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Characterisation of the rehydration behaviour of milk protein concentrates in the presence of sugar

M.Phil Thesis

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Characterisation of the rehydration behaviour of milk protein concentrates in the presence of sugar

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Abstract:

The main focus of this study was to characterize the hydration of milk protein in high protein powders to provide insights and strategies that might improve the use of these powders in foods. While the majority of hydration studies reported in the literature have been conducted on systems where there is an excess of water there has been little research characterising hydration in models that more closely approximate powder hydration in real food systems. This study investigates the impact on protein powder hydration of one of the most common ingredients in food systems: sugar. Results from this study show that rehydration of MPC85 powders is much more sensitive to aging compared to rehydration in water. An aged MPC85 powder was found to have the same solubility profile with respect to temperature in pure water compared to the fresh powder. However the degree of solubility was markedly reduced when the same powder was rehydrated in water containing sugar (20%). This should also be kept in mind while calculating the solubility of milk powders during the processing as other ingredients will interfere with them to affect solubility of milk powders which in turn will affect the shelf life of the food products.

It was also shown that the specific volume of the insoluble material sedimented during solubility studies increased as the solubility of the overall increased to about 50%. At higher degrees of solubility the specific volume of the sediment material decreased. Microscopy showed that in the lower solubility range predominantly small particles dissolved and the large particles retained their structural integrity through centrifugation and thus the volume of sediment was relatively unchanged despite material dissolving and becoming part of the supernatant. At higher levels of solubility the large particles dissolve primarily through the outer particle surface which therefore resulted in a progressive decrease in volume with solubility. The rate of change in the specific volume of the sediment and mass of the sediment with the increase in the solubility and temperature was also dependent on the solvent. The rate of change in water was higher than in the 20% sugar solution.

A new mechanism for MPC powder rehydration was also proposed wherein water ingress into the particles occurs over a very short time scale. Water ingress equilibrium was assumed to occur when the concentration of sodium ions reached equilibrium on the assumption that all sodium salts are highly soluble and that the sodium salts are evenly dispersed throughout the primary powder particle. This occurred over a time-period of a
couple of minutes compared with about thirty minutes for the bulk total soluble solids as measured through centrifugation. Increases in solubility with increasing rehydration temperature is proposed to result from shrinkage of the micelles in the particles due to increased hydrophobic bonding that in turn separates the micelles from each other allowing further ingress of water and solubilisation.
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1 Introduction

Proteins are one of the main constituents of foods which perform several critical functions in food systems. The protein structure plays a very important role in defining the functionality of food proteins (Damodaran, 1997).

Milk proteins are valuable component of milk and through consumer demand for high protein foods are often concentrated using separation techniques such as membranes or ion exchange to remove non-protein constituents and then spray dried and sold as a powder to extend their shelf life, facilitate their use and reduce transportation costs (Mimouni et al., 2009).

In the food industry, applications of milk powders as ingredients generally require the powder to be dissolved back into an aqueous medium (Gaiani et al., 2007; Martin et al., 2007). They are usually added to a formulation for a particular nutritional or functional purpose. They are used in a vast range of food systems which include health drinks, infant formulae products, cheese and bakery products (Baldwin and Pearce, 2005). Traditional milk powders such as skim milk powder or whole milk powder are readily dispersible due to their high concentration of highly soluble components such as lactose and thus are relatively easy to handle during food manufacture. With the development of newer high protein powder like milk protein concentrate the ease of dissolution is markedly decreased and when coupled with their use in low moisture food systems complex hydration properties that are not fully understood. These milk powders are rehydrated in different water environments which affect their functional properties.

There are two types of water environments present in foods. Excess water environment where there is enough water available for all the ingredients to be fully hydrated e.g., beverages. The other is limited water environment where there is not enough water available for the ingredients to be fully hydrated e.g., high protein bar systems. The level of hydration depends on the amount of water available for the ingredient’s dissolution. Protein hydration is defined as the average amount of water carried by unit weight of protein when the protein molecules migrate through solution (e.g., in diffusion, ordinary electrophoresis and sedimentation experiments) (Wang, 1954). Due to the incomplete hydration of ingredients there are different problems associated with limited water environments. Thus, in the limited water environment such as protein bars, the knowledge of the mechanism of
powder rehydration is required which effect dissolution and the consumer’s perception of the overall product quality.

1.1 Research Problem

Milk powders are used in different food product formulations and of particular interest for this study is the incorporation of concentrated milk protein powders in high protein nutrition bars. Due to the low water activity of nutrition bars there is insufficient water to enable all components to be fully hydrated. The kinetics of rehydration of the components varies to the extent that the time-scale for equilibration is in the order of weeks. In contrast, in a beverage system with excess water the system equilibrates within minutes which allows for relatively simple quality control at the point of manufacture.

One of the main quality problems associated with high protein nutrition bars is hardening of the bars over a period of time. Protein bar is composed of different ingredients and moisture level in all parts of the bar is not equal resulting in the difference in the water activity. So water diffusion may take place from the high water activity portion to lower water activity portion and due to this water diffusion the portion from which the water diffuses hardens over a period of time. This water activity problem is present in almost all limited water environments in foods. Another possible physico-chemical reaction is the Maillard browning reaction taking place between lactose, protein and water.

Much research has been reported attempting to determine the causes of protein bar hardening over time however, current knowledge implies that the main cause is the water migration within the protein bar and thus mobility of bar components. Hardening of protein bars is the result of the water migration from one ingredient to another. Protein aggregation and carbohydrate crystallization are the main driving forces for the water migration (Boice et al, 2004).

There are different types of milk powders which are commercially used in manufacturing of different food products. Due to the difference in the composition of these powders, each powder has a unique hydration property and therefore unique functional properties. Milk powders consist of different components like protein, moisture, lactose, minerals and fat. During the rehydration of milk powder these components acquire unique hydration characteristics that may be dependent on other ingredient components and/or the hydrating environment.
The main objective of this study is to characterize the hydration of milk protein in different high protein powders to provide insights and strategies to improve the use of these powders in limited water environment foods.
2 Literature Review

The main impetus for the initiation of this study was to characterize the hydration of milk protein in different high protein powders to provide insights and strategies that might improve the use of these powders in limited water environment foods.

To this end the focus of this literature review is to firstly classify the characteristics of commercially available limited moisture food systems. This is followed by a review of the main components and structure of high protein milk powders and their chemistry with a particular emphasis on their interactions with water. The review then expands its focus from the milk powders themselves to their interaction with the main solvents in food (hydrophilic and hydrophobic) and the food product ingredients that may modify the solvent. In addition to ingredients a further important aspect to consider are the processing conditions during which hydration occurs. After establishing the system, that is the subject of this thesis, the review then analyses potential methods that may be of value for this study. The review concludes with recommendations for the direction of the study.

2.1 Commercially Available Limited Moisture Environment in Foods

Food products can be categorized based on their moisture content. One category is the food system containing excess water like beverages and the other a food system with less water content such as protein bars. In excess water system, equilibration occurs within minutes and the ingredients will become fully hydrated whereas in systems containing less water, like protein bars, there is insufficient water to enable all components to be fully hydrated. These systems typically exhibit long time scales for equilibration.

A simple food system/environment may only contain water and sugar but foods are often complex systems. A food system consisting of both solid and liquid phases cannot be described as limited water environment without considering the hydration of its components. We can say that when there is not enough water available for ingredients to fully hydrate then this is the limited water environment.

2.1.1 Quality Issues in Limited Moisture Foods

As with most food systems the key quality attributes associated with limited moisture food systems are organoleptic characteristics. The organoleptic characteristics of most importance with limited water foods, those of texture and flavour, are discussed below.
2.1.1.1 Texture and Sensory Evaluation

Texture is a multi-parameter attribute. The attribute of a substance resulting from a combination of physical properties and perceived by the senses of touch, sight and hearing is known as sensory perceived texture (Brennan, 1988).

