of β-lac (Kontopelis, 2004; Mensi, 2013; Le Maux et al, 2014). This calyx is made of two sheets of strands A-D and strands E-H. The loops of strands E-F act as a seal over the calyx (Kontopelis, 2004; Tavel et al, 2008, Domínguez-Ramírez et al, 2013,) (figure 1.12). The α-helices made of strand I and part of A are at the interface of dimer formation (figure 1.12). At low pH the loop is closed and is open at neutral pH and above. β-lac exists as a non-covalent dimer at pH 7, with a net charge of -8 (Simons et al, 2002). The oligomerisation of β-lac is partly due to the hydrophobic effect, peptide-peptide interactions and salt bridging. The hydrophobic effect appears to be the driving force for oligomerisation by causing a decrease in water accessible area upon dimer formation. Peptide-peptide interactions occur using the proteins I strand by antiparallel pairing, while salt bridging occurs between the A and B loops. (Sakurai, 2001; Mercandante et al, 2012; Shin, 2015).

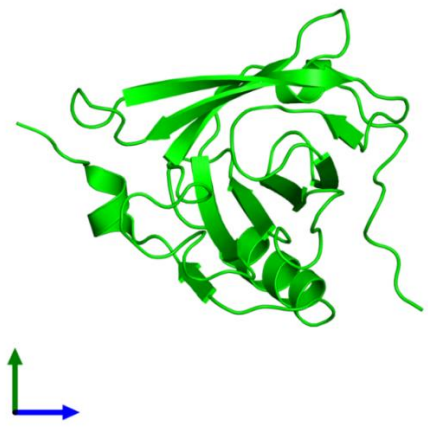


Figure 1.11: *β-lac from bovine Taurus (cow) (protein data bank (europe) PDB database: ID 1dv9.*

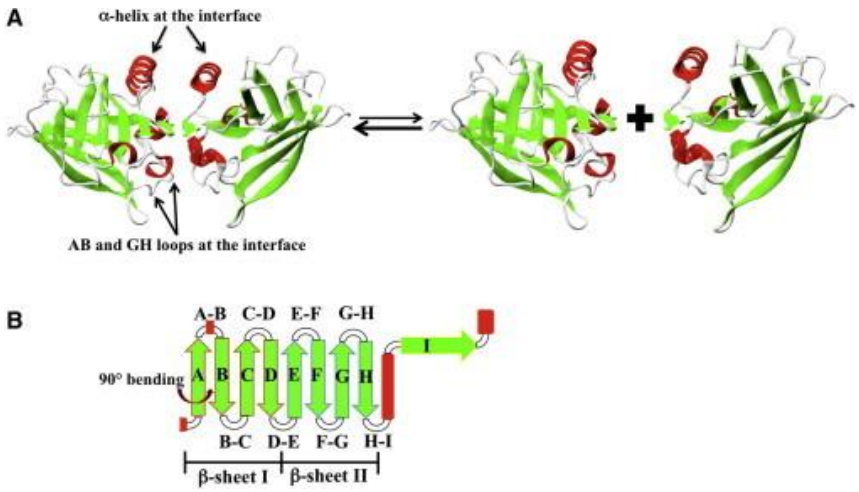


Figure 1.12: *Image indicating the α-helices and β-sheets of β-lac and its dimer interface*

1.6.3 Casein

Casein protein accounts for 80 % (w/w) of the total protein content in bovine milk. It is a phosphoprotein, in that it specifically binds calcium phosphate molecules (Holt el al, 2013; Buggy et al, 2017; Glab & Boratynski, 2017). Caseins present in bovine milk include α_{s1}, α_{s2}, β and κ-casein, the proportions of which depend on the mammal it is derived from. For bovine derived casein the proportion is roughly 4 : 1 : 4 : 1 for α_{s1} : α_{s2} : β : κ. In humans however β-casein is in the highest concentration, with a small amount of κ (Lonnerdal, 2003) and negligible amounts of α_{s1} and α_{s2}. Casein exists as a micelle with α_{s1}, α_{s2} and β located on the interior and κ circling its circumference. This thermodynamically stable structure forms a complex with nanoclusters of calcium phosphate (figure 1.13). The M_w of casein varies from 19-24.5 kDa depending on which type of casein (α, β, κ) and the degree of phosphorylation. The average molecular weight and chain lengths are detailed in table 1.6.

Table 1.6: *Summary of data regarding casein variants.*

Casein fraction (phosphorylated)	M_w (kDa)	Chain length (A.A)	pI	No. of cyst residues	No. of phosphorylated residues
α_{s1}	24.5	199	4.4	1	8-9
α_{s2}	24.3	207	4.9	2	13
β	24.0	209	4.7	1	4-5
κ	19.0	169	5.6	1-3	2

1.6.3.1 α_{s1} casein

α_{s1} casein makes up roughly ~40 % (w/w) of the casein component of bovine milk (Huppertz et al, 2018). It's composed of 199 amino acid residues, 8-9 of which are phosphorylated serines. The phosphorylation that occurs allows the stabilisation of calcium phosphate nanoclusters which are present throughout the casein micelle. Pre-phosphorylation the M_w of α_{s1} casein is 23.0 kDa, which increases to 23.6 kDa after phosphorylation. The pI changes from 4.9 to 4.4 after phosphorylation. Calcium phosphate binding maintains the structure of the micelle under thermal stress as well as providing a source of calcium to the consumer. α_{s1} casein contains one cysteine group and as such cannot form an intramoleuclar disulphide bridge (Huppertz et al, 2018).

1.6.3.2 α_{s2} casein

α_{s2} casein encompasses roughly 8-10 % (w/w) of the protein content of bovine milk. It is composed of 207 amino acids, with up to 11 phosphorylated residues. Pre-phosphorylation, the M_w of α_{s1} casein is 24.3 kDa, which increases to 25.2 kDa after phosphorylation. Concurrently, its pI shifts from 8.3 to 4.9. Its structure is stabilised by a disulphide bond and α_{s2} contains the highest amount of phosphorylated resides of any casein present in bovine milk (Farrell et al, 2009; Huppertz et al, 2018).

1.6.3.3 β-casein

30-35% (w/w) of casein protein content is made of β-casein. It is composed of 209 amino acids. Pre-phosphorylation, its M_w is 23.6 kDa, which increaes to 24.0 kDa after phosphorylation and the pI changes from 5.1 to 4.7. β-casein contains 1 sulphur containing amino acid and as such isn't stabilized by a sulphur bridge. β-casein is less calcium sensitive than the α strains, even so, it is prone to calcium precipitation at 20 °C and above, while at 20 °C and below it can remain soluble at calcium concentrations of 400-500 mM. Under physiological conditions it can bind 7 calcium ions for every one casein molecule (de Kruif & Holt, 2003; Huppertz et al, 2018).

1.6.3.4 κ-casein

κ-casein is present in milk in the smallest concentration, at about 10-12 % (w/w). Its molecular weight is 19.0 kDa, made of 169 amino acid residues and has a pI of 5.6 when phosphorylated. Generally, κ casein contains one phosphorylated group, but double and triple phosphorylated variants do exist. Casein contains two sulphur containing amino acids meaning its structure is stabilised by a sulphur bridge. Unlike the other forms of casein, κ-casein is not sensitive to

calcium and as such can be present alongside other caseins that are sensitive to calcium in forming a micelle (Huppertz et al, 2018).

1.6.3.5 The casein micelle

α, β, κ casein mentioned above all combine into a single structure termed a micelle when in milk (figure 1.13 and 1.14). The casein micelle can be defined as a sterically stabilized association colloid, where 95 % of its structure is protein and the 5 % remainder are minerals, mainly calcium phosphate. It is a near spherical structure in the size range of 50-600 nm, where the size variance depends on the organism the milk comes from and stage of lactation. Casein micelles are stable at concentrations of up to 0.2 M calcium phosphate and 50°C at neutral pH, however alkaline pH can induce its collapse. 65% of total calcium in milk is bound as calcium phosphate to a casein micelle. The micelle can harbour huge amounts of water, with the hydration values of 3.0 - 3.5 g of water per gram of dry protein.

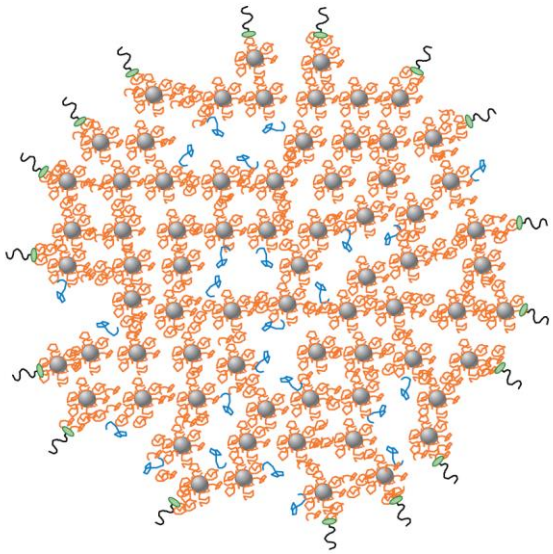


Figure 1.13: *A casein micelle with α_{s1}, α_{s2} and β casein in the interior and κ-casein on the exterior. $Ca_3(PO_4)_2$ nanoclusters are coloured in grey (image taken from Dalgeish & Corredig, 2012).*

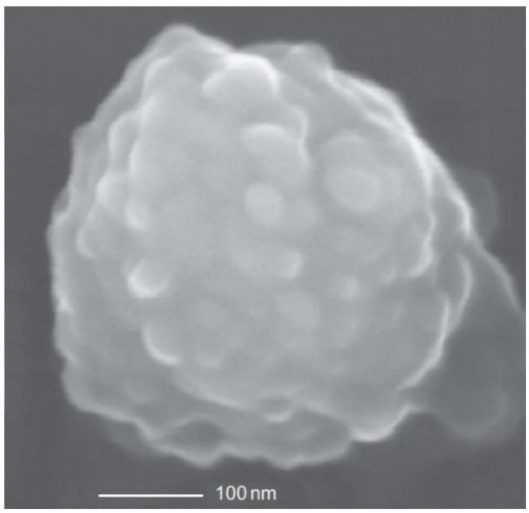


Figure 1.14: *Scanning electron micrograph of a casein micelle (taken from Huppertz et al, 2018).*

1.6.4 Minor proteins present in bovine milk

1.6.4.1 Lactoferrin

Lactoferrin (LF) is an iron binding protein present in the milk of bovine and human animals (~0.1-0.8 g L^{-1} and 2.0-6.0 g L^{-1} respectively). It is composed of 700 amino acids, 17 intramolecular disulphide bonds and has a molecular weight of 76.2 kDa. The concentration of LF in human milk is high at 6 g L^{-1} in the early lactation stages and declines to roughly 1.8 g L^{-1} in the later stages. Its concentration in bovine species however is much lower ranging from highs of 0.8 g L^{-1} to lows of 0.1 g L^{-1} depending on lactation stage (Adlerova et al, 2008; Giansanti, 2016).

Despite being present in relatively low concentrations, LF plays a role in the human innate immune system. Its purpose is to prevent bacteria from using free iron by binding iron ions beforehand. When iron is bound to LF it is known as holo-LF and when it is not bound it's known as apo-LF. Bacteria pathogenesis occurs by a pathogen binding to a host cell surface. LF prevents this by binding to the lipopolysaccharide of the bacterial wall leading to cell lysis of the bacterial cell. Finally, LF has anti-inflammatory properties where it can be administered orally to downregulate an inflammatory response (Dupont, 2011).

1.6.4.2 BSA

BSA is a protein present in the milk of cows (~0.4 g L^{-1}) (Raikos, 2010) and is present as a result of the leakage of protein from blood serum into the milk at the tight junctions in the cells of the mammary gland (Boland, 2011). It is composed of 583 amino acids, including 17

intracellular disulphide bonds (and a single free sulfhydryl group), giving it a molecular weight of ~66 kDa (Zhao et al, 2013; Bocedi, 2016). BSA has no explicit function in milk and its concentration in bovine milk changes depending on the stage of lactation, with greater concentrations present in in the early stages and lower concentrations in the later stages (Benavidez & Garcia, 2013).

1.6.4.3 Immunoglobulins

Immunoglobulins are divided into subclasses, of which IgG, IgA and IgM are found in mammalian milk (Brummitt et al, 2011), in concentrations less than 1 % (w/v). IgG is present in the highest concentrations in colostrum and milk. Every monomeric subclass of immunoglobulins has the same structure – two heavy and light chains with a molecular weight of roughly 160 kDa. These heavy and light chains are linked together by a disulphide bond, which gives rise to the classic Y-shape. (Hurley & Theil, 2011; Giansanti, 2016).

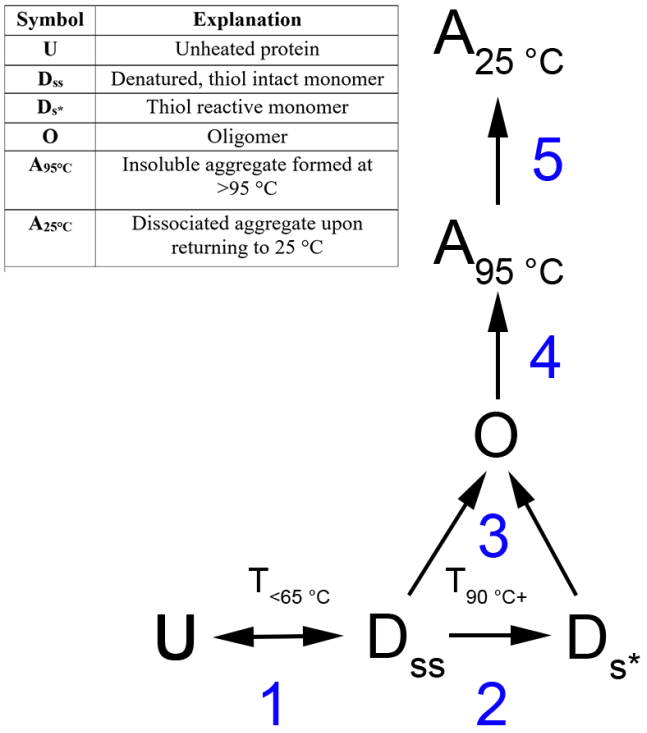
1.6.5 Heat induced aggregation of whey proteins

Preventing the self-association of whey proteins in a high salt/protein concentration solution is the central aim of this work. The prevention of irreversible heat induced aggregate formation is of equal importance, given the high temperatures these colloidal solutions are exposed to during processing. β-lac is the key driver of heat induced aggregation in whey solutions, due to its free thiol (Trivedi et al, 2009) and inability to refold after heat induced unfolding. α-lac on the other hand can refold after unfolding and has no free thiol groups, making it less problematic in producing aggregates. High salt and protein concentrations are conducive to α-lac/β-lac self-association and coagulation in an unheated system, and when heated, these solutions are also more likely to produce irreversible aggregates. β-lac denaturation can induce gelation when heated (when present in even low concentration), increasing the solution viscosity and produces irreversible aggregates containing both α-lac and β-lac, thereby preventing the refolding of α-lac Other proteins present also contribute to colloidal instability, albeit to a much lesser extent than β-lac such as BSA and lactoferrin. Here I outline the main whey protein self-association mechanisms, as well as the process that produce heat – induced aggregation in milk.

1.6.5.1 α-lac

In depth analysis of a pure solution of the aggregation mechanism of α-lac is a topic that has not been explored extensively in the literature. This is because highly purified α-lac is not produced commercially. While there are studies performed using minimum 92% purity α-lac (McGuffey et al, 2005), even small quantities of β-lac contamination can lead to β-lac –

induced irreversible aggregation, making it challenging to understand the unfolding and aggregation behaviour of α-lac alone. McGuffey (McGuffey et al, 2005) have outlined the heat induced aggregation mechanisms for α-lac (figure 1.15).



Symbol	Explanation
U	Unheated protein
D_{ss}	Denatured, thiol intact monomer
D_{s*}	Thiol reactive monomer
O	Oligomer
$A_{95°C}$	Insoluble aggregate formed at >95 °C
$A_{25°C}$	Dissociated aggregate upon returning to 25 °C

Figure 1.15: *A flow chart describing the heat induced aggregation of holo-α-lactalbumin (bovine) (adapted from McGuffey et al, 2005)., where step 1 indicates the equilibrium between unheated α-lac* (**U**) *and heat denatured monomer (thiols buried)* (**D_{ss}**)*, assuming temperatures do not exceed 90 °C. Step 2 however indicates the complete denaturation of α-lac beyond 90 °C and presents the formation of a monomer with a reactive thiol* (**D_{s*}**)*. This leads to the formation of oligomers via thiol exchange of reactive monomers (mainly dimers and trimers)* (**O**) *step 3, of which some will lead to large irreversible aggregates (step 4,* **$A_{95°C}$***) and renatured monomers (step 5,* **$A_{25°C}$***).*

α-lac denatures at relatively low temperature (65 °C) and doesn't readily aggregate due to the absence of free thiol groups. Hong & Cremer (Hong & Cremer, 2005) **propose** a mechanism for steps 1-3 for α-lac that was not purified, (containing ~8 % β-lac). However,

the step 1-3 mechanism was repeated by McGuffey and others (McGuffey et al, 2005) by comparing α-lac with a similar purity to that used by Hong & Cremer to a high purity one (Hong & Cremer, 2002) (purified via IEX, however still contains a small amount of β-lac), yielding minimal difference. McGuffey and others expanded this with steps 4 and 5, indicating that heating to 95 °C leads to irreversible aggregate formation, while step 5 indicates some refolded monomer is also formed. They demonstrated this mainly using turbidity measurements and micro DSC by heating and cooling where step 5 occurs when upon cooling some aggregate returns to soluble and some remains insoluble aggregate (step 4). Based on the experimental evidence available therefore, heat denaturation followed by cooling of α-lac can lead to both irreversible aggregation and refolding of protein to monomer, which based on previous studies at up to 8% β-lac, seems to be insensitive to β-lac content.

1.6.5.2 β-lac

In a heated α-lac/β-lac protein solution the primary source of aggregation is disulfide bonding. Disulfide bonding is the dominant cause of increased solution viscosity and precipitation, during whey thermal processing (Petit et al, 2011; Wijanyanti et al, 2014). β-lac contains a free thiol located at CYS121, and aggregation arises by disulfide exchange of these free thiols between molecules of β-lac protein. β-lac exists as a native dimer at neutral pH and 20°C; however, these dimers can dissociate into monomers and unfold once heated to 70 °C or more, providing the protein concentration is low enough (<25 mg ml^{-1}) (De Wit, 2009). The free thiol of β-lac is revealed at this point and facilitates disulfide bridging by the interaction between unfolded polypeptide chains of β-lac, leading to aggregation. Considering this, removal of β-lac entirely from a whey protein mixture could contribute to fewer issues during thermal processing.

1.8 Milk additives

1.8.1 Calcium and Amino acid addition

Calcium in milk is needed to enhance the functional and nutritional properties of milk products (Deeth and Lewis, 2017, Balk et al, 2017). The addition of extra calcium is often done to enhance these properties in milk (Lartillot & Philippe, 2004). Addition of calcium also carries some drawbacks such as increasing the solution ionic strength, lowering colloidal stability and aggregation, leading to increases in solution viscosity, making dairy processing more difficult.

Addition of amino acids such as arginine have been shown to inhibit protein aggregation, in low salt protein solutions, both in a simulation and experimental setting (Das et al, 2007, Bozorgmehr & Monhemi, 2015) and has been demonstrated to increase solubility

of protein partially folded states (Arakawa et al, 2007). This occurs by it strengthening the hydration layer surrounding proteins and by extension increasing the strength of repulsion between these proteins in solution, becoming more colloidally stable. Therefore, amino acids may offer some advantages as an additive to dairy protein beverages, i.e. enhanced stability and enhanced amino acid profile.

Glycine addition has been studied in β-lac solutions to assess its role in maintaining colloidal stability (Arakwaka, 1987; Arakwaka, 1989). The proposed mechanism by which this occurs is that β-lac is preferentially hydrated as opposed to the glycine in solution, when β-lac is in its native state. When β-lac is denatured however, its charge distribution is more heterogeneous and consequently, ion binding is reduced, making this state more unfavourable. Hence as the denatured state is so energetically unfavourable, that should lead to the stabilisation of the native state.

Given this earlier work, here we investigate if adding calcium as an amino acid salt, e.g. ($Ca(AA)_2$) could be a useful way of both adding calcium and increasing colloidal stability. $Ca(AA)_2$ salts can be prepared with relative ease due to their relatively high calcium binding when dissociated in solution (Ho et al, 2007). Here we investigate $Ca(Gly)_2$ and $Ca(Lys)_2$ (figure 1.16).

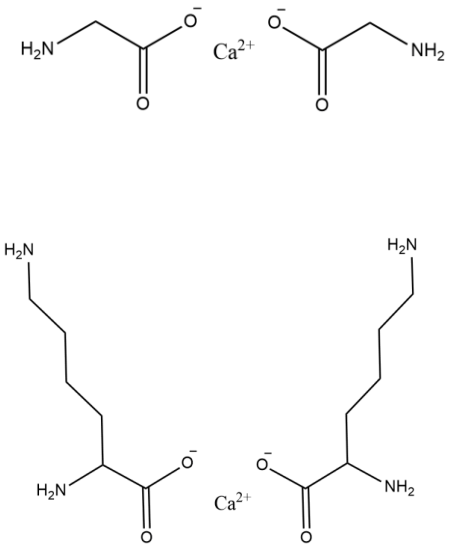


Figure 1.16: *The structure of $Ca(Gly)_2$ (top) and $Ca(Lys)_2$ (bottom).*

1.8.2 The Hofmeister series

The Hofmeister series ranks the influence of specific ions on the physical behaviour of a wide variety of aqueous processes, including protein folding and colloidal assembly (Zhang & Cremer, 2006). The typical ranking of the Hofmeister series is summarised in figure 1.17. Ions

to the left of Cl^- are termed kosmotropes and ions to the right are known as chaotropes. Kosmotropes are strongly hydrated and have a stabilising effect on proteins and chaotropes are known to destabilise folded proteins (Zhang & Cremer, 2006). Ionic calcium is a strong chaotrope in the Hofmeister series, third only to barium and guanidinium. Hence, adding additional calcium to a high protein concentration solution is problematic as protein solubility generally decreases with increased salt concentration (Cohn, 1943; Curtis et al, 2002). Specific ion-macromolecule interactions can be more important for ion specific/protein than the bulk ion properties (Pegram & Record, 2008; Kunz, 2010; Salis et al, 2014; Schwierz et al, 2016), for example a protein that binds an explicit ion i.e. α-lactalbumin and calcium.

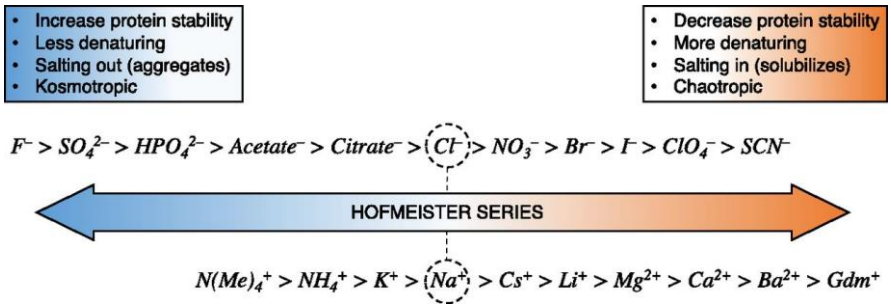


Figure 1.17*: The Hofmeister series general trend (Taken from Wicky et al, 2017).*

1.9 Mineral ions binding to milk protein.

A number of monovalent and divalent metals are present in milk ultrafiltrate (Gaucheron, 2005; Haug et al, 2007; Lyklema, 2009; Li et al, 2016) . All serve the function of providing adequate nutrition to the consumer. A surplus of these salts in the bulk milk solution however can cause formulation issues (de Wit & Kessel, 1996; Arnaudov & de Vries, 2006; Kennedy & Mounsey, 2009) related to electrostatic screening at high ionic strengths causing insoluble aggregate formation when heated. Naturally, the milk protein casein is what binds excess serum calcium and reduces the amount of free calcium in solution (O'Kennedy, 2009; Holt et al, 2013).

A whey protein solution however does not have the advantage of excess calcium being bound by casein as it is present in half the concentration in IMF compared to bovine milk (40 % w/w vs 80% w/w). This is the case for the most stable IMF's (Lien, 2003; Buggy et al, 2016), which mainly consist of whey proteins (specifically α-lac). In cow's milk, Ca^{2+}, K^+ and Na^+ are present in the highest concentrations, with lesser amounts of Mg^{2+}, Mn^{2+}, Sr^{2+} and Zn^{2+} (Gaucheron, 2005). All of these salts can bind to proteins present in milk both specifically and non-specifically, explicitly α-lac, β-lac and casein.

Table 1.7: *Table showing the concentrations of specific metals present in cow's milk (adapted from Gaucheron, 2005)*

Mineral salt	Concentration (mg kg^{-1})	Concentration (mmol kg^{-1})
Calcium	1043-1283	26-32
Magnesium	97-146	4-6
Sodium	391-644	17-28
Potassium	1212-1681	31-43
Chloride	772-1207	22-34

Several different metals ions bind to both α-lac (Kronman et al, 1981; Noyelle & van Dael, 2002;) and β-lac (Simons et al, 2002; Kontopolis et al, 2004; Navarra et al, 2007) both specifically and non-specifically. Specific binding indicates selective ion binding to a specific amino acid residue by chelation (Cao et al, 2017; Kumar, 2017), that has an explicit ion association constant, whereas non-specific binding indicates likely ion binding at away from the protein isoelectric point. α-lac has a negative net charge (-4.5) at pH 7.0 (Gao et al 2008) and has a primary calcium binding site (Permyakov, 2000; Permyakov, 2001), with an association constant orders of magnitude higher for calcium than for other ions (Permyakov et al, 1981; Noyelle & Van Dael, 2002). (Boye et al, 1997; Hendrix et al, 2000; Permyakov & Berliner, 2000; Atri et al, 2010; Nicoleta & Rapeanu, 2010).

1.9.1 Ion binding and α-lac

α-lac has a distinct calcium binding site and several sites which bind Zn^{2+} (Permyakov, 1991; Vegarud et al, 2000; Yasui et al, 2006). Its calcium binding site is composed of the amino acid residues of Asp 82, 87, 88, carbonyl groups of Trp79 and Gln84, with 1-2 water molecules in coordinating calcium, forming a pentagonal bipyramid construct (Anderson et al, 1997; Permyakov, 2000). This calcium binding site binds calcium at a greater rate the higher the calcium ion concentration present. The calcium binding site can also bind Mn^{2+}, Cd^{2+}, and Mg^{2+}, albeit at a much lower association energies than for calcium (10^9 vs 10^6 M^{-1})(Table 1.8)(Permyakov & Kreimer, 1986; Gaucheron, 2005; Shinozaki, & Iwaoka, 2017). The same can be said for the Zn^{2+} site with the highest association constant for Zn^{2+}, where it can bind Co^{2+} and Hg^{2+} also at a reduced association constant relative to Zn^{2+}. The primary Zn^{2+} binding site is made up of the amino acid residues of Glu7, Glu11, Asp11 and Asp37, where the metal ion bound forms a tetrahedral structure between itself and these amino acid residues. These binding constants change with temperature and pH.

Sodium binds to the primary calcium site of α-lac albeit with a significantly lower binding constant (K = 1.4 × 10^3 M^{-1} vs 1 × 10^9 M^{-1} at ~pH 7.0) (Permyakov & Berliner, 2000; Noyelle & Van Dael, 2002). The metal binding constants for α-lac are shown in Table 1.8.

Table 1.8: *Table illustrating the respective metal binding constants to α-lac. Where the association constant of an ion (at several concentrations) to α-lactalbumin is indicated by* **(k_{IA})**, *the rate at which specific ions bind* **(k_R)** *and the activation energy* **(E_a)** *associated with an ion binding is measured in kJ* mol^{-1} *(Adapted from Noyelle & Van Dael, 2002).*

Metal ion Ca^{2+} binding site	***Ion association constant*** **(k_{IA}) (M^{-1})**	***Rate constant*** **(k_R) (mol^{-1} s^{-1}) at 20 °C**	***Activation energy*** **(E_a) (kJ mol^{-1})**
Ca^{2+} (5 mM)	1 × 10^9	18.3	70 +/-2
Ca^{2+} (2 mM)		15.3	73 +/-2
Ca^{2+} (0.5 mM)		14.4	70 +/-3
Ca^{2+} (0.2 mM)		9.8	65 +/-3
Ca^{2+} (0.1 mM)		9.3	70 +/-3
Ca^{2+} binding to Na^+ bound α-lac	2.7 × 10^6	11.0	71 +/-4
Sr^{2+} (5 mM)	7.3 × 10^5	6.63	69 +/-5
Sr^{2+} (2.5 mM)		4.60	73 +/-2
Sr^{2+} (0.5 mM)		2.30	73 +/-2
Mn^{2+} (20 mM)		1.6	73 +/-5
Mn^{2+} (2.5 mM)		1.3	72 +/-3
Mn^{2+} (0.25 mM)		0.13	73 +/-5
Cd^{2+}(0.25 mM)	4.0 × 10^5	0.096	65 +/-3
Zn^{2+} binding site			
Co^{2+} (43 mM)	1.3 × 10^6	0.140	97 +/-2
Co^{2+} (2.5 mM)		0.058	108 +/-5
Co^{2+} (0.25 mM)		0.031	105 +/-6
Mg^{2+} (50 mM)	2.5 × 10^3	0.236	99 +/-5
Mg^{2+}(2.5 mM)		0.091	97 +/-5
Mg^{2+} (0.25 mM)		0.036	97 +/-2
Zn^{2+} (0.20 mM)		0.032	101 +/-5

1.9.2 Ion binding and β-lac

β-lac has a single free thiol (located at CYS121) that mediates the formation of disulphide linked aggregates (Kontopolis et al, 2004; Invernizzi et al, 2008; Petit et al, 2012). Hoffman & van Mill, (Hoffman & van Mill, 1999) studied the effect of thiol blocking agents on β-lac specifically and confirmed that rendering free thiols inert reduced the amount of disulphide linked aggregates, but aggregation proceeded afterwards via non-specific interactions between ions and proteins. While the addition of salt/minerals accelerates the process of heat induced aggregation, there are not necessary for oligomerisation (Verheul et al, 1998; Carrotta et al, 2001; Jung et al, 2008; Ako et al, 2010). Majhi and others, (Majhi et al, 2006) studied this, where they examined electrostatic driven protein aggregation of β-lac in low ionic strength buffers. They indicated that while aggregation is hindered in the absence of calcium, it occurs regardless once heat is applied albeit at a slower rate.

Native β-lac has a single ligand binding site per monomer. This site is called a calyx and binds hydrophobic ligands (Martins et al, 2008; Dominguez et al, 2013; Bello & Garcia-Hernandez, 2014). Ions however interact via non-specific interactions with the β-lac surface by the formation of intermolecular salts bridges (between the amino acids from 33 – 40 depending on the amino acid qequence of the protein) and this mediates dimer/oligomer formation (Sakurai et al, 2001; Sakurai & Goto, 2002; Hinzman et al, 2016). Through this mechanism, two molecules of β-lac can bind 1-3 ions of calcium (at equivalent binding affinities of 2.9×10^2 M^{-1}) (Deng et al, 2010) at physiological pH to form a dimer, depending on the Ca: protein ratio. Higher order oligomers can be formed by the same mechanism. Greater calcium concentrations mediate oligomer formation by the lowering of electrostatic barrier between proteins and decreasing the distance between them to allow bridging to occur (Mulvihill & Kinsella, 1988; Simons et al, 2002; Arnaudov & de Vries et al, 2006).

Sodium as well as other metals have the same effect on dimer formation of β-lac as calcium except that higher concentrations are needed for the dimerization to occur. This is logical considering calcium ranking higher on the Hofmeister series as a chaotrope (Zhang et al, 2006; Thormann, 2012; Okur et al, 2017). Regardless of the ability of β-lac to bind metals, high ionic strengths in β-lac solutions generally cause gelling, which leads to insoluble aggregate formation can lead to manufacturing difficulties for IMF.

1.9.3 Ion binding and Casein

Casein has the ability to harbour concentrated nano-clusters of calcium as calcium phosphate throughout its internal structure (McMahon & Oommen, 2008; de Kruif et al, 2012; Lenton et al, 2015). It has an association constant for calcium at 5.0×10^2 M^{-1}. Every one mole of casein

being capable of binding five moles of calcium (Parker & Dalgeish, 2009; Mekmene & Gaucheron, 2011). Caseinate has a similar association for calcium to casein. Assuming no calcium is bound, its association constant for calcium is 1.54×10^2 M^{-1}, with being capable of binding 15 mol of calcium per mole of caseinate (Alvarez et al, 2007; Mekmene & Gaucheron, 2011). The majority of aggregation caused in dairy processing is a result of casein/β-lac aggregates, where they aggregate due do heat denaturation, screening caused by high ionic strengths or a combination of both (Cho et al, 2003; Kehoe & Foegedling, 2011; Pinto et al, 2014; Gaspard et al, 2017).

1.10 Thesis motivation

The primary motivation for this work is to understand the role of specific and non-specific calcium binding to α-lac and its role in the colloidal and structural stability of the protein. Furthermore, we examine the role of calcium amino acids salts as an alternative to $CaCl_2$ for the supplementation of whey protein or IMF solutions using a range of biophysical characterisation tools.

Chapter 2: Materials and Methods

2.1 Preparation of buffers and reagents

2.1.1 Buffers

All buffers were prepared using ultrapure water (Milli-Q) and at a minimum, analytical grade reagents. Concentrated hydrochloric acid solution (HCl, M_w = 36.46 g mol^{-1}) (Fisher Scientific, UK) or concentrated sodium hydroxide solution (NaOH, M_w = 40.00 g mol^{-1}) (Fisher Scientific, UK) was used to adjust pH, unless otherwise stated. To prevent microbial growth, 0.02% (w/v) sodium azide (NaN_3, M_w = 65.0 g mol^{-1}) (Fisher Scientific, UK) was added to all buffers. All buffers were filtered either under vacuum with a 0.45 μm filter; or using a syringe driven 0.45 μm Millex-HV filter (Millipore, Ireland).

2.1.1.1 Sodium Acetate

0.275 M Sodium acetate buffer (CH_3COONa, M_w = 82.03 g mol^{-1}) was prepared at pH 4.0 and pH 5.5. Sodium Acetate buffer at pH 4.0 was prepared using 16.50 g of glacial acetic acid (CH_3COOH, M_w = 60.05 g mol^{-1}) (Fisher Scientific, UK) and 4.48 g of NaOH (Fisher Scientific, UK) per litre of water. Sodium acetate buffer at pH 5.5 was prepared using 16.50 g of CH_3COOH and 6.70 g of NaOH per litre of water.

2.1.1.2 Tris-HCl

0.05 M of Tris-HCl was prepared by mixing of Tris hydrochloride (M_w = 157.6 g mol^{-1}) (Fisher Scientific, UK) and Tris base (M_w = 121.1 g mol^{-1}) (Fisher Scientific, UK) in the quantities outlined in the table 2.1.

Table 2.1: *Quantities of Tris Acid/Base used to make Tris-HCl buffer*

pH	Mass of Tris hydrochloride (g)	Mass of Tris Base (g)	Final volume (ml)
6.5	0.76	0.01	100
7.0	0.73	0.04	100
8.0	0.43	0.26	100
9.0	0.08	0.53	100

2.1.2 Preparation of reagents

2.1.2.1 Preparation of Calcium chloride stock solutions

A 1.0 N calcium chloride ($CaCl_2$, M_w = 111.11 g mol^{-1}) (Fisher Scientific, UK) stock solution was made by dissolving 11.1 g of calcium chloride in 100 ml of water. The calcium content in the solution was determined by measurement of conductivity. The conductivity measurements were standardised by volumetric analysis (see section 2.2.2).

A $CaCl_2$ solution in buffer was prepared in the same manner, by replacing water with 0.05 M Tris-HCl, pH 7.0. The calcium concentration was determined volumetrically (*see* 2.2.2). A standard curve for Flame Atomic Absorption Spectroscopy (FAAS) was also generated from this stock solution for calcium concentration determination.

2.1.2.2 Preparation of volumetric analysis reagents

A 0.1 M Ethylenediaminetetraacetic acid (EDTA, M_w = 372.24 g mol^{-1}) (Fisher Scientific, UK) stock was made by dissolving 3.72 g of EDTA in 50 ml of water and brought to 100 ml in a volumetric flask. 30 % Ammonium hydroxide (NH_4OH, M_w = 35.046 g mol^{-1}) (Fisher Scientific, UK) was used to maintain alkaline pH. Erichrome black T (EBT) ($C_{20}H_{12}N_3NaO_7S$, M_w = 461.38 g mol^{-1}) (Sigma-Aldrich, Ireland) was used as an indicator.