The sensory method of texture evaluation includes several steps outside and inside the mouth from the first bite through to mastication, swallowing and residual feel in the mouth and throat (Szczesniak, 2002). Hardness of food can be judged by the forces needed to break down the food structure. Oral processes have a significant effect on the breakdown of the food in the mouth (Wilkinson et al, 2000).

Measuring the texture of a food system by consumers involves the mastication of the food. Mastication is where the ingested food is transported to post-canine teeth by the tongue; here it is made into bolus suitable for swallowing. This involves the breakdown of solid food into smaller particles, the incorporation of saliva, shaping the particles into a bolus, and finally transportation to the pharynx where it is swallowed and enters the esophagus (Mioche et al, 1993).

The important factors having the influence on the sensory evaluation of texture and flavour of a food are listed as follows:

- Rate and mode of production of a new food surface.
- The production rate of saliva.
- Dissolution and dispersion of the food.
- Release of involatile tastants and volatile odorants.
- Transportation of volatiles to the nasal cavity (Kilcast, 2004).

Mioche et al (1993) reported to measure the texture of food by using electromyographic (EMG) signals to analyze the chewiness of the food product during mastication.

The sensory evaluation of whey protein gels as reported by Gwartney et al., (2004) was done in following steps.

1. Prefracture, where the properties of surface smoothness, slipperiness and springiness of gels prior to fracture were measured.
2. First Bite, where the properties of compressibility, firmness, moisture release and crumbliness from the first bite were measured.
3. Chew-down properties, where the properties of the particles were evaluated after the first 8 to 10 chews.
4. Pre-swallowing, the moisture in the mouth prior to swallowing.

They also suggested measuring the number of chews and time to swallow as a useful evaluation of the bar texture.

Kilcast (2004) found that sensory testing can be used to evaluate the attributes of food products by:

- Using a trained sensory panel as an analytical instrument where panelists are trained on the relevant sensory properties of the food.
- Using a hedonic test to determine the properties of the food in terms of liking or acceptability from untrained consumers.

A compression test is a common method for the objective evaluation of texture obtained from the resulting force-displacement curve. Oh et al., (1983) investigated the relationship between the texture measurements from an instron force-displacement curve and those produced from sensory evaluation. It was observed that there was a high correlation between the maximum cutting stress and the work on cutting with sensory evaluation of firmness.

Poysa et al. (2005) conducted another study on the quality of soy protein in tofu, the hardness of tofu was measured using Instron Texture Measuring system. The samples were compressed to 50% using a 10 kg load cell with a 57 mm diameter cylinder probe, at speed of 10 mm/min. Turgeon and Beaulieu (2001) used Texture Analyzer TA.XT-2 to test the texture of whey protein gels. A 12 mm diameter cylinder probe was used to penetrate the gels created. From this a force-time curve was produced with a crosshead speed of 1 mm/s. All measurements were carried out in triplicate. A TA.XT-2 Texture Analyzer was also used to test gels through compressing them twice with 50% compression using a 40 mm diameter cylindrical probe, at a speed of 1 mm/s (Martins and Netto, 2005). A Texture Analyzer can be used to measure the chewiness, hardness, crispness, gumminess and stickiness of products. The hardness value is the peak force of the first compression of the product. The hardness does not always occur at the point of deepest compression, although typically does for most products (Bourne, 1978).

From these methods it was decided to use the Texture Analyzer TA.XT-2 for calculating the interactions between the sediments rehydrated at different temperatures.
Flavour

How a consumer perceives the flavour of a food is a major factor determining their acceptance of the product. The quantity of flavour released into the oral cavity depends on the retention of flavour compounds in the food matrix. This is influenced by the nature of the ingredient flavour interactions (Dattatreya et al., 2002). Hydrophobic and hydrogen bonds are responsible for the interactions between flavours and proteins (Lubbers et al., 1998).

Flavour is a combination of mouth feel, taste and aroma. The texture of the product can have a major impact on the flavour release through its influence on mass transfer. Flavour release in the mouth is different to those produced from a machine in several ways. As flavour assessed in the mouth is diluted through the addition of saliva and swallowing, as well as a continuous change in volume, composition and viscosity of the food during consumption (Dattatreya et al., 2002). Variable factors that may cause problems within sensory testing are the differences in chewing efficiency among people and the effect of mastication on the flavour release from solid and thickened foods (De Roos and Wolswinkel, 1994).

Damodaran (1996) reported that temperature has a very little effect on flavour binding unless significant thermal unfolding of the protein occurs. Flavour binding is usually enhanced at alkaline pH, due to protein’s tendency to denature extensively at alkaline pH rather than at an acidic pH.

Overbosch et al., (1991) concluded that the sensory perception of flavour is influenced by the following factors:

- Amount of bound flavour in relation to its overall concentration.
- Time scale of mastication and swallowing of a bite.
- Solubility characteristics of the product.

Gremli (1974) conducted a research on the interactions between flavour compounds and soy protein and reported that an aqueous dispersion of soy protein reduced the volatility of aldehydes. This shows that the magnitude of the interaction was a function of the chain length of the protein.

Dattatreya et al., (2002) found that there are several mechanical systems that are used to determine the flavour within a food system. They include gas chromatography-olfactometry, charm-technique and aroma extract dilution analysis. However, Szczesniak
(1987) reported that these models are inefficient for the determination of the true sensory appeal of the consumers. Therefore sensory evaluation is the best method for the determination of the true flavour of the product and its acceptance to the consumer.

**Sensory Panel**

It is a group of testers who have exceptional sensory abilities and can describe products on the basis of taste, smell or feel. The panellists are trained to describe their sensory experiences using words they generate in previous training sessions. It is the most appropriate tool to determine when a food product reaches the end of its shelf life (Hough et al., 2002).

The advantages in running sensory panels are as follows:

- They give an idea of what consumers may experience.
- These are more rapid than most non-sensory methods.
- Panelists are able to measure more than one attribute.
- They are very sensitive and good at detecting minute differences in food.

There are also several disadvantages to have sensory panels which are listed below:

- As panelists can suffer sensory fatigue.
- People within the panel can bias each other.
- It can be expensive.
- It is time-consuming.
- Replacement of the panelists is difficult.
- Some panelists may not be good at scoring an attribute.
- Some attributes may be open to interpretation.

Many of these disadvantages can be overcome through the use of sensory booths to reduce the risk of bias among the panellists. Panellists are screened and trained to ensure that they are able to sense all major attributes of a food. This includes creating attribute descriptors that everyone understands.

Untrained consumer testing and hedonic testing can be used to determine properties of food related to liking or the acceptability of a product (Hough et al., 2002; Kilcast, 2004).

**2.1.1.2 Sensory and Texture Correlation**

When the texture is compared with other sensory properties it is found that there are no single receptors for texture. Rather texture is a multi-parameter as a number of tissues
(periodontal, skin) and receptors (kinaesthetic) are involved. Moreover, sensory response is subject to individual differences like dentures, age, hunger etc.

**Factors Affecting Sensory and Texture Correlation**

- Similarity of physical aspects e.g., squeezing a loaf of bread vs compression test.
- Differences in the material composition make the test material difficult for heterogeneous materials e.g., muscle of meat.
- Complexity of the sensory terms like consistency, body etc which are difficult to be defined based on single criterion.
- Complexity of sensory scales e.g., category vs ratio scales. Ratio scales are better at finding larger difference.

So it is not very easy to correlate the sensory and texture analysis due to the different factors in the sensory analysis. For correlation we have to be more specific in defining the different terms used in sensory analysis.

2.1.2 **An example of a changes in the quality attributes in a limited moisture food – the protein bar system**

The development of higher protein dairy powders has arisen from consumer demand for specialised nutrition. To develop such foods formulators require “pure” ingredients so that the inclusion of non-required components that dilute the composition with regard to the desired nutrients is reduced. A key market that is currently experiencing growth is that of sports nutrition (International Market Bureau, 2010). Within this sector dairy proteins are sought after for their inclusion in protein bars.

**Table 1. Formulation of Protein Bar Recipe**

<table>
<thead>
<tr>
<th>Ingredient</th>
<th>Percentage</th>
</tr>
</thead>
<tbody>
<tr>
<td>Protein Powder</td>
<td>30</td>
</tr>
<tr>
<td>Glycerol</td>
<td>15</td>
</tr>
<tr>
<td>Water</td>
<td>15</td>
</tr>
<tr>
<td>Sugar</td>
<td>30</td>
</tr>
<tr>
<td>Fat</td>
<td>6</td>
</tr>
<tr>
<td>Minerals</td>
<td>4</td>
</tr>
</tbody>
</table>
Table 1 shows the typical formulation of athlete bars designed for performance nutrition. In protein bar the main ingredients are protein (whey, soy and MPC), glycerine, polyols, corn syrup, oil and highly branched carbohydrate. Some vitamins and minerals are also added in commercial bars. A brief description of the function of each ingredient is given below.

As mentioned at the start of this review the equilibration times for hydration and hence component mobility in limited moisture foods can be in the order of weeks. In the protein bar system such equilibration is correlated with increases in the texture and is commonly referred to as ‘bar hardening’. Protein bar systems contain small quantity of water of which only about 10% interacts with the 30-50% protein and relatively little fat present in the bar. For this reason, there is little moisture available to dissolve the ingredients in the bar. Moreover, initially the water is equally divided between the protein and sugar systems, then sugar system will give up the water and crystallize, and the protein will absorb the water. This causes the water activity to drop with water absorption by the protein resulting in bar hardening (Gallo-Torres, 2003).

The ingredients in a typical bar formulation are discussed below in reference to their contribution to the ‘bar-hardening’ phenomenon.

2.1.2.1 Protein Bar Ingredients

Protein

The properties of protein are dependent on its type in food systems. The physico-chemical properties of protein are affected by the fraction and distribution of hydrophilic and hydrophobic residues specially the interaction taking place between the protein and water. The hydrophobic residues fill about 40-50% of the water accessible surface in most globular proteins (Fennema et al., 2008). The proteins having more hydrophobic residues will be having more insolubility due to increased interaction between the hydrophobic residues themselves. Whey protein peptides contribute towards the softness of the bars as they do not draw moisture from other ingredients within the bar (Gerdes, 2005).

Protein aggregation is caused by the interactions between proteins and formation of complexes with a higher molecular weight (Sanchez and Paquin, 1997). During the storage, protein aggregation takes place which causes the decrease in its solubility (Li-Chan, 1983). Protein aggregation can take place through mainly hydrophobic interactions and also
covalent aggregation by disulphide bonds and non-reducible cross-links. Aggregation of individual particles is the result of hydrogen bond formation which occurs in hydrophobic interactions, dipole-dipole and charged-dipole interactions (Tolstoguzov, 1997).

High temperature can favour protein aggregation in foods having high moisture content (De Graaf, 2000). Moreover, the severe heat treatment of foods having low carbohydrate and high protein content results in isopeptide cross-linking. The extent of these isopeptide linkages is decreased in presence of reducing sugars but increased with intensity of heat treatment (Singh, 1991).

Moreover, different functional properties of food like gelling, emulsification, viscosity, texture and solubility can be influenced by cross-linking (Kuraishi, 2000). The oxidative coupling of two adjacent cysteine residues forms disulphide bonds within a food protein matrix (Zayas, 1997). Disulphide bonds or hydrophobic interactions favour aggregation.

Turgeon and Beaulieu (2001) found that protein aggregation in protein gels was highest at the isoelectric point of the protein and at high temperatures protein aggregation is associated to lower the water-holding capacity of the protein.

Moreover, Sanchez and Paquin (1997) reported that the viscoelastic properties are due to micro-particulate proteins and protein-polysaccharide complexes. During storage at low temperatures, due to disturbance in the balances between the elastic and viscous components, an increase in the firmness of the product occurs through cold gelation.

**Glycerol**

Glycerol molecules are larger than water molecules. Due to the glycerol’s property of not retaining moisture the excess moisture within the food system migrates from the glycerine and is reabsorbed by the protein resulting in the hardening of the product over time. Glycerol helps in lowering the water activity of protein bar.

**Polyols**

Polyols are not contributing to the sugar content of bars. Polyols are composed of glycosyl units with three hydroxyl groups. Each of these hydroxyl groups has the possibility of forming a hydrogen bond with one or more water molecules. The sugar rings are connected by a ring of hydrogen atoms (Fennema et al., 2008). They function as humectants and reduce the water activity of the bar formulation.
**Corn syrup**

The softness of the bars can be influenced by corn syrup. High fructose corn syrups are used for different purposes which include retaining moisture and preventing drying out, controlling crystallization, aiding the blending in of sweeteners, acids and flavouring (Coleman and Harbers, 1983). The hardness of the bar is effected by the Dextrose Equivalent (DE) of both corn syrup solids and corn syrup i.e., the higher the DE the softer the texture of bar. DE is defined as percentage of reducing value of pure dextrose and calculated on a dry weight basis.

Addition of highly branched carbohydrate such as Fibersol-2(E) prevents water loss from protein bars. Fibersol-2(E) is composed of maltodextrin, fructooligosaccharides and polydextrose which are soluble fibres with poor digestibility (Boice et al., 2004).

**Oil**

Oil in protein bar formulations acts as a substitute for fat which is removed from other ingredients aiding to create a softer bar.

### 2.1.2.2 Causes of Protein Bar Hardening

**Carbohydrate Crystallization**

Due to the formation of hydrogen bonds between the polysaccharide molecules and water molecules, carbohydrate is able to retain water within a food system. These hydrogen bond formation increases the hydrophilic behaviour of the polysaccharides which also results in its enhanced flexibility. Therefore the overall flexibility of the food system is affected (Sanchez and Paquin, 1997). Moreover, water mobility within the food system can be controlled by incorporating modified polysaccharides (BeMiller and Whistler, 1996).

BeMiller and Whistler (1996) confirmed about the relationship between the amount of sugar and the amount of water within a food system. Glycans within the sugar are highly hydrophilic and therefore quickly hydrate resulting in sugar chains having the capacity to hold water molecules.

Due to the hygroscopic nature of amorphous sugars, they have the property to absorb water from the food system. However, carbohydrate crystallization decreases the amount of moisture absorbed by sugars (Burea et al, 2005). During storage this results in crystallization above a critical temperature-dependent relative humidity (Levine and Slade,
The process of solid-liquid separation where a solidifying substance assumes the form and structure of a crystal is known as Crystallization (BeMiller and Whistler, 1996).

Burea et al (2005) reported the interaction between the hydroxyl groups of sugars with the hydrophilic sites of proteins within a food system. However, sugar crystallization eliminates protein-sugar interactions, the proteins are excluded from the sugars crystals. In this case, sugar will give up water in a system, crystallize and the protein will absorb water. However, protein will not absorb water to the same extent as other ingredients due to its hydrophobic nature (Gallo-Torres, 2005).

Roos and kare (1999) found that crystallization of sugar not only release water but also creates a concentrated matrix, causing the molecules to become more tightly packed with less water therefore creating an overall firmer food product.

The rate of crystallization within a food system is also affected by the type of sugar used within the syrup. Anhydrous sugars such as sucrose, lactose can crystallize at room temperature after absorbing the amount of water needed to decrease their glass transition temperature (Tg) to below room temperature. Sugars that form hydrated crystals (trehalose dehydrate and raffinose tri, tetra or penta hydrates) retain higher amounts of water without crystallization since they need certain amount of water to get the Tg value below room temperature, also need to have enough water to form the hydrated crystals (Iglesias et al, 1997).

The heating temperature of the syrup during production can affect the hardening of the bars over time. At higher temperatures such as those used during heating of the syrup, carbohydrate crystallization is likely to occur (Levine and Slade, 1986).

**Water Migration**

The way the water migrates within a food system is dependent on the amount of water within the food system (Godbillot et al, 2006). The orientation, location and amount of water within the food system affect its properties. These factors are governed by its reactions by the reactions with the ingredients within the food system such as sugars and proteins, which are able to absorb significant amounts of water at certain water activities (Fennema et al, 2008).