2.1.2.3 Preparation of calcium bound amino acid salts

Calcium glycinate ($C_4H_8CaN_2O_4$, M_w = 188.20 g mol^{-1}) and calcium lysinate ($C_{12}H_{26}CaN_4O_4$, M_w = 330.44 g mol^{-1}) were prepared by Dr. Denise Rooney via an acid-base reaction between calcium hydroxide ($Ca(OH)_2$, M_w = 74.10 g mol^{-1}) (Fisher Scientific, UK) and either glycine ($C_2H_5NO_2$, M_w = 75.07 g mol^{-1}) (Millipore, Ireland) or lysine ($C_6H_{14}N_20_2.H_2O$, M_w = 164.21 g mol^{-1}) (Millipore, Ireland) (Haa) using reflux.

$$Ca(OH)_{2(s)} + 2Haa_{(aq)} \rightleftharpoons Ca(aa)_{2(aq)} + 2H_2O_{(l)} \quad \textbf{2.1}$$

Equation 2.1 shows the reaction that took place. Equilibrium lay on the right, where $Ca(OH)_2$ was the strong base. $Ca(aa)_2$ is aqueous and unreacted $Ca(OH)_2$ is solid. Solid $Ca(OH)_2$ allowed the amino acid salt to be isolated by filtration. Purity was evaluated by IR spectroscopy and H-NMR. Calcium content was measured by EDTA titration and FAAS. Amino acid content was measured using refractometry (against standards for the pure amino acid).

2.1.2.4 Preparation of protein stock solutions

α-lactalbumin (α-lac) was purified as described in section 2.4.3. Once purified, protein was washed of salt repeatedly by ultrafiltration using an Amicon Ultra-4 MWCO 10 kDa (Millipore, Ireland) at 7000 × g with Milli-Q water. It was then dialysed exhaustively against the desired buffer. The protein concentration was determined by UV-Vis absorbance at 280 nm using an extinction coefficient of 2.01 ml mg^{-1} cm^{-1} (Nuchuchua et al, 2014). The purification of β-lactoglobulin (β-lac) followed the same procedure, except the fractions that were collected were the ones assigned as β-lac. The extinction coefficient used to determine concentration for β-lac by UV absorbance at 280 nm was 0.96 ml mg^{-1} cm^{-1} (Perez- Fuentes et al, 2017). Whey Protein Isolate (WPI) is a mixture of α-lac and β-lac, with minor amounts of the protein BSA, as well as <1% of is mass consisting of lactose, ash and fat. The proportions of α-lac and β-lac were determined by size exclusion high performance liquid chromatography (SE-HPLC) and sodium dodecyl sulfate–polyacrylamide *gel* electrophoresis (SDS-PAGE).

2.1.2.5 Preparation of amino acid/salt solutions with protein

Amino acid solutions with protein were prepared using a 1:1 dilution method. For a 10 mg ml^{-1} α-lac solution with 20 mg ml^{-1} of glycine present; first a 20 mg ml^{-1} α-lac solution was made as per method 2.1.2.4 in 50 mM Tris-HCl, pH 7.0 (**A**). Next a 40 mg ml^{-1} glycine solution was made by dissolving ~ 40 mg of glycine in 50 mM Tris-HCl (any pH drift was brought back to pH 7.0, before bringing to the final volume) (**B**) – its amino acid concentration was confirmed using refractometry. Finally, equal volumes of solution A + B were mixed to acquire a final solution of 10 mg ml^{-1} α-lac in 50 mM Tris-HCl, pH 7.0 with 20 mg ml^{-1} glycine present. This was also done using lysine. pKa values of the amino acids are listed in table 2.2.

For $CaCl_2$ containing solutions; the same method 1:1 dilution method above was applied, except using $CaCl_2$ instead of an amino acid salt. If samples were to be using for QELS, anatop filtration was also done.

Table 2.2: *Amino acids used throughout experientsation and the pKa of the associated ionisable groups.*

Amino acid	pKa (NH_2 group)	pKa (COO^- group)
Glycine (Gly)	9.6	2.3
Lysine (Lys)	2.2	8.9

2.2 Quantifying calcium in a solution

2.2.1 Conductivity

2.2.1.1 Sample preparation

A 1.0 N $CaCl_2$ standard was prepared by dissolving 11.1g of $CaCl_2$ in 50 ml of water and then making up to the mark in a 100 ml volumetric flask. To generate a standard curve, conductivity values for a serial dilution of the $CaCl_2$ stock (Haynes, 2009) (Table 2.3) were compared with those measured for the freshly prepared solutions and used to create a standard curve (figure 2.2).

In general, a 1.0 N calcium chloride solution was found to have a concentration in the region of 0.92 M once standardised. This was further confirmed volumetrically, with < 1% variance.

Table 2.3: *Conductivity values used to standardise Calcium chloride solutions.*

Mass %	g/100ml	g/L	M	mS
0.5	0.5	5	0.045	8.1
1	1	10	0.09	15.7
2	2	20	0.18	29.4
5	5	50	0.45	67
10	10	100	0.9	117
15	15	150	1.35	157
20	20	200	1.80	177

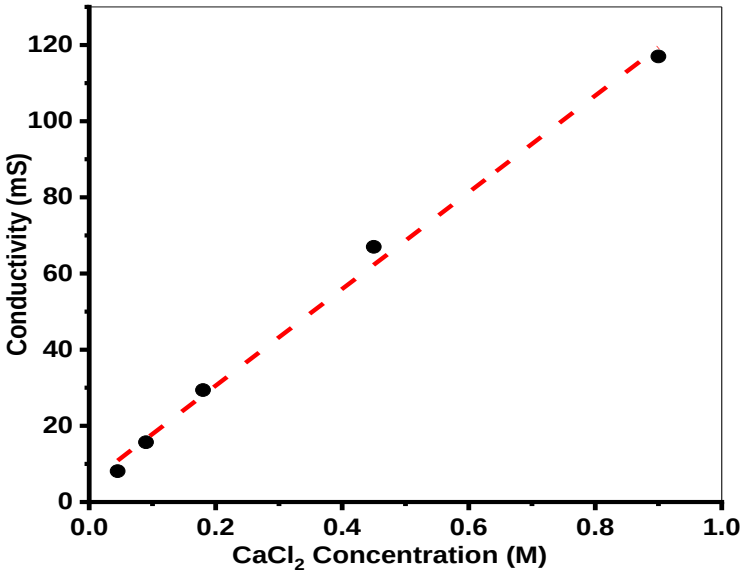


Figure 2.2: *Calibration of conductivity meter to determine calcium chloride concentrations in water (Haynes, 2009).*

2.2.2 Volumetric analysis

2.2.2.1 Sample preparation

The calcium chloride solution in 0.05 M Tris-HCl, pH 7.0 (0.92 M) (section 2.2) was diluted 1:5 and added to a conical flask with 10 ml of ammonium hydroxide and 1 mg of EBT. 0.1 M. EDTA was then added to the solution in the conical flask via burette until the colour changed from pink, to purple. Once the solution turned purple, 0.1 M EDTA was added dropwise until the colour changed from purple to blue. The volume of 0.1 M EDTA was recorded for each experiment. This procedure was repeated four more times. A reference solution (using buffer or water) was also titrated in the same way five times and the titre subtracted from the values determined in the calcium solutions. All replicates were within 0.1 ml of one another.

2.2.3 Flame atomic absorption spectroscopy (FAAS)

2.2.3.1 Background

Flame atomic absorption spectroscopy (FAAS) can be used to determine the concentration of a metal in a solution. FAAS involves the atomisation of metal ions via a flame. This allows solid crystalline calcium to be dissociated into individual ground state atoms, where they can be excited to a higher energy state, and subsequently return to the ground state and this emission can be measured spectroscopically. The concentration of calcium is proportional to the amount of light absorbed. This allows the generation of standard curve relating absorbance

to calcium concentration. The initial calcium concentration was determined via volumetric analysis. A serial dilution was performed for the lower concentration samples. If dissolved in a different buffer, a new reference is taken and subtracted accordingly.

2.2.3.2 Instrumentation

A Perkin Elmer AAnalyst 200 was used for measurements. The setup was equipped with a double-beam optical system, hollow cathode lamp and acetylene and air gas-based nebulizer. A calcium specific lamp was used (P/N N305-0114) (Perkin Elmer, Ireland).

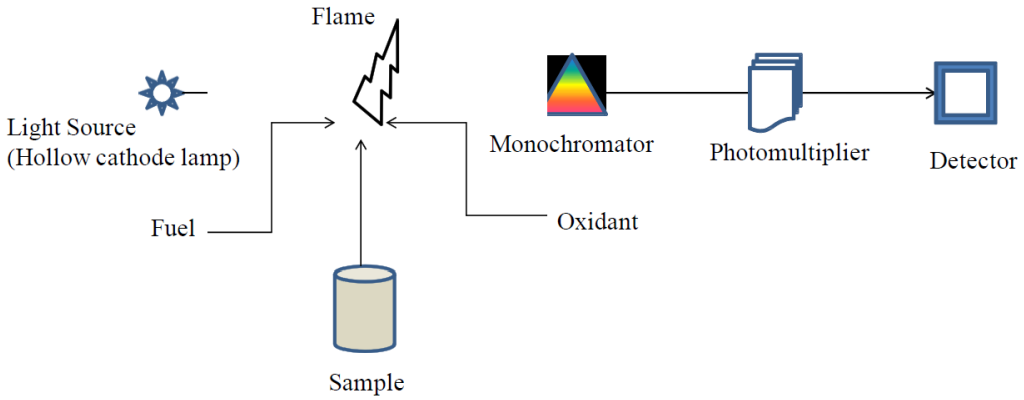


Figure 2.3: *Figure illustrating the principal of FAAS*

2.2.3.3 Sample preparation

Standards were prepared from a 50 mM calcium chloride standard stock and serial dilution was performed to acquire more dilute standards (25, 12.5, 6.25, 3.13, 1.56, 0.78, 0.39 mM). After this, all stocks were diluted 1:100 to acquire an absorbance < 1.000. A standard curve was generated plotting calcium concentration vs absorbance (figure 2.4).

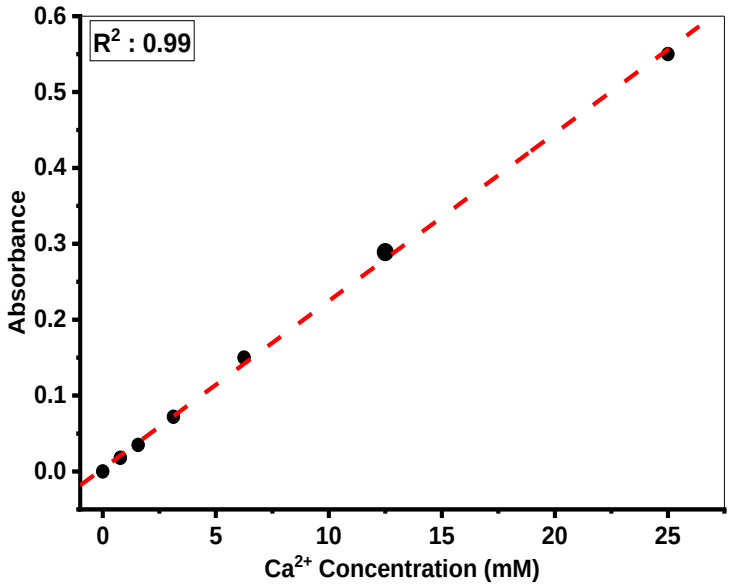


Figure 2.4: *Standard curve determined for calcium by FAAS.*

2.3 Quantifying amino acids amount in a solution

2.3.1 Refractometry

2.3.1.1 Background

The refractive index describes how fast light moves through a medium relative to a reference medium (in this case water or buffer). The lower the optical density is for a solution, the greater the speed of light is through the medium and thus the lower the refractive index. Any material that can interact with light has a refractive index (*n*). This refractive index depends on the wavelength (λ) of the light and the temperature. Determining the refractive index of a solution is based on Snell's law where the ratio of sines of angles of the incidence and reflection of a wave is constant between two mediums (one medium colour in white, the other in blue) (Figure 2.5).The components of a refractometer are also summarised in figure 2.6.

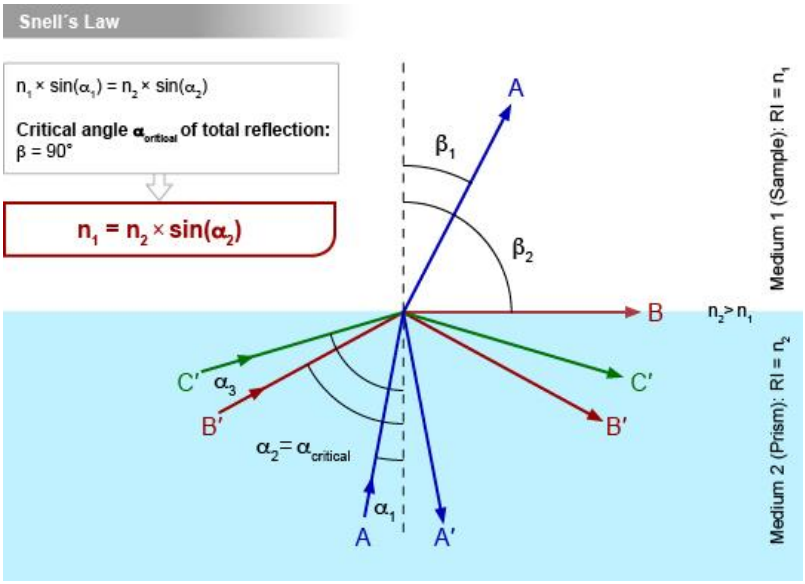


Figure 2.5: *Figure illustrating the principal of Snell's law. A, B, C indicate incident light rays and A', B' and C' indicate refractive light rays.*

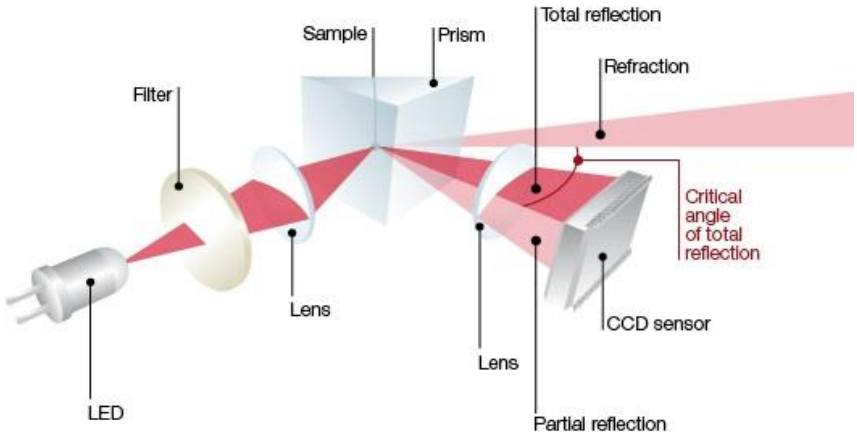


Figure 2.6: *Key features of a refractometer.*

2.3.1.2 Instrumentation

A PTR 46 refractometer was used in all experiments. The temperature was set to 20 °C and allowed to equilibrate for 5 min before measurements. The refractive index of buffer only was measured in triplicate. The sample to be measured is directly applied to the measuring prism. The sensor array measures the refractive index.

2.3.1.3 Sample preparation

Three 50 mg ml^{-1} stock solutions of L-lysine monohydrate and Glycine were prepared in 10 ml volumetric flasks in triplicate. The solution pH was re-checked at this point. Lower

concentrations were prepared by serial dilution. The average refractive index and standard deviation were determined for each amino acid (figure 2.7 A+B). These were used to determine the concentration of amino acid solutions of unknown concentration.

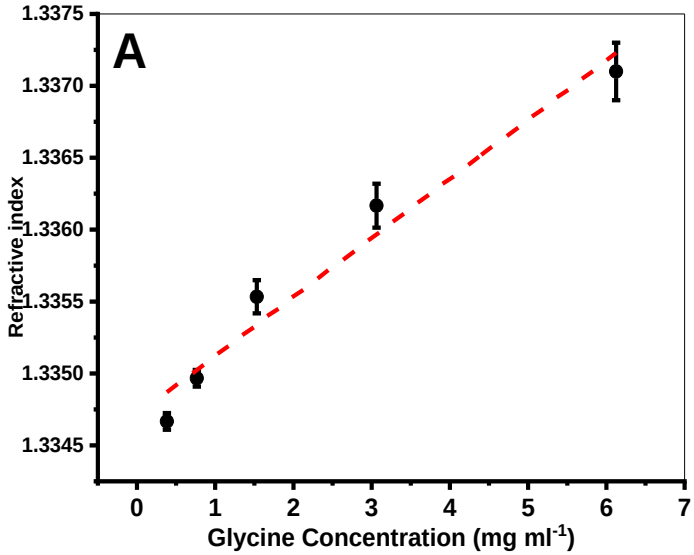


Figure 2.7A: *Refractive index values for Glyine (A) at increasing concentration. These values were used to determine the amino acid content in samples prior to use.*

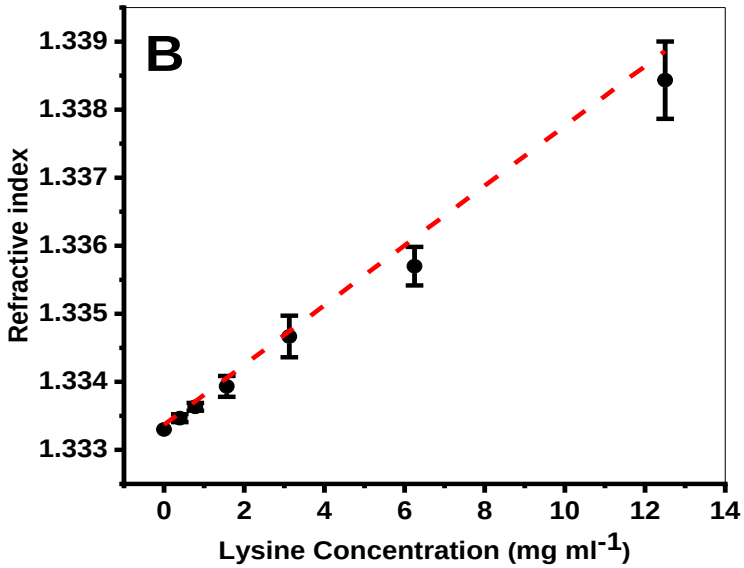


Figure 2.7B: *Refractive index values for Lysine (B) at increasing concentration. These values were used to determine the amino acid content in samples prior to use.*

2.4 Chromatography

2.4.1 Background

Size exclusion chromatography (SEC) is a method to separate molecules based on their size. (Stiegel, 2009). Throughout the run, molecules are separated based on their size with the largest molecules (figure 2.8, green in colour) interacting the least with the stationary phase and eluting faster. The smaller molecules/particles (figure 2.8, pink in colour) elute slower as they are retained on the stationary phase for longer (Hong et al, 2012). Most methods of chromatographic separation are driven by enthalpy of adsorption, whereas SEC is unique in that this variable is assumed to be 0, thus the relationship between retention and entropy are related by:

$$lnK_A = -\Delta S^0/R \qquad \textbf{2.2}$$

where $\boldsymbol{K_D}$ the thermodynamic retention factor in SEC, defined by:

$$K_A = \frac{V_R - V_0}{V_i} \qquad \textbf{2.3}$$

where $\boldsymbol{V_R}$ is the retention volume, $\boldsymbol{V_0}$ is the interstitial volume and $\boldsymbol{V_i}$ is the intraparticle volume. A large value for $\boldsymbol{K_A}$ implies that the analyte in question is completely accessible to the stationary phase pores and the opposite is true for smaller values of $\boldsymbol{K_A}$.

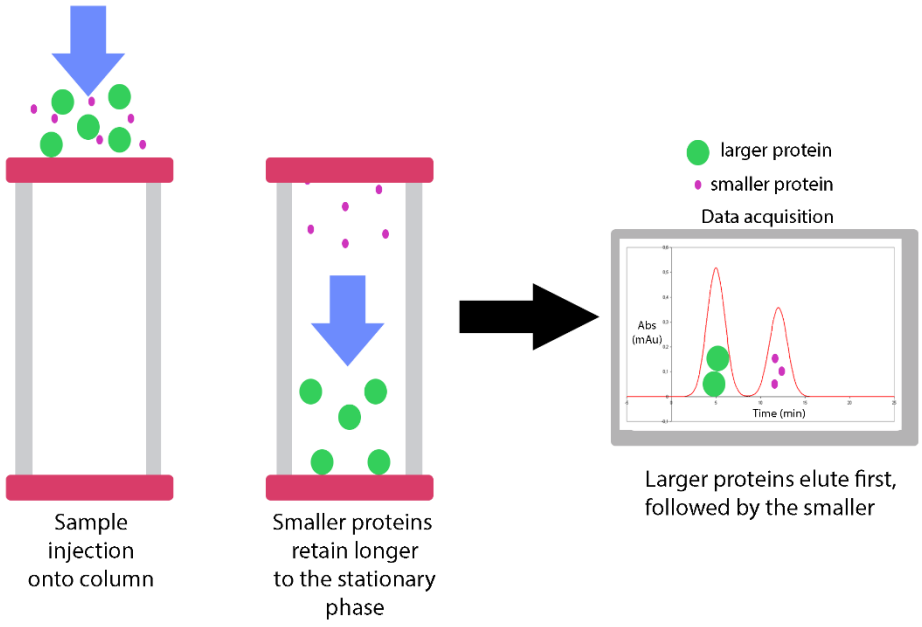


Figure 2.8: *The principle of size exclusion chromatography (SEC).*

Ion exchange chromatography (IEX) is a method of separating molecules that exploits the variations in net electrical charge as a function of pH (Tsonev & Hirsh, 2008). The stationary phase can either strongly interact with positively charged molecules (cation exchange resin) or negatively charged molecules (anion exchange resin). By applying either a salt or pH gradient to alter the effective charge of each analyte, they will elute when the interaction has been screened (salt gradient) or when the net charge no longer favours interaction (pH gradient) (figure 2.9).

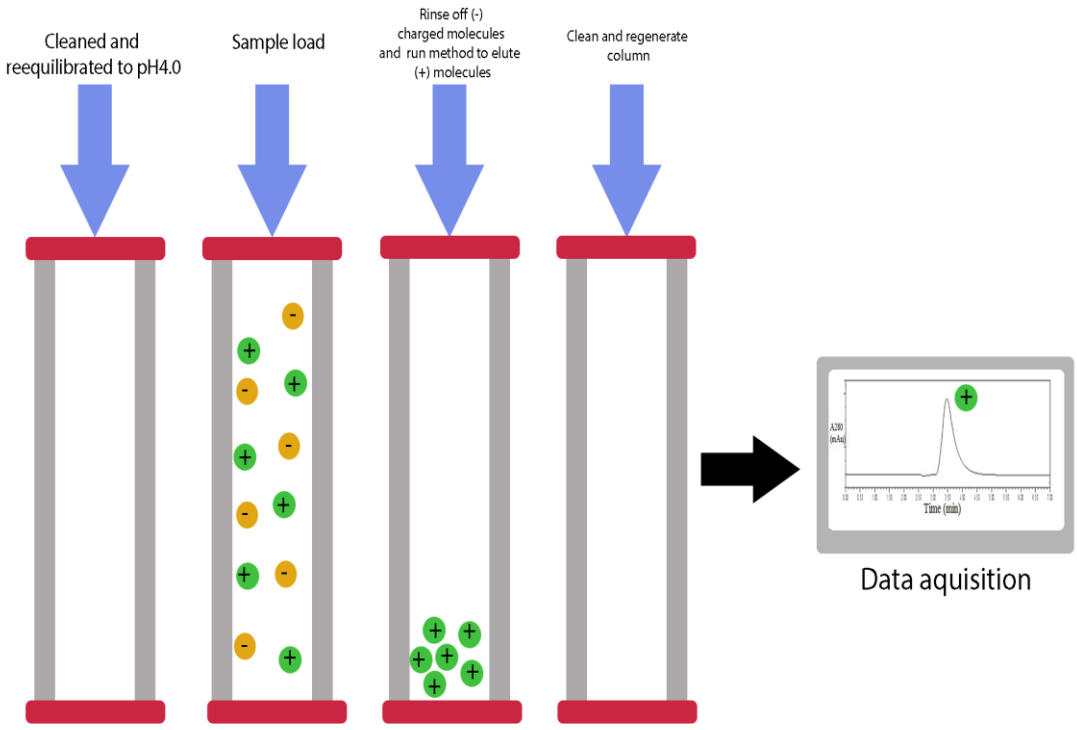


Figure 2.9: *The principle of Ion Exchange Chromatography (IEX)*

High performance liquid chromatography (HPLC) is an analytical technique that enables the identification, separation and purification of components in a mixture for qualitative and quantitative analysis. It is based on the principle where molecules in a mixture are applied to and separated from each other on a stationary phase by passing a mobile phase over it at a constant flow rate.

2.4.2 Protein Purification Instrumentation

All protein purifications were performed using either an AKTA Purifier or AKTA Prime chromatography system (GE Healthcare Bio-Sciences AB, Sweden). An XK 50/100 column (SEC) (GE Healthcare Bio-Sciences AB, Sweden) and a XK 50/60 column (IEX) column were used for all purifications. Data was collected using UNICORN software v.5.31.

The SEC column was packed with Sephacryl S-200 high resolution media (GE Healthcare Bio-Sciences AB, Sweden). The stationary phase was a hydrophilic cross-linked allyl dextran and N,N'- methylene bisacrylamide co-polymer. The media has the features of minimising non-specific adsorption while maximising rigidity and chemical stability. The particle size distribution allows for molecules to be resolved in the molecular weight ranges between 5 – 250 kDa. After every five runs the column was cleaned using one column volume of 0.2 M NaOH and equilibrated with five column volumes of 0.275 M sodium acetate, pH 4.0. The IEX column was packed with SP Sepharose Fast flow media (GE Healthcare Bio-Sciences AB, Sweden). The media was comprised of sulphopropyl strong cation exchange groups attached to the matrix via ether linkages. After every run the column was cleaned using one column volume of 1.0 M NaOH, followed by re-equilibration with five column volumes of 0.275 M sodium acetate, pH 4.0.

2.4.2.1 HPLC Instrumentation

HPLC analysis was carried out using a Shimadzu CBM-20A system controller attached to a Shimadzu LC-20AT solvent delivery model, DGU-20A degasser, SIL-20AHT autosampler and a SPD-M20A UV-Vis detector. Shimadzu LC solution software was used for real-time analysis. A Superdex 200 Increase 10/300 GL size exclusion column composed of cross-linked agarose and dextran (GE Healthcare, Sweden) was used for the analysis.

2.4.3 Protein Purification

2.4.3.1 Whey protein dissolution

Whey proteins, α-lac and β-lac were purified from Whey Protein Isolate (WPI) (BiPro, Davisco Foods Intl). 1.00 g of WPI was added to a conical flask containing 30 ml of 0.275 M sodium acetate, pH 4.0. The suspension was stirred occasionally over the course of 2 hr to dissolve the protein in solution.

2.4.3.2 Size exclusion chromatography (SEC)

Both α-lac and β-lac can be isolated by the following method. The hydrated WPI solution was added to a 50 ml falcon tube and centrifuged at 7000 × g for 2 hr to remove large, undissolved particulates. The supernatant was decanted off and filtered through a 0.22 μm Millex syringe driven filter. The filtered solution was loaded onto the Sephacryl S-200 XK 50/100 column at a flow rate of 2 ml min^{-1}. An isocratic elution was performed using a mobile phase of 0.275 M sodium acetate, pH 4.0. The run time was 20 hr. Fractions were collected using an automated fraction collector, with each fraction volume being 22 ml. The programme

sequence is outlined in Table 2.4. Each breakpoint is a point in the run where at least one parameter changes; either flow rate or whether fractions are collected or not.

Table 2.4: *SEC steps for α-lac purification. Breakpoint indicates a point which a run parameter changes; volume indicates the amount of buffer ran through the column at this breakpoint; flow rate indicates the rate at which buffer runs through the column and fraction volume is the volume collected in each fraction.*

Breakpoint	Volume (ml)	Flow Rate (ml min^{-1})	Fraction Volume (ml)
1	0	2.00	0
2	400	2.00	22
3	2300	2.00	22
4	2300	0	22

2.4.3.3 Ion exchange chromatography (IEX)

The same IEX method is used to isolate α-lac and β-lac. The only difference between the two methods is the fractions that are collected. The fractions corresponding to the correct protein (either α-lac or β-lac) were collected from the SEC step and loaded onto the equilibrated (with 0.275 M NaAc, pH 4.0 buffer) IEX column at a flow rate of 10 ml min^{-1}. Unbound protein was washed off the column after the loading step with five column volumes of the same buffer at flow rate of 10 ml min^{-1}. A pH gradient was used to elute the purified protein (using 0.275 M buffer NaAc, pH 4.0 to pH 5.5). The mobile phase composition described in table 2.5 was used for protein elution, where mobile phase pH changed from 4.0 to 5.5 and fractions were collected using an automated fraction collector.

Table 2.5: *Ion exchange protocol for α-lac/β-lac purification.*

Breakpoint	Volume (ml)	Buffer concentration (%)	Flow Rate (ml min^{-1})	Fraction Volume (ml)
1	0	60	10.00	22
2	1600	100	10.00	22
3	1900	100	10.00	22

2.4.3.4 High performance liquid chromatography (HPLC)

Protein samples for HPLC were prepared by dialysing protein against 50 mM Tris-HCl, pH 7.0 using an Amicon Ultra-4 filter with a MWCO of 10 kDa (Millipore, Ireland). The protein concentration used was 5 mg ml^{-1}. Samples were filtered using a 0.22 µm Millex-GV syringe driven filter before adding to a glass HPLC vial (Chromacol, Germany) and sealed using a rubber septum cap (Thermoscientific, UK). Parameters for the run are outlined in table 2.6.

Table 2.6: *Characterisation of purified protein by SE-HPLC sample details.*

Stationary phase	Superdex 200 Increase 10/300 GL
Mobile phase	0.05 M Tris-HCl, pH 7.0 + 0.15 M NaCl
Flow rate	0.75 ml min^{-1}
Runtime	60 min
Sample volume	25 µl

Sample absorbance at 280 nm was measured and the chromatograms were exported as ASCII files and analysed using OriginPro 2017 data analysis software.

2.4.3.5 Calibration curve: Molecular weight

A calibration curve (figure 2.10) was constructed to determine the relationship between the protein molecular weight and retention time (R_t). The proteins used ranged between 44 to 669 kDa and were taken from the calibration kit recommended by the column manufacturer (P/N:

28403842, GE healthcare). The buffer used was 0.05 M Tris-HCl, pH 7.0 with 0.15 M sodium chloride (NaCl, M_w = 58.44 g mol^{-1}) (Fisher Scientific, UK).

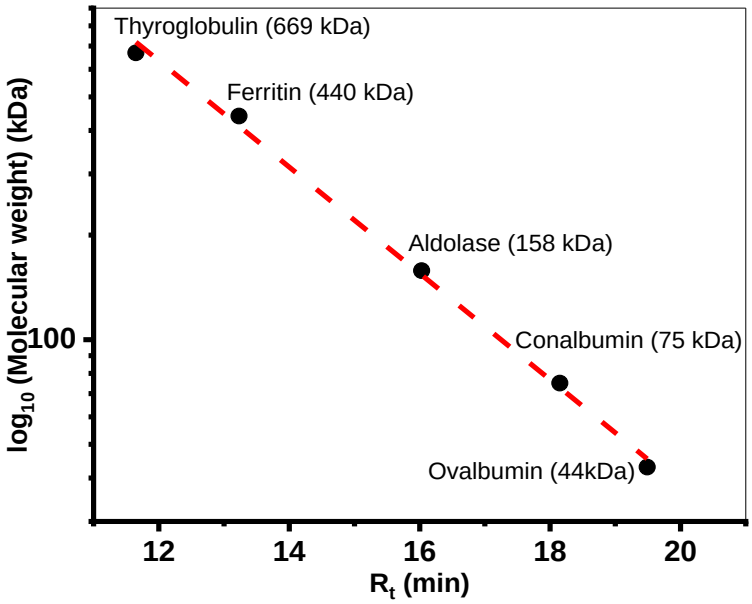


Figure 2.10: *Protein Molecular Weight Calibration curve for HPLC Superdex 200 Increase column*

2.5 Polyacrylamide Electrophoresis

2.5.1 Background

Polyacrylamide gel electrophoresis (PAGE) refers to the transport of ions through a solution in an electric field. Considering how pH affects the net charge of a protein, this allows separation of biomolecules by the number of ionisable groups they contain. As proteins migrate at a constant velocity, there is a constant balance between electrical force and viscous drag. The rate at which these molecules migrate differs depending on the size, charge and shape of them. This allows the size of proteins in a solution to be identified by running a sample alongside a molecular weight ladder. (Hayes & Stockman, 1889).

2.5.2 Instrumentation

12.6% gels were prepared using the protocol outlined in table 2.7. All reagents were mixed and degassed for 30 min. All buffers were prepared using ultrapure water. Sodium dodecyl sulphate (SDS, $NaC_{12}H_{25}SO_4$, M_w = 288.37 g mol^{-1}) was purchased from Riedel-de Haen (Sigma Aldrich, Germany). 30% acrylamide/Bis (37.5:1) was purchased from Biorad (USA).

Table 2.7: *Reagents used for the preparation of the running gel*

Reagent	Volume (ml)
MilliQ water	3.15
1M Tris-HCl, pH 8.8	2.50
10 % (w/v) SDS	0.10
Acrylamide/Bis	4.20

A 10 % (w/v) solution of ammonium persulphate ($(NH_2)_2S_2O_8$), M_w = 228.18 g mol^{-1}) (Sigma Aldrich, UK) was prepared and 50 µl was added to a conical flask. 5 µl of tetramethylethylenediamine (TEMED, $C_6H_{16}N_2$, M_w = 116.24 g mol^{-1}) was added to the mixture. The gels were prepared by using a casting frame and stand, with a spacer plate and 1 mm spacer sheet (Bio-Rad, USA). Tert-amyl alcohol was added to eliminate air bubbles. Gels were left for 40 min to set at room temperature. 4% stacking gels were prepared as per table 2.8. Components were degassed for 30 min prior to use.

Table 2.8: *Reagents used for the stacking gel*

Reagent	Volume (ml)
MilliQ water	3.15
0.5 M Tris-HCl, pH 6.8	1.25
10% w/v	0.05
Acrylamide/Bis	0.65

25 µl of 10% (w/v) ammonium per sulphate and 5 µl of TEMED were added to the mixture (Table 2.7). The tert-amyl alcohol from the running gel was poured off and the gel top was

rinsed using ultrapure water. The stacking gel was added to this. 10-lane ladders were placed on top and the gel was allowed to set over the course of 1 hour.

2.5.3 Sample buffer preparation

Sample buffer was prepared following the table 2.9. Glycerol was purchased from Sigma Aldrich (USA). Bromophenol blue was purchased from (Riedel-de Haen Sigma Aldrich, Germany).

Table 2.9: *Reagents used for sample buffer used for SDS-PAGE*

Reagent	Volume (ml)
MilliQ water	4.80
0.5 M Tris-HCl, pH 6.8	1.20
Glycerol	1.00
10 % (w/v) SDS	2.00
Bromophenyl blue	1 mg

In the case of preparing reducing buffer, 475 µl of the above solution was mixed with 25 µl of β-mercaptoethanol (Sigma Aldrich, Belgium). For the running of the gel, 10X Tris-glycine (Bio-RAD, USA) diluted 1:10 was used.

2.5.4 Molecular weight markers preparation

Molecular weight ladders were purchased from Bio-Rad (Bio-Rad, USA) (figure 2.11). The markers were diluted 1:20 with reducing buffer and boiled at 95 °C for 5 min before use.