Bell and Labuza (2002) reported that water migration within the system could be identified through an increase in the water activity of the food. As moisture is lost from one
food ingredient it would be absorbed by another ingredient, possibly causing an increase in the water activity of the food.

Due to migration of water molecules to the protein, molecular linkages are formed which reduce the flexibility of the bar texture.

2.2 Protein

The word protein was derived from the Greek word “proteois” meaning “the first” or “primary” due to its fundamental role in sustaining life (Fennema et al., 2008). Proteins are made up of chains of amino acids linked by peptide bonds. These polypeptides consist of more than 100 amino acid residues. Proteins are mainly composed of 20 different amino acids; however, some proteins do not contain all 20 amino acids. The largest known proteins are composed of nearly 10,000 amino acids (Milewski, S., 2001). The differences in structure and function of proteins arise from the sequence in which amino acids are linked together. The properties of proteins can be changed by varying the amino acid sequence, the type and ratio of amino acids and the chain length of polypeptides (Damodaran and Paraf, 1997). Nucleoproteins (ribosomes), glycoproteins (ovalbumin and κ-casein), phosphoproteins (α- and β-caseins, kinases and phosphorylases), lipoproteins (proteins of egg yolk and several plasma proteins), and metallo-proteins (haemoglobin, myoglobin and several enzymes) are the examples of the conjugated proteins.

In glyco- and phospho-proteins, the proteins are linked covalently to carbohydrate and phosphate groups respectively, whereas the other conjugated proteins are non-covalent complexes containing nucleic acids, lipids or metal ions. The dissociation of these attached groups can be carried out under appropriate conditions (Milewski, 2001).

2.3 Protein Chemistry

In this section the overview of the protein structure and its classification based on the properties of the protein will be discussed.

2.3.1 Amino Acids Commonly Found in Proteins

There are 20 amino acids having different sizes, shapes and polarity, which are incorporated into the polypeptide chains. These amino acids are also known as proteinogenic (Milewski, 2001). Table 2 shows the list of proteinogenic amino acids.
Table 2. Proteinogenic Amino Acids (Milewski, S., 2001)

<table>
<thead>
<tr>
<th>Abbreviated Names</th>
<th>Hydropathy Index*</th>
<th>Occurrence in Proteins (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Glycine Gly</td>
<td>-0.4</td>
<td>7.5</td>
</tr>
<tr>
<td>Alanine Ala</td>
<td>1.8</td>
<td>9.0</td>
</tr>
<tr>
<td>Valine Val</td>
<td>4.2</td>
<td>6.9</td>
</tr>
<tr>
<td>Leucine Leu</td>
<td>3.8</td>
<td>7.5</td>
</tr>
<tr>
<td>Isoleucine Ile</td>
<td>4.5</td>
<td>4.6</td>
</tr>
<tr>
<td>Proline Pro</td>
<td>-1.6</td>
<td>4.6</td>
</tr>
<tr>
<td>Phenylalanine Phe</td>
<td>2.8</td>
<td>3.5</td>
</tr>
<tr>
<td>Tyrosine Tyr</td>
<td>-1.3</td>
<td>3.5</td>
</tr>
<tr>
<td>Tryptophan Trp</td>
<td>-0.9</td>
<td>1.1</td>
</tr>
<tr>
<td>Serine Ser</td>
<td>-0.8</td>
<td>7.1</td>
</tr>
<tr>
<td>Threonine Thr</td>
<td>-0.7</td>
<td>6.0</td>
</tr>
<tr>
<td>Cysteine Cys</td>
<td>2.5</td>
<td>2.8</td>
</tr>
<tr>
<td>Methionine Met</td>
<td>1.9</td>
<td>1.7</td>
</tr>
<tr>
<td>Asparagine Asp</td>
<td>-3.5</td>
<td>4.4</td>
</tr>
<tr>
<td>Glutamine Gln</td>
<td>-3.5</td>
<td>3.9</td>
</tr>
<tr>
<td>Aspartate Asp</td>
<td>-3.5</td>
<td>5.5</td>
</tr>
<tr>
<td>Glutamate Glu</td>
<td>-3.5</td>
<td>6.2</td>
</tr>
<tr>
<td>Lysine Lys</td>
<td>-3.9</td>
<td>7.0</td>
</tr>
<tr>
<td>Arginine Arg</td>
<td>-4.5</td>
<td>4.7</td>
</tr>
<tr>
<td>Histidine His</td>
<td>-3.2</td>
<td>2.1</td>
</tr>
</tbody>
</table>

*= Scale combining hydrophobicity and hydrophillicity which can be used to predict amino acid location in aqueous environment (- values) and amino acid location in hydrophobic environment (+ values).

The number of each kind of amino acid in proteins is different. These 20 amino acids never occur in equal amounts in proteins. However, some general observations can be made. Some amino acids, especially methionine and tryptophan are relatively rare whereas some others can frequently be found in proteins such as alanine, glycine, lysine, or glutamate (Milewski, S., 2001)

2.3.2 Classification of Amino Acids

There are four main groups into which we can classify the amino acids on the basis of their properties and these are described below.

2.3.2.1 Hydrophobic Amino Acids

Those amino acids can participate in polar interactions not only amongst themselves but also with the solvent molecules which have oxygen or nitrogen attached in their side chain and due to this they can participate in hydrogen bonding which is extremely important
in non-covalent interactions in proteins and due to these bonds a protein or protein chain or amino acid chain folded together. Polar amino acids are ones which are likely to interact with solvent and in this interaction they can allow oxygen and nitrogen atoms to interact with the solvent and among themselves to form a network and remain in the solvent (Fennema et al., 2008).

### 2.3.2.2 Polar Amino Acids

The amino acids which have oxygen or nitrogen attached in their side chain and due to this they can participate in polar interactions not only amongst themselves but also with the solvent molecules. So they can participate in hydrogen bonding which is extremely important in non covalent interactions in proteins which is what holds a protein or protein chain or amino acid chain folded together. Polar amino acids are ones which are likely to interact with solvent and in this interaction they can allow oxygen and nitrogen atoms to interact with the solvent and among themselves to form a network and remain in the solvent (Fennema et al., 2008).

### 2.3.2.3 Acidic Amino Acids

Asparagine and Glutamine are the amino acids containing Aspartic acid and Glutamic acid in this group (Milewski, 2001).

### 2.3.2.4 Basic Amino Acids

Lysine and Arginine are the amino acids which are grouped under basic amino acids (Milewski, 2001). Table 3 gives a classification summary of the basic amino acids.

<table>
<thead>
<tr>
<th>Group</th>
<th>Amino Acid (Symbol)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Aliphatic</td>
<td>Ala, Val, Leu, Ile</td>
</tr>
<tr>
<td>Aromatic</td>
<td>Phe, Tyr, Trp, His</td>
</tr>
<tr>
<td>Charged</td>
<td>Asp, Glu, Lys+, Arg+, His+, Cys</td>
</tr>
<tr>
<td>Uncharged (Somewhat polar)</td>
<td>Asn, Gln, Ser, Thr, Tyr</td>
</tr>
<tr>
<td>Sulfur containing</td>
<td>Cys, Met</td>
</tr>
</tbody>
</table>

Note = Glycine as a simple amino acid and Proline is the only imino acid due to imine group instead of amine group. Moreover, some belong to more than one category.

### 2.3.3 Protein Structure

Protein is a polypeptide chain of a set of amino acids linked together by peptide bonds and it has a definite three dimensional structure. Amino acids are considered as the basic
structural units of proteins. These amino acids consist of a central $\alpha$-carbon atom which is covalently attached to hydrogen atom, carboxyl group and side chain $R$ group. The only thing which makes each amino acid different from another is the difference in the side chains as all amino acids have same hydrogen atom, amino group and carboxyl group. The central $\alpha$-carbon atom is actually a chiral atom and is asymmetric as four different groups are attached to it. General structure of amino acid is shown in Figure 1.