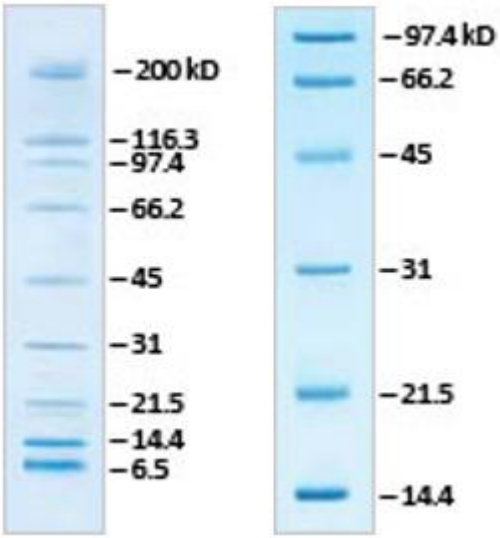


Figure 2.11: *High and low range molecular weight markers and their molecular weights run on 12.6% SDS polyacrylamide gel, stained with Coomassie R-250.*

2.5.5 Preparing the stain/destain solution

The staining solution used was Coomassie brilliant blue R-250 (Biorad, USA) 100%. The destaining solution used was ultrapure water, methanol (Sigma Aldrich, UK) and acetic acid in the ratio 60:30:10 (v/v).

2.5.6 Sample preparation

All protein samples were dialyzed with the desired buffer using Amicon-4 Ultra centrifugal device and mixed with sample buffer. Every sample contained 0.5 – 10 µg of protein depending on the experiment aim. The final volume of each sample was 20 µl. All samples were heated at 95 °C for 5 min, allowed to cool and centrifuged before being loaded on the gel.

2.5.7 Running a sample

A Mini-PROTEAN Tetra cell systems (Biorad, USA) was used to run all SDS-PAGE gels. Gels were run using a 200 V external power source until the dye front was ~1 cm above the end of the glass slide (roughly 40 min). Gels were then removed from the plates and left to stain overnight. Gels were destained the following day, replacing the destain solution

periodically. Gels were imaged using an Epson perfection scanner. Band intensities were analysed using ImageJ software.

2.6 Spectroscopy

2.6.1 Theory

Spectroscopy involves the measurement and interpretation of the signal arising from the interaction of electromagnetic radiation with matter. Different forms of spectroscopy can be used depending on what is being analysed. For biomolecules such as proteins the most widely used forms are UV-Vis, fluorescence and nuclear magnetic resonance (Penner, 2010). The radiation that arises from these processes can be related to related to both frequency and wavelength by the equation:

$$E = h\nu = hc/\lambda \qquad \textbf{2.4}$$

where $\boldsymbol{E}$ is energy, $\boldsymbol{\nu}$ is frequency, $\boldsymbol{\lambda}$ is wavelength, $\boldsymbol{h}$ is Planck's constant and $\boldsymbol{c}$ is the speed of light.

2.6.2 UV-Vis absorbance spectroscopy

Protein concentration can be determined using UV-Vis spectroscopy. Absorption of radiation in the UV range is dependent on the tyrosine and tryptophan content of a protein (and to a lesser extent phenylalanine). The absorbance (at A280 nm) reading from one protein to the next can differ greatly due to the differences in the number of amino acids that contain aromatic side chains. This is normalised by accounting for the protein standard extinction coefficient. (Aitken, 2002).

Protein concentration was using the Beer-Lambert Law:

$$A = \epsilon c l \qquad \textbf{2.5}$$

where $\boldsymbol{A}$ is absorbance at 280 nm, $\boldsymbol{\epsilon}$ is the extinction coefficient in mg ml^{-1} cm^{-1}, $\boldsymbol{c}$ is protein concentration in mg ml^{-1} and $\boldsymbol{l}$ is the path length in cm.

An extinction coefficient of 2.01 mg^{-1} ml cm^{-1} and 0.96 mg ml cm^{-1} for α-lac and β-lac respectively was used for concentration determination (Gill & Von Hippel, 1989).

2.6.3 Second derivative UV/Vis absorbance spectroscopy

Derivative spectroscopy is a method of analysing a broad absorbance peak that arises when a spectrum is recorded from 240 – 300 nm. It reduces peak overlap. Specifically, peaks arising from tyrosine (265-285 nm), tryptophan (265-295 nm) and less significantly phenylalanine (245-270 nm) can be identified and studied (Equation 2.6-2.8).

Zero order $$A = f(\lambda)$$ **2.6**

First Order $$\frac{dA}{d\lambda} = f'(\lambda)$$ **2.7**

Second Order $$\frac{\mathrm{d}^2\mathrm{A}}{\mathrm{d}\lambda^2} = \mathrm{f}''(\lambda)$$ **2.8**

where $\boldsymbol{A}$ is absorbance and $\boldsymbol{\lambda}$ is wavelength

With each differentiation step, the signal to noise ratio (S/N) decreases, yielding superior resolution between peaks on the absorbance spectrum. To further increase the resolution, a Savitzky-Golay function is used to smooth out the dataset, while not compromising on data resolution.

2.6.4 Fluorescence Spectroscopy

Fluorescence spectroscopy can be used to study the structure, conformation and interactions of proteins in solution. Intrinsic sources of fluorescence in proteins arise from the amino acid residues of tyrosine, tryptophan and phenylalanine. When a protein is excited at 295 nm, it can reside in one of many vibrational levels of an excited singlet state (for example going from S1 to S2). Once an electron is excited from this state to a higher singlet state, molecules relax by internal conversion to a higher vibration level than the initial singlet state. Molecules that reside in a high vibrational level will fall to the lowest vibrational level via relaxation and release energy through collisions. Energy is then released in the form of heat or fluorescence. Both of these processes can be more easily visualised by the Perrin-Jabonski plot (figure 2.12) (Sauer, 2011)

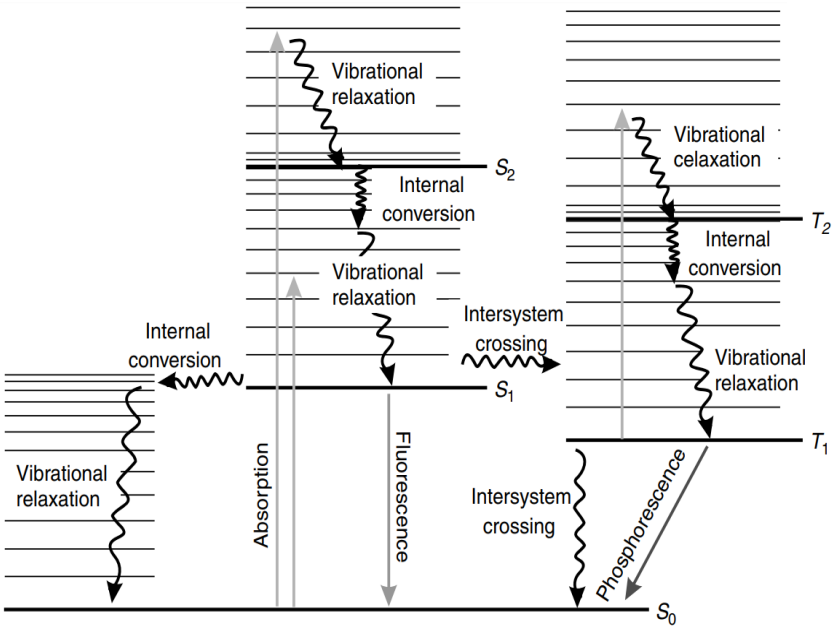


Figure 2.12: *Perin-Jabonski diagram demonstrating electronic state transitions.*

2.7 High throughput assays

All high throughput assays were done using a Spectramax M2e (Molecular Devices USA) plate reader using 96 well plates. Protein aggregates scatter light in the visible wavelength region when formed in an aqueous solution. This makes solution turbidity a method of quantifying the relative loss in intensity of scattered light and by extension, protein aggregation (Hall, 2016; Wang et al, 2016).

2.7.1 Protocol

Samples were measured in a transparent 96-well plate (Costar, USA). Stock protein samples were diluted in the desired buffer to give a final protein concentration of 10 mg ml^{-1}. The final volume for each sample was 200 µl. Samples were heated using an oven at 105 °C for 60 min. Solution turbidity was measured at 450 nm. Turbidity was calculated with equation 2.10.

$$A = 2 - log_{10}(\%T) \quad \textbf{2.9}$$

$$\%T = 10^{(2-A)} \quad \textbf{2.10}$$

where $\boldsymbol{A}$ is absorbance and $\boldsymbol{\%T}$ is % transmission

2.8 Protein aggregation

2.8.1 Removal of large precipitated aggregates

One of methods used to determine the degree of protein aggregation in this work was to measure and characterise protein solutions after separation from large aggregates. To characterise the supernatant/precipitate, samples were centrifuged at 13,000 × g for 30 min using a micro centrifuge (VWR Galaxy, USA). The purpose of this was to adequately separate supernatant from precipitated protein following aggregation. The supernatant was aspirated using a micropipette (Gilson, UK) from the microcentrifuge tube (figure 2.13). Protein concentration in the supernatant was measured by UV spectroscopy to determine the protein lost to precipitated aggregates and further characterised by HPLC and SDS-PAGE.

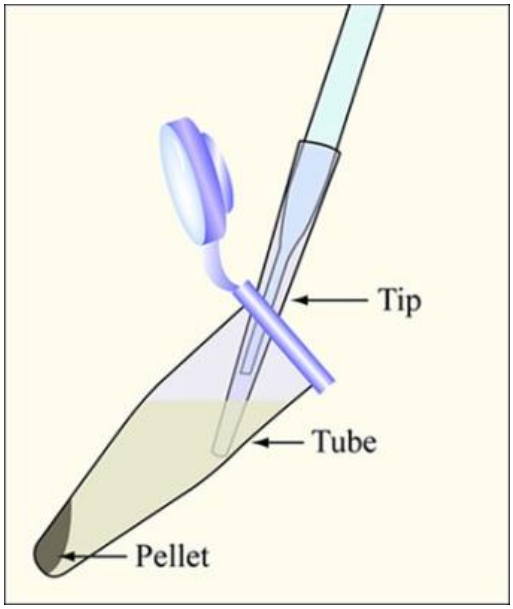


Figure 2.13: *Illustration of the method of removing the supernatant*

2.8.2 Characterising protein aggregation in the supernatant.

Once the precipitated protein was removed and the protein concentration in the supernatant determined by UV absorbance, the oligomeric states in the supernatant were then determined by a combination of SE-HPLC and non-reducing SDS-PAGE.

2.9 Differential scanning calorimetry (DSC)

2.9.1 Instrumentation

A Perkin Elmer Pyris-6 Differential Scanning Calorimeter (Perkin Elmer, Ireland) was used to perform all measurements. 55 µl of protein solution was added to a large volume stainless

steel pan (Perkin Elmer, Ireland). Pyris software was used to view data. All analysis was performed using OriginPro 2017.

2.9.2 Running a sample

Two DSC samples are required per run. One containing the analyte suspended in buffer and the reference sample, containing only buffer of the exact same mass. Sample pans were hermitically sealed and placed into the DSC furnace. Samples were allowed to equilibrate at 25 °C for 5 min and temperature was increased from 25 – 95 °C at a scan rate of 1 °C min^{-1}. The temperature was then decreased at the same scan rate from 95 °C - 25 °C. This was repeated two more times.

2.9.3 Data analysis

2.9.3.1 Baseline subtraction

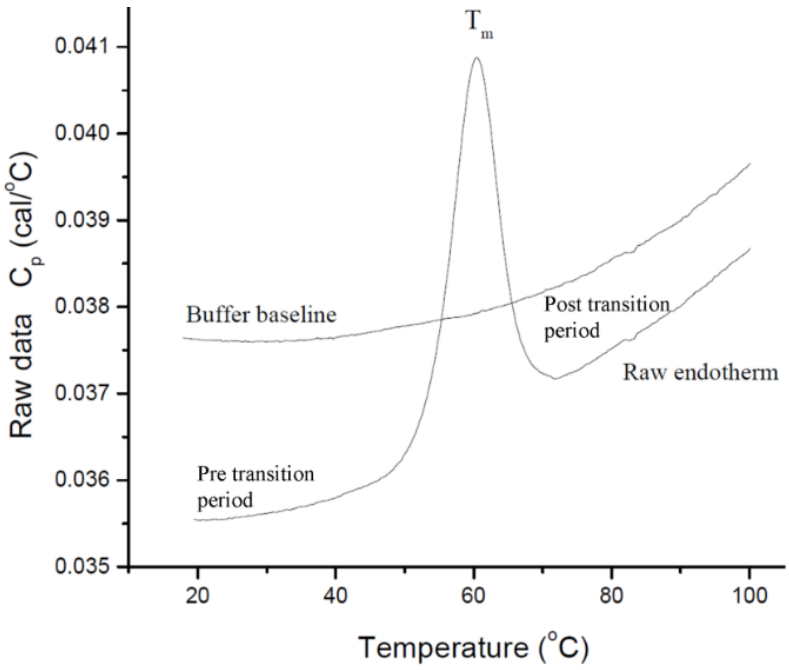


Figure 2.14: *A thermogram pre-baseline subtraction (Cooper, 1999)*

A DSC thermogram for a protein solution is composed of three states. These include the pre-transition baseline, the endothermic unfolding transition and the post-transition baseline (figure 2.14). When analysing a thermogram, it is important to know that a protein solution will have a lower heat capacity than water. This is seen as a negative value for heat capacity on the thermogram. Once the protein begins to unfold, the thermogram will begin to increase in heat capacity, which is logical considering a protein requires excess heat capacity to unfold. The maximum point of this peak is termed the melt transition temperature which is the point at which half the protein molecule has unfolded. The post-transition period is the point at

which the protein has completely unfolded. A cubic function was used for baseline correction. A typical post baseline subtraction is shown in figure 2.15

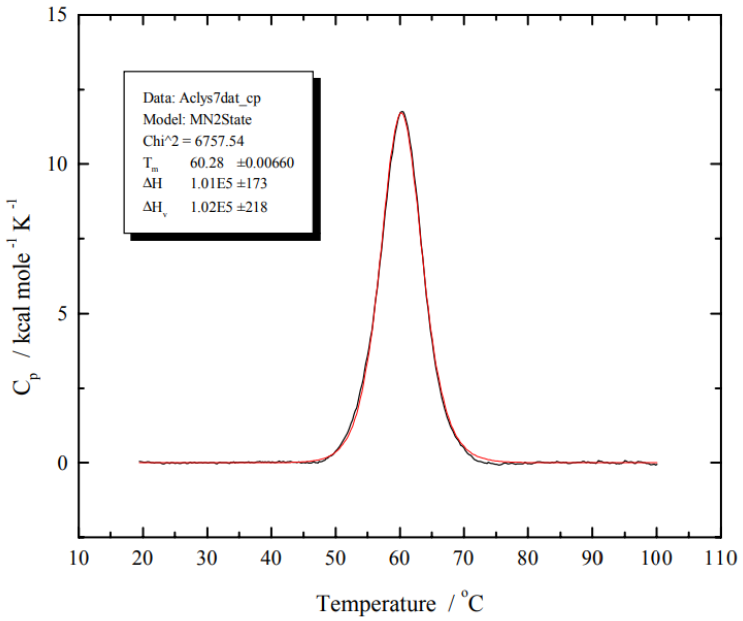


Figure 2.15: *A thermogram post baseline subtraction (Cooper, 1999)*

2.9.3.2 Van't Hoff plot

Van't Hoff enthalpy (ΔH_{VH}) is a measure of enthalpy associated with an unfolding event. Calculating ΔH_{VH} begins with a thermogram and assumes a two-state model (the protein is either fully folded or fully unfolded, no intermediate states exist), and using equation 2.11. For this analysis, the area under the peak at a specific temperature (figure 2.16, fraction unfolded) is divided by the total peak area. This is repeated several more times to acquire a linear relationship between ln (K) and 1/T. The slope of this line is the van't Hoff enthalpy. Comparing the ΔH to ΔH_{VH} allows the likeness of the system to an ideal 2-state system to be compared.

$$\frac{d(lnK)}{d(\frac{1}{T})} = -\frac{\Delta H^0}{R^0} \qquad \textbf{2.11}$$

where $\boldsymbol{K}$ is the equilibrium constant, $\boldsymbol{\Delta H}$ is enthalpy, $\boldsymbol{T}$ is temperature and $\boldsymbol{R}$ is the ideal gas constant.

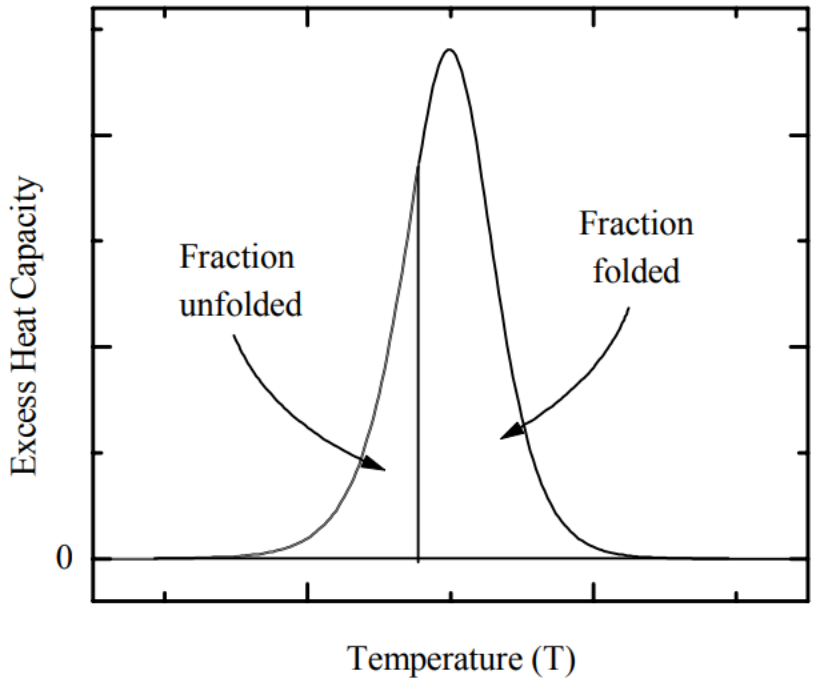


Figure 2.16: *Van't Hoff analysis using the area under a thermogram peak (Cooper, 1999)*

2.9.3.3 Reversibility index (RI)

The Reversibility Index (Blumlein & McManus, 2013) is a method of quantifying how much monomeric protein remains after a heating and cooling cycle during a DSC experiment. Following the first cycle, some protein mass is lost to irreversible aggregation, therefore in the following heating cycle, less will unfold. The differences in the areas under the endothermic peaks allows the calculation of RI by equation 2.12.

$$RI = \frac{\Delta H_{me}\,(n+1)}{(\Delta H_{me}(n)} \qquad \textbf{2.12}$$

Where $\boldsymbol{\Delta H_{me}}$ is the integrated area under the unfolding peak from the melt transition temperature, $\mathbf{T_m}$, to the end of an endothermic transition, $\mathbf{T_e}$, during an unfolding event, $\boldsymbol{n}$.

2.10 Quasi elastic light scattering (QELS)

2.10.1 Background

QELS is used for studying the properties of colloidal solutions that is both non-invasive and non-destructive. This technique is among the most popular in determining the size of particles and can be applied in the size range of 0.001 µm to several microns in size, as well as determining net particle interactions in a solution.

QELS measures the speed at which particles are diffusing by Brownian motion. The velocity of this Brownian motion is defined by the translational diffusion coefficient (D), which can be converted into a particle hydrodynamic radius (r_h) by using the Stokes-Einstein equation (equation 2.13).

$$r_h = \frac{k_B T}{6\pi\eta D_\tau} \qquad \textbf{2.13}$$

where $\boldsymbol{D_\tau}$ is the translational diffusion coefficient, $\boldsymbol{k_B}$ is the Boltzmann coefficient (1.38×10^{-23} kg.m^2.s^{-2}.K^{-1}), $\boldsymbol{T}$ is temperature, $\boldsymbol{\eta}$ is the viscosity of the medium and $\boldsymbol{r_h}$ is the hydrodynamic radius (nm).

Hydrodynamic radius can be defined as the radius of a hard sphere that diffuses at the same average speed as a particle being measured. The value of which is strongly dependant on ionic strength, surface structure and particle shape.

2.10.2 Instrumentation

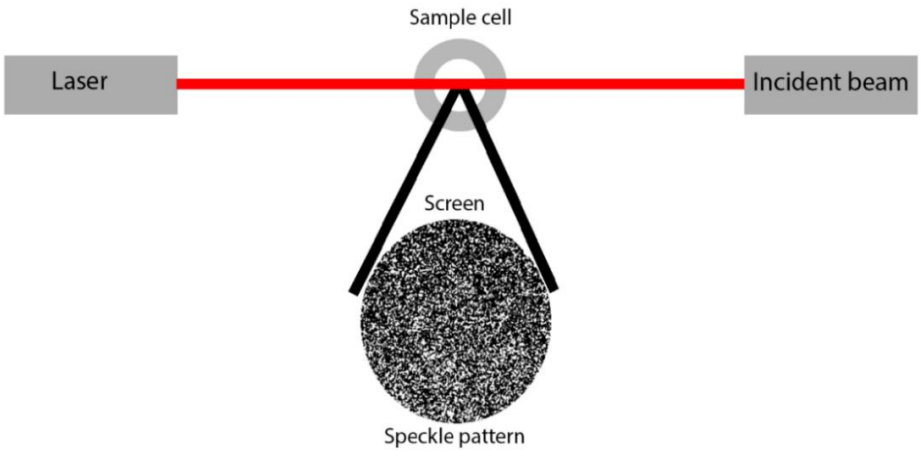


Figure 2.17: *Schematic representation of a QELS setup*

The QELS instrument consisted of an ALV/CGS-3 Goniometer in a self-contained system with a HeNe laser at a wavelength of 632 nm, an ALV/LSE-5004 light scattering electronics and multiple Tau digital correlator. The temperature of the sample was maintained using an external water bath (Thermo Scientific DC30-K20) at 293.15 K. All measurements were taken at a scattering angle of 90 °.

2.10.3 Sample preparation

Samples were prepared by 1:1 dilution method as described in the DSC section. Samples were filtered with a 10 mm Anatop 0.02 µm (Whatman, USA) syringe driven filter prior to measurements. The final volume used was 200 µl. All samples were added to a 5 mm diameter cylindrical glass tube (Hilgenberg, GmbH, Germany). Post experiment concentration of each protein solution was re-determined via UV-Vis, using a quartz cell (Hellma Analytics) with a pathlength of 1 cm.

2.10.4 Experimental process

Once a sample is made and placed into the instrument, a laser is passed through it and the particles will scatter light at all angles, however the light was detected at a 90° angle to the laser. The photon counter used here is what produces data that measures photons detected (intensity) as a function of time. The larger the particle, the slower the diffusion, reflected by the change in intensity and frequency of fluctuations by the size of the particle (Roosen-Runge et al, 2011; Yu et al, 2019) (figure 2.18).

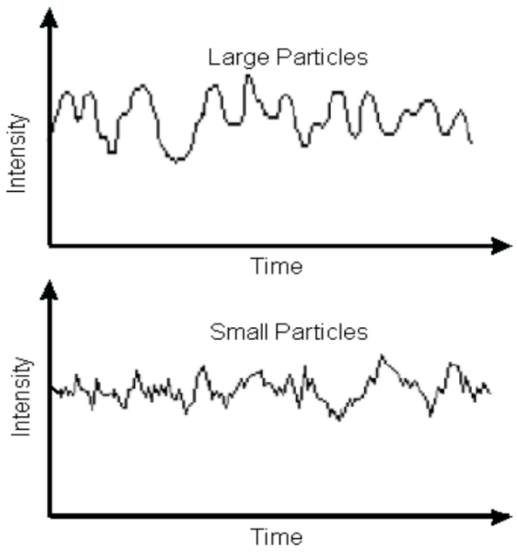


Figure 2.18: *Standard QELS intensity fluctuations for a large and small particle as a function of time.*

As light scatters from the particle, its motion contributes a certain amount of randomness to the scattered lights phase, so that the scattered light from two bodies is added together causing destructive or constructive interference. Bright patches in a speckle pattern are the result of light scattering constructively and dark patches are the result of destructive interference (see figure 2.17 for a typical speckle pattern). The speckle pattern observed appears to be in constant motion, this is because particles are constantly moving, forming new speckle patterns and new intensity fluctuations. This is illustrated in figure 2.19, where intensity changes with time. Smaller particles move faster as they are influenced more by the solvent molecules that surround them. Large particles generally have a longer time to decay as opposed to smaller particles having a short time to decay.

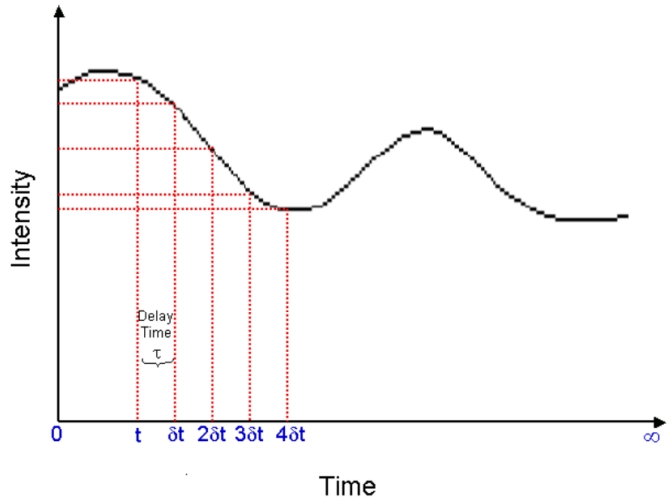


Figure 2.19: *Light intensity fluctuations with time.*

Experimentally, light intensity fluctuations can be calculated by an intensity correlation function. Post analysis, this provides the diffusion coefficient of the particles. The particles velocity is defined by the translational diffusion coefficient, denoted by *D*. Where in this case *D* is the experimentally measured diffusion coefficient and is used to solve for hydrodynamic radius (r_h).

QELS can give insight into colloidal stability of protein solutions by calculation of the net interaction parameter (k_D). Depending on the sign and magnitude of this value, it is possible to measure if particle (or protein) interactions are mainly dominated by attractive (negative k_D) or repulsive (positive k_D) interactions. This information can then be used as an indicator of structural stability. This is calculated using equation 2.14:

$$\frac{D_c}{D_0} = 1 + k_D\phi \qquad \textbf{2.14}$$

where $\boldsymbol{D_c}$ is a diffusion coefficient at a set concentration $\boldsymbol{c}$, $\mathbf{D_0}$ is diffusion coefficient assuming a concentration of 0, $\boldsymbol{k_D}$ is the net interaction parameter and $\boldsymbol{\phi}$ is volume fraction of the protein in solution.

Chapter 3: Impact of calcium on α-lactalbumin: Colloidal and structural stability

3.1 Introduction

Over the last decade, increasing demand for more mineral dense and nutritionally viable whey protein solutions has emerged due to their use in infant formula and sports drink products. Much of this has been motivated by the removal of milk quotas for farmers in Ireland and a need to develop new uses for the increased milk production. For baby formula, a more mineral dense solution is required to match the composition of human milk for improved infant health (Fox et al, 2006; Eugenia et al, 2006; Molska et al, 2014). Whey protein also provides a convenient source of protein for athletes, that aids exercise recovery (West et al, 2017). Both bovine and human milk consist of similar proteins, with a high degree of amino acid sequence homology (Kamau et al, 2010; Layman et al, 2018), however the proportions of whey proteins present in human and bovine milk differ. Both forms of milk also contain calcium, with bovine milk having roughly 25 - 30 mM total calcium and human milk total calcium concentration is 6 - 10 mM (Vitolo, 2004; Gaucheron, 2005; Lewis, 2011). Human milk whey content largely consists of α-lactalbumin (α-lac), with smaller amounts of β-lactoglobulin (β-lac) and low amounts of the non-whey protein casein. Their whey:casein ratios are 60:40 and 20:80 respectively (Lien, 2003; Buggy et al, 2016) depending on the lactation stage. Therefore, significant processing of bovine milk is required to match the composition of human milk more closely, in terms of both protein and mineral content.

An important objective in designing an adequate human milk substitute is to match the protein and mineral quantities (Goedhart, 1994; Aly, 2013). Elevated protein concentration (1-12 wt% w/v) is required during industrial processing of dairy protein products, particularly those prepared by spray drying **(**Taneja & Singh, 2015**).** This process involves adjustments of pH, ion types, ionic strength and temperature (Raikos, 2010; Brodkorb et al, 2016). Elevating ionic strength and protein concentration are not conducive to maximum protein structural and colloidal stability.

These processing steps can lead to the formation of protein aggregates and possibly heat exchanger fouling, reduction in runtimes and ultimately leaves the final product to be un-aesthetic to buyers (Bansal, 2006; Mahdi et al, 2009) Sadeghinezhad et al, 2015). The subsequent aggregation that occurs can reduce the shelf life and stability of the product. These products often require supplementation by the addition of salts, which can affect the colloidal stability and optimal viscosity during processing and after re-constitution (Hussain et al, 2012; Uluko, 2016). As such it is an imperative to quantify the effect of solution conditions on both the colloidal and structural stability of these proteins.

Whey protein beverages enriched with α-lac and depleted in β-lac are a feasible method of supplementing the amino acid profile and altering calcium concentration of infant

formula to a composition closer to that of human breast milk (Heine, 1996; Lien, 2003). Processing of solutions depleted in β-lac improves the processing characteristics, since β-lac undergoes irreversible aggregation on heating due to the formation of disulphide-linked aggregates following unfolding (Liu et al, 1991; Majhi et al, 2006). The extent of aggregation is dependent on environmental conditions such as pH, temperature, ionic strength and protein concentration. (Chi et al, 2003; Ishikawa et al, 2005). Salt ions can bind to proteins, which can lead to a change in net-charge on the protein surface. In addition, high concentrations of salt (starting at a minimum concentration of 150 mM salt, depending on the exact ionic strength of the ions involved) can contribute to charge screening and a decrease in colloidal stability of proteins in solution (Arakawa & Timasheff, 1984; Perez-Jimenez et al, 2004). In terms of the processing of whey proteins, two types of stability are important to consider; thermal stability and solution stability. Thermal stability is a measure of the how well a protein can maintain its structure under thermal stress. Solution stability of a protein however can be thought of its ability to resist aggregation in solution by self-association – its colloidal stability (James & McManus, 2012).

Quasi elastic light scattering (QELS) can be used to determine whether colloidal particle interactions in a solution are predominantly repulsive or attractive using the net interaction parameter (k_D) (Curtis et al, 2002; James & McManus, 2012; Roberts et al, 2014; Lorber, 2018). Generally speaking, a positive k_D indicates a more colloidally stable system, whereas a negative k_D indicates net attraction in the system.

k_D can be determined by measuring the collective diffusion of colloidal scale solutions using the following relationship (3.1):

$$D_c = D_0 \left[1 + k_D \phi\right] \qquad \textbf{3.1}$$

where $\boldsymbol{\phi}$ is the volume fraction of protein (calculated from the partial specific volume of α-lac (0.703 cm^3/g), $\boldsymbol{D_c}$ in the collective diffusion coefficient at a protein concentration $\boldsymbol{c}$ and $\boldsymbol{D_0}$ is the viscosity corrected infinite diffusion coefficient.

Thermal denaturation of a protein in solution can be monitored using differential scanning calorimetry (DSC). It can be used as a general analytical tool and as a direct route to fundamental data about intermolecular and intramolecular forces (Cooper, 1999). This technique is widely used to measure the melt transition temperature of a protein (T_m) and the enthalpy associated with an unfolding event (ΔH_{cal}). DSC is the most widely applied method to determine structural stability of a protein (Yasui et al, 2006; Liu et al 2011). The ability of a protein to refold to its native form after heat-induced unfolding can be quantified by the refolding index (R.I.) (Blumlein & McManus, 2013). Here we assess the thermal stability of

α-lac at a range of calcium chloride concentrations using DSC to determine the thermal stability of the protein as the calcium concentration changes. By determining a refolding index for the same solutions, we can estimate the amount of protein that refolds after an initial unfolding event over a range of calcium concentrations and hence the amount of protein lost to irreversible aggregation after heating. Significantly, these measurements can be made at relatively high protein concentrations, in the region of 100 mg ml^{-1}, which is close to the concentrations that proteins are processed (12 wt % (w/v)).

Apo-α-lac, with no bound calcium, is less structurally stable than its calcium bound counterpart (holo-α-lac) (Ewbank Creighton, 1993; Nicoleta & Rapeanu, 2010). Apo-α-lac has a significantly lower T_m (32 °C vs 67 °C for holo-α-lac) and a lower ΔH (40-50% lower), when analysed by DSC compared to holo-α-lac (Atri et al, 2010). Many studies have already been conducted demonstrating how low calcium concentration affects the solution and structural stability of α-lac (Greene et al, 1999; Hendrix et al, 2000) at low protein concentrations, generally < 3 mg ml^{-1}. However higher concentrations of protein are required for processing and little research analysing the impact of calcium on α-lac at high protein concentration has been conducted, particularly for highly purified protein. There are number of challenges in understanding the stability of α-lac in the presence of calcium, specifically, commercial sources of α-lac contain small, but significant quantities of β-lac, which strongly influence the heat induced aggregation of α-lac. Here, we prepare ultra-pure solutions of α-lac and perform studies at high protein concentrations (100 mg ml^{-1}), providing new insights into the solution and structural stability of the protein at processing-relevant protein concentrations.

The major challenge in understanding how calcium affects the stability of α-lac is the use of impure starting material. Specifically, α-lac protein powder that contains other proteins (β-lac), is used ‘’without further purification’’ (Chrysina et al, 2000; Wang et al, 2006; Gao et al, 2008) and includes co-precipitated buffer salts when lyophilised. In these studies, samples are generally prepared by re-hydration of the protein powder with buffer. Small concentrations of β-lac however can drastically reduce the thermal stability of the system through the formation of large aggregates and gelling (Kavanagh et al, 2000; Havea, 2001). α-lac at only 85% purity is used in many studies (Nicoleta & Rapeanu, 2010; Nielsen et al, 2017; Nielsen et al, 2018). However, higher purity product is available and is also widely used (Liu et al, 2011; Buggy et al, 2016; Brodkorb et al, 2016). Given how strongly β-lac influences the system behaviour, studies conducted on highly purified α-lac, would provide quantitative and more useful data in understanding its behaviour. In an industrial setting the aim is more directed to characterisation of the end product after a stress is applied to a milk solution, without assessment of the reason the product itself changed and what caused it

(Alomirah & Alli, 2004; Antoine & De Souza, 2007; Kamau et al, 2010; Lam & Nickerson, 2015). However, more detailed tuning of solution conditions and ultimately, the production process could be achieved with more detailed insight into the role of interactions of individual components of the system.

3.1.1 Aim of the study

In this study, the aim is to determine the impact of calcium concentration on an α-lac solution at high concentration. Using highly purified α-lac, the thermal and solution stability of the protein was assessed. Low concentration experiments were then done using QELS to establish the effect of calcium concentration on the colloidal stability of α-lac. After establishing which calcium concentrations give a more colloidally stable α-lac solution, high concentration experiments (DSC) are performed to directly measure the thermal stability of α-lac and assess the optimum conditions for formulating a solution with high protein and calcium concentration.

3.2 Results

3.2.1 Purification and characterisation of α-lactalbumin

α-lac was purified from α-lac whey powder (Davisco Foods Intl, 80% α-lac by weight) using a combination of size exclusion chromatography (SEC) (figure 3.1) and ion exchange chromatography (IEX) (figure 3.2). The purified protein was than characterised using SE-HPLC (figure 3.3), with the correct molecular weight determined by calibration against standard molecular weight markers (figure 3.4) and SDS-PAGE (figure 3.5).

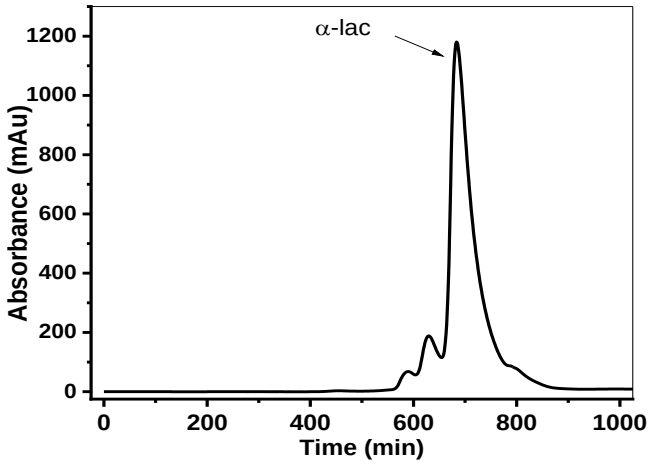


Figure 3.1: *Size exclusion chromatogram showing the separation of the components of the α-lac whey powder on a Sephacyrl S-200 column.*

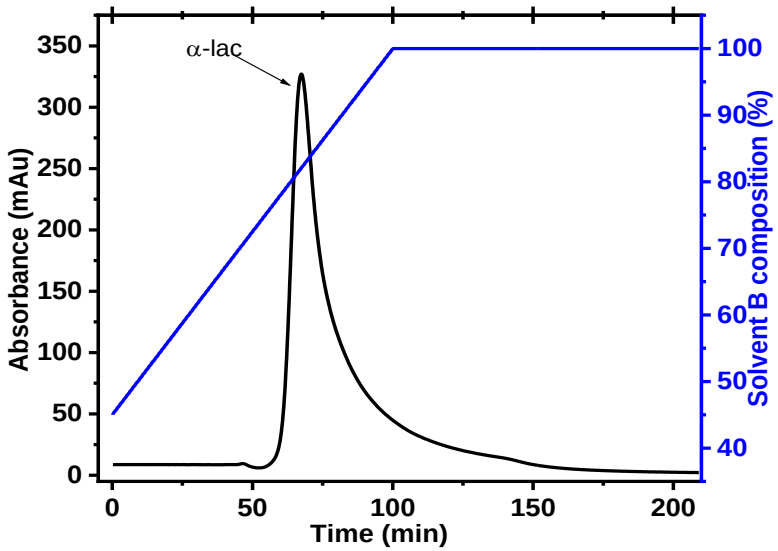


Figure 3.2: *Ion exchange chromatogram after purifying pooled fractions from the size exclusion step. The major peak contains α-lac, with a yield of ~30% from SEC to IEX.*

SE-HPLC (figure 3.3) and non-reducing SDS-PAGE (figure 3.5) were used to determine protein purity of the product after the two-stage purification.

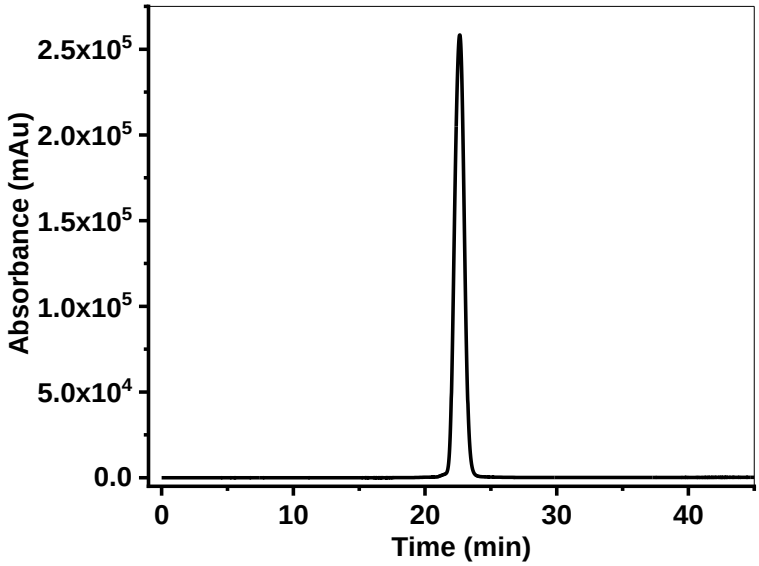


Figure 3.3: *SE-HPLC chromatogram for α-lac in 0.05 M Tris-HCl with 0.15 M NaCl at pH 7.0 after purification by size exclusion chromatography and ion exchange chromatography on a Superdex S-200 column using a flow rate of 0.75ml min^{-1}.*

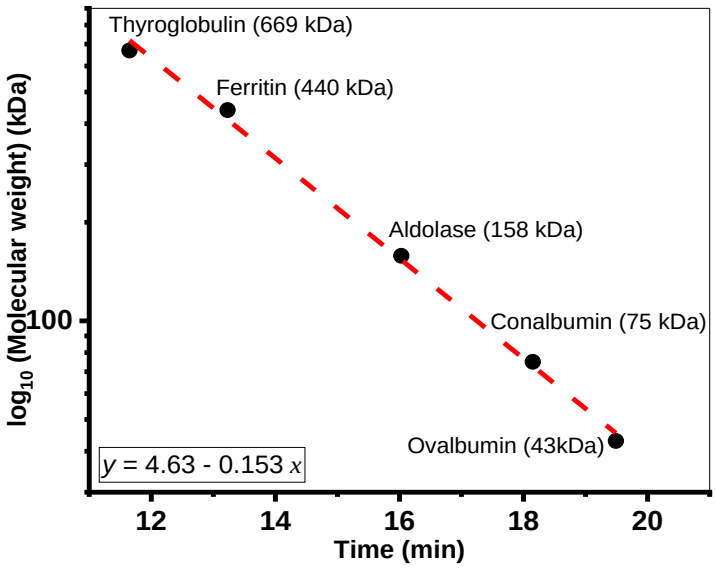


Figure 3.4: *Calibration curve for SE-HPLC relating protein molecular weight to retention time in 0.05 M Tris-HCl with 0.15 M NaCl at pH 7.0 on a Superdex 200 column using a flow rate of 0.75 ml min^{-1}.*

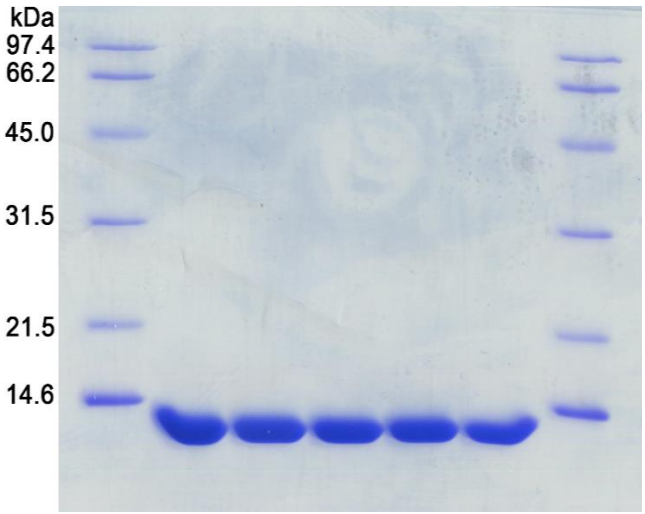


Figure 3.5: *12.6 % non-reducing SDS-PAGE gel showing a single band corresponding to α-lac. No protein oligomers or other bands are present, indicating a highly purified protein.*

SE-HPLC produced a single peak that eluted at 22.6 minutes, with a purity of ≥ 98% (figure 3.3). Using the calibration curve for this column, under the same buffer conditions, the protein was found to have a molecular weight of ~14.8 kDa (figure 3.4). This value was relatively consistent with the literature molecular weight for α-lac (14.2 kDa) (Spolaore et al, 2010; Buggy et al, 2016). SDS-PAGE show a single band located below the

lysozyme marker was recorded, indicating a protein with a molecular weight slightly lower than that of lysozyme at 14.6 kDa (figure 3.5).

To fully control the solution conditions for all measurements performed, we have ensured that all samples are fully dialysed against the required buffers, and calcium concentrations are determined for each measurement.

3.2.2 The benefits of using purified α-lac

To demonstrate clearly the impact of using industrial quality, rather than purified α-lac, we performed DSC to measure the reversibility of heat induced protein unfolding. Sample A, figure 3.6A was prepared by dissolving stock (as supplied) α-lac (80% α-lac by weight) in buffer (without dialysis). Here we see a number of endothermic peaks associated with the unfolding process. Once the sample is cooled and allowed to unfold for a second time, little protein remains in the monomeric state and only a small portion of protein refolds. Sample B, figure 3.1B was purified using SEC and IEX, and prepared by repeated dialysis against the sample buffer and the calcium content (<1 mM) was determined using both F.A.A.S and volumetric analysis. In the purified sample, more information about the thermodynamics of protein unfolding and refolding can be determined, including melt transition temperature ($\mathbf{T_m}$), refolding index (**R.I.**), calorimetric enthalpy ($\mathbf{\Delta H_{cal}}$) and van't Hoff enthalpy ($\mathbf{\Delta H_{VH}}$) as opposed to only T_m in sample A (summarised in table 3.1). Our analysis indicates that once the α-lac sample is sufficiently pure there is a dramatic change in reversibility (15 % vs 53% for impure and pure solutions respectively) and the number of reaction states of the system (2+ state vs 2 state, for impure and pure samples respectively), providing insights not currently available in the literature to our knowledge.

Table 3.1: *Table indicating the thermodynamic parameters associated with an impure/pure sample of α-lactalbumin.* ***Sample A:*** *α-lac dissolved in 0.05 M Tris-HCl, pH 7.0.* ***Sample B:*** *α-lac purified and dialysed into 0.05 M Tris-HCl, pH 7.0.*

	Sample A (impure)	**Sample B (pure)**
T_m	67 °C	65 °C
R.I.	<0.15	0.53
ΔH_{cal}	74.0 kcal mol^{-1}	67.0 kcal mol^{-1}
Reaction states	2+ state	2-state

In addition to the thermodynamic parameters that can be determined by DSC, a highly purified starting material allows measurement of the net interaction parameter (k_D) using QELS. This measurement can determine if the interaction potential in an α-lac solution is attractive or repulsive and hence it's potential solution stability.

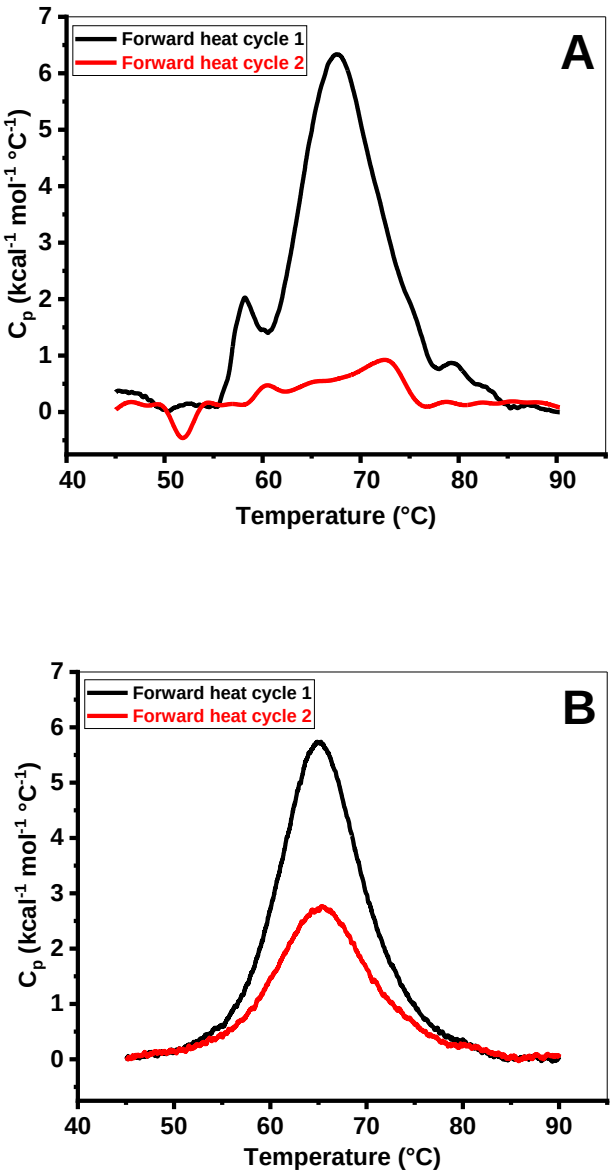


Figure 3.6: Sample A: *Thermogram of unpurified α-lac powder (80% by weight), prepared simply by dissolution at 100mg/ml in 0.05 M Tris, pH 7.0 with concentration determined by powder mass*. **Sample B:** *Thermogram of 100mg ml^{-1} α-lac after purification, dialysed against 0.05 M Tris-HCl, pH 7.0 with a calcium content of <1 mM. Calcium concentration was measured by FAAS and by titration and protein concentration measured by UV absorbance at 280nm.*

3.2.3 Quasi elastic light scattering (QELS)

QELS was used to determine the net interaction parameter, k_D for α-lac at a range of calcium concentrations at constant pH. Protein concentrations in the range 10-60 mg ml^{-1} were measured (figure 3.7). α-lac at all calcium concentrations has a negative k_D value, indicating net-attractive interactions in solution. The k_D for purified α-lac in buffer, which has a calcium concentration less than 1 mM is -2.0. As the calcium concentration is increased to 60 mM, k_D decreases to a minimum of -6.5 and then increased slightly at higher calcium concentrations. At 500 mM $CaCl_2$, k_D is -4.5. The increasingly negative k_D values (-2.0 to -6.5) from <1 mM Ca^{2+} to 60 mM calcium is indicative of increased attraction between proteins. A similar k_D was recorded for α-lac (Gast et al, 1998) where a k_D of -7.0 +/- 1.0 was recorded in 12 mM $CaCl_2$ in 0.05 M Sodium Cacodylate with 0.05 M NaCl. The slightly more negative k_D in this case, is most likely due to the higher ionic strength of the sample.

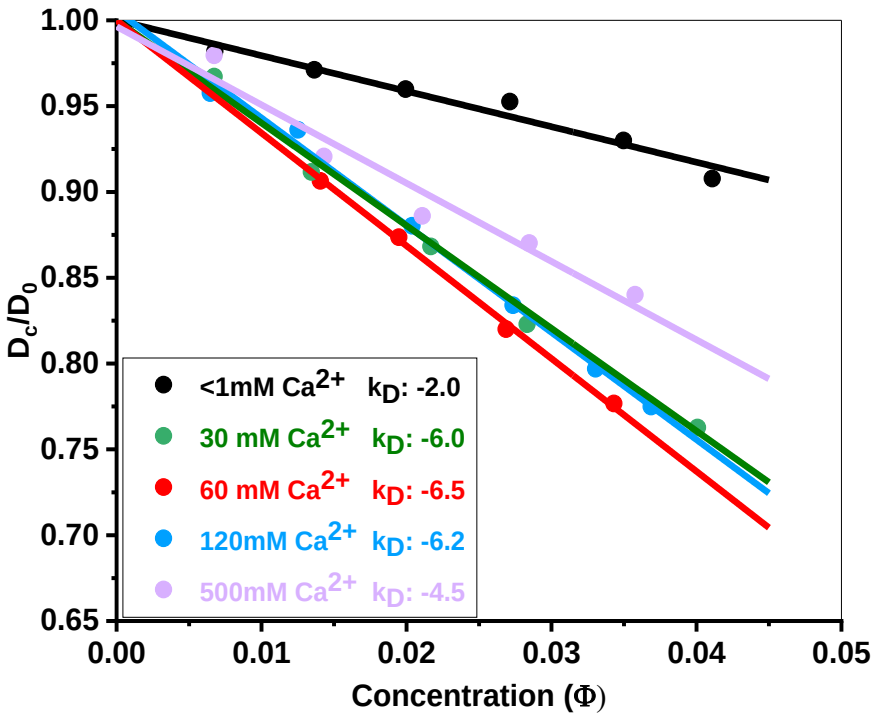


Figure 3.7: *D_c/D_0 as a function of volume fraction for α-lac with increasing calcium concentrations. All samples were prepared 0.05 M Tris-HCl, pH 7.0. All polydisperity indexes were all calculated to be <0.1. The D_0 value calculated was 1.92 × 10^{-9} m^2/s.*

3.2.4 Differential scanning calorimetry (DSC)

3.2.4.1 A standard thermogram and baseline subtraction

During all DSC experimentation, the heat flow was increased/decreased at a scan rate of 1°C min^{-1} while the change in heat capacity with increasing temperature of the given solution was measured alongside a reference sample of the buffer.

For every sample containing protein, as temperature increases across the forward scan, a temperature value is reached where the proteins in solution begin to unfold. This unfolding is an endothermic process. All thermograms recorded here were relatively consistent with the work of Wang et al, where they also performed DSC experiments on α-lac. The difference however is the material used by Wang and others (figure 3.10) contained β-lac alongside α-lac in the ratio 1:10. This is evident by a significant reduction in their reversibility and heat capacity.

For each thermogram, a baseline was first established and subtracted to measure calorimetric enthalpy by integrating the area under the curve. The baseline was fitted using a cubic function in OriginPro 2018. An example of a standard DSC is shown in figure 3.8 and 3.9, as well as a baseline generation both before and after it is performed. All thermograms consisted of three heat cycles (solid line) and three cool cycles (dashed line).

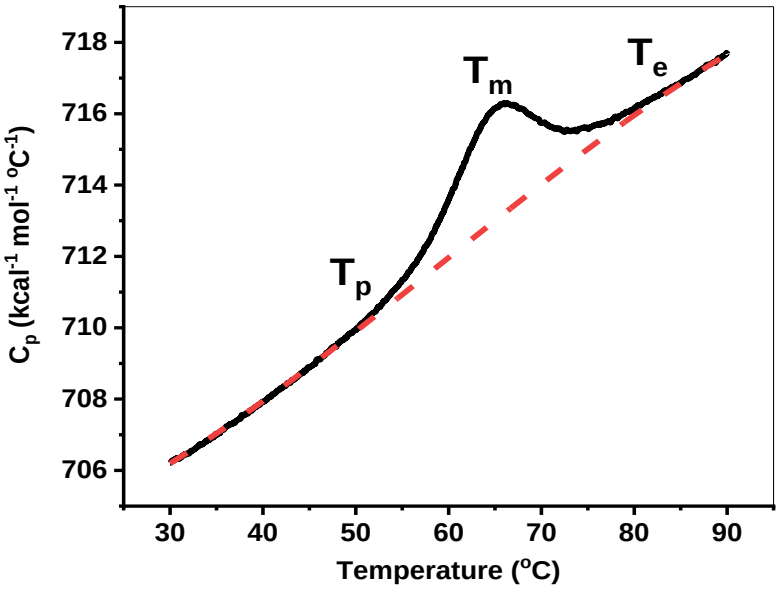


Figure 3.8: *A typical DSC thermogram for 100 mg ml^{-1} α-lac in 0.05 M Tris-HCl, pH 7.0 prior to baseline subtraction (red). Baseline was determined by cubic interpolation using OriginPro 2019.*

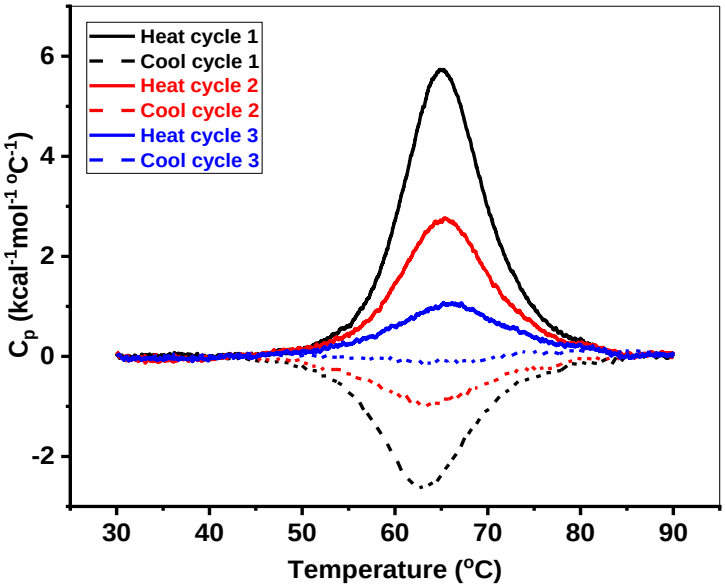


Figure 3.9: *Normalised and baseline subtracted DSC thermogram for α-lac (100 mg ml^{-1}) in 0.05 M Tris-HCl, pH 7.0 for three heating and cooling cycles (<1 mM Ca^{2+}).*

3.2.4.2 DSC and α-lac reversibility

Throughout all DSC experimentation, samples were sealed inside a capsule, under a constant flow of Nitrogen to ensure no loss/gain in protein material via evaporation. Thermal denaturation occurs during a heat cycle and some protein will be lost to irreversible aggregation (in the range of conditions used), which is why the area under the exotherm after an unfolding event is less than the endotherm. When a second unfolding occurs, this is seen as a reduced ΔH compared to the first heat cycle endotherm due to the loss of protein to irreversible aggregation after thermal unfolding. When a third cycle is done ΔH is reduced again. Essentially the more cycles done, the less native protein available for the next heating cycle to unfold. The refolding index (R.I.) was calculated via equation 3.4.

$$RI = \frac{\Delta H_{me}\,(n+1)}{\Delta H_{me}(n)} \qquad \textbf{3.4}$$

where $\boldsymbol{\Delta H_{me}}$ is the integrated area under the unfolding peak from the melt transition temperature, T_m, to the end of an endothermic transition, $\boldsymbol{T_e}$, during an unfolding event, $\boldsymbol{n}$.

In any sample where refolding was observed, an exothermic trough was observed when cooled. When heated again (cycle 2) this trough was seen as an endotherm with a lower ΔH than the first endotherm. While some data was been done in the literature indicating some reversibility (Wang et al, 2006) (Figure 3.10) the protein material used contained α-lac: β-lac in the ration 10:1, reducing the RI and making data analysis more complicated.

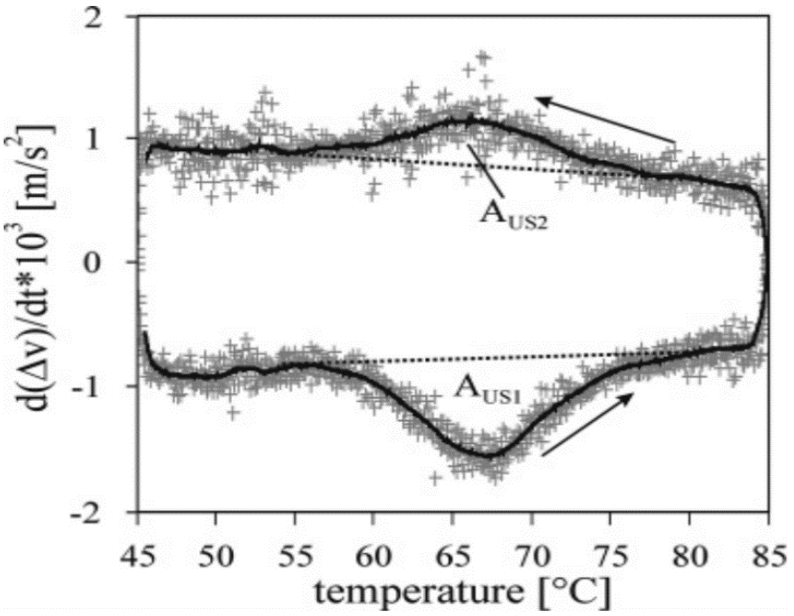


Figure 3.10: *Thermogram taken from Wang et al, 2006. 40 mg/ml α-lac (α-lac:β-lac 10:1) at pH 6.5. Crosses indicate raw data; the solid black line indicates smoothed data.*

3.2.4.3 The effect of increased calcium concentration on T_m and R.I.

The aim of this study was to determine the effect of increased calcium concentration on the thermal stability of α-lac. A single buffer at a fixed ionic strength and pH (0.05 M Tris-HCl, pH 7.0) was used so that only calcium concentration was varied from one DSC thermogram to the next. After purification, α-lac was suspended in 0.275 M Sodium acetate buffer as such was extensively dialyzed against MilliQ water and dialyzed again into 0.05 M Tris-HCl, pH 7.0 to exclude any changes in ionic strength. Table 3.2 summaries the data shown in figure 3.11 which indicates that an increase in T_m is observed as calcium concentration is increased from 0 - 250 mM (65-68/69 °C) with a small decreased in T_m observed at higher calcium concentrations.

Calcium concentration (as $CaCl_2$) was increased to determine its impact on the melt transition temperature (figure 3.11) and refolding index (R.I.). Conditions were chosen to resemble a milk ultrafiltrate environment. Here pH was kept constant at the neutral range, as was protein concentration at 100 mg ml^{-1}. The same general trend (with the exception of the 18 mM $CaCl_2$ sample) was observed when comparing ΔH values, which indicates that as calcium concentration is increased, it allowed higher temperatures to be achieved and more energy is required to unfold the protein, albeit the gain in ΔH is negligible after 60 mM. At $CaCl_2$ concentrations higher than 250 mM, there is a reduction in T_m, ΔH and R.I. Similar results were observed in the literature where there was an apparent increase in all these values up to 100-200 mM calcium, where a plateau is reached and at $CaCl_2$ concentrations higher

than 200 mM, there was a steady reduction in T_m (Hendrix et al, 2000; Engel et al, 2002; Liu et al, 2011).

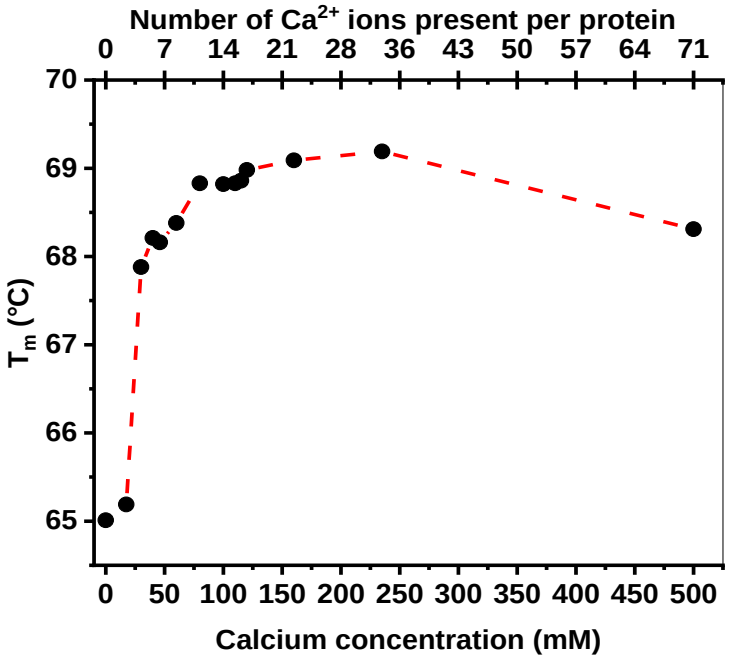


Figure 3.11: *Change in T_m of α-lac with increasing calcium concentrations. Protein concentration was 100 mg ml^{-1} in 0.05 M Tris-HCl, pH 7.0. The graph line is present to guide the eye.*

Table 3.2: *Data used in figures 3.11 & 3.12, showing changes in T_m, ΔH_{cal} and R. I with increasing Ca^{2+} concentration. The protein concentration was 100 mg ml^{-1} α-lac in 0.05 M Tris-HCl, pH 7.0.*

Ca^{2+} added (mM)	T_m (°C)	ΔH_{cal} (kJ/mol)	R.I.
0	65.0	67.5	0.56
18	65.2	59.4	0.54
30	67.9	78.0	0.54
40	68.0	78.5	0.56
46	68.0	93.6	0.21
60	68.0	79.0	0.18
100	68.8	73.3	0.19
110	68.9	89.3	0.39
115	68.9	76.1	0.3
120	68.9	80.5	0.55
160	69.2	72.9	0.48
235	69.3	70.9	0.4
500	68.3	70.9	0.27

The variance in ΔH values for samples 46 mM and 110 mM were slightly outside the accepted instrument uncertainty, where generally the values can vary 6 - 8 % on the same instrument. Peak integration error was minimised by not using any 'smoothing' functions during calculation. Protein solution sample preparation where a 1:1 dilution method was used to minimise error and UV-Vis to determine protein concentration. Alternatively, different calcium :protein ratios could increase or decrease the amount of energy required for unfolding to occur by the overcharging effect for the 110 mM value (expanded upon in discussion).

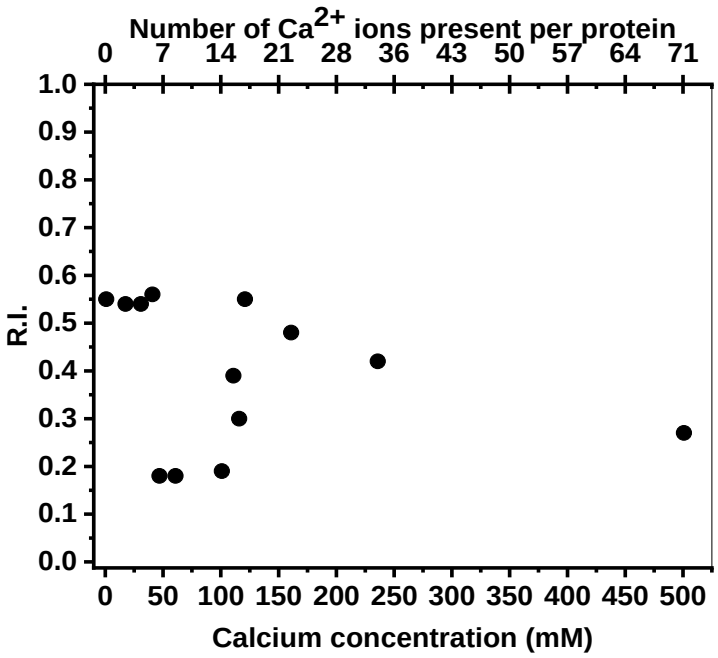


Figure 3.12: *R.I. with increasing $CaCl_2$ concentration for α-lac at a protein concentration of 100 mg ml^{-1} in 0.05 M Tris-HCl, pH 7.0.*

3.2.5 Confirmation of aggregation and return to monomeric protein state after heating and cooling

Samples used in DSC measurements are sealed inside stainless seal pans. To further examine the protein solution after heating, the experiment was repeated in a 1.5 ml microcentrifuge tube using a water bath for heating. Visual inspection of the sample showed that after a heating and cooling cycle, the solution changed from completely transparent to somewhat turbid. This is indicative of aggregate formation. Large aggregates were separated from the solution by centrifugation at 13,000 × g. The supernatant was removed via aspiration for further analysis by SDS-PAGE, SE-HPLC and intrinsic fluorescence.

When a protein is heated to 95 °C, this can cause the breaking of chemical bonds formed between the amino acids of a protein. As such not 100 % of the protein mass heated will be able to refold upon cooling and a certain amount of aggregate will form. SDS-PAGE (figure 3.13) and SE-HPLC (figure 3.14) were run in tandem consisting of three samples which were: unheated, heated for 1 heat/cool cycle and heated for 2 heat/cool cycles. These measurements were performed to determine the quantity of fragments/aggregates formed during this process and to confirm that refolding to a monomeric state had occurred. One band was seen in all samples, located below the lysozyme molecular weight standard (14.6 kDa), indicative of monomeric α-lac remaining in the supernatant after removal of irreversible aggregates. No higher molecular weight aggregates were detected up to 97.4 kDa.

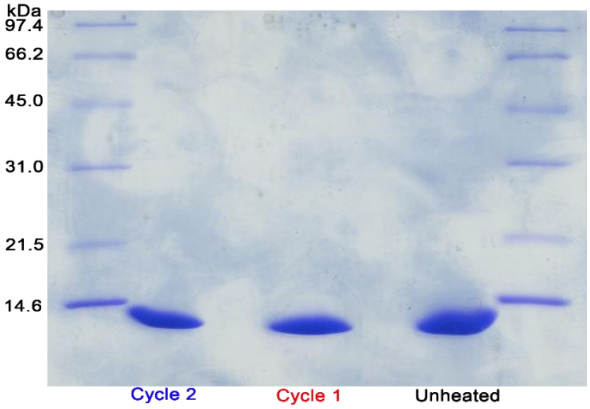


Figure 3.13: *α-lac samples (in 0.05 M Tris, pH 7.0) (<1 mM Ca^{2+}) resolved on a 12.6 % SDS-PAGE (non-reducing).*

SE-HPLC was performed on the same protein sample as to further confirm that the molecular weight of the refolded protein was consistent with the unheated sample. Some low molecular weight aggregates (~11 min) were also present in the samples that had undergone two heat/cool cycles. These aggregates where too small to be removed by centrifugation.

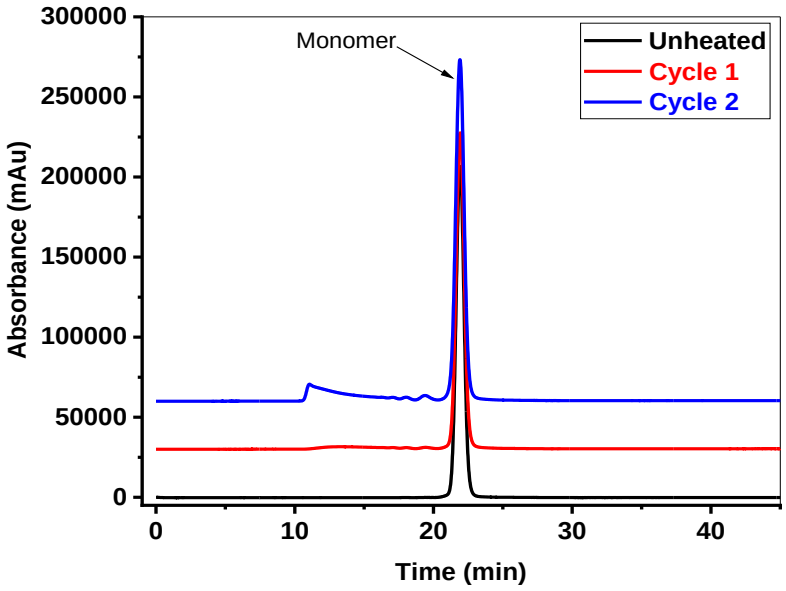


Figure 3.14: *SE-HPLC chromatogram demonstrating the quantity of monomer remaining in solution after heat is applied*

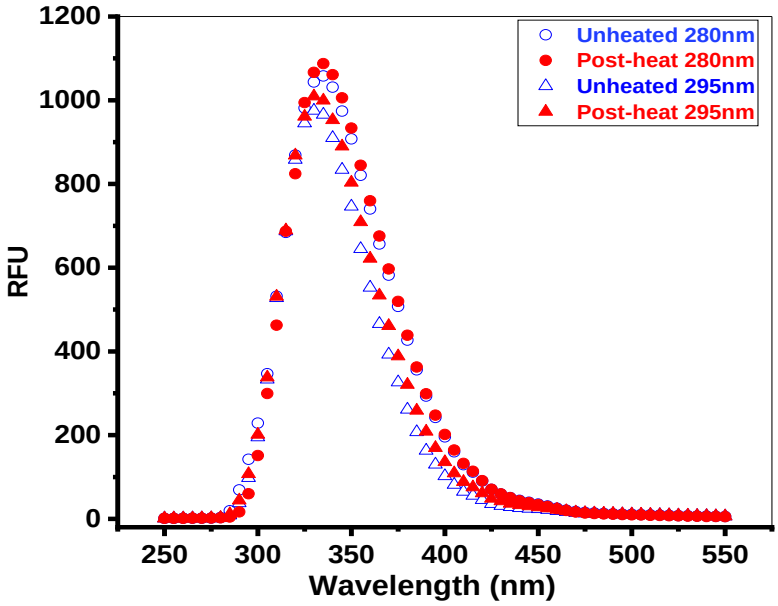


Figure 3.15: *Intrinsic fluorescence emission spectra for α-lac samples excited at 280 nm and 295 nm. Spectra for unheated and post-heated samples are shown. Samples were prepared at 1 mg ml^{-1} α-lac in 0.05 M Tris-HCl, pH 7.0 with <1 mM Ca^{2+}.*

Intrinsic fluorescence was performed to determine if secondary and tertiary structure were retained once the protein had refolded after a heat/cool cycle. After a microcentrifuge sample separated soluble and insoluble protein material, the soluble portion was probed with intrinsic fluorescence (the same as SDS-PAGE and SE-HPLC above). This same sample had previously been shown to contain monomeric, refolded α-lac via SDS-PAGE and SE-HPLC. These fluorescence spectra were compared with an unheated sample of α-lac. A sample of α-lac (1 mg ml^{-1}) was excited at both 280 and 295 nm and the fluorescence intensity and maximum emission wavelength (λ_{max}) were recorded. Post excitation at 280 nm and 295 nm, there was similar overlap of the emission spectra between the heated and unheated sample. This overlap of emission spectra indicates that monomeric α-lac (supernatant) had indeed the same conformation as an unheated sample, indicating that its secondary and tertiary structure were retained after a heat/cool cycle.

3.2.6 Assessment of the effect of high calcium concentrations on the structure of α-lac before heat is applied

The DSC data indicates that when heat is applied, when the calcium to α-lac ratio is high it leads to higher R.I. values. To ensure that protein structure was not perturbed by high calcium concentrations prior to heating, we used 2^{nd} derivative UV spectroscopy (figure 3.16) to determine if adding calcium to an unheated protein had any effect on its structure before heat is applied. A sample of 1 mg ml^{-1} in 500 mM $CaCl_2$ was prepared. The spectra of the low calcium and high calcium samples indicate that no significant alteration of structure is observed at high calcium concentrations. This is in good agreement with the literature (Chrysina et al, 2000), that demonstrates the binding of calcium does not specifically alter its structure, but does make its tertiary structure more resistant to unfolding.