![Amino Acid Structure](http://en.wikipedia.org/wiki/File:AminoAcidball.svg)

Figure 1. The general structure of an alpha amino acid, with the amino group (left) and the carboxyl group (right)

The peptide linkage results from the condensation of the carboxyl group of the first amino acid and the amino group of the second amino acid with the removal of a water molecule. In this linear sequence all the amino acid residues are in the L-configuration. The amino acid side chains vary in size, shape and polarity. A protein with $n$ amino acid residues contains $n-1$ peptide linkages (Fennema et al., 2008). The physicochemical, structural, biological properties and functions of a protein can be explained by the chain length and the sequence of amino acid residues linkages. The molecular weight of proteins is in the range of 10 kDa to more than 1000 kDa (Milewski, 2001).

In conjugated proteins other side groups become covalently bonded to some amino acid residues. This may concern phosphorylation (mostly of Ser or Thr); glycosylation, which takes many forms; hydroxylation (mostly of Pro or Lys); as well as attachment of some other groups. Metalloproteins contain one or more, bivalent or trivalent, cations, that are tightly bound, but not by covalent bonds; in other words, they are not part of the protein, though the biological function of the protein generally depends on their presence. A still weaker association is involved in the formation of lipoproteins. Most of these are not proteins but complexes of several protein molecules and several lipid molecules, held together by weak forces like van der Waals, hydrophobic, etc.
The side groups determine chemical reactivity, i.e., the possibility of forming covalent bonds. Also much of the physicochemical behaviour follows from the nature of the side groups. Some can be involved in hydrogen bonding, either as a hydrogen donor (-OH and =NH) or as an acceptor (=O, -O-, =N- and –S-). The bulkiness greatly varies, group molar mass ranging from 1 (Gly) to 130 (Trp).

Some groups can be ionized, i.e., carry an electric charge. A proton can dissociate from a carboxyl group above a certain pH, giving a negative charge; and several other groups become protonated below a certain pH, giving positive charges. Several proteins are glycosylated, and some of these glucides contain carboxyl groups. Other proteins are phosphorylated, especially the caseins, which contain phosphoserine residues (R is -CH₂O-PO₃H₂). Two protons can dissociate from a phospho group, giving pK values of about 1.5 and 6.5.

The structure of proteins is traditionally described in a hierarchy of four levels. The primary structure of a protein simply consists of its linear sequence of amino acids. Secondary structure is concerned with local morphology. Some combinations of amino acids will tend to curl up in a coil called an α-helix or into a sheet called a β-sheet. Tertiary structure is the entire three-dimensional shape of the protein. This shape is determined by the sequence of amino acids. In fact, a single change can change the entire structure. Finally quaternary structure is concerned with the structure of a protein with multiple peptide subunits, like hemoglobin with its four subunits. Not all proteins have more than one subunit (Fennema et al., 2008).

2.3.3.1 Primary Structure
The primary structure of a protein represents the linear sequence of the amino acids in which they are linked together by peptide bonds. The three dimensional structure of the protein in solution is dependent on the amino acid sequence and the chain length of the polypeptide.

The primary structure determines the higher structures, which in turn determine properties like conformational stability and solubility. Nevertheless, it is mostly not possible to predict the higher structures from the primary structure.

The actual variation in primary structure really is very great. Some proteins have a fairly regular structure, like collagen, where the greater part of the amino acid sequence consists
of repeats of Gly-Pro-Pro or Gly-Pro-Lys, of which the third residue may be hydroxylated. By far most proteins have much more intricate primary structures.

Despite the flexibility of the peptide chain, most proteins do not assume random conformations in solution. Contrariwise, the conformation mostly is highly ordered.

### 2.3.3.2 Secondary Structure

Generally, there are two forms of secondary structures found in proteins namely helical structures and the extended sheet-like structures.

**Helical Structures**

The formation of the protein helical structures takes place when the $\phi$ (phi) and $\psi$ (psi) angles of the consecutive amino acid residues are twisted to a same set of values. Theoretically, it is possible to create different types of helical structures by choosing different combinations of $\phi$ (phi) and $\psi$ (psi) angles. However, only three types of helical structures are found in proteins namely, $\alpha$-, $3_{10}$- and $\beta$-helix. Among these three forms, the $\alpha$-helix is the most occurring and stable form in proteins due to the hydrogen bonding as shown in figure 2.

![Figure 2. Representation of an ideal $\alpha$-helix made up of 16 amino acids (without side chains) linked in a short polypeptide (guweb2.gonzaga.edu/.../CHEM440pub/L05-index.cfm)](https://guweb2.gonzaga.edu/.../CHEM440pub/L05-index.cfm)

The binary code is responsible for the $\alpha$-helix formation and is related to the arrangement of the polar and non-polar residues in the amino acid sequence. In most of the proteins, the non-polar surface of the helix is at the interior region of the protein and participates in the hydrophobic interactions with other non-polar surfaces.

The other helical protein structures are $3_{10}$-helix and $\beta$-helix which are less stable than $\alpha$-helix. These structures consist of short segments of few amino acids residues and are present as the minor portions in most of the proteins.
Due to special structure properties of proline, the segment containing it is not able to form α-helices. Proteins which contain proline residues tend to assume a random or aperiodic structure (Fennema et al., 2008).

**β-Sheet Structure**

The β-Sheet is an extended structure with specific geometries. Due to the perpendicular orientation of C=O and N-H groups to the direction of the chain, hydrogen bonding is possible only between segments and not within the segment. The β-Strands are usually 5-15 amino acid residues long.

In proteins, hydrogen bonding is responsible for the interaction between two β-Strands of the same molecule resulting in a sheet like structure known as β-pleated sheet. In the sheet like structure the side chains are oriented perpendicular to the plane of sheet. Two types of β-pleated sheet structures are formed by considering the N→C directional orientations of strands, namely parallel and antiparallel β-Sheets. Due to the differences in β-Strands directions the geometry of hydrogen bonds is affected. As the location of N-H···O atoms are in a straight line in antiparallel sheets causing them to be more stable than the parallel sheets and enhance the stability of hydrogen bonds whereas in parallel sheets they lie at angle and reduce the stability of hydrogen bonds.

The polypeptide segments contain alternate polar and non-polar residues in their structure and segments rich in hydrophobic side chains have high tendency to form β-Sheet structures.

The β-Sheet structure is more stable than the α-helix. Proteins containing large fractions of β-Sheet structure usually exhibit high denaturation temperatures. The conversion of α-helix type proteins into β-Sheet takes place when the former are heated and then cooled. However, the conversion of latter into the former is not yet observed in proteins.

β-turn or β-bend is another structural form found in proteins due to result of 180° reversal of polypeptide chain involved in β-sheet formation. The antiparallel β-sheet formation results in hairpin type bend in β-sheet structure whereas the parallel β-sheet formation results in the crossover bend (Fennema et al., 2008).

**Tertiary Structure**

It is the spatial arrangement which is attained by the folding of linear protein chain with secondary structure segments resulting into a compact three dimensional form. The
formation of tertiary structure from primary structure is a very complex process and its
details are present in its amino acid sequence at the molecular level. The optimization of
various interactions (hydrophobic, electrostatic, van der waals and hydrogen bonding)
between different groups in proteins and the conformational entropy of the polypeptide
chain results in the formation of tertiary structure from thermodynamic point of view, as
the net free energy of the molecule is reduced to the minimum possible value.

During the formation of tertiary structure, the relocation of the most of the
hydrophobic residues at the interior of the protein structure away from the water
environment and relocation of most of the hydrophilic residues, especially charged residues,
at the protein-water interface is the most important rearrangement causing reduction in
free energy. As the hydrophobic residues tend to be buried in the protein interior, so this
often can be accomplished partially due to steric constraints. The nonpolar residues occupy
about 40-50% of the water accessible surface of the protein molecules in most of globular
proteins. Moreover, some of the polar groups are buried in the interior of proteins. These
buried polar groups are hydrogen bonded with other polar groups in such a way that their
free energies are minimized in non-polar environment of the protein interior. The ratio of
non-polar and polar residues on protein’s surface has influence on several of its
physicochemical properties.

The folding of protein during formation of tertiary structure also results in the
reduction of protein-water interfacial area as protein is forced to fold in order to minimize
the said area.