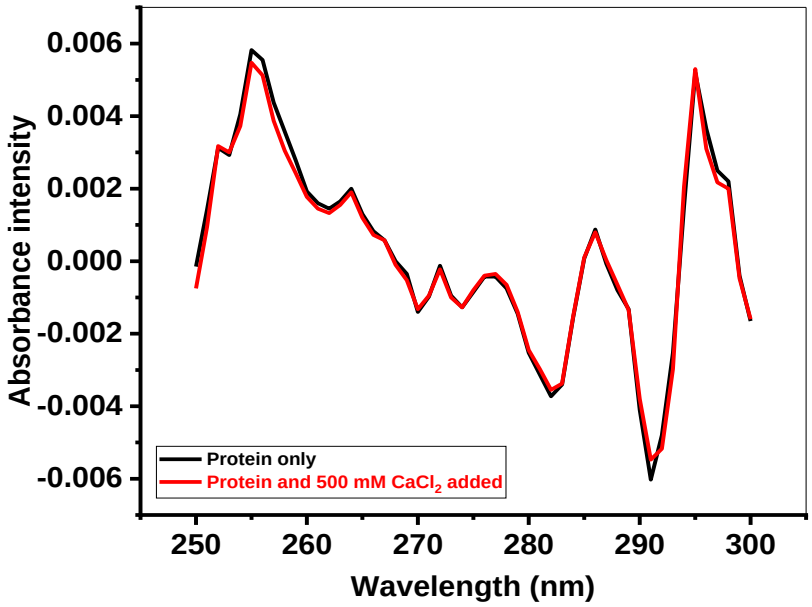


Figure 3.16: *Second derivative absorbance spectra of α-lac with and without calcium. Both samples were prepared in 0.05 M Tris-HCl, pH 7.0 (With 500 mM Ca^{2+} present in the Tris buffer).*

3.2.7 van't Hoff enthalpy

Protein unfolding can proceed either by a 2-state unfolding (protein state is either in folded or unfolded) or by a 3-state mechanism that involves a number of partially unfolded intermediate states (Wijesinha et al, 2007; Durowoju et al, 2017). This assessment is determined by measurement of the van't Hoff enthalpy (ΔH_{VH}). Van't Hoff enthalpy is a measure of the ΔH associated with an unfolding event. This measurement is completely dependent on the shape

of the endotherm and is performed by measurement of the area under the endotherm peak at a specific temperature and divided by total area of the peak. This allows the extent of unfolding that has occurred to be calculated for a range of temperatures using equation 3.2 to determine K_{unfold} (equilibrium of unfolding)

$$K_{unfold} = \frac{f_{unfolded}}{f_{folded}} \qquad \textbf{3.2}$$

where $\boldsymbol{f_{unfolded}}$ is fraction of unfolded protein and $\boldsymbol{f_{folded}}$ is the fraction of folded protein at a given temperature. Then by using the equation:

$$\frac{d(lnK)}{d(\frac{1}{T})} = -\frac{\Delta H}{R} \qquad \textbf{3.3}$$

where ***K*** is the area unfolded divided by the total area. ***R*** is the ideal gas constant and ***T*** is temperature in Kelvin (K). This allows a plot of ***ln K* vs *1/T*** (using data from figure 3.10) to be constructed where the slope is equal to the van't Hoff enthalpy (***ΔH***) divided by ***R*** (figure 3.17).

For a 100 mg ml^{-1} α-lac sample in 0.05 M Tris-HCl, pH 7.0 with <1.0 mM Ca^{2+}, the van't Hoff enthalpy was 65.00 kcal mol^{-1} compared to a ΔH_{cal} of 67.5 kcal mol^{-1} measured by DSC for a sample at the same calcium concentration. Since $\Delta H_{cal}/\Delta H_{VH} \sim 1$, we can assume that unfolding occurs via a 2-state mechanism, as does lysozyme, which is a closely related protein (Blumlein & McManus, 2013).

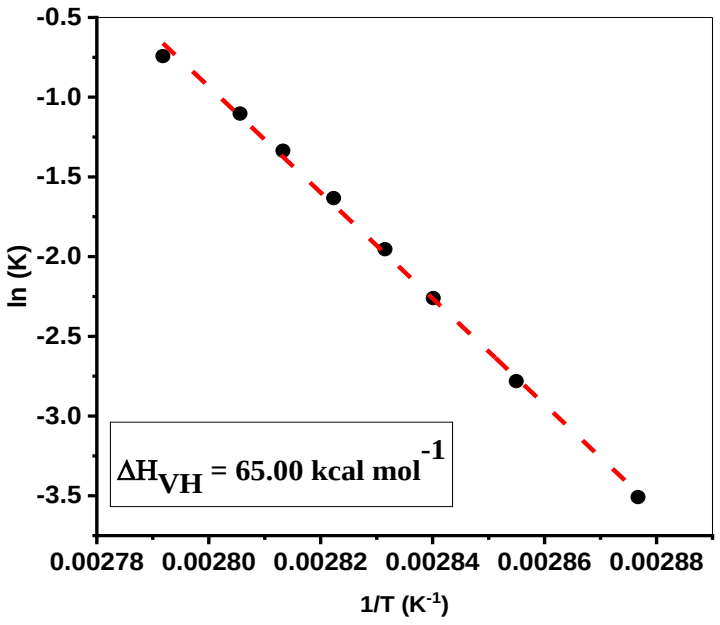


Figure 3.17: *Calculation of the van't Hoff enthalpy for 100 mg ml^{-1} α-lac in 0.05 M Tris-HCl, pH 7.0 with <1.0 mM Ca^{2+}.*

Table 3.3: *Data used for van't Hoff plot. The sample measured was 100 mg ml^{-1} α-lac in 0.05 M Tris-HCl, pH 7.0.*

Temperature (°C)	Temperature (K)	Temperature 1/T (K^{-1})	Fraction unfolded (Area)	Fraction unfolded/total area (K_{unfold})	Ln K_{unfold}
65.0	358.2	0.00279	32.1	0.47	-0.74
63.2	356.4	0.00281	22.4	0.33	-1.10
62.3	355.5	0.00281	17.7	0.26	-1.34
61.1	354.3	0.00282	13.2	0.19	-1.63
60.0	353.2	0.00283	9.5	0.14	-1.95
58.9	352.1	0.00284	7.0	0.10	-2.26
57.1	350.3	0.00285	4.2	0.06	-2.78
54.5	347.6	0.00287	2.0	0.03	-3.51

3.3 Discussion

Calcium can bind to α-lac in both specific positions and non-specifically on the protein**.** Specific binding of calcium to α-lac occurs at one primary ligand binding site (the carboxylic oxygen of amino acids Asp82, Asp87 and Asp88 with the carbonyl oxygen of amino acids Trp79 and Trp84) and one secondary site (consisting of Thr38, Gln39, Asp83 and Leu81) (Permyakov et al, 2001). Hence at a minimum, two calcium ions are required per protein to saturate the specific calcium binding sites on the protein. Given that the binding constants are 2.0×10^7 M^{-1} (primary binding site) (Permyakov & Berliner, 2000) and 3.1×10^4 M^{-1} (secondary binding site) (Kronman et al, 1981), in reality, the calcium concentration required to achieve full binding at both sites is indeed higher than that. If we examine the DSC data, we find that there is a significant shift in T_m and ΔH between 18 mM and 30 mM $CaCl_2$. This is consistent with a transition between apo and holo-α-lac, and suggests that by 30 mM $CaCl_2$ (corresponding to 4 calcium ions per protein), specific binding of calcium to the protein is complete.

Non-specific calcium binding however appears to continue, since changes to RI and k_D occur above calcium chloride concentrations of 30 mM. Ca^{2+} k_D reaches a minimum of -6.5 in QELS and also the R.I. decreases to 0.18 at 60 mM Ca^{2+} in DSC (fig 3.18); maintaining this R.I until 100 mM Ca^{2+}. k_D becomes more negative and increasing amounts of protein undergo irreversible aggregation, which indicates a decrease in stabilising repulsive interactions (i.e. the net charge on the protein approaches zero).

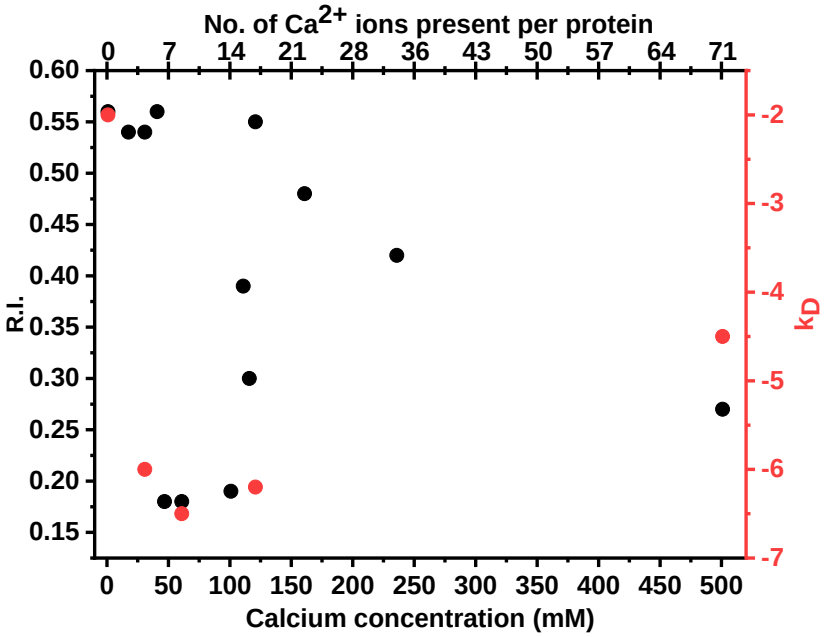


Figure 3.18: *Comparision of DSC (R.I.) and k_D data from QELS of α-lac in 0.05 M Tris-HCl, pH 7.0, with increasing concentrations of calcium.*

Above 100 mM $CaCl_2$, R.I. begins to increase again, indicating less irreversible aggregation as calcium concentration increases, which is suggestive of overcharging, i.e. the amount of calcium bound to the protein is sufficient to provide a positive charge on the protein surface, contributing to repulsive interactions and hence increased solution stability. This would be consistent with the observations of other investigators **(**Quesada-Perez et al, 2003; Lyklema, 2006; Zhang et al, 2008, Zhang et al, 2010; Kubickova et al, 2012; Lund et al, 2013, Roosen-Runge et al, 2013), where the 2003 and 2006 papers reviewed the concept of overcharging and the relation to the Hofmeister series and the later studies demonstrated the overcharging concept practically in biological systems using a diverse range of different proteins and multivalent cations.

At calcium concentrations higher than 120 mM, R.I. values begin to decrease again, which is suggestion of charge screening at calcium concentrations between 120 - 500 mM. The change in behaviour, which is reflected in protein-protein interactions measured by

QELS and by the degree of irreversible protein aggregation with increasing calcium concentrations are summarized in Table 3.4.

Table 3.4: *Summary of α-lac interactions with calcium*

Ca^{2+} Concentration (mM)	**Ca^{2+} ions present per protein**	**Ca^{2+}/protein interactions occurring**
10 - 30 mM	2 - 4	Specific
30 - 100 mM	4 - 14	Non-specific
100 – 120 mM	14 - 18	Overcharging
120 mM +	18 +	Screening

At pH 7.0, α-lac has a net negative charge of -4.5 (Gao et al, 2008) and as such positively charged species which interact its surface can either be protons, metal cations or metal hydroxides (Schubert et al, 2019). In addition to the specific binding of calcium to α-lac, non-specific binding leads to overcharging. Overcharging can occur by the deprotonation of surface exposed amino acid residues and adsorption to these residues by oppositely charged ions.

In this work, we make a clear distinction between colloidal stability, which is measured by QELS and structural stability, measured by DSC. QELS was used to quantify the colloidal stability of α-lac. A decrease in diffusion coefficient was measured as protein concentration was increased across all calcium concentrations, resulting in negative k_D values, indicating increasingly attractive protein-protein interactions with increasing calcium concentrations up to ~ 60 mM $CaCl_2$. However, at 120 to 500 mM calcium less negative k_D values were measured. Since calcium is considered a chaotropic salt on the Hofmeister series, it will disrupt water structure and will accumulate at the surface of the protein non-specifically by interaction with negatively charged amino acid residues (Lee et al, 2017). This interaction between amino acid residues and calcium can reduce the negative charge they contribute to net surface charge. A combination of increased protein and calcium concentration creates an environment where proteins are slower moving, the distance between proteins is reduced, and the degree of electrostatic repulsion is decreased. However, this has the most impact on solution behaviour between 0 - 60 mM calcium as the 120 mM + values appear to be more colloidally stable than the slightly lower ionic strengths indicating that protein-protein interactions are different in the 120 - 500 mM range and that this is most likely due to

overcharging. As more calcium was added to the protein solution (up to 100 mM), it resulted in a more structurally stable protein, given the greater temperatures required to unfold it and increasing values for ΔH. However, while the protein may become more structurally stable with increasing $CaCl_2$ concentrations, the solution stability (as determined by k_D) does not. Figure 3.18 shows a strong correlation between the two parameters at calcium concentrations <120 mM Ca^{2+}, where a more negative k_D value is strongly associated with a lower R.I. value. This would indicate that a more negative k_D value for an α-lac solution correlates to a reduced ability to refold after unfolding via heat denaturation. The more negative k_D values with this would suggest that proteins are self-associating as the effect of attractive interaction potentials is greater before heat is applied and this is negatively affecting the refolding ability of α-lac, once heated.

The processing of dairy proteins requires heating whey proteins to >100 °C, with added calcium salts present. Predominantly, this is during the processing of cow's milk with calcium concentrations in the range of 30 mM. However, there is a demand for dairy drinks with higher calcium concentrations as well as higher protein concentrations. Here we show that under the right conditions, both could be achieved with reasonable solution and thermal stability, potentially opening routes to new dairy drinks or sports recovery beverages. This could be achieved by increasing the proportion of α-lac in dairy protein solutions. Other work has shown that this strategy works on industrial processing scale (Buggy et al, 2016).

3.4 Conclusions

α-lac was purified from a whey protein powder consisting primarily as α-lac. Colloidal stability was assessed using QELS. k_D values were determined for α-lac at various calcium concentrations ranging from a k_D value of -2.0 to -6.0, reaching a minimum at 60 - 120 mM calcium chloride. After this the k_D becomes more positive, indicating increasing colloidal stability, most likely due to overcharging.

The structural stability of purified α-lac with increasing calcium concentration was assessed with DSC at processing relevant concentrations. The highest refolding index (R.I.) for α-lac was recorded at ~30 - 50 mM and 100 - 120 mM. At the lower calcium concentrations (30 - 50 mM), this is due to electrostatic repulsion because of charged amino acids on the surface of the protein. In the higher calcium concentration regime, our data suggests that non-specific calcium binding to the protein surface, results in overcharging, again contributing a repulsive component to the interaction potential, resulting in greater ability to resist irreversible aggregation after unfolding. These observations were consistent with DLS measurements.

By careful tuning of solution conditions, it may be possible to create high protein and high mineral content beverages that are cost effective to processing and are shelf stable. The calcium:protein ratio of 14-18:1 appears to allow a high calcium concentration to be present (100-120 mM) and also maintain α-lac structural stability at temperatures up to 95 °C. This could be particularly useful for dairy processing by LTLT (low temperature long time) processing and some forms of HTST (high temperature, short time) processing, which all occur below 100 °C. More experimentation would be required for assessing the protein:calcium ratio effect on temperature >100°C however which is expanded upon in chapter 4. Finally using QELS could possibly be a method of correlating colloidal stability to structural stability during whey formulation.

Chapter 4: Amino acid calcium salts: Colloidal & structural stability of α-lactalbumin

4.1 Introduction

During dairy processing, supplementation with salts is necessary to ensure the healthy growth of an infant fed with IMF (Cockell, 2003; Molska et al, 2014) and adequate recovery for athletes using whey based sports drinks (Tipton, 2015). The addition of calcium to IMF is usually in the form of calcium chloride and calcium hydroxide (Riou et al, 2011; On-nom, 2012; Kaushik et al, 2014). Little exploration of alternate calcium sources for use in IMF and other dairy based beverages has been conducted. Hence, studies on protein stability in the presence of other calcium additives are potentially valuable. Many studies have assessed the impact of calcium on the solution and thermal stability of α-lac (Hendrix et al, 2000; Atri et al, 2010; Albis-Arrieta et al, 2011; Crowley et al, 2016). In other contexts, (i.e. the biopharmaceutical industry) amino acid are used as excipients to improve protein structural stability (Golovanov et al, 2004; Das et al, 2007; Bozormehr & Monhemi, 2015) by increasing the amount of thermal energy required to unfold it (Platts & Falconer, 2015; Bozorgmehr & Monhemi, 2015). While not a standard additive in dairy processing, the use of amino acids may offer advantages not currently exploited in this sector, offering both nutritional supplementation and possibly a route to more convenient processing.

In chapter 3, we demonstrated that high concentration solutions of α-lac retain a significant amount of stability at higher calcium concentrations; here, we assess if adding calcium in the form of a calcium amino acid salt could increase stability and improve processibility of dairy beverages (Fox et al, 2007; Tang & Skibsted, 2016).

The use of amino acid calcium salts ($Ca(AA)_2$ could potentially have two advantages when added to a high protein concentration solution. One, as an additional source of nitrogen for athletes to aid muscle recovery (Weinert, 2009) and also to aid the healthy growth of infants using baby formula (Agostoni et al, 2000). The second, a convenient method to add calcium without compromising heat stability. The amino acids glycine and lysine were selected, mainly due to their high affinity for calcium in the deprotonated state (Ho et al, 2007; Tang & Skibsted, 2016). Glycine and Lysine also have been shown to improve protein thermal stability by a preferential hydration effect (Timasheff, 2002; Ghosh et al, 2009; Bye et al, 2014; Shah et al, 2016; Sudrik et al, 2017). Using purified α-lac solutions, we added calcium in the form of calcium amino acid salts of glycine and lysine ($Ca(AA)_2$) (figure 4.1) and assessed the thermal and solution stability of the mixtures and also the aggregation profile of α-lac after heat induced denaturation of the protein in the presence of the amino acid salts.

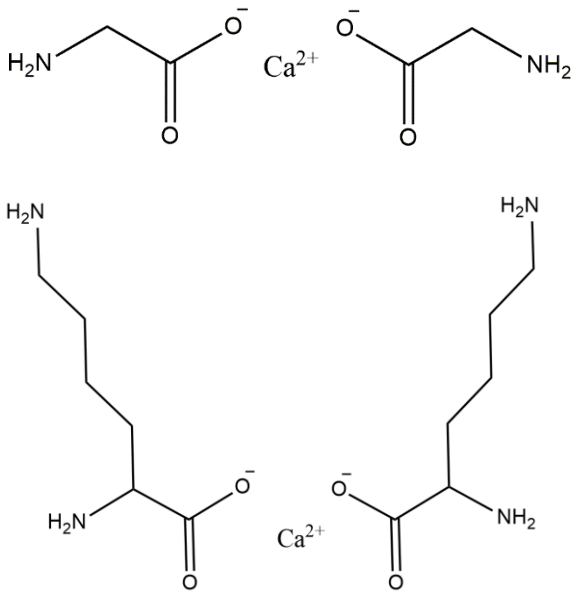


Figure 4.1: *Structure of calcium glycinate $Ca(Gly)_2$ (top) and calcium lysinate $Ca(Lys)_2$ (bottom).*

In this chapter, we describe the synthesis of calcium amino acid salts and characterise them using H-NMR, C-NMR and IR-spectroscopy (Kremer et al, 2001). Calcium glycinate ($Ca(Gly)_2$) and calcium lysinate ($Ca(Lys)_2$) (figure 4.1) can be prepared by redox reaction using the desired amino acid and calcium hydroxide ($Ca(OH)_2$), exactly as described by Fox (Fox et al, 2007). The calcium content of the product was also determined using F.A.A.S and volumetric analysis and the amino acid content was determined by refractive index. The thermal stability of α-lac was determined by DSC and the solution stability was measured using QELS for unheated solutions and using QELS, HPLC and SDS-PAGE to quantify aggregation in α-lac solutions that have been heat denatured.

4.1.2 Aim of the study

The aim of the chapter is to determine the impact of $Ca(AA)_2$ concentration on a high α-lac concentration protein solution. This will first be assessed in terms of colloidal stability, then thermal stability to associate a relationship between the two. Aggregates that do form will be assessed in terms of solubility, size and distribution using SE-HPLC and SDS-PAGE across a broad calcium concentration range using calcium from $CaCl_2$, $Ca(Gly)_2$ and $Ca(Lys)_2$.

4.2 Results

4.2.1 Characterisation of calcium bound amino acids and amino acid reactants

(All synthesis and characterisation experiments were performed by Dr. Denise Rooney, Maynooth University)

4.2.1.1 Glycine & $Ca(Gly)_2$ H-NMR

Calcium glycinate ($Ca(Gly)_2$) and calcium lysinate ($Ca(Lys)_2$) were synthesised in an acid-base reaction (section 2.1.2.3 in methods). The pure amino acid and calcium bound products were characterised using H-NMR, C-NMR and IR-spectroscopy (figures 4.2-4.7).

Glycine was dissolved in D_2O for H-NMR and spectra were collected from 0 – 7.5 ppm. This was repeated using $Ca(Gly)_2$ and peaks were assigned as per figure 4.2.

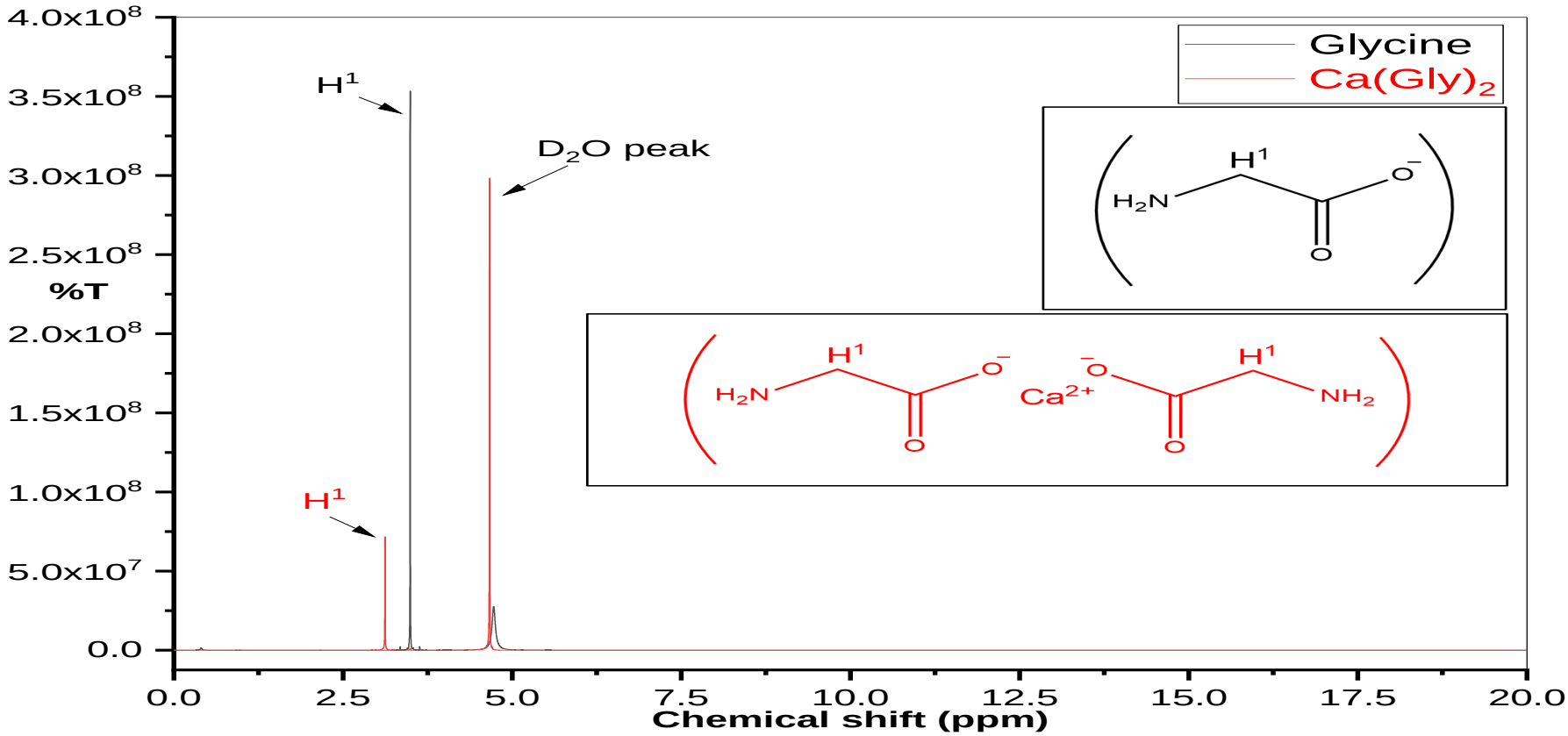


Figure 4.2: *H-NMR spectra of glycine & $Ca(Gly)_2$ dissolved in D_2O.*

Two peaks were recorded in both the glycine and $Ca(Gly)_2$ H-NMR spectra (figure 4.2). The ~4.8 ppm peak is likely to correspond to either a H_2O contaminant from D_2O or proton exchange with the D_2O solvent, forming H_2O. No peaks corresponding to NH_2 were recorded possibly due this proton exchange with the deuterium ions of the D_2O solvent, which commonly occurs in protein, peptide and amino acid solutions, in which Deuterium: Hydrogen ion displacing also occurred in the characterisation of 10 different globular proteins in D_2O (Suvorina et al, 2012) and free amino acids (Wang et al, 2014; Yamazaki et al, 2017).. This spectra was in good agreement with the glycine standard of BMRB (Biological Magnetic Resonance Data Bank, code bmse000089) where H-NMR conditions were the same as here (using 500 Mhz spec NMR) and glycine was suspended in 100% D_2O giving two peaks, assigned the same way as indicated here. The exact bond angles of glycine are also indicated in table 4.1. This experiment was repeated using C-NMR to verify the reactant and product purity.

Table 4.1: *Table indicating the bond angles associated with protonated glycine. Adapted from Iijima et al, 1991.*

Bond	Angle (°)
H – N – H	110.3
H – N – C	113.3
N – C – C	112.1
H – C – H	107.0
C – C – O	111.6
C – C = O	125.1
C – O – H	112.3

4.2.1.2 Glycine & $Ca(Gly)_2$ C-NMR

Glycine was dissolved in D_2O for C-NMR and spectra were collected from 0 – 230 ppm. This was repeated using $Ca(Gly)_2$ and peaks were assigned as per figure 4.3.

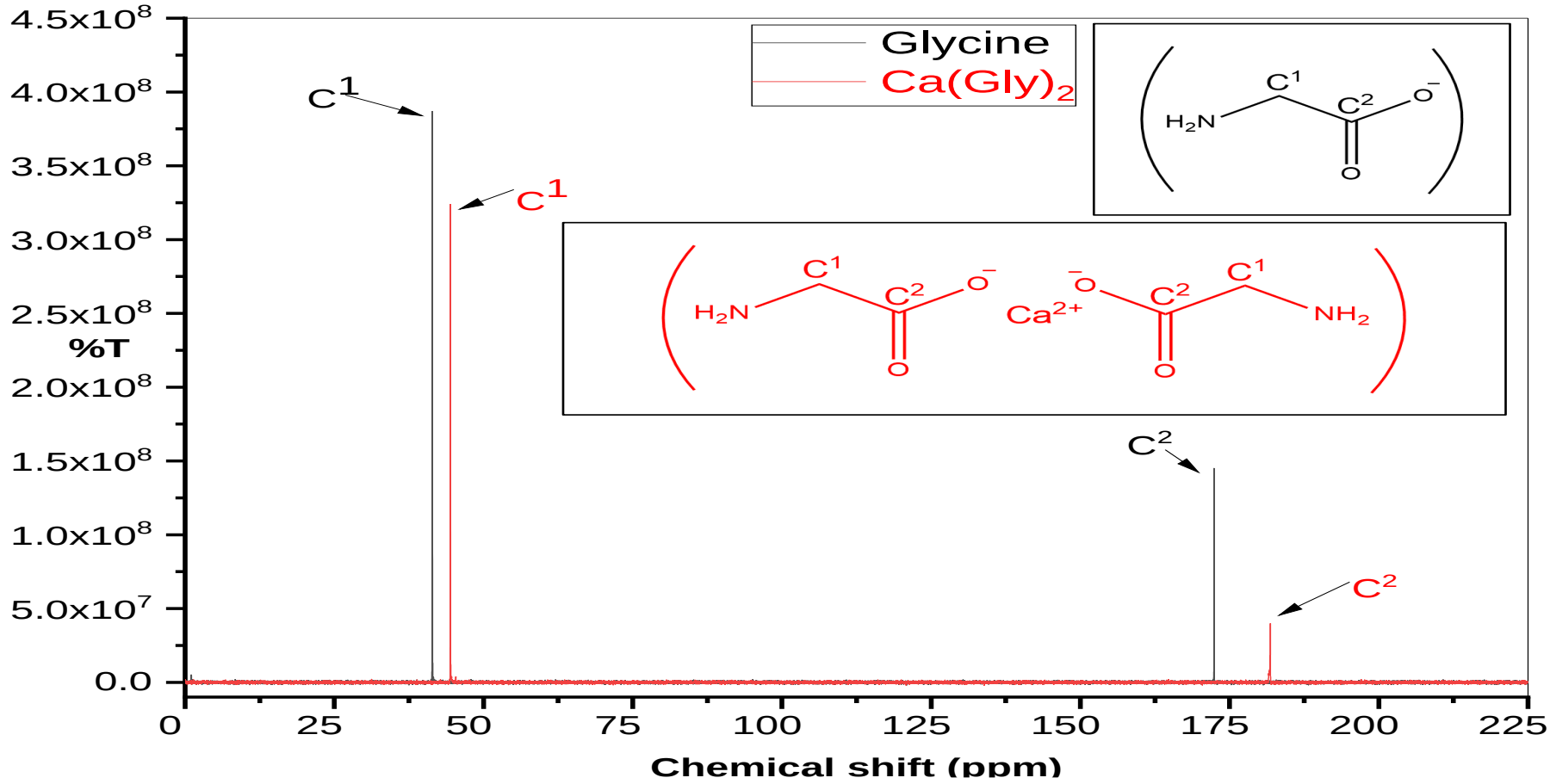


Figure 4.3: *C-NMR spectra of glycine & $Ca(Gly)_2$ dissolved in D_2O.*

Two peaks were recorded in both the glycine and $Ca(Gly)_2$ C-NMR spectra (figure 4.3). The peak located at ~42 - 45 ppm was due to the C^1 carbon (indicated in the inset of figure 4.3). The peak located at 170 - 180 ppm was due to the C^2 carbon corresponded to the COO^- group of glycine, this peak is the same for all C-NMR conducted here. This spectrum was in good agreement with the glycine standard of BMRB (Biological Magnetic Resonance Data Bank, ID bmse000089), where glycine was also suspended in 100% D_2O. Here two peaks were produced, one indicating the COO^- group (at 175 ppm) and the α-carbon (44 ppm). Though the chemical shifts were marginally different compared to the BMRB standard, the peak orders were the same.

4.2.1.3 Glycine & $Ca(Gly)_2$ IR-Spectroscopy

Solid state IR-Spectroscopy were performed on glycine & $Ca(Gly)_2$ and spectra were collected in the 400 - 4000 cm^{-1} range (figure 4.4).

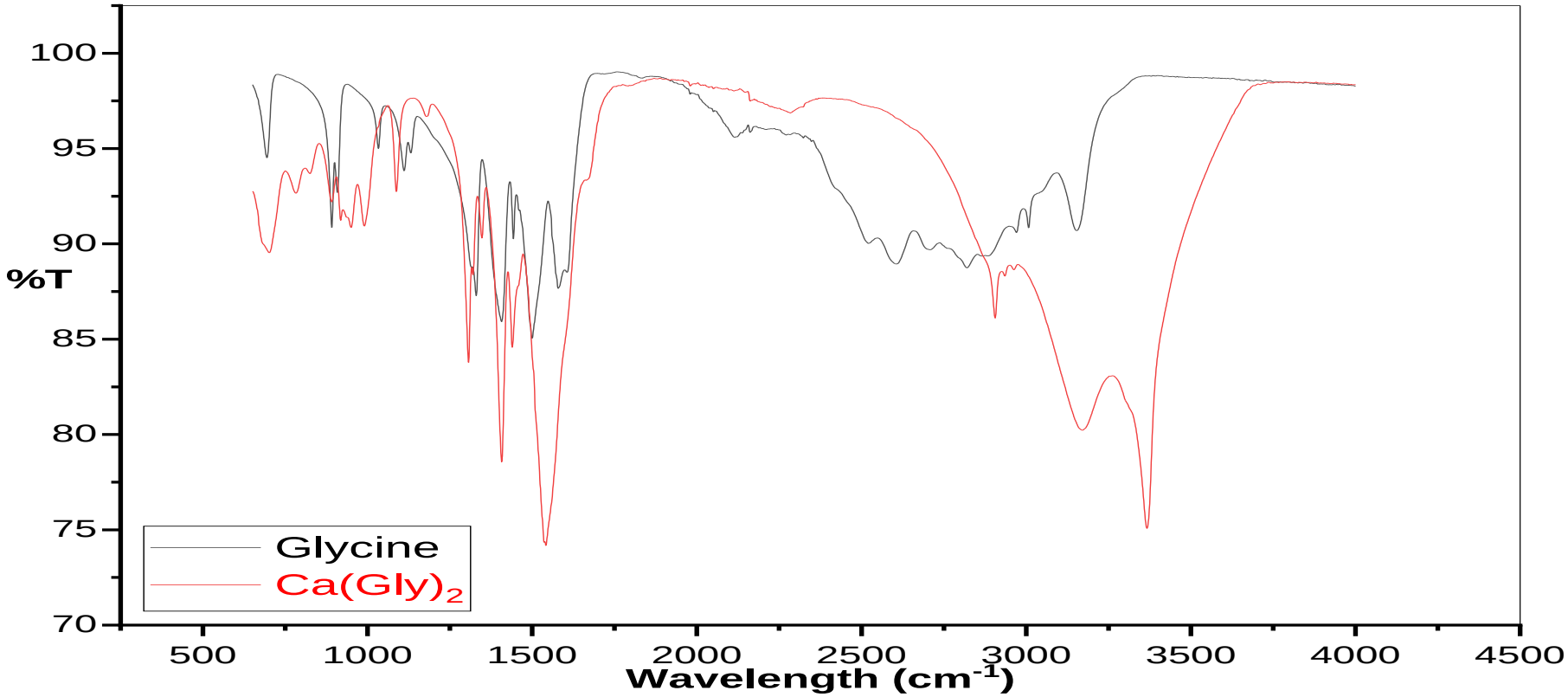


Figure 4.4: *IR spectrum of glycine & $Ca(Gly)_2$.*

IR-Spectra comparing glycine and $Ca(Gly)_2$ (figure 4.4) were performed to confirm product purity. The IR spectrumof glycine, indicates that it is present in the deprotonated state, evident by NH_2^+ and NH_3^+ absorptions at ~2500 and 2750 cm^{-1}, which are absent in the calcium complexes. A strong absorption in the 1500- 1600 cm^{-1} range indicative of the COO^- stretches also appears in the complex when the free amino acid bond stretches to a calcium ion (similar to Fox et al, 2007). The carboxyl stretches measured here in the 1410-1541 cm^{-1} region also compare well to literature values of 1406-1567 cm^{-1} (Fox et al, 2007). The H-NMR, C-NMR and IR data together indicate that the reactants used were not contaminated with other components and that the $Ca(Gly)_2$ product was formed.

4.2.1.4 Lysine and $Ca(Lys)_2$ H-NMR

Lysine was dissolved in D_2O for H-NMR and spectra were collected from 0 – 7.5 ppm. This was repeated using $Ca(Lys)_2$ and peaks were assigned as per figure 4.5.

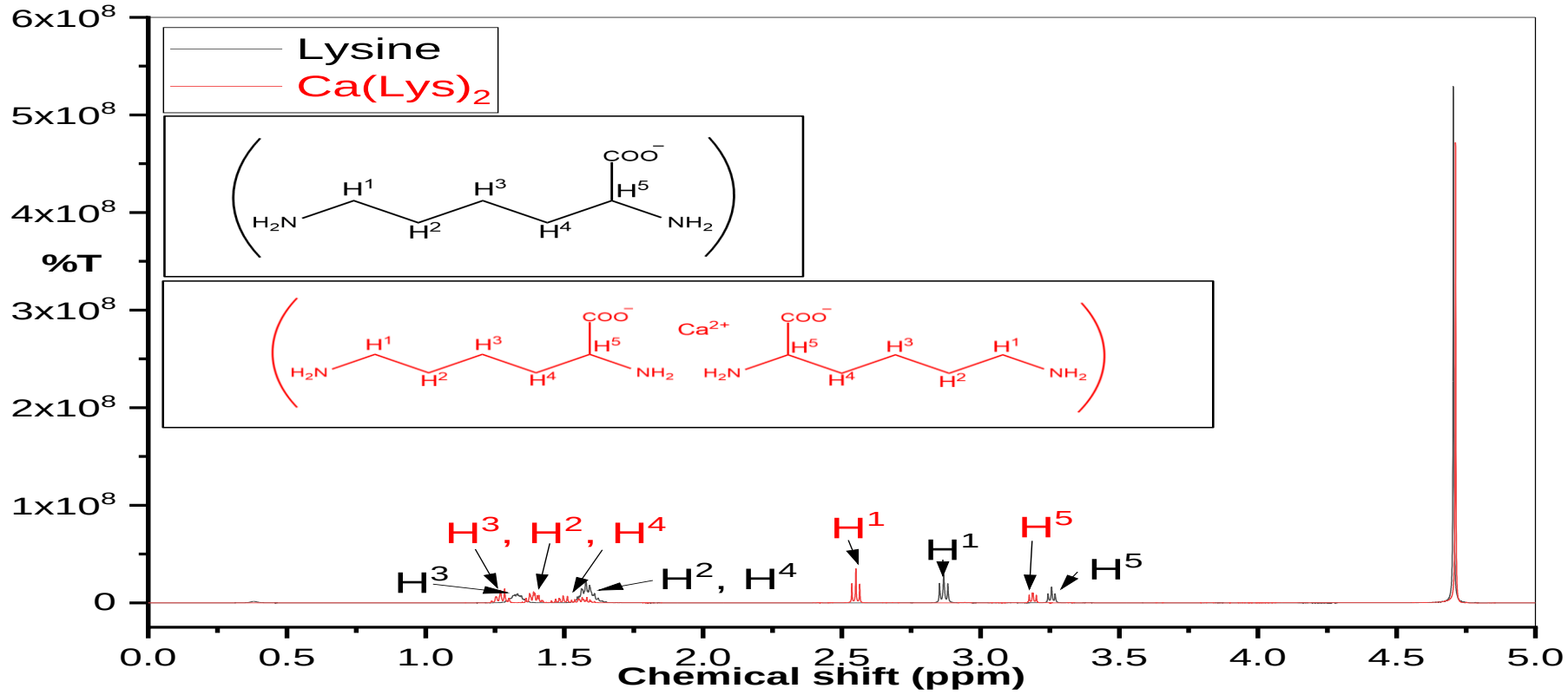


Figure 4.5: *H-NMR spectra of lysine & $Ca(Lys)_2$ dissolved in D_2O.*

Several peaks were recorded in both the lysine and $Ca(Lys)_2$ H-NMR spectra (figure 4.5), summarised in table 4.2. All peaks were assigned as per figure 4.5. This spectrum was in agreement with the lysine standard of BMRB (Biological Magnetic Resonance Data Bank) code (bmse000043, lysine in D_2O), with the exception of the H^2 and H_4 peaks which did not resolve as well here compared to the BMRB standard. Though the chemical shifts were slightly different compared to the BMRB standard, the peak elution orders were the same.

Table 4.2. *Comparision of H-NMR data between lysine and $Ca(Lys)_2$ in D_2O, relative to BMRB standard*

Proton number	**Chemical shift (ppm) (Lysine)**	**Chemical shift (ppm) (Lysine) BMRB standard**	**Chemcial shift (ppm) $Ca(Lys)_2$**
H^1	2.86	3.0	2.5
H^2	1.57	1.6	1.4
H^3	1.3	1.3	1.3
H^4	1.6	1.7	1.5
H^5	3.3	3.5	3.2

4.2.1.5 Lysine & $Ca(Lys)_2$ C-NMR

Lysine was dissolved in D_2O for C-NMR and spectra were collected from 0 – 230 ppm. This was repeated using $Ca(Lys)_2$ and peaks were assigned as per figure 4.6.

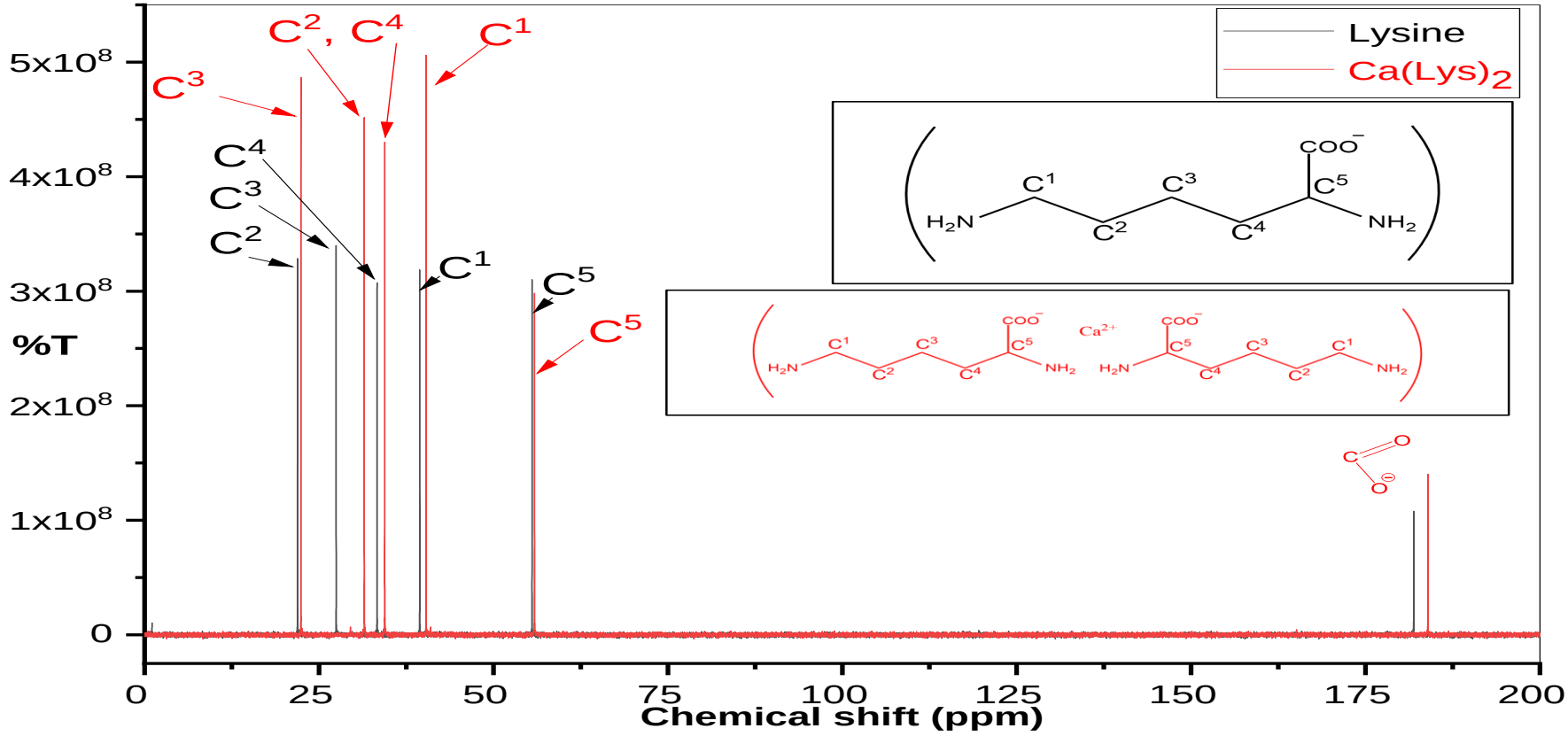


Figure 4.6: *C-NMR spectra of lysine & $Ca(Lys)_2$ dissolved in D_2O.*

Several peaks were recorded in both the lysine and $Ca(Lys)_2$ C-NMR spectra (figure 4.6), summarised in table 4.3. These spectra are consistent with the lysine standard of BMRB code bmse000043, where solvent conditions were the same as here (lysine in 100 % D_2O) and measurements were also performed using a 500 Mhz NMR. Though the chemical shifts were slightly different compared to the BMRB standard, the peak elution orders were the same.

Table 4.3. *Comparision of C-NMR data between lysine and $Ca(Lys)_2$ in D_2O relative to BMRB standard.*

Proton number	**Chemical shift (ppm) (Lysine)**	**Chemical shift (ppm) (Lysine) BMRB standard**	**Chemcial shift (ppm) $Ca(Lys)_2$**
C^1	40.3	41.0	40.0
C^2	21.9	23.0	31.2
C^3	27.3	28.0	22.5
C^4	33.6	32.5	34.2
C^5	55.9	56.0	55.9

4.2.1.6 Lysine & $Ca(Lys)_2$ IR-Spectroscopy

Solid state IR-Spectroscopy were performed on glycine & $Ca(Gly)_2$ and spectra were collected in the 400 - 4000 cm^{-1} range (figure 4.7).

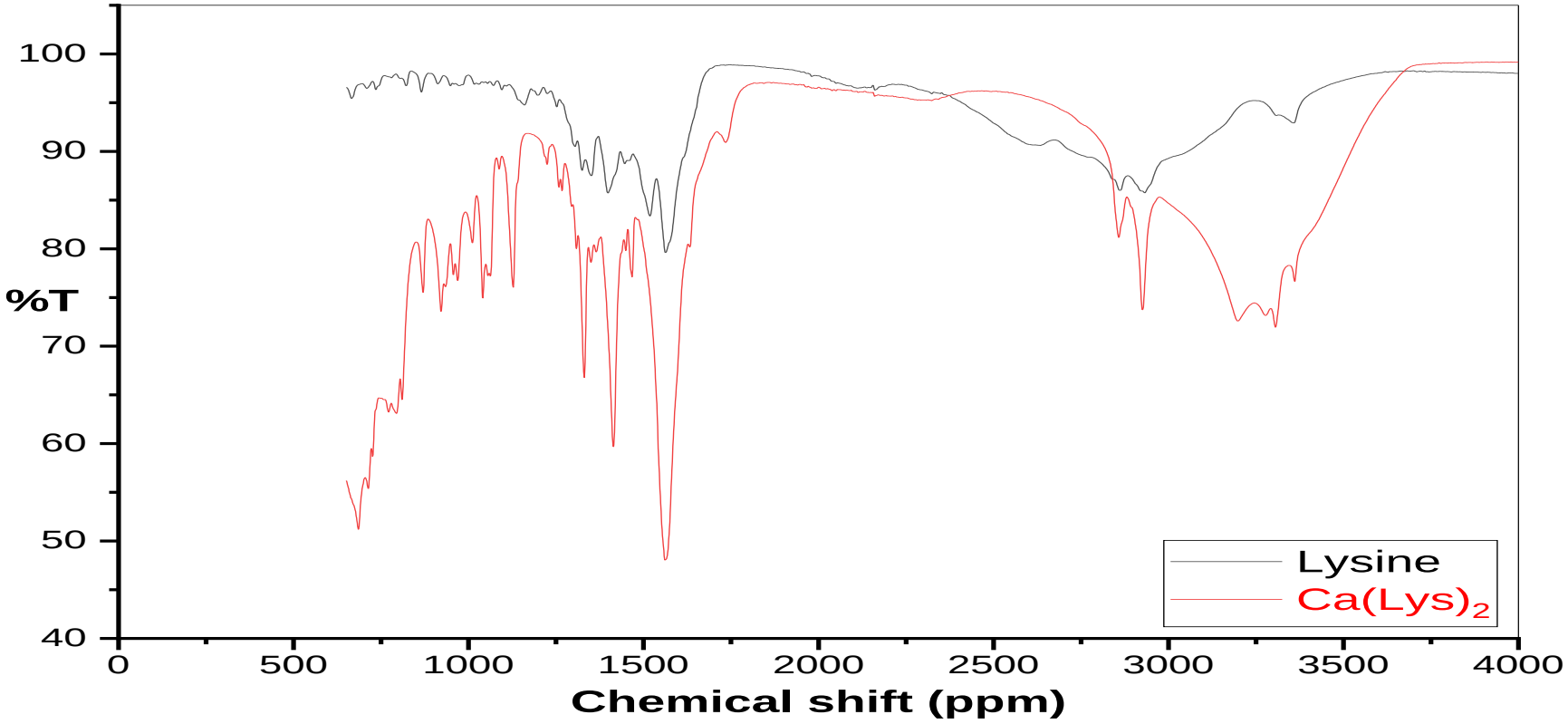


Figure 4.7: *IR spectrum of $Ca(Lys)_2$*

IR-Spectra were acquired comparing both lysine and $Ca(Lys)_2$ (figure 4.7). The IR spectrum of lysine indicates that it is present in the deprotonated state, evident by NH_2^+ and NH_3^+ absorptions at ~2500 and 2750 cm^{-1}, which are absent in the metal complexes. A strong absorption in the 1560 - 1570 cm^{-1} range indicative of the COO^- stretches also appears in the complex when the free amino acid bond stretches to a calcium ion (similar to Fox et al, 2007). The carboxyl stretches measured here of 1410-1541 cm^{-1} compare well to the literature values of 1406-1567 cm^{-1} (Fox et al, 2007).

4.3 Quasi elastic light scattering (QELS)

QELS was used to determine the net interaction parameter (k_D) for α-lac in the presence of $Ca(Lys)_2$ and $Ca(Gly)_2$ at a constant calcium concentration (30 mM) (figure 4.8). These values were compared with k_D values for α-lac in 30 mM $CaCl_2$ and glycine alone (at the same concentration present in the $Ca(Lys)_2$ sample) over a protein concentration range of 10 – 60 mg ml^{-1} at pH 7.0.

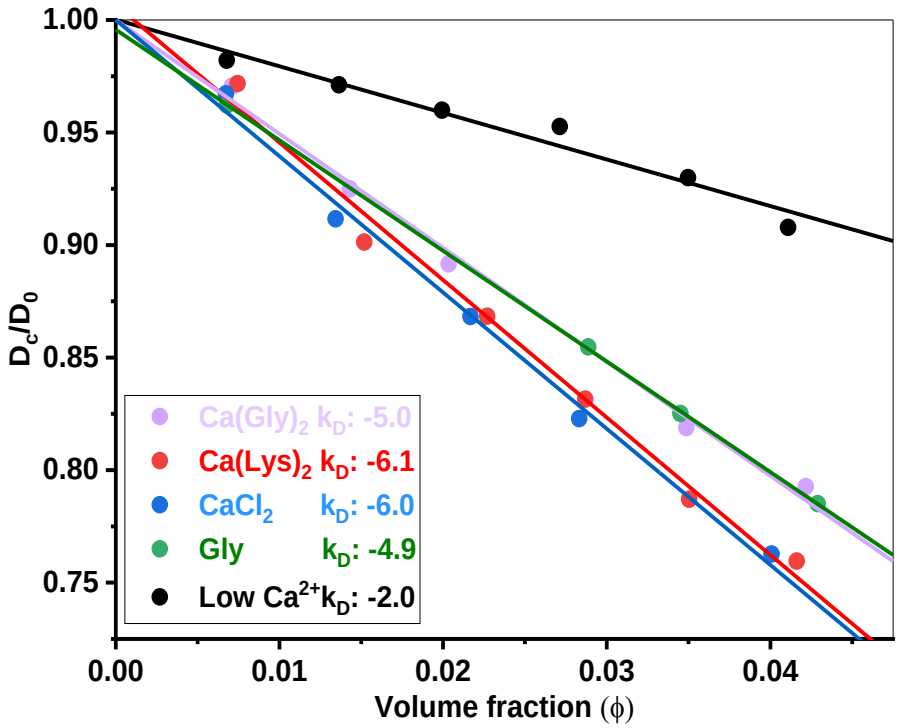


Figure 4.8: *D_c/D_0 as a function of volume fraction for α-lac with calcium, amino acid and $Ca(AA)_2$. The $CaCl_2$, $Ca(Gly)_2$ and $Ca(Lys)_2$ samples contained 30 mM calcium. The buffer used was 0.05 M Tris-HCl, pH 7.0. The D_0 value calculated was 1.92 × 10^{-9} m^2/s. The error in the k_D value for the low calcium reference sample (black) was +/- 0.18, determined by the Microsoft Excel function LINEST.*

The k_D for purified α-lac in buffer, which has a calcium concentration of less than 1 mM is -2.0, which decreases to – 6.0 in 30 mM calcium chloride. α-lac in the presence of 33 mg ml^{-1} glycine had a k_D value of – 4.9, which is approximately the same as in a solution containing $Ca(Gly)_2$. α-lac in the presence of $Ca(Lys)_2$ (30 mM calcium) had a k_D of -6.1 vs 6.0 in the $CaCl_2$ solution.

The hydrodynamic radius (nm) of α-lac in solution was measured using QELS (figure 4.9 – 4.11) and used to measure aggregate formation in α-lac solutions containing calcium after heat induced denaturation. The size distribution for α-lac in low calcium

indicates a hydrodynamic radius of ~2 nm before heating (figure 4.9A). All solutions were heated for 5 min at 105 °C and the hydrodynamic radius measured for samples containing <1 mM calcium, 30mM $CaCl_2$, $Ca(Gly)_2$ and $Ca(Lys)_2$. Measurement of the hydrodynamic radius was also done in the presence of $(Ca(AA)_2$ without heat applied. A baseline was established using α-lac with <1 mM $Ca^{2+,}$ which was unheated.

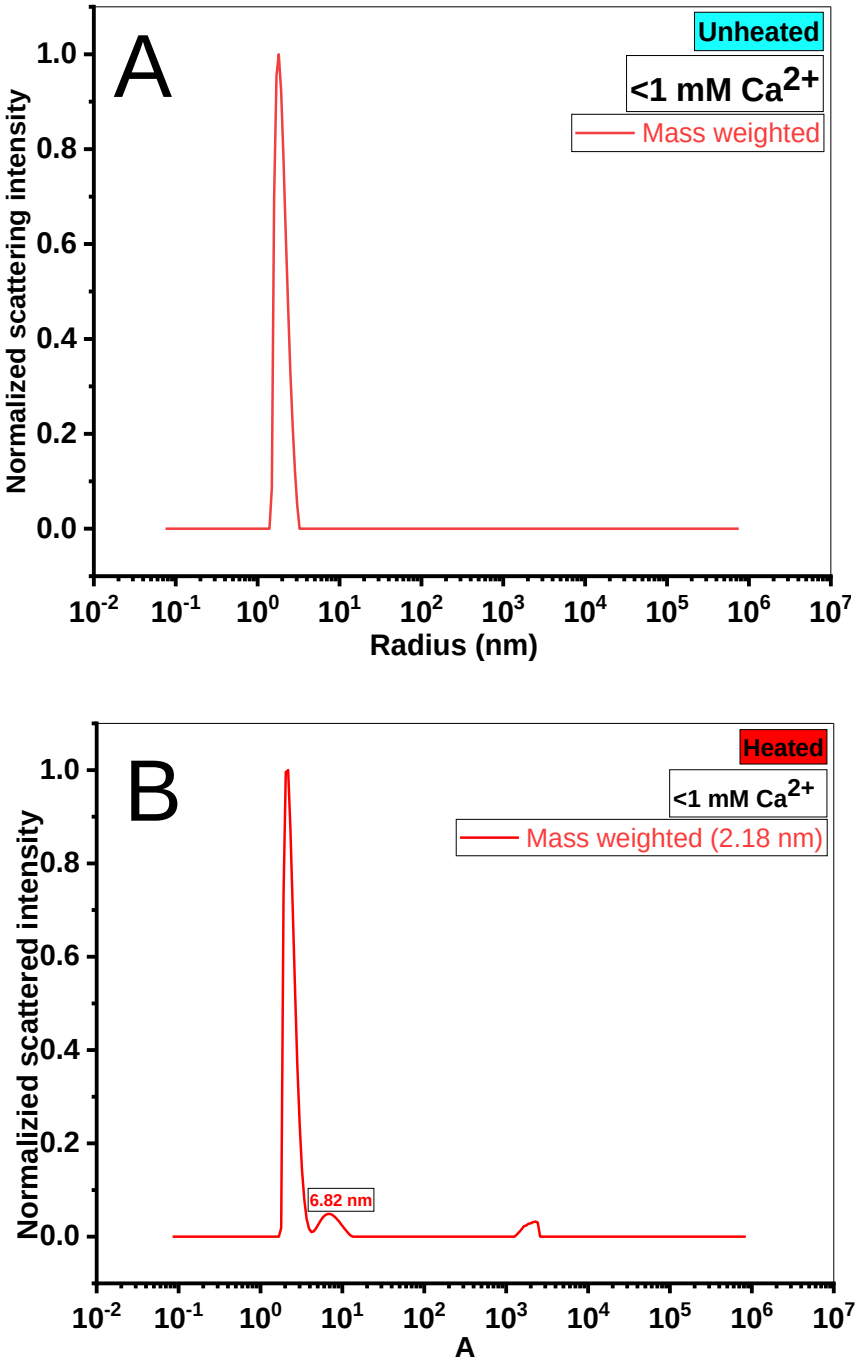


Figure 4.9: *QELS runs indicating the average radius of unheated (A) and heated (B) solutions of 10 mg ml^{-1} α-lac in 0.05 M Tris-HCl, pH 7.0 (<1 mM Ca^{2+}), indicating the average size distribution obtained from a mass weighted fit to the autocorrelation function.*

The hydrodynamic radius measured was 1.82 nm for the sample pertaining to figure 4.9A (α-lac in 0.05 M Tris-HCl, pH 7.0, unheated), which was in good agreement with the literature value measurement (1.88 nm) (Gast et al, 1998). After heat was applied, an increase in average aggregate size was measured (to 2.18 nm) as well as larger aggregate formation (>6.82 nm). This experiment was then repeated using $Ca(AA)_2$ as a calcium source.

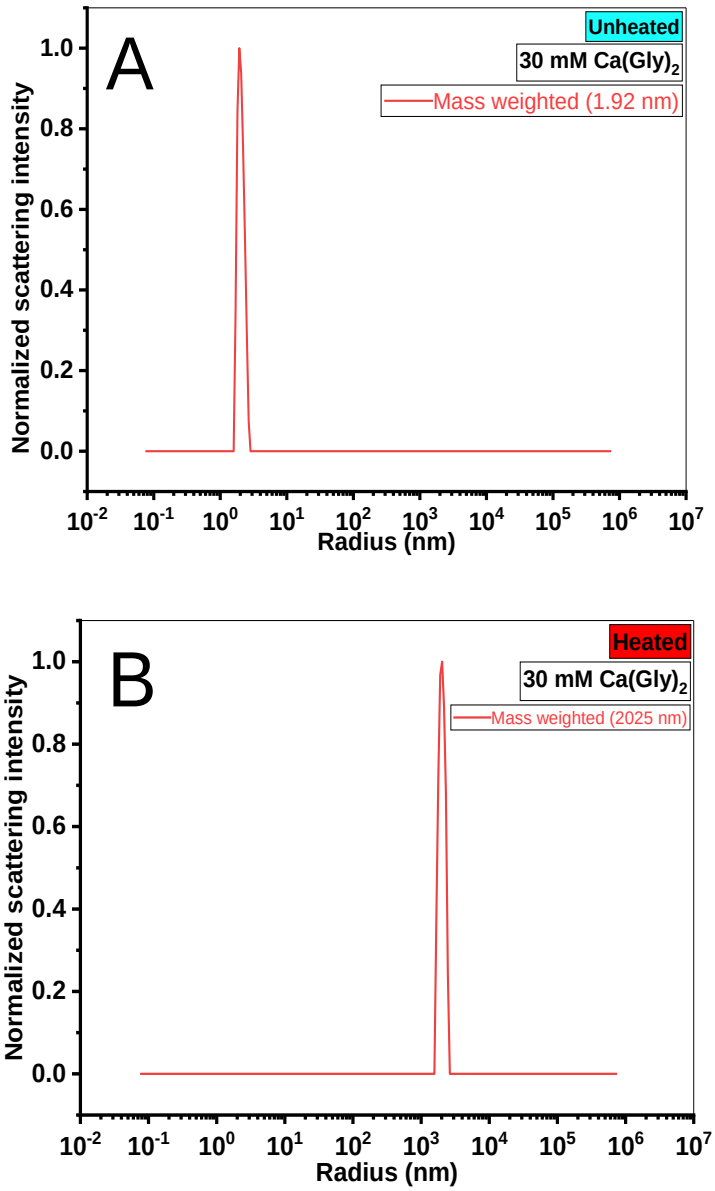


Figure 4.10: *QELS runs indicating the average radius of unheated* ***(A)*** *and heated* ***(B)*** *solutions of 10 mg ml^{-1} α-lac in 0.05 M Tris-HCl, pH 7.0 (with 30 mM of calcium from $Ca(Gly)_2$) indicating the average size distribution obtained from a mass weighted fit to the autocorrelation function.*

The addition of the $Ca(Gly)_2$ salts increased the average hydrodynamic radius to 1.92 nm, which may be indicative of the formation of low molecular weight aggregates, increasing the average radius. After heating the average radius increased in all solutions to ~ 2500 nm, which is indicative of the presence of large aggregates.

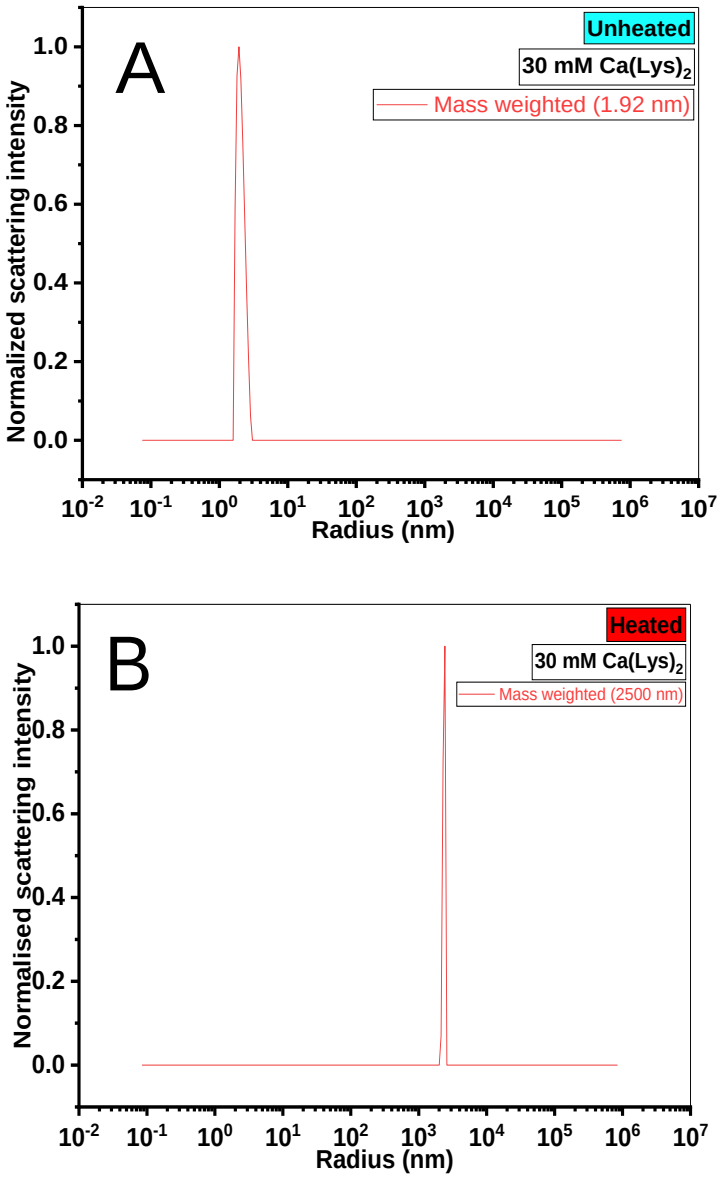


Figure 4.11: *QELS runs indicating the average radius of unheated **(A)** and heated **(B)** solutions of 10 mg ml^{-1} α-lac in 0.05 M Tris-HCl, pH 7.0 (with 30 mM of calcium from $Ca(Lys)_2$) indicating the average size distribution obtained from a mass weighted fit to the autocorrelation function.*

The addition of the $Ca(Lys)_2$ salts increased the average radius to 1.92 nm, which may be indicative of the formation of low molecular weight aggregates, increasing the average

radius. After heating the average radius increased in all solutions to ~ 2500 nm, which is indicative of the presence of large aggregates.

Clearly, after heating, both aggregated protein and a significant protein of monomer/small oligomer remain in solution, regardless of the calcium salt present. To further characterise differences between the different samples, we used a high throughput approach (measuring sample turbidity after heating) to examine the effect of increasing calcium concentration on the aggregation of α-lac when heated. In addition, following heating (and subsequent aggregate formation), precipitated protein was sedimented by centrifugation, and the supernatant removed for further analysis. In the supernatant, total protein content was determined by UV absorbance and a combination of SE- HPLC and SDS-PAGE was used to determine the size distribution of oligomers remaining in solution in the supernatant.

4.4 High throughput Assay

A high throughput assay was used as a method of screening a broad range of calcium and $Ca(AA)_2$ concentrations (figure 4.12 - 4.13). This assessed which source of calcium and at what concentration resulted in a loss of total protein mass (wt %), after heat denaturation (105 °C for 60 min). For all high throughput assays, purified α-lac was used (3.2.1 method). Protein concentration was kept constant at 10 mg ml^{-1}. All work was performed in 0.05 M Tris-HCl, pH 7.0 using a transparent 96 well plates. Before each measurement set, the entire plate was shaken for 30 seconds.

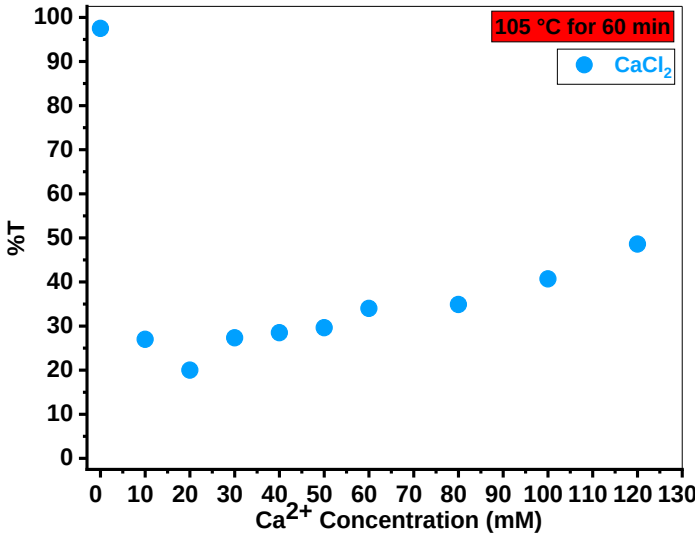


Figure 4.12: *% transmittance as a function of calcium concentration for α-lac with increasing $CaCl_2$ concentration. All samples were prepared in 0.05 M Tris-HCl, pH 7.0.*

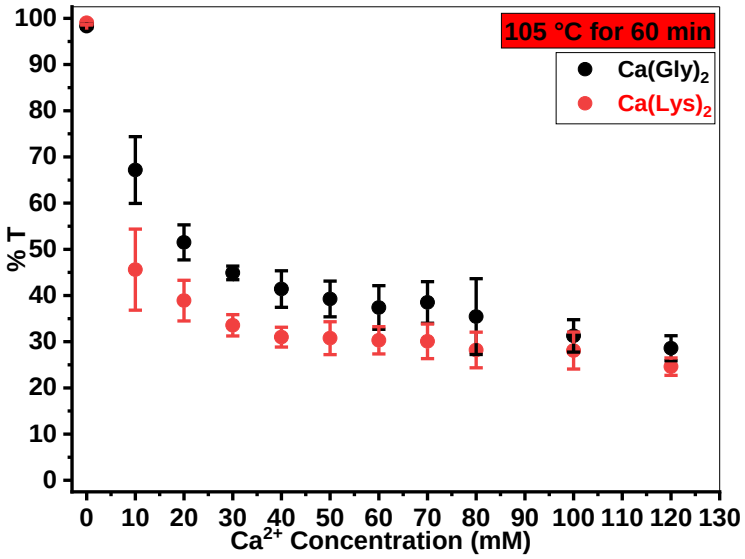


Figure 4.13: *% transmittance as a function of calcium concentration for a-lac with increasing calcium concentration from $Ca(Gly)_2$ and $Ca(Lys)_2$. All samples were prepared in 0.05 M Tris-HCl, pH7.0.*

The change in turbidity with added $CaCl_2$ is shown in figure 4.12. These results indicate that significant amounts of protein aggregation occur at all calcium concentrations and that this appears to be most aggregated at 20 mM $CaCl_2$. If we compare this to data collected for α-lac in the presence of Ca $(AA)_2$ (figure 4.13), we see that the % transmission decreased less for the samples containing calcium as amino acid salts, but that aggregation was also observed in these samples. However, the data does suggest that there may be a difference in either the total amount of aggregated protein or the size ranges of the aggregates, depending on the form of the calcium salt added.

4.5 UV-Vis Spectroscopy

UV-Vis Spectroscopy was used to quantify total protein in the supernatant of samples separated from precipitated protein, and indirectly, the amount of protein lost to irreversible aggregation was every sample (figure 4.14). After heat treatment and centrifugation to remove larger aggregates, the supernatant was analysed. The concentration of all samples used for SE-HPLC was determined using α-lac extinction coefficient of 2.01 ml mg^{-1} cm^{-1}.

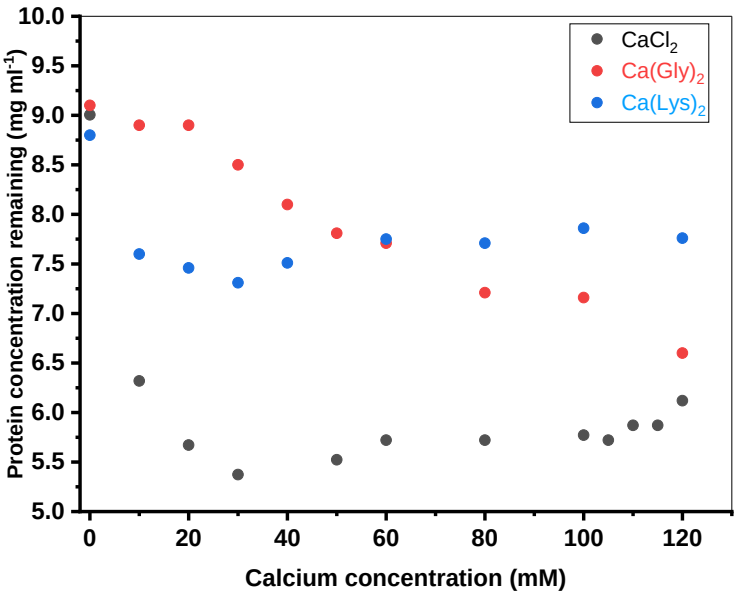


Figure 4.14: *Change in α-lac concentration in sample supernatant with increasing calcium concentration (added as amino acid salts) in 0.05 M Tris-HCl, pH 7.0. Concentration was determined by UV absorbance and indicates the concentration of monomer and small oligomer remaining after heat induced denaturation (starting protein concentration was 10 mgml^{-1}).*

In figure 4.14 we see significant differences between the effects of the two amino acid salts. While approx. 25 % of protein is in the form of large aggregates for the Ca $(Lys)_2$ sample in the 10 – 40 mM calcium range, this varies from 10 – 20% with increasing calcium concentration over the same 10 – 40 mM range for the $Ca(Gly)_2$ salt. Both are different to the $CaCl_2$ sample by allowing a greater concentration of α-lac to remain soluble and by extension caused less insoluble aggregates to form.

4.6 SE-HPLC

SE-HPLC was used to quantify the remaining monomer and small oligomers in the protein solutions (supernatant) after heating to compare the effects on aggregation for the different calcium salts. For comparison, a heated sample of α-lac in 0.05 M Tris-HCl, pH 7.0, with 30 mM calcium chloride was measured (figure 4.15). Monomer/dimer/trimer peaks were assigned by comparison with molecular weight standards used to calibrate the column. The same procedure was then repeated using increasing amounts of $CaCl_2$, $Ca(Gly)_2$ and $Ca(Lys)_2$ (figures 4.16 - 4.18). The proportions of each component were determined by integration of the area under the curve.