Several physicochemical properties of the protein are affected by the fraction and
distribution of the hydrophilic and hydrophobic residues in the primary structure. Among
globular proteins, it is generally found that the larger molecules contain larger fractions of
nonpolar amino acids as compared to smaller molecules (Fennema et al., 2008).

**Quaternary Structure**

This structure refers to the spatial arrangement of a protein when it contains more than
one polypeptide chain. Any of the quaternary complexes can be made up of protein
subunits that are homogeneous or heterogeneous. The formation of oligomeric structures is
the result of specific protein-protein interactions. These interactions are driven by non-
covalent interactions such as hydrogen bonding, hydrophobic and electrostatic interactions.
Hydrophobic amino acids appear to influence the tendency to form oligomeric proteins.

Proteins containing more than 30% hydrophobic amino acids residues show a greater tendency to oligomeric structures.

The thermodynamic requirement in the formation of quaternary structure is to bury exposed hydrophobic surfaces of subunits. It is physically impossible to form a tertiary structure that will bury all the non-polar residues when the hydrophobic amino acid content is more than 30%. Hence, there are greater chances of hydrophobic patches to exist on the surface and interaction of these patches between adjacent monomers which leads to the formation of dimers, trimers and so forth.

Most of the food proteins exist as oligomers of different polypeptides. These proteins typically contain 35% hydrophobic amino acid residues and also they contain 6-12% proline (Fennema et al., 2008).

2.3.4 Interactions in the Protein Structure Stability

The process of folding of a random polypeptide chain into a unique three-dimensional structure is quite complex. The basis for the biologically active conformation is in the amino acid sequence of the protein. The native conformation of a protein is a thermodynamic state in which various favorable interactions are maximized and the unfavorable ones are minimized such that the overall free energy of the protein molecule is at the lowest possible value. There are two types of forces that contribute to the protein folding.

1. Intramolecular interactions originating from forces intrinsic to the protein molecule. Examples are Van der waals and steric interactions.

2. Intramolecular interactions affected by the surrounding solvent. Examples are hydrogen bonding, electrostatic and hydrophobic interactions.

2.3.4.1 Steric Interactions

These interactions are caused by the electrons in between the different groups. Their effect cannot be lessened by rotating the molecule around a bond. Due to the steric hindrance from side-chain atoms, segments of polypeptide chain can assume only a limited number of configurations. Any change in the planar geometry of the peptide unit will also bring increase in the free energy of the molecule. Hence, folding of the polypeptide chain can take place only in such a way that deformation of bond lengths and bond angles are avoided (Fennema et al., 2008).
2.3.4.2 Van der waals Interactions

These interactions are due to the dipole-induced dipole and induced dipole-induced dipole interactions between neutral atoms in protein molecules. When two atoms come close to each other, each atom induces a dipole in the other through polarization of the electron cloud. The interaction between these induced dipoles has an attractive as well as a repulsive component. The magnitude of these forces is dependent on the distance between the two atoms.

Van der waals interactions are very weak, decrease rapidly with distance and become negligible beyond 6Å. In proteins, however, since numerous pairs of atoms are involved in van der Waals interactions, the sum of its contribution to protein folding and stability is very significant (Fennema et al., 2008).

2.3.4.3 Hydrogen Bonds

These bonds involve the interaction of a hydrogen atom which is covalently attached to electronegative atom with another electronegative atom. Schematically, a hydrogen bond may be represented as D-H····A, where D and A are donor and acceptor electronegative atoms, respectively. The strength of hydrogen bond ranges between 2-7.9 kcal/mol, depending on the pair of electronegative atoms involved and the bond angle.

There are several groups in proteins which are capable of forming hydrogen bonds. Among these groups the greatest number of hydrogen bonds are formed between the N-H and C=O groups of the peptide bonds in the α-helix and β-sheet structures. The peptide hydrogen bond can be considered as a strong permanent dipole-dipole interaction between the N δ−H δ+ and C δ+=O δ− dipoles.

At longer distances weak dipole-dipole interaction between the N-H and C=O groups decreases the strength of the hydrogen bond. The strength of N-H····O=C hydrogen bonds in the interior of proteins, where the dielectric constant is close to 1, is typically about 4.5 kcal/mol. The “strength” refers to the amount of energy needed to break the bond.

The formation of each hydrogen bond in protein reduces the free energy by about -4.5kcal/mol. Hydrogen bond is an ionic interaction and its stability depends on the dielectric constant of the environment. The reason behind the low stability of hydrogen in secondary structures is a low dielectric caused by the interaction between nonpolar residues. These bulky side chains serve as a hurdle for access of water to the N-H····O=C hydrogen bonds (Fennema et al., 2008).
2.3.4.4 Electrostatic Interactions

Proteins contain several amino acid residues with ionizable groups. Depending on the pH, different amino acids behave differently, like Asp and Glu residues as negatively charged and Lys, Arg and His are positively charged. At alkaline pH Cys and Tyr residues are negatively charged. So depending on the number of negatively and positively charged residues, proteins assume either a net negative or positive charge. The pH at which the net charge is zero is called isoelectric pH (pI) and the pH of the protein solution in the absence of electrolytes is known as the isoionic point.

All charged groups in proteins are distributed on the surface of the protein molecule with few exceptions. At neutral pH proteins assume either a net positive or a net negative charge, so the net repulsive interaction between same charges will destabilize protein structure. Also the attractive interactions between oppositely charged groups at certain critical locations may contribute to stabilize the structure of protein. In reality, however, the strength of these repulsive and attractive forces is minimized in aqueous solutions due to high permittivity of water.

The attractive and repulsive interactions between charges located on the protein surface do not contribute significantly to the protein stability. However, charged groups are partially buried in the interior of protein, where the permittivity is lower than water, usually with salt bridges with strong interaction energy. The electrostatic interaction energy depends on the distance and the local permittivity. The electrostatic interactions influence the protein folding as the charged groups remain exposed to the aqueous environment (Fennema et al., 2008).

2.3.4.5 Hydrophobic Interactions

In aqueous solutions, hydrogen bonding and electrostatic interactions between various polar groups in a polypeptide chain are not able to act as driving force for protein folding due to less energy. These polar interactions lose their stability in aqueous environment and this stability is dependent on maintenance of nonpolar environment. Hydrophobic interactions among nonpolar groups provide the main driving force for protein folding.

In aqueous solutions, the hydrophobic interaction between nonpolar groups is the result of thermodynamically unfavourable interaction between water and nonpolar groups. Moreover, nonpolar groups tend to aggregate, so that the area of direct contact with water is minimized. This water-induced interaction between nonpolar groups in aqueous solutions
is known as hydrophobic interaction. In proteins, hydrophobic interaction between nonpolar side chains of amino acid residues is the major reason that proteins fold into unique tertiary structures in which a majority of nonpolar groups are removed from the aqueous environment.

Hydrophobic interactions are endothermic unlike other non-covalent bonds i.e., hydrophobic interactions are stronger at high temperatures and weak at low temperatures (reverse of hydrogen bonding). Unlike electrostatic, hydrogen bonding and Van der Waals interactions, hydrophobic interaction follows an exponential relationship with distance between interacting groups whereas the former follow a power law relationship with distance between interacting groups. Thus it is more effective over relatively long distances.

The hydrophobic free energy of a molecule is directly proportional to the non-polar surface area that is accessible to water. It is evident that hydrophobic free energy contributes significantly to the stability of protein structure. The average hydrophobic free energy per amino acid residue in globular proteins is approximately 2.5 kcal/mol (Fennema et al., 2008).

**2.3.4.6 Disulphide Bonds**

These are the only covalent side chain cross-links found in proteins. They can occur both intramolecularly and intermolecularly. In monomeric proteins, disulphide bonds are formed as a result of protein folding. When two cys residues are brought into proximity with proper orientation, oxidation of the sulfhydryl groups by molecular oxygen results in disulfide bond formation. Once formed, disulfide bonds help stabilize the folded structure of proteins.