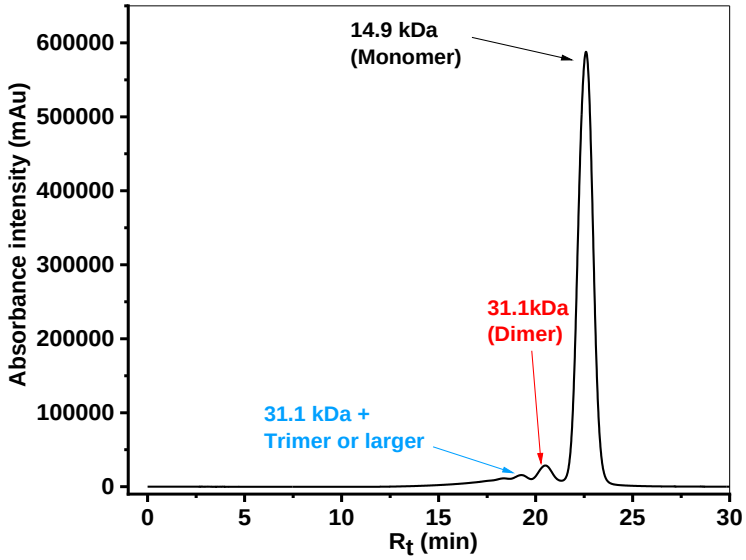


Figure 4.15: *SE-HPLC chromatogram showing the separation of the oligomer components of heated α-lac in 0.05 M Tris-HCl, pH 7.0 w/30 mM $CaCl_2$.*

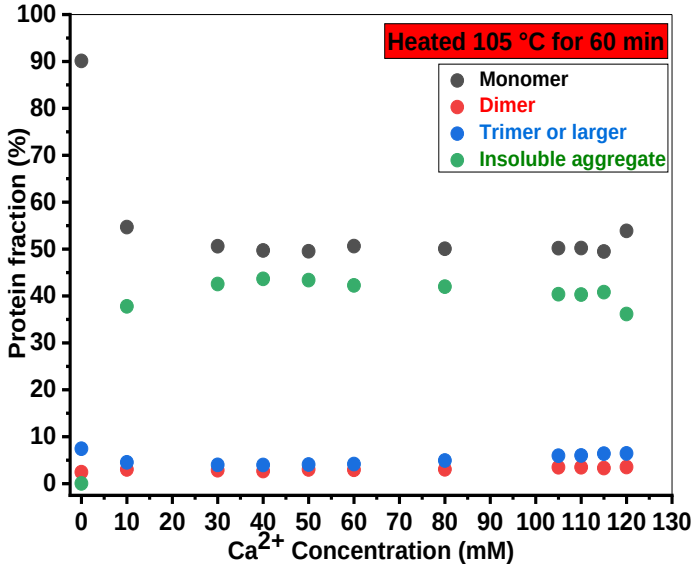


Figure 4.16: *Quantities of insoluble aggregates (from UV absorbance), monomer and oligomers present in heated α-lac solutions in 0.05 M Tris-HCl, pH 7.0 with increasing calcium concentration ($CaCl_2$).*

As calcium was increased in samples containing $CaCl_2$ (figure 4.16), the lowest % monomer retention (~ 50 %) occurred at 20-30 mM calcium and this was maintained as calcium concentration was increased further, indicating no additional calcium added after 30 mM resulted in increased oligomer formation (consistent with the observation of Hendrix et al (Hendrix et al 2000). Insoluble aggregates were in the region of 40%, with small oligomers

(dimer/trimer) comprising the other 10%. Increases in dimer and larger small oligomers quantity were also recorded, the more calcium present in solution.

The same experiment was repeated by increasing calcium concentration using $Ca(Gly)_2$ (figure 4.17). Across the entire calcium concentration range, higher proportions of monomer and smaller amounts of insoluble aggregates are present, representing a significant improvement over $CaCl_2$. The degree of oligomer formation remains constant at less than 5% of the total. As monomer concentration increases, this is reflected in greater proportions of insoluble aggregates.

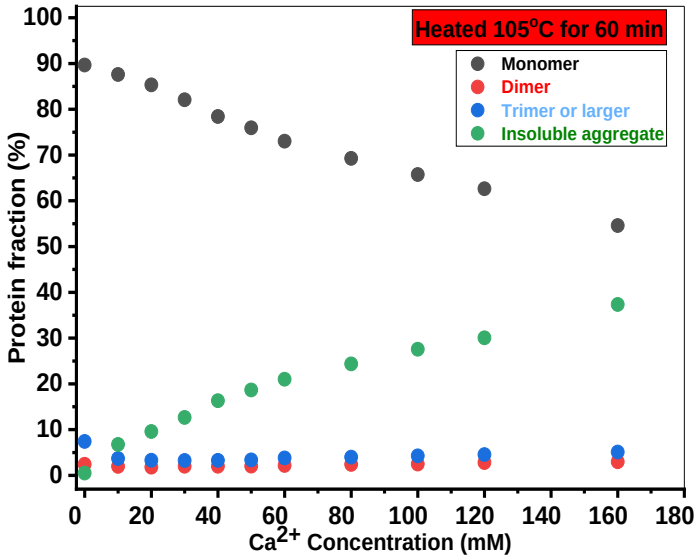


Figure 4.17*: Graph comparing the quantities of oligomer present in heated α-lac solutions with increased calcium concentration ($Ca(Gly)_2$)*

There are few studies on $Ca(AA)_2$ salts and their effect of the stability of α-lac (or any other protein). However, some work has been done examining the role of glycine on the aggregation thermodynamics for proteins, where a higher concentration of amino acid contributes to a higher T_m and C_p (Sabulal & Kishore, 1995; Sabulal and Kishore, 1997; Tang and Skibsted, 2016). The protein concentration in those studies was lower (<1.0 mg ml^{-1}) compared to the higher concentration experiments here.

Repeating this experiment using $Ca(Lys)_2$ (figure 4.18) resulted in a similar profile to α-lac solutions containing $CaCl_2$, but these solutions do yield a greater monomer retention of 65-70 % compared to 50 % and a lower insoluble aggregate formation.

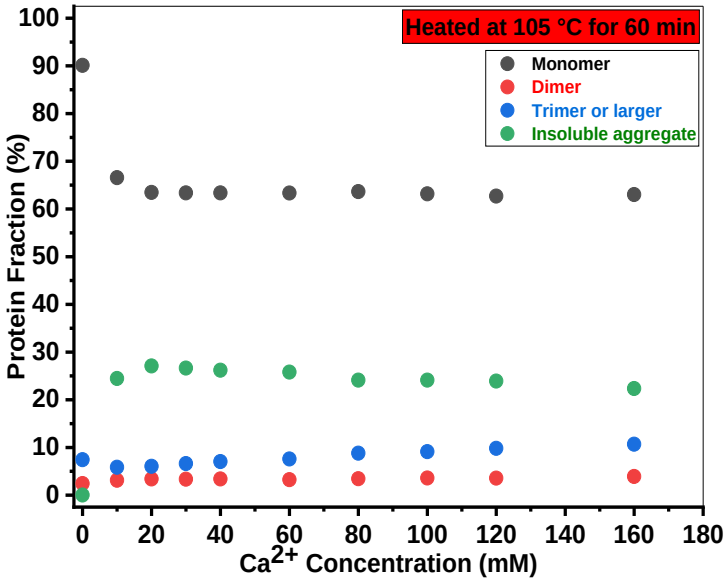


Figure 4.18: *Graph comparing the quantities of oligomer present in heated α-lac solutions with increased calcium concentration ($Ca(Lys)_2$)*

$Ca(Lys)_2$ did result in the largest amount of soluble aggregates formed (dimer and trimer). Almost no research has been conducted using $Ca(Lys)_2$ as a method of adding calcium to an α-lac solution. However, for lysine as an additive, a considerable amount of work has been performed on globular proteins, which indicates that lysine contributes to greater soluble protein concentrations in stability studies, which is consistent with the effects that we observe here (Sabulal & Kishore, 1995; Shiraki et al, 2002). All data from fig 4.16-4.18 is summarised in table 4.4.

Table 4.4: *Table summarising the data from figures 4.16 – 4.18.*

Ca^{2+}Concentration (mM)	% Monomer	% Dimer	%Trimer or Larger	% Insoluble Aggregate
$CaCl_2$				
0	90.1	2.4	7.4	0
10	54.7	3.0	4.5	37.7
30	50.6	2.9	4.0	42.5
40	49.7	2.7	4.0	43.6
50	49.5	3.0	4.0	43.3
60	50.6	2.9	4.2	42.2
80	50.0	3.0	4.9	42.0
105	50.1	3.5	5.9	40.4
110	50.2	3.3	6.0	40.3
115	49.5	3.3	6.4	40.8
120	53.9	3.5	6.4	36.2
Ca^{2+}Concentration (mM) $Ca(Gly)_2$	% Monomer	% Dimer	%Trimer or larger	% Insoluble aggregate
0	89.6	2.4	7.4	0.5
10	87.6	2.0	3.7	6.8
20	85.3	1.8	3.3	9.6
30	82.1	2.0	3.3	12.7
40	78.4	2.0	3.3	16.3
50	75.9	2.0	3.4	18.7
60	73.0	2.1	3.8	21.0
80	69.3	2.4	4.0	24.4
100	65.73	2.5	4.3	27.6
120	62.6	2.8	4.5	30.0
160	54	3.0	5.1	37.3
Ca^{2+}Concentration (mM) $Ca(Lys)_2$	% Monomer	% Dimer	%Trimer or larger	% Insoluble aggregate
0	90.1	2.4	7.4	0.0
10	66.5	3.1	5.8	24.5
20	63.5	3.4	6.0	27.1
30	63.4	3.4	6.6	26.7
40	63.4	3.4	7.0	26.2
60	63.3	3.3	7.6	25.8
80	63.6	3.4	8.8	24.1
100	63.2	3.6	9.1	24.1
120	62.7	3.6	9.8	24.0
160	63.0	3.9	10.7	22.3

4.7 SDS-PAGE

SDS-PAGE was also performed to measure the quantity of oligomers present (figure 4.19-4.20) and to confirm the data acquired using SE-HPLC. The samples used were the same ones for SE-HPLC.

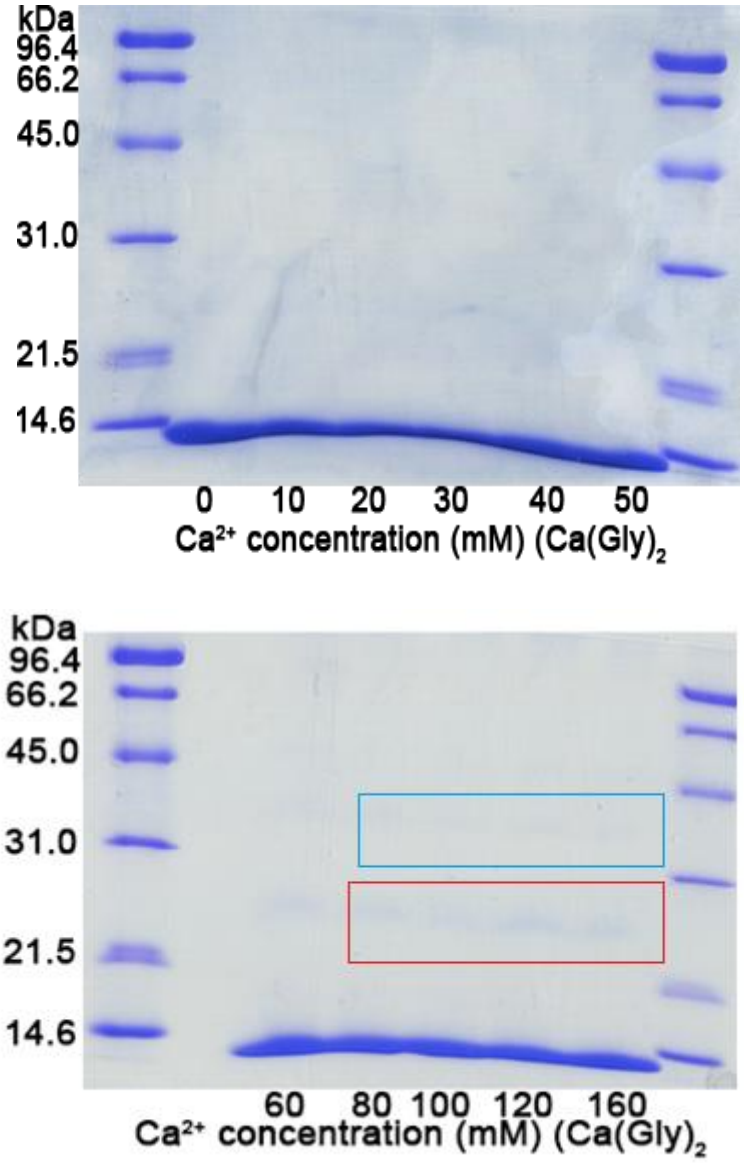


Figure: 4.19: 12.6 % *non-reducing SDS-PAGE gel of α-lac showing a band below the 14.6 kDa standard and weak bands indicative of dimer (red) and trimer(blue) at the higher calcium concentrations (Concentrations in mM are* Ca^{2+} *from* $Ca(Gly)_2$*).*

SDS-PAGE indicates that at all $Ca(Gly)_2$ concentrations, the majority of α-lac exists primarily as monomer, with slightly greater instances of dimer/trimer at 80 mM+ calcium concentrations.

For Ca $(Lys)_2$, higher levels of oligomers are observed across all calcium concentrations, which confirms the results observed by SE-HPLC.

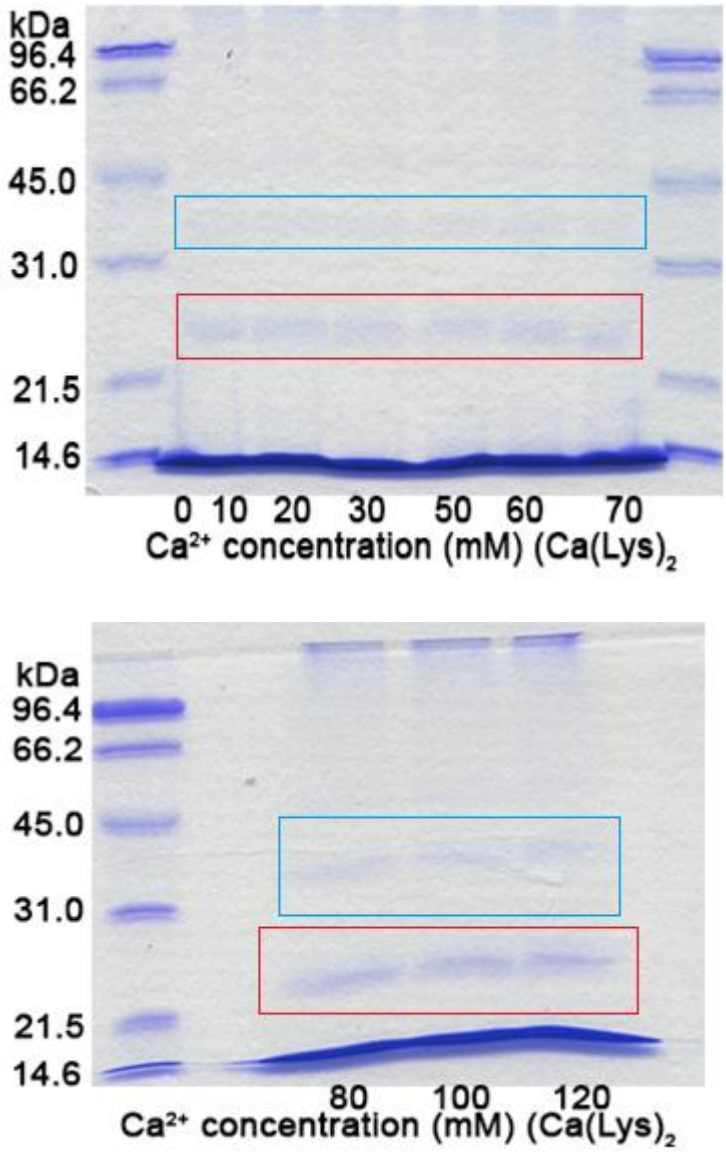


Figure 4.20: *12.6 % non-reducing SDS-PAGE gel of α-lac showing a band below the 14.6 kDa standard and bands indicative of dimer (red) and trimer (blue) at the higher calcium concentrations. (Concentrations in mM are Ca^{2+} from $Ca(Lys)_2$)*

SDS-PAGE indicates that at all $Ca(Lys)_2$ concentrations, the majority of α-lac exists primarily as monomer, with slightly greater instances of dimer/trimer at 80 mM+ calcium concentrations. This was also observed in SE-HPLC where dimer /trimer % was the highest at 80 mM+ $Ca(Lys)_2$.

4.8 DSC

DSC was performed on α-lac samples at a constant calcium concentration to acquire thermodynamic data regarding the aggregation/unfolding of α-lac in the presence of $Ca(AA)_2$ salts (T_m, R.I, ΔH_{me}) (figure 4.21-4.23). Where applicable, the separate components (amino acid and calcium) were also performed to assess if $Ca(AA)_2$ salts are superior in providing additional structural stability to α-lac.

All samples excluding the glycine sample had a calcium concentration of 30 mM (DSC solution parameters summarized in table 4.1). Both the ΔH_{me} and R.I. values were measured for all experiments. A reference point was first established using α-lac with 30 mM calcium to give an R.I. and T_m of 0.54 and 67.8 °C, in good agreement with the literature (Hendrix, 2000; Mittal & Singh, 2014). This experiment was then repeated using glycine as an additive (in low calcium < 1 mM) giving a R.I./T_m of 0.26 and 66.1 °C. This indicates that glycine has a reduced ability to allow refolding in α-lac compared to the sample with calcium chloride, however the ΔH value was reduced, indicating that less thermal energy is required to cause unfolding.

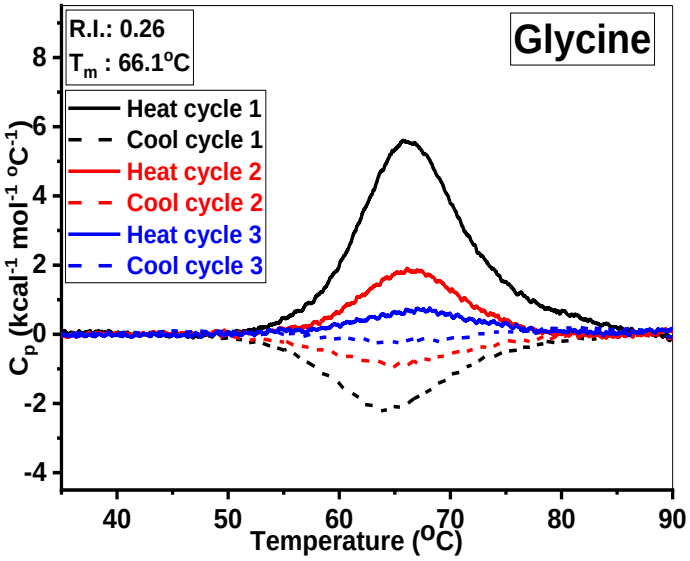


Figure 4.21: *Normalised and baseline subtracted DSC thermogram for α-lac (100 mg ml^{-1}) in 0.05 M Tris-HCl, pH 7.0 (<1 mM Ca^{2+}), with glycine for three heating and cooling cycles*

A sample containing $Ca(Gly)_2$ (fig 4.22) was measured for α-lac with 30 mM Ca^{2+} (from $Ca(Gly)_2$) giving a T_m and R.I. of 0.35 and 68 °C, hence a greater R.I was achieved for the calcium amino acid salt than for glycine alone. A greater T_m than $CaCl_2$ was also obtained. ΔH value for both samples were similar.

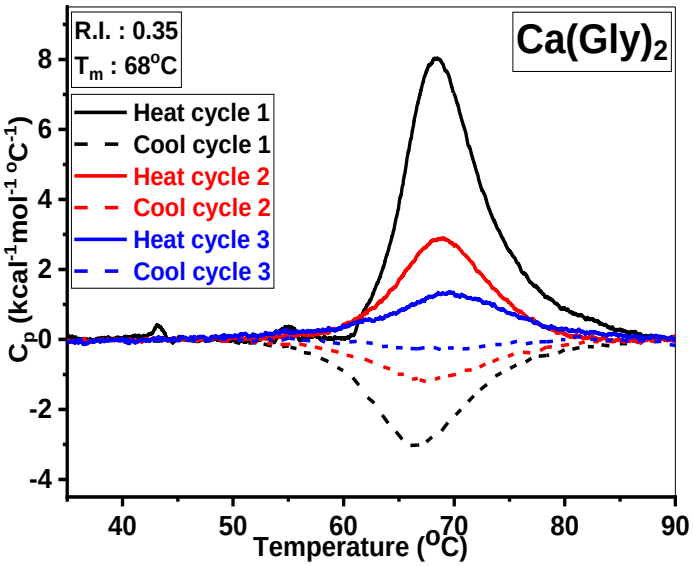


Figure 4.22: *Normalised and baseline subtracted DSC thermogram for α-lac (100 mg ml^{-1}) in 0.05 M Tris-HCl, pH 7.0, with $Ca(Gly)_2$ (30 mM Ca^{2+}) for three heating and cooling cycles.*

An α-lac sample containing $Ca(Lys)_2$ (fig 4.23) was performed containing the same concentration of calcium (30 mM) C giving a T_m and R.I. of 0.38 and 68.4 °C respectively, values that are similar to those obtained for the $Ca(Gly)_2$ salt. ΔH was higher than for any other sample (55.0) and maintaining an adequate R.I. (0.38). All thermodynamic data is summarised in table 4.5.

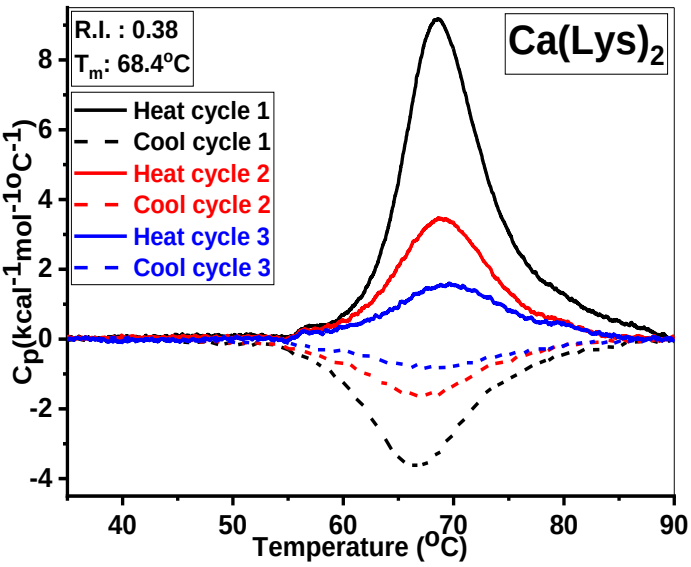


Figure 4.23: *Normalised and baseline subtracted DSC thermogram of α-lac (100 mg ml^{-1}) in 0.05 M Tris-HCl, pH 7.0, with(Ca(Lys)$_2$ (30 mM Ca^{2+}) for three heating and cooling cycles.*

Table 4.5 *Summary of DSC experimental data measured*

Excipient added	Ca^{2+} Concentration (mM)	ΔH_{me} (kJ/mol)	R.I.	T_m (°C)
$CaCl_2$	30	45.0	0.54	67.9
Glycine only	<1 mM	38.8	0.26	66.1
$CaGly_2$	30	45.0	0.35	68.0
$CaLys_2$	30	55.0	0.38	68.4

4.9 Discussion

A comparison between three calcium salts was made on both the colloidal and thermal stability of the whey protein α-lac at pH 7.0; specifically $CaCl_2$, $Ca(Gly)_2$ and $Ca(Lys)_2$. The impact of increasing calcium concentration from these sources on the colloidal stability of α-lac was quantified using QELS, while thermodynamic parameters were measured using DSC. A combination of SE-HPLC, SDS-PAGE and UV-Vis was used to determine the quantity of protein remaining in solution and the oligomeric state of which was quantified into % monomer, % dimer and % trimer or larger.

QELS was performed at 30 mM calcium as it is the calcium concentration in bovine milk. A k_D value of -6.0 for samples containing 30 mM $CaCl_2$, was measured indicating net attractive interaction potentials are predominant in this solution. This was compared with $Ca(Gly)_2$ and $Ca(Lys)_2$ at the same calcium concentration, giving a k_D of -5.0 and -6.1 respectively, implying that $Ca(Gly)_2$ gives a more colloidally stable solution than the $CaCl_2$ baseline by a slightly less negative k_D, suggesting less protein-protein attraction. $Ca(Lys)_2$ however had a near equally stabilising effect to $CaCl_2$, allowing also lysine to be added to the solution, without changing k_D. This isn't surprising however as glycine is well documented as a stabiliser in protein solutions (Sabulal & Kishore, 1995; Platts & Falconer, 2015).

A calcium chloride concentration series was performed in the range 0 – 120 mM in heated α-lac solutions. This indicated that for $CaCl_2$, most aggregation is observed between 20 - 120 mM However, when calcium is added as amino acid salts, less total insoluble aggregate formation occurs (indicated by lower % transmission) over the entire calcium concentration range. However, there significant differences observed for each amino acid salt. While for $Ca(Gly)_2$, the least amount of aggregation is observed, the degree of aggregation increases with the addition of more of the salt (hence both the calcium and amino acid concentrations increase). For $Ca(Lys)_2$, aggregation is relatively insensitive to the amino acid salt concentration.

SE-HPLC and SDS PAGE data indicate that there are significant differences in the aggregate size profile between all three calcium salts. The most aggregation and most insoluble aggregates occur in samples containing $CaCl_2$. The least total aggregation is observed in sample containing the $Ca(Gly)_2$ salt. Particularly at low calcium concentrations, the degree of aggregation is low, at least in the 10 mg/ml samples used for the HPLC experiments. While total aggregation for the $Ca(Lys)_2$ salts was also relatively low, this sample had a higher content of oligomer than the other samples.

Finally DSC was done to compare all three salts to better replicate a high protein concentration processing environment. Both $Ca(AA)_2$ salts appeared to have a reduced ability to allow α-lac refolding, which differed from the results obtained by SE-HPLC, likely attributed to the fact that DSC protein concentration was done at 100 mg ml^{-1}, compared to SE-HPLC done at 10 mg ml^{-1}. Hence, it will be important to understand the role of protein concentration in mediating the interactions in solution.

4.10 Conclusion

$Ca(AA)_2$ salts were synthesised to use as alternate calcium sources to $CaCl_2$. Colloidal stability was assessed using QELS. k_D values indicated no significant change relative to $CaCl_2$, at 30 mM calcium throughout.

Structural stability was assessed by conducting a calcium concentration series ranging from 0- 120 mM Calcium in all three salts and the quantity of protein lost to insoluble aggregation was measured. The remaining soluble protein material was then assessed by quantifying the amount of monomer/dimer/trimer. This indicated that both $Ca(AA)_2$ salts allowed greater quantity of α-lac to remain soluble after heat denaturation.

Finally, DSC was then done to measure the R.I., T_m and ΔH to better replicate a large-scale industry processing setting. This inferred that the ability of α-lac to refold was reduced by using $Ca(AA)_2$ salts, $Ca(Lys)_2$ increased the amount of energy required to unfold. However, this may be able to circumvent by adjusting concentration.

This chapter makes an implication that in a high temperature processing environment, CaAA salts could be a superior method of adding calcium to a milk solution than traditional calcium sources such as $CaCl_2$. Specifically, $Ca(Gly)_2$ allows more monomer retention in whey solutions predominatly consisting of α-lac, in the calcium concentration range of both human (6 - 10 mM) and bovine (25 - 30 mM) milk. $Ca(Lys)_2$ can also be used across a broad range of calcium concentrations during processing, given monomer retention remained steady (70%) regardless of calcium concentration.

Chapter 5: Thermal stability in β-lac dominant whey protein solutions

5.1 Introduction

The two main consumer product applications of powdered bovine whey are infant milk formulae (IMF) and athlete food supplements (Petroczi et al, 2008; Murphy et al, 2015; Crowley et al, 2016). The protein content of both products is predominantly whey based, however the ratio of α-lac:β-lac is different in both products. IMF whey content emulates the composition of human milk using the bovine derived whey protein α-lac and the majority of whey sports supplements consist mainly of β-lac (Haug et al, 2007; Roy, 2008; Fox et al, 2011; Martin et al, 2016). Where chapter 3 and 4 focused on infant formula and α-lactalbumin (20% of the whey content of bovine milk), chapter 5 will concentrate on the primary protein component of supplements and sports drinks, specifically β-lactoglobulin (80% of the whey content of bovine milk) and β-lac dominant whey mixtures (Murphy et al, 2014; Corrochano et al, 2018; Orru et al, 2018).

When whey sports drinks are reconstituted, the desired end product specification is a high protein concentration (>80 % of dry weight is whey) beverage, that supplies the entire range of amino acids to ensure adequate muscle recovery as well as a sufficient source of nitrogen to facilitate muscle protein synthesis (Hoffman & Falvo, 2004; Coker et al, 2012; Orru et al, 2018; Cintineo et al, 2018). The processing differences are small when compared to IMF, where fewer additives are included, but the thermal processing is almost identical, but IMF processing is slightly more stringent to account for increased infant vulnerability to illness. Hence it is important to understand the physical and chemical determinants of β-lac stability during processing. A complication persists where a high β-lac concentration can be causal for increasing aggregate size and occurrence as well as an increase in solution viscosity during thermal treatment (El-Shibiny et al, 2007; Erabit et al, 2014; Crowley et al, 2016). This is particularly evident at high protein concentrations (>10 % w/v) and high ionic strength (>0.15 M) such as at processing environment (Pouzot et al, 2004; Meza-Nieto et al, 2007; Bu et al, 2013: Kastelic et al, 2015). Whey based sports drinks (Abella et al, 2016) from powdered bovine milk contain mostly β-lac and is widely regarded as the limiting factor in thermal stability (Jeyarajah &Allen, 1994; Wijayanti et al, 2014) during whey processing. Issues such as a high β-lac and salt concentration are a direct cause in heat exchanger fouling (Jasch et al, 2007; Sadeghinezhad et al, 2015), as well as reducing end product yield by irreversible protein aggregation. The T_m of β-lac is near equal (70 °C) to the T_m of α-lac (~65°C) depending of the calcium concentration present) and β-lac does not refold to a monomeric state after heat induced unfolding (Owusu-Apenten et al, 2002; McGuffey et al, 2005; Halkau et al, 2008). Hence a method is required to supplement the nitrogen and calcium content of the solution and maintain stability and this can possibly be done by adding amino acids, calcium or

$Ca(AA)_2$ salts. Here we assess calcium amino acid salts as a potential additive to β-lac dominant whey protein solutions.

5.1.1 Aim of the study

The aim of this study is to determine the impact of calcium and $Ca(AA)_2$ concentration on β-lac. Using purified β-lac, a range of calcium salts at increasing concentrations is assessed in terms of thermal stability. A high throughput assay was used to screen a broad range of solution conditions when heated, SE-HPLC is then used to assess the remaining protein material aggregation status and if they can remain colloidally stable. These experiments were then repeated using a β-lac dominant whey mixture (WPI) as the protein source, given it is the starting material in nearly all dairy processing and is the intended application of this research.

5.2 Results

5.2.1 Purification and characterisation of β-lactoglobulin

β-lac was purified from WPI whey powder (Davisco Foods Intl, ~79% β-lac by weight) using a combination of SEC (figure 5.1) and IEX (figure 5.2), using the exact same purifcation conditions as that of α-lac. The purified protein was then characterised using SE-HPLC (figure 5.3), with the correct molecular weight determined by calibration against standard molecular weight markers and SDS-PAGE (figure 5.4).

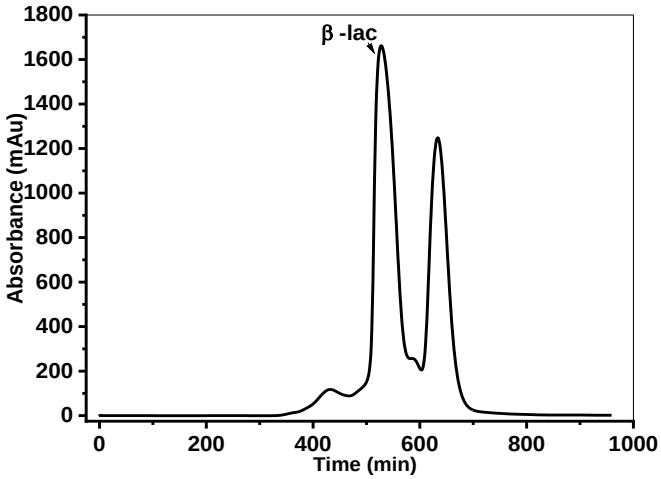


Figure 5.1 *Size exclusion chromatogram showing the separation of the components of the WPI powder on a Sephacyrl S-200 column.*

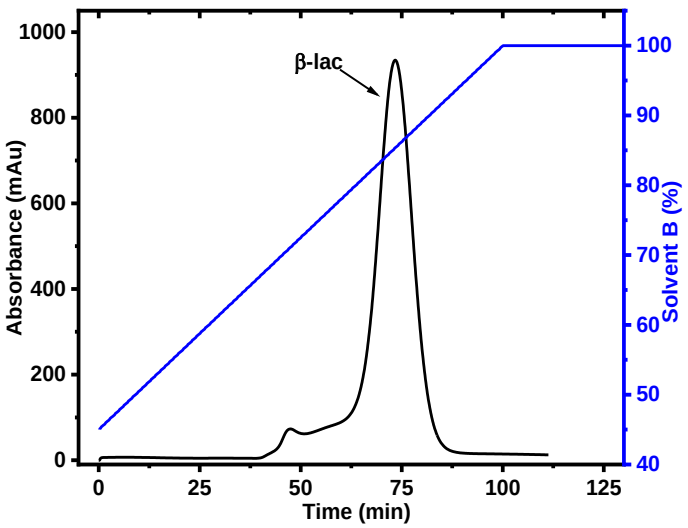


Figure 5.2: *Ion exchange chromatogram after purifying fractions from the size exclusion step. The major peak contains β-lac, with a yield of ~50% from SEC to IEX.*

SE-HPLC (figure 5.3) and non-reducing SDS-PAGE (figure 5.4) were used to determine protein purity of the product after the two-stage purification.

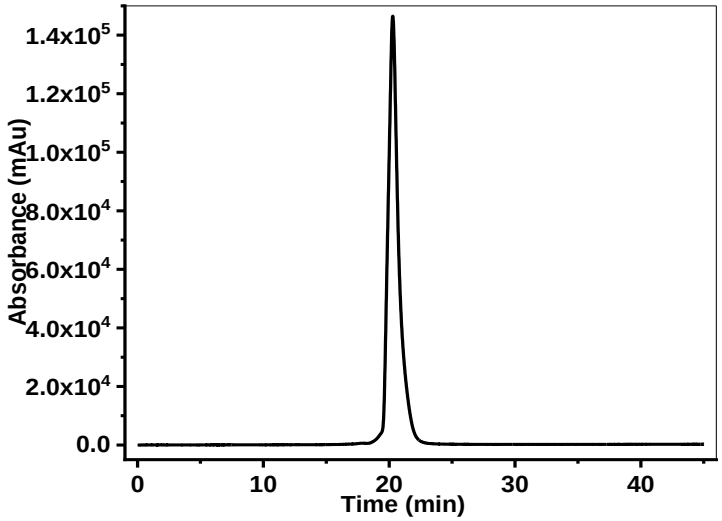


Figure 5.3: *SE-HPLC chromatogram for β-lac in 0.05 M Tris-HCl, pH 7.0 with 0.15 M NaCl after purification by size exclusion chromatography and ion exchange chromatography on a Superdex S-200 column using a flow rate of 0.75ml min^{-1}.*

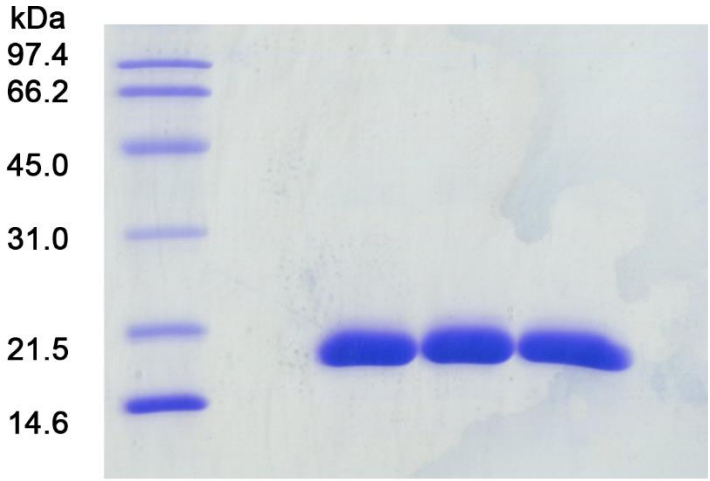


Figure 5.4: *12.6 % non-reducing SDS-PAGE gel showing a single band corresponding to β-lac. No protein oligomers or other bands are present, indicating a highly purified protein*.