Protein mixtures containing cystine and Cys residues are able to undergo sulfhydryl-disulfide interchange reactions. This interchange reaction also can occur within a single denatured protein if it contains a free sulfhydryl group group and disulfide bond. The formation of a unique three dimensional protein structure is the net result various attractive and repulsive noncovalent interactions and any covalent disulfide bonds.

For the milk powders having particle size about 150 μm most of the particles are sensitive to van der Waals forces (about $10^{-7}$ N), capillary forces (about $10^{-5}$ N) and electrostatic forces. In a particular system, the number of contact points between particles is very significant.
If a protein has enough exposed hydrophobic groups at the isoelectric point, it aggregates due to the lack of the electrostatic repulsion through hydrophobic bonds and isoelectric precipitation will occur. Also the low intermolecular hydrophobic interactions will compel the protein to stay in solution even at isoelectric point due to hydration and steric repulsion.

2.3.5 **Protein Folding**
Anfinsen (1973) was the first to find that protein sequence can spontaneously fold into the native three dimensional (3D) structure with the corresponding function as a protein with broken disulphide bonds unfolded by urea can spontaneously fold again, restoring its native disulphide bonds and full native activity. The protein native three dimensional structures are under thermodynamic control and the native structure is the result of some definite pathway, i.e., it is under kinetic control.

The hydrogen bonds play vital role in protein folding which are formed between main chains, main and side chains and side-side chains of polar groups. Also the hydrophobic interaction between the non-polar regions also plays vital role in protein folding as these interactions are responsible for the folding of non-polar groups to greater extent towards the interior of the protein. Disulphide bonds slow down the rate of protein folding and this is not limited by the reaction rate of disulphide formation.

2.3.6 **Food Proteins**
Food proteins are those proteins that are easily digestible, nontoxic, nutritionally adequate, and functionally useable in food products (Fennema et al., 2008). Foods vary in their protein content and also in the properties of their proteins (Li-chan, 2004). Milk, meats (including fish and poultry), eggs, cereals, legumes and oilseeds have been found to be the major sources of food proteins (Fennema et al., 2008).

2.3.7 **Milk Proteins**
Milk proteins are considered best of all food proteins (Fox and Mcsweeney, 2003). Milk proteins are nutritionally important and provide a wide range of functional properties to milk powders (Oldfield and Singh, 2005). Milk protein represents 3-4% of milk composition (Vaclavik and Christian, 2005) and is a heterogeneous mixture of proteins. The main proteins present in milk are casein, whey and lipoproteins (Walstra et al., 2006). Table 4 presents an overview of the milk proteins.
### Table 4. Proteins in Milk (Walstra et al., 2006)

<table>
<thead>
<tr>
<th>Protein</th>
<th>mmol/ml Milk</th>
<th>g/kg Milk</th>
<th>g/100 g Protein</th>
<th>Molar Mass</th>
<th>g Protein/g N</th>
<th>Remarks</th>
</tr>
</thead>
<tbody>
<tr>
<td>Casein</td>
<td>1120</td>
<td>26</td>
<td>78.3</td>
<td>~23600</td>
<td>6.36</td>
<td>IEP = 4.6</td>
</tr>
<tr>
<td>αs1-Casein</td>
<td>450</td>
<td>10.7</td>
<td>32</td>
<td>~25200</td>
<td>-</td>
<td>Phosphoprotein</td>
</tr>
<tr>
<td>αs2-Casein</td>
<td>110</td>
<td>2.8</td>
<td>8.4</td>
<td>~19550</td>
<td>-</td>
<td>Same, contains –S–S</td>
</tr>
<tr>
<td>β- Casein</td>
<td>360</td>
<td>8.6</td>
<td>26</td>
<td>23983</td>
<td>-</td>
<td>Phosphoprotein</td>
</tr>
<tr>
<td>κ- Casein</td>
<td>160</td>
<td>3.1</td>
<td>9.3</td>
<td>~20500</td>
<td>-</td>
<td>“Glycoprotein”</td>
</tr>
<tr>
<td>γ-- Casein</td>
<td>40</td>
<td>0.8</td>
<td>2.4</td>
<td>~26</td>
<td>-</td>
<td>Part of β-casein</td>
</tr>
<tr>
<td>Whey proteins</td>
<td>~320</td>
<td>6.3</td>
<td>19</td>
<td>-</td>
<td>~6.3</td>
<td>Soluble at IEP</td>
</tr>
<tr>
<td>β-Lactoglobulin</td>
<td>180</td>
<td>3.2</td>
<td>9.8</td>
<td>18283</td>
<td>6.29</td>
<td>Contains cysteine</td>
</tr>
<tr>
<td>α-Lactalbumin</td>
<td>90</td>
<td>1.2</td>
<td>3.7</td>
<td>14176</td>
<td>6.25</td>
<td>Part of lactose</td>
</tr>
<tr>
<td>Serum albumin</td>
<td>6</td>
<td>0.4</td>
<td>1.2</td>
<td>66267</td>
<td>6.07</td>
<td>synthase</td>
</tr>
<tr>
<td>Proteose peptone</td>
<td>~40</td>
<td>0.8</td>
<td>2.4</td>
<td>40000</td>
<td>~6.54</td>
<td>Blood protein</td>
</tr>
<tr>
<td>Immunoglobulins</td>
<td>~4</td>
<td>0.8</td>
<td>2.4</td>
<td>40000</td>
<td>~6.20</td>
<td>Heterogeneous</td>
</tr>
<tr>
<td>IgG1, IgG2</td>
<td>-</td>
<td>0.65</td>
<td>1.8</td>
<td>-</td>
<td>-</td>
<td>Glycoproteins</td>
</tr>
<tr>
<td>IgA</td>
<td>-</td>
<td>0.14</td>
<td>0.4</td>
<td>~150000</td>
<td>-</td>
<td>Several types</td>
</tr>
<tr>
<td>IgM</td>
<td>-</td>
<td>0.05</td>
<td>0.2</td>
<td>~385000</td>
<td>-</td>
<td></td>
</tr>
<tr>
<td>Miscellaneous</td>
<td>-</td>
<td>0.9</td>
<td>2.7</td>
<td>-</td>
<td>-</td>
<td>Part is cryoglobulin</td>
</tr>
<tr>
<td>Lactoferrin</td>
<td>~1</td>
<td>0.1</td>
<td>-</td>
<td>86000</td>
<td>6.14</td>
<td>Glycoprotein, binds</td>
</tr>
<tr>
<td>Transferrin</td>
<td>~1</td>
<td>0.01</td>
<td>-</td>
<td>76000</td>
<td>6.21</td>
<td>Fe</td>
</tr>
<tr>
<td>Membrane proteins</td>
<td>-</td>
<td>0.7</td>
<td>2</td>
<td>-</td>
<td>~7.1</td>
<td>Glycoprotein, binds</td>
</tr>
<tr>
<td>Enzymes</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>Predominantly</td>
</tr>
</tbody>
</table>

Note: ~ = Approximate composition. IEP = isoelectric pH.

### 2.4 Types of Milk Proteins

Milk proteins are broadly classified as either casein or whey proteins.

#### 2.4.1 Casein Chemistry and Structure

The major protein present in milk is casein representing approximately 80% of the milk protein. Casein is a phosphoprotein with an ester-bound phosphate and contains a high phosphorous (0.85%) and proline content (Zayas, 1997). Caseins are insoluble at pH 4.6. In milk, the caseins form spherical colloidal structures by associating together known as casein micelles having a diameter range of 50 to 300 nm with an average diameter of 150 nm. Casein micelles consist of water, protein and salts. The micelle is composed of 92% casein and 8% inorganic salts, which are largely in the form of colloidal calcium phosphate (CCP) (Oldfield and Singh, 2005). These casein micelles together with fat globules, impart the
white color to milk (Zayas, 1997). There are three main casein fractions, known as alpha\textsubscript{s-}, beta- and kappa-casein (\(\alpha\)-, \(\beta\)- and \(\kappa\)-casein) where alpha\textsubscript{s-} casein itself consists of \(\alpha\textsubscript{s1}\)-casein and \(\alpha\textsubscript{s2}\)-casein. However, these two fractions are difficult to separate from each other (Vaclavik and Christian, 2005). The casein proteins can be subdivided into two categories based on their sensitivity to calcium. The non-calcium sensitive group which is \(\kappa\)-casein whereas \(\alpha\textsubscript{s1}\), \(\alpha\textsubscript{s2}\) and \(\beta\)-casein are the three calcium-sensitive members (Horne, 2006).