SE-HPLC produced a single peak that eluted at 20.3 minutes, with a purity of ≥ 98% (figure 3.3). Using the calibration curve for this column, under the same buffer conditions, the protein was found to have a molecular weight of ~33.4 kDa (figure 5.4). This value was relatively consistent with the literature molecular weight for dimeric β-lac (~36 kDa). A similar molecular weight for dimeric β-lac was also recorded by (Mercadante et al,

2012), where in a comparable pH and ionic strength regime (pH 7.0, 0.1 M NaCl), a molecular weight of 33.0 kDa β-lac was recorded by X-Ray diffraction and also a slightly lower than anticipated molecular weight was recorded (31.0 kDa) at pH 7.0 when analysed by SE-HPLC (Perez-Moral et al, 2011), using a similar column (Superose 12HR, GE –Healthcare).

A single band was also observed by SDS PAGE, located between the 14.6 and 21.5 kDa standard indicating a protein with a molecular weight slightly lower than that of soybean trypsin inhibitor (21.5 kDa) and greater than lysozyme at 14.6 kDa (figure 3.5), indicating dimers had dissociated to monomers after the SDS-PAGE sample prep process, which commonly occurs when heated from 75-95 °C (Wijayanti et al, 2013). The confirmation of dimeric β-lac in these buffer conditions was further confirmed via QELS.

5.2.2 QELS

QELS was used to confirm the dimer state of unheated β-lac in 0.05 M Tris-HCl, pH 7.0 (figure 5.5). The hydrodynamic radius was ~3.0 nm. This was consistent with the literature value of 3.0 nm at pH 7.0 reported by Parker and others (Parker et al, 2005), indicating dimeric β-lac.

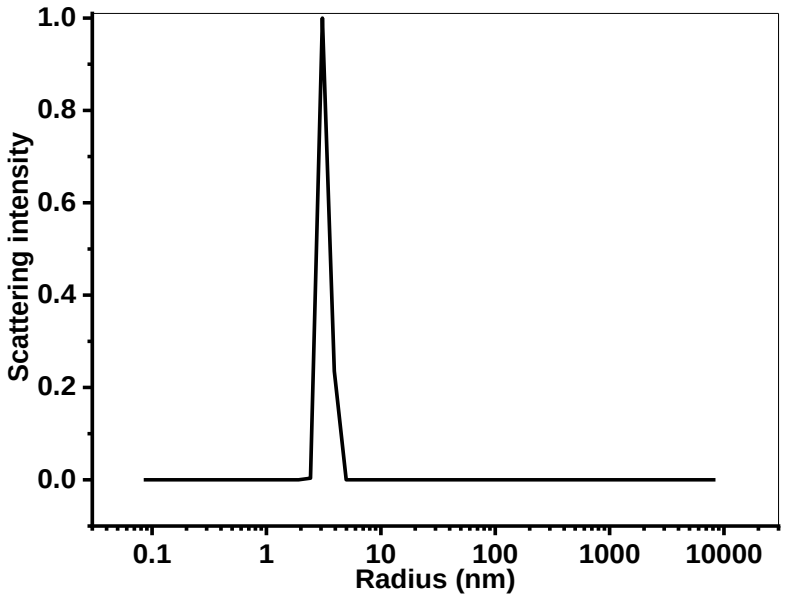


Figure 5.5: *QELS run indicating the average hydrodymnamic radius of an unheated solutions of β-lac added. The solution was prepared in 0.05 M Tris-HCl, pH 7.0.*

5.3 β-lac

5.3.1 High throughput assay

A high throughput assay to measure % transmission for β-lac samples in the presence of calcium salts after heating was used as a method of screening a broad range of β-lac concentrations and pH's (figure 5.6). Protein concentration was increased in 10 mg ml^{-1} increments. All work was performed in 0.05 M Tris-HCl, pH 7.0 using a transparent 96 well plate. Before each measurement set, the entire plate was shaken for 30 seconds. This assessed which pH and protein concentration resulted in greatest turbidity (an indicator of aggregation). For all high throughput assays, purified β-lac was used (method 3.2.1).

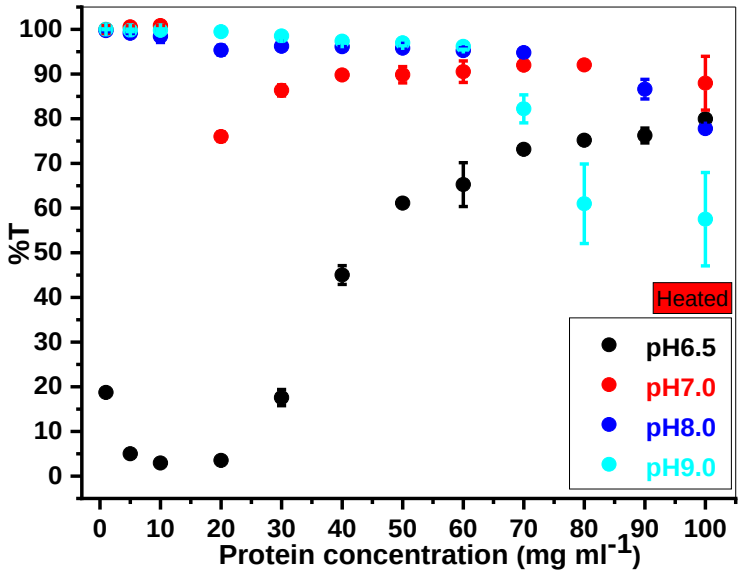


Figure 5.6: *The relationship between % transmission (%T) and protein concentration at different pH values for heat denatured β-lac samples.*

Across the protein concentration range examined, an initial glance would suggest that with the exception of pH 6.5, relatively little aggregation occurs. However, visual inspection revealed that many samples in-fact formed transparent gels and no hence little change in turbidty occurred in these samples, even though irreversible aggregation had occurred. Hence the remainder of the study was performed at neutral pH.

5.3.2 SE-HPLC

SE-HPLC was used to determine protein loss after heat-denaturation of β-lac. This technique also allowed determination of the oligomers present and their respective quantities (figures 5.7-5.9). The solution variables were set as 10 mg ml^{-1} in 0.05 M Tris-HCl, pH 7.0. The experiment was performed in 1.5 ml microcentrifuge tubes and samples were heated at 85 °C for 45 min. Larger aggregates were separated via centrifugation and filtered using a 0.22 µm filter. The supernatant was then further analysed using SE-HPLC and non-reducing SDS-PAGE. The protein concentrations for monomer and oligomers were determined by integration of the chromatogram peaks. The amount of insoluble protein was determined by subtracting total soluble protein detected by HPLC from the total protein in the solution before heating.

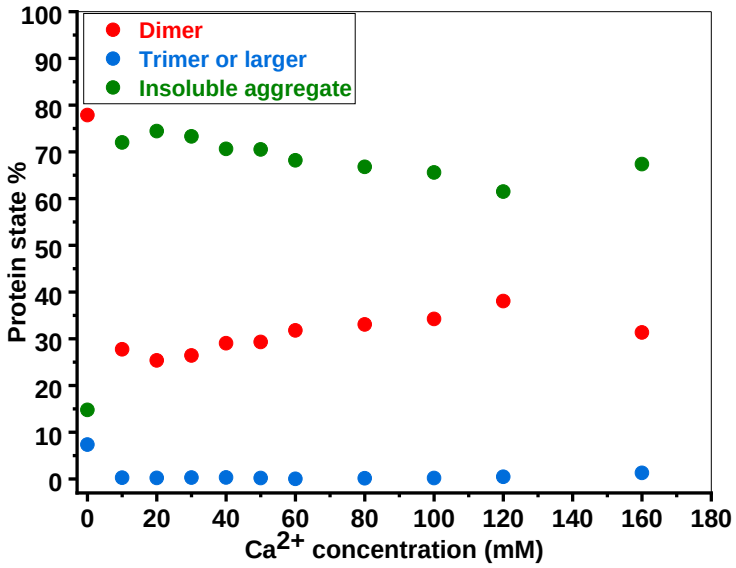


Figure 5.7: *Quantities of monomer, oligomer and insoluble protein present in heated β-lac solutions with increased calcium concentration ($CaCl_2$) in 0.05 M Tris-HCl, pH 7.0.*

As calcium chloride concentration increased, an initial reduction in the quantity of β-lac dimer was recorded up to 20 mM after which, as more calcium was added, an increase in β-lac dimer was observed up to 120mM $CaCl_2$. Very low proportions of other oligomers were detected, and the majority of aggregated protein was in the form of insoluble aggregate. (figure 5.7).

The experiment was then repeated using $Ca(Gly)_2$ as a calcium source (figure 5.8). In this case, there is significantly more soluble aggregate (dimer) present over the calcium concentration range, and significantly less insoluble aggregate, which in fact decreases with increasing calcium concentration.

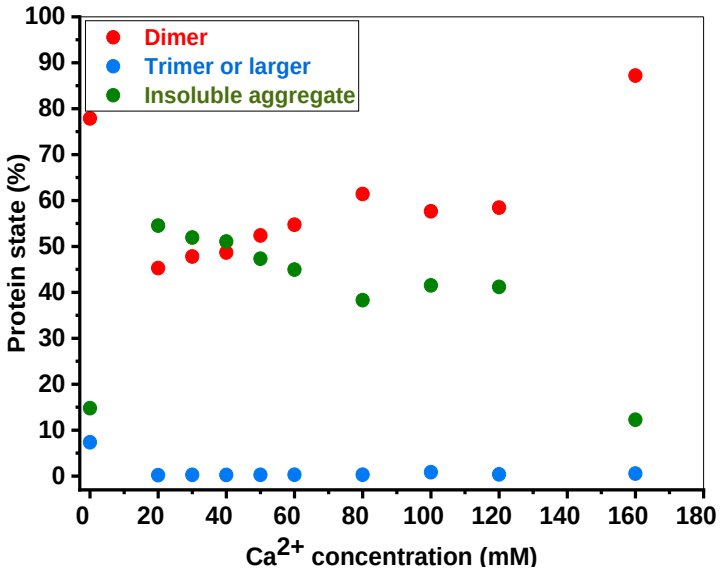


Figure 5.8: *Quantities of monomer, oligomer and insoluble aggregate present in heated β-lac solutions with increasing calcium concentration ($Ca(Gly)_2$) in 0.05 M Tris-HCl, pH 7.0.*

Numerous aggregation studies have already been conducted examining the role of glycine in with β-lac solutions and these demonstrate that glycine does increase β-lac solubility after heating.

Finally the additive $Ca(Lys)_2$ appeared to yield the highest soluble protein in β-lac solutions by improving the amount of protein retained after heat treatment (figure 5.9). There was an increase of ~30 % of soluble protein compared to $Ca(Gly)_2$. Across all calcium concentrations there was a small quantity of trimer or larger oligomers recorded indicating that insoluble aggregates predominantly form when heated.

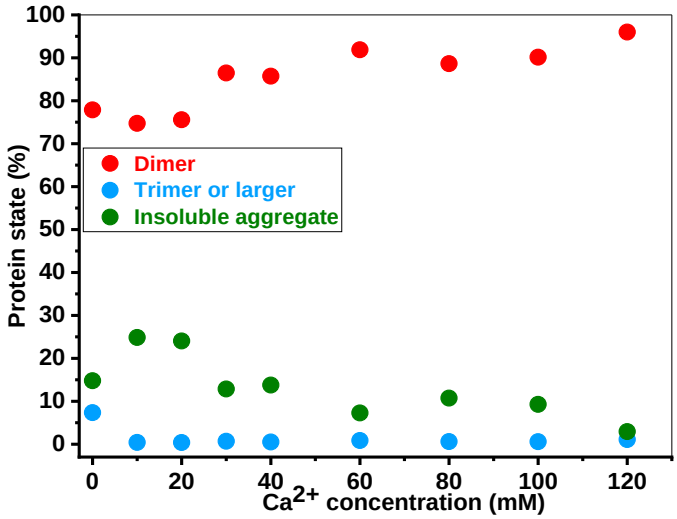


Figure 5.9: *Graph comparing the quantities of oligomer present in heated β-lac solutions with increased calcium concentration ($Ca(Lys)_2$ in 0.05 M Tris-HCl, pH 7.0.*

5.3.3 SDS-PAGE

SDS-PAGE was performed to further confirm SE-HPLC results. The samples used for SDS-PAGE were the same ones from SE-HPLC (i.e. prepared at the same time). As calcium concentration as $Ca(Gly)_2$ and $Ca(Lys)_2$ is increased, there is an increased proportion of soluble protein present after heating. This can be observed in figure 5.10, with a marginal increase in band intensity, the more of either $Ca(AA)_2$ salt added (the same volume of supernatant is loaded in each well). A single band located between the 14.6 and 21.5 kDa molecular weight marker indicates a β-lac in its monomeric form (18.3 kDa). The presence of only monomer here as opposed to dimer in SE-HPLC, is likely due to β-lac dimer dissociating at temperatures greater that 70 °C, but below 150 °C assuming the protein concentration is low (<25 mg ml^{-1}) as noted by De Wit (De Wit, 2009).

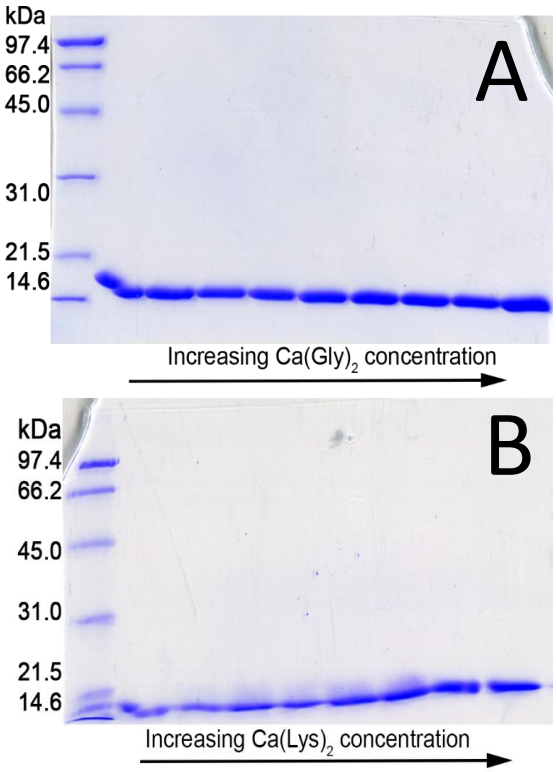


Figure 5.10: *12.6 % non-reducing SDS-PAGE gel of β-lac in 0.05 M Tris-HCl, pH 7.0 with increasing calcium concentration. Gels show a band between the 14.6 and 21.5 kDa standard and correspond to calcium from $Ca(Gly)_2$(A) and to $Ca(Lys)_2$(B) respectively.*

5.4 WPI

5.4.1 SE-HPLC

SE-HPLC was used to determine the total amount of protein lost to irreversible aggregation. A WPI stock solution of 20 mg ml^{-1} (by weight) in 0.05 M Tris-HCl, pH 7.0 was prepared. A small volume of this solution was taken and was diluted to 10 mg ml^{-1} with buffer and characterised via SE-HPLC (fig 5.11) to establish a baseline total protein amount before heat denaturation. This demonstrated that WPI consisted predominantly of β-lac dimer (51%) and α-lac monomer (16 %) after repeating the run using protein standards, with some smaller quantities of BSA (<1 %) as well as aggregates, from β-lac and α-lac (32%) corresponding to the peaks from 10 – 18 min.

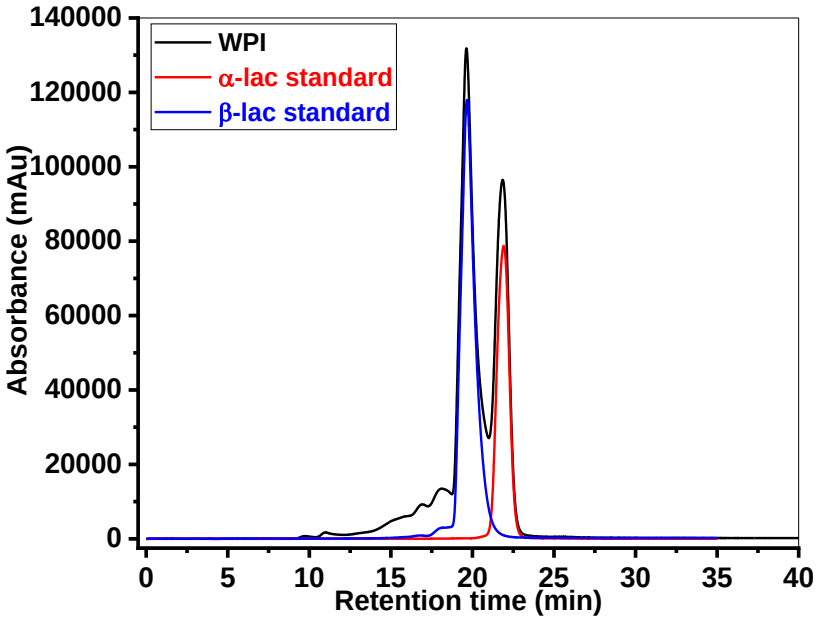


Figure 5.11: *SE-HPLC chromatogram of WPI in 0.05 M Tris-HCl, pH 7.0. α-lac and β-lac standards (purified protein) were run in the same conditions.*

Three experiments, with WPI across a calcium concentration series were performed using $CaCl_2$, $Ca(Gly)_2$ and $Ca(Lys)_2$ (figure 5.12). In this case, because this is a protein mixture, it is difficult to determine what the identity of all chromatogram peaks are after heating, so here we quantify total soluble protein, which includes monomer and oligomers for both α-lac and β-lac after heating. All solutions were heated/cooled and large aggregates were sediment by centrifugation and the supernatant separated by aspiration, filtered and loaded onto the SE-HPLC column to quantify the total amount of soluble protein, and hence, indirectly, total protein lost to irreversible aggregation (fig 5.12).

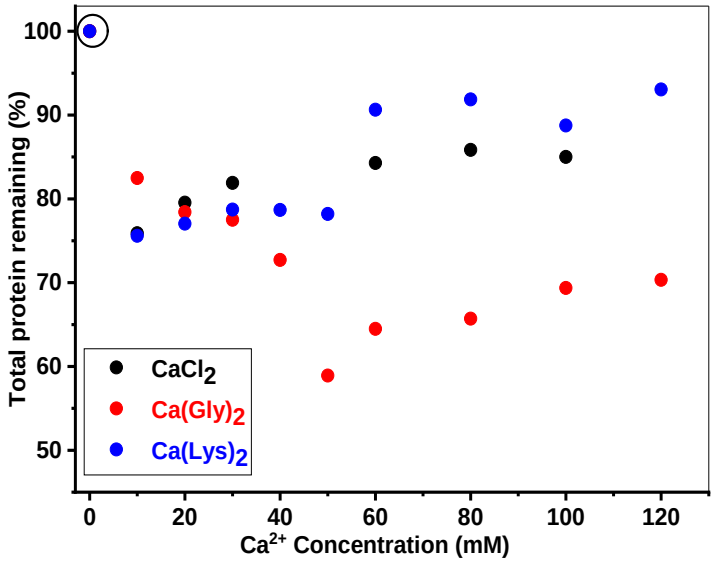


Figure 5.12: *Total protein remaining in solution after removal of precipitated protein following heat denaturation. protein concentration was determined by SE-HPLC. The black circle indicates a sample which was not heated. n =1 in this case.*

All samples retained ~75 - 80% of total protein at calcium concentrations up to 40 mM indicating that by using $Ca(AA)_2$ sources, it's possible to also supplement amino acids as well as calcium while retaining an equal amount of protein in the soluble supernatant. However at higher calcium concentrations, $Ca(Lys)_2$ results in more soluble protein than for the other calcium salts.

5.4.2 SDS-PAGE

The same SE-HPLC samples were also analysed by SDS-PAGE (fig 5.13). SDS-PAGE used to determine the ratio of α-lac to β-lac in all SE-HPLC samples by comparing band intensities for the separated proteins found in the supernatant after heating. A concentration series for purified α-lac and β-lac was performed on separate gels (fig 5.14-5.17) and the band intensities at each concentration were used to construct a concentration calibration curve for each protein. For the heated samples, comparisons of the band intensities for each protein with the calibration curves were used to estimate protein content. An unheated WPI sample was run on every gel for comparison (figure 5.13). The quantities of α-lac and β-lac present in each sample are summarised in table 5.1.

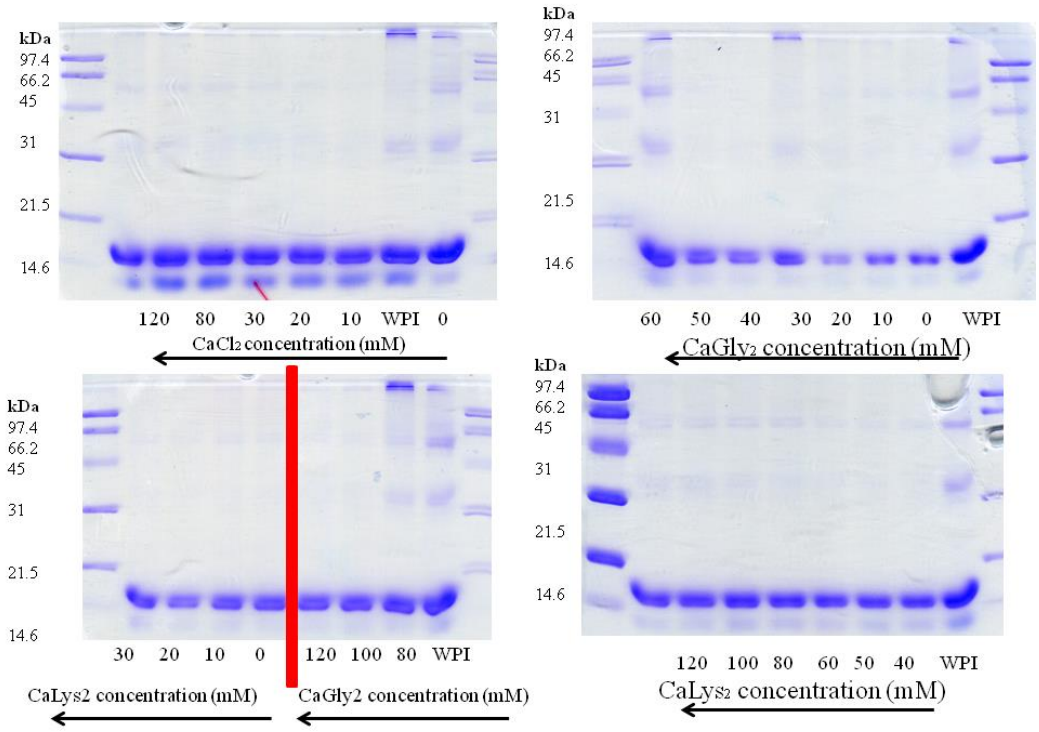


Figure 5.13: *12.6 % non-reducing SDS-PAGE gels with increasing calcium concentrations from three different calcium sources in 0.05 M Tris-HCl, pH 7.0. All lanes contain α-lac and β-lac from WPI. All gels also contains a WPI standard lane.*

5.4.2.1 α-lac concentration series

Five samples of increasing α-lac concentration were measured, as well as two WPI samples to determine the concentration of α-lac in the WPI sample. The WPI sample loaded had a α-lac concentration of 5.3 µg, calculated by comparison with calibration standards using purified protein on the same gel (by relating band intensity to protein concentration).

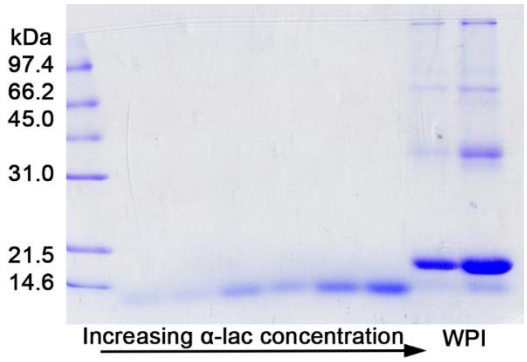


Figure 5.14: *12.6 % non-reducing SDS-PAGE gel with increasing α-lac concentrations in 0.05 M Tris-HCl, pH 7.0.*

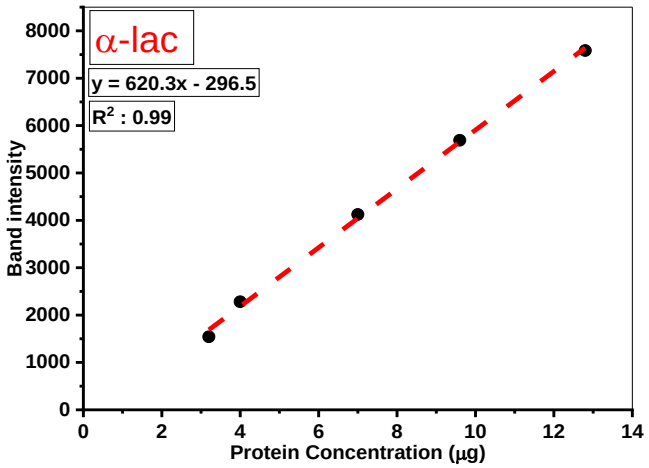


Figure 5.15: *Calibration curve demonstrating the relationship between protein concentration and SDS-PAGE band intensity using the gel 5.14.*

5.4.2.2 β-lac concentration series

Five samples of increasing β-lac concentration were measured, as well as two WPI samples to determine the concentration of β -lac in the WPI sample. The WPI sample loaded had a β -lac concentration of 14.5 µg, calculated by comparison with calibration standards of purified protein on the same gel (by relating band intensity to protein concentration).

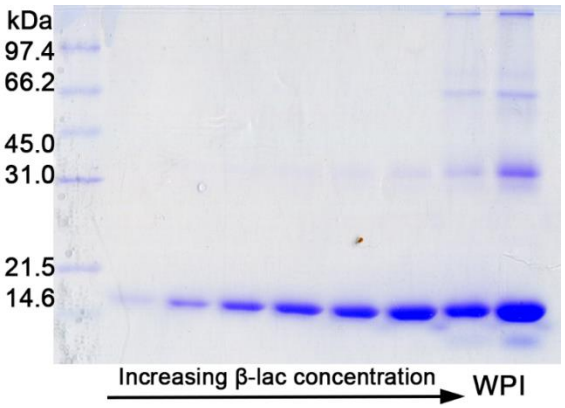


Figure 5.16: *12.6 % non-reducing SDS-PAGE gel with increasing β-lac concentrations in 0.05 M Tris-HCl, pH7.0.*

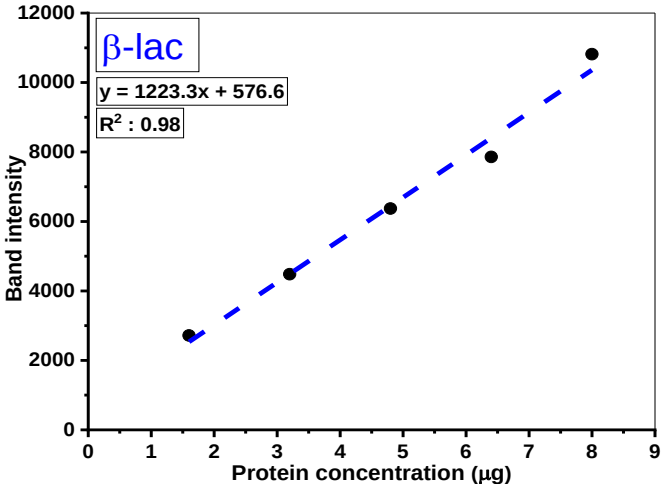


Figure 5.17: *Calibration curve demonstrating the relationship between β-lac concentration (ug) and SDS-PAGE band intensity using the gel 5.16.*

α-lac:β-lac ratio was determined to be 1:2.7 or ~26% α-lac and ~74% β-lac. This corresponded to the manufacturer's specification of this WPI (Davisco, BiPro), which has a composition of ~ 25 % α-lac and 75 % β-lac exactly (also determined by Douglas et al, 2018).

Table 5.1: *Table summarising α:β lac ratio with calcium concentration based on SDS-PAGE band intensities in figure 5.13.*

Calcium source	Calcium (mM)	α-lac quantity in lane (µg)	β-lac quantity in lane (µg)	α-lac: β-lac
WPI ref (unheated)	**<1.0**	**5.3**	**14.5**	**1:2.7**
$CaCl_2$	10	4.9	8.5	1: 1.7
$CaCl_2$	20	4.5	8.3	1: 1.8
$CaCl_2$	30	3.9	8.6	1: 2.2
$CaCl_2$	80	5.3	8.6	1: 1.6
$CaCl_2$	120	5.3	9.7	1: 1.8
WPI ref (unheated)	**<1.0**	**5.3**	**14.5**	**1: 2.7**
$CaGly_2$	10	1.2	4.5	1: 3.8
$CaGly_2$	20	1.1	3.8	1: 3.5
$CaGly_2$	30	1.2	2.4	1: 2.0
$CaGly_2$	40	1.6	6.3	1: 3.9
$CaGly_2$	50	2.1	5.4	1: 4.5
$CaGly_2$	60	2.4	6.4	1: 2.6
$CaGly_2$	80	5.2	8.4	1: 1.6
$CaGly_2$	100	4.1	8.0	1: 1.9
$CaGly_2$	120	3.8	7.5	1: 1.9
WPI ref (unheated)	**<1.0**	**5.3**	**14.5**	**1: 2.7**
$CaLys_2$	10	1.8	4.8	1: 2.6
$CaLys_2$	20	3.8	7.2	1: 1.9
$CaLys_2$	30	3.7	7.05	1: 1.9
$CaLys_2$	40	2.6	7.1	1: 2.7
$CaLys_2$	50	2.3	7.5	1: 3.2
$CaLys_2$	60	2.6	7.9	1: 3.0
$CaLys_2$	80	2.8	8.8	1: 3.1
$CaLys_2$	100	2.3	8.4	1: 3.6
$CaLys_2$	120	1.9	7.9	1: 4.1

5.5 Discussion

5.5.1 β-lac

The three calcium salts assessed produce three separate ranges of aggregates in pure β-lac solutions that have been exposed to heat denaturation. All β-lac solutions regardless of calcium sources consisted of dimer, trimer or larger and insoluble aggregates, the proportions of which depend on the calcium source.

β-lac solutions with increasing concentrations of $CaCl_2$ demonstrated that the majority of protein exists as insoluble aggregate (~70-75 %), with the soluble portion being mainly dimer (~25-30 %) and a negligible amount of trimer or larger. A point of minimum dimer concentration (at 20 mM calcium) is reached going from 0-20 mM and dimer : insoluble aggregate ratio amount increases with $CaCl_2$ concentration thereafter up to 120 mM, with trimer or larger aggregates remaining consistent throughout.

$Ca(Gly)_2$ as a calcium source was superior relative to $CaCl_2$ where across the entire calcium range more β-lac remained dimeric and less insoluble aggregates were formed. A point of minimum dimer was also reached at 20 mM calcium here of 45% dimer, and ~50% insoluble aggregate. This shifted to 90 % dimer and <10% insoluble aggregate at calcium concentrations >120 mM).

$Ca(Lys)_2$ β-lac solutions consisted mainly of dimer, regardless of the calcium concentration used. A trough at 20 mM calcium was also recorded, giving 75 % dimer β-lac and the remainder being trimer and insoluble aggregates. The ratio shifted to nearly entirely dimer (90%) as calcium concentrations increased to 120 mM.

This would indicate that $Ca(AA)_2$ salts allow heated β-lac solutions to exists primarily as a native dimer regardless of the calcium concentration present, opposed to $CaCl_2$, where aggregates formed were larger (insoluble). To apply this to an industry setting, aggregation studies were also done on WPI solutions.

5.5.2 WPI

The three calcium salts assessed produce three separate ranges of aggregates in WPI solutions that have been heat denatured. Total proteins remaining in solution was assessed first using SE-HPLC. This was done in tandem with SDS-PAGE to indicate the ratio of α-lac : β-lac aggregates remaining in solution.

WPI solutions of $CaCl_2$ demonstrated that 80-85 % of protein material remained soluble after heat treatment, where nearly all of the α-lac protein remained in solution and the 15-20 % of

insoluble aggregates that did occur were β-lac, shifting the α-lac : β-lac ratio from 1:2.7 to 1:1.6-1.8, depending on the calcium concentration.

$Ca(Gly)_2$ solutions formed a greater quantity of insoluble aggregates, beginning with ~80% remaining soluble at 10 mM $Ca(Gly)_2$, reaching a low pint of 60% at 50 mM and returning to 70% when increased further to 120 mM. Soluble aggregates consisted mainly of α-lac at low calcium concentrations and this ratio shifted to β-lac as $Ca(Gly)_2$ concentration increased

Finally $Ca(Lys)_2$ formed the least amount of insoluble aggregates by allowing more protein mass to remain in solution as calcium concentration was increased. The aggregates that did form were mainly β-lac, but retaining ~50% α-lac in solution throughout.

5.6 Conclusion

β-lac was purified from a whey protein powder consisting mainly of β-lac. The impact of calcium concentration across three sources ($CaCl_2$, $Ca(Gly)_2$ and $Ca(Lys)_2$ was assessed by quantifying the aggregates caused due to heat denaturation into soluble (dimer, trimer, trimer or larger) and insoluble (precipitate). $Ca(AA)_2$ sources of calcium when heated, lead to a greater quantity of soluble aggregates than insoluble relative to $CaCl_2$.

WPI used as the protein source, determined that even in whey mixtures, $Ca(AA)_2$ sources of calcium gave more soluble aggregates than insoluble, where depending on the calcium source, the ratio of α-lac:β-lac changes , with $CaCl_2$ retaining most α-lac, $Ca(Gly)_2$ retaining the least and $ca(Lys)_2$ retaining roughly 50% of α-lac.

This would open up new supplementation possibilities, reducing the amount of insoluble aggregates caused from the formulation weak-point of the protein β-lac.

Summary and concluding remarks

The main focus of this work was to create a high protein concentration whey solution with added mineral salts, which is both colloidally and thermally stable. This was investigated by extensively purifying whey powder into its main constituents of α-lactalbumin and β-lactoglobulin. The impact of the addition of calcium to whey as $CaCl_2$, $Ca(Gly)_2$ and $Ca(Lys)_2$ was assessed by a variety of characterisation tools, including quasi-elastic light scattering, size exclusion high performance liquid chromatography, electrophoresis, spectroscopy and differential scanning calorimetry. The aim was to better understand the cause of aggregation as well as probing new methods of preventing it, while still providing a nutritionally viable solution.

The impact of calcium concentration on the stability of purified α-lactalbumin was assessed in chapter 1. Colloidal stability was assessed by QELS, while thermal stability was assessed by DSC. These techniques together indicated two calcium: α-lac ratio's where the solution is relatively colloidally and thermally stable. One at low calcium concentration (30 mM) and a second at higher concentrations (80 – 100 mM), although the explanations as to why stability is achieved in each case is different. For the higher calcium concentrations, we propose that overchanging of the protein by non-specific binding to α-lactalbumin, introduces electrostatic repulsion between proteins in this calcium regime.

Alternate calcium sources were then explored by synthesis of calcium amino acid salts and their impact on whey protein stability assessed. A concentration series for each salt was performed to assess the effect on α-lac stability and to quantify the amount and sizes of aggregates formed after heat denaturation. Both $Ca(Gly)_2$ and $Ca(Lys)_2$ were found to increase the concentration of α-lactalbumin remaining in solution after heating and by extension reduce the formation of large aggregates that precipitate, being a significant improvement over $CaCl_2$ formulations. Total protein aggregation was also reduced.

β-lactoglobulin was then investigated using these $Ca(AA)_2$ salts in the same way as for α-lactalbumin. We determined that both salts also reduced the quantity of non-soluble aggregates in highly purified β-lac solutions relative to $CaCl_2$ formulations, increasing end product soluble protein concentration. We also probed the impact of calcium amino acid salts on whey protein mixtures (WPI) after heat treatment. $Ca(AA)_2$ salts also proved to be superior to traditional sources of calcium by again reducing the amount of insoluble aggregates formed. The consistency of the remaining aggregates in solution depending on the salt used to supply calcium, either containing more α-lac or β-lac.

This work makes an implication in whey product formulation, demonstrating that it is possible to supplement whey protein solutions with high concentrations of calcium without decreasing product quality. For $CaCl_2$, this can be achieved in α-lactalbumin solutions, providing that the protein is pure. For both whey proteins and their mixtures, calcium amino acid salts allow calcium to be added at high concentration with less insoluble aggregate formation after heat treatment.

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