The structure of casein micelle was first studied forty five years ago by a group of Waugh in the 1960’s and early 1970’s. They hypothesized different structures on the basis of physico-chemical studies of casein aggregation which lead to “coat core” and “submicellar” models which appeared in various different forms. While considering the physico-chemical studies, electron microscopy was also used to study the overall structure of the micelles directly. These studies showed that the casein micelles are almost spherical but they have a non-homogeneous structure. The studies involving electron microscopy were reviewed and re-investigated and it was reported that many of the electron micrographs of micelles contained artefacts of different types and that electron microscopy alone is not enough to justify the apparent “submicellar” structure.

There are eight sites of post-translational phosphorylation in \(\alpha\textsubscript{s1}\)-casein which are important in their interactions with the calcium. Clustering of polar and non-polar residues is another important property of caseins. From these characteristics a unique dipolar structure which is composed of a highly solvated, charged domain and a hydrophobic globular domain. It appears as the polar domain approaches the random coil type behavior and the hydrophobic domain has a mixture of \(\alpha\)-helix, \(\beta\)-structure, \(\beta\)-turns and unordered structure. Due to the flexibility of the polar domain the molecular dimensions become very sensitive to ionic strength and to binding of ions, particularly protons (\(H^+\)) and Ca\textsuperscript{2+}. Moreover, intermolecular interactions between hydrophobic domains results in self association or association with other caseins. Due to the binding of the Ca\textsuperscript{2+} to orthophosphate the polar domain is discharged which increases the importance of these hydrophobic interactions. Precipitation of isolated \(\alpha\textsubscript{s1}\)-casein or micelle formation by \(\kappa\)-casein interaction is possible outcome of these intermolecular interactions (Carr, 1999).

The same properties are exhibited by the other two calcium sensitive caseins i.e., \(\alpha\textsubscript{s2}\)-casein and \(\beta\)-casein. Charged polar and hydrophobic domains are present in both \(\alpha\textsubscript{s2}\)-casein and \(\beta\)-casein. Based on the presence of phosphoseryl clusters \(\alpha\textsubscript{s2}\)-casein is the most
hydrophilic and its properties are more sensitive to ionic strength than to temperature whereas β-casein is the most hydrophobic of all the proteins due to a single phosphoseryl cluster in N-terminal sequence and its characteristics are mostly temperature dependent (Carr, 1999).

Due to the amphipathic nature of κ-casein, it has the ability to stabilize the milk casein micelle. K-casein lacks clusters of phosphoseryl residues in its polar domain as compared to other calcium sensitive caseins. This reduces the capacity of Ca^{2+} binding and polar domain is neither discharged nor dehydrated by Ca^{2+} addition. Moreover, it is not precipitated by Ca^{2+}. The isolated K-casein is present in the form of large spherical aggregates similar to soap micelles in physiological buffers. Each of these is held by lateral interactions among the hydrophobic domains. These hydrophobic domains of κ-casein interact with similar domains of other caseins when they are present (Carr, 1999).

As reported by Fox and McSweeney (2003) the location of κ-casein must be such that it is able to stabilize the calcium-sensitive caseins, allow rapid and specific hydrolysis by chymosin and similar proteases and permit complex formation with whey proteins when heated in normal milk. According to the above conditions and also to the observation that κ-casein content is inversely proportional to micelle size across the micelle size distribution in bovine milk the most suitable location of κ-casein is as the surface layer (Horne, 2006).

The κ-casein appears to be present on the surface of the casein micelles. The hydrophilic, C-terminal part of κ-casein is assumed to protrude 5 to 10 nm from the micelle surface into the surrounding solvent, giving the micelle a hairy appearance. The highly charged flexible hairs physically prevent the approach of other micelles thus providing the micelles with their stability (Oldfield and Singh, 2005). Figure 3 shows a typical casein micelle model. There are many other models.

Casein does not experience denaturation. Heating at temperatures above 120°C causes the casein to slowly become insoluble due to chemical changes (Walstra et al., 2006). The casein micelle structure is relatively heat stable. Milk can withstand heating for 14 to 19 min at 140°C before coagulating. This property is useful for applications where solubility and heat stability are required, for example, recombined evaporated milk, hot beverages and soups (Oldfield and Singh, 2005). On the other hand, casein micelle stability is easily destroyed by the addition of acid or by enzymatic hydrolysis (rennet). Acid dissolves the CCP and causes dissociation of individual casein proteins from the casein micelle; enough acid
causes casein to precipitate out at pH 4.6, which is the basis of yoghurt and fresh cheese manufacture. Moreover, addition of rennet destabilizes the micelle by removing the charged portion of κ-casein responsible for micelle stability. This subsequently allows the micelles to aggregate and a curd is formed (cheese production) (Vaclavik and Christian, 2005).

The form in which the caseins exist in milk products depends on the processing conditions used. Acid precipitation will result in non-micellar form, whereas skim milk or MPC contains the micellar caseins (Mulvihill and Ennis, 2003).

There are two types of casein models in the literature; sub micelle models (Slattery and Evard, 1973; Schmidt, 1982; Walstra and Jenness, 1984; Walstra, 1990) and non-sub micelle models (visser, 1992; Holt, 1992; Horne, 1998).

Sub-micelle models are based on the electron micrographs showing composition of casein micelles as sub-micelles of 10 nm in diameter (Carr, 1999). Schmidt (1982) reported the colloidal calcium phosphate linkage between the sub-micelles of varying composition as shown in figure 4. The sub-micelles enriched in κ-casein are present on the surface while κ-casein depleted sub-micelles are buried inside the micelle. In non-sub micelle model the dual bonding model has been formulated which is based on the electrostatic and hydrophobic interactions as shown in figure 5 (Horne, 1998). In this model bonding takes place between the hydrophobic regions and linking of hydrophilic regions containing phosphoserine clusters to colloidal calcium phosphate clusters. K-casein molecules limit further growth and are described in figure by letter ‘K.’
Researchers have yet to agree on any single micelle model and this debate is still going on. However, they have agreement on the concept of casein micelle electrostatically and sterically stabilized by a ‘hairy layer’ coat of the κ-casein (Walstra, 1990; Swaisgood, 1992; Horne, 1998).

2.4.2 Whey Protein Chemistry and Structure
The second protein fraction present in milk is the whey proteins representing approximately 20% of milk protein (Vaclavik and Christian, 2005). These are the globular proteins which can be isolated from the whey (Cayot and Lorient, 1997) representing...
approximately 20% of milk protein. It includes lactalbumin and lactoglobulin. Whey proteins are more hydrated than casein and are denatured and precipitated by heat rather than by acid (Vaclavik and Christian, 2005).

Whey proteins are composed of a uniform distribution of proline, polar and non-polar amino acid residues along their respective polypeptide chains and so exhibit a compact, globular conformation with substantial helical content in their native state. These proteins fold intermolecularly with most of hydrophobic residues buried in such a way that it prevents the whey proteins from extensive self association or interaction with other proteins. However, they are heat sensitive which results in denaturation and protein-protein interactions involving disulphide interchange and calcium mediated aggregation. Moreover, they undergo intermolecular interactions with κ-casein during heat processing of milk through disulphide interchange. Whey proteins are very important in the determination of heat stability of milk protein concentrates (Carr, 1999).

Whey proteins do not form micelles; instead they exist in solution as globular proteins. The main whey proteins are: β-lactoglobulin (50% by weight), α-lactalbumin (20%), bovine serum albumin (5%), and immunoglobulins (10%). β-lactoglobulin is the major whey protein and it tends to govern the general behaviour of the whey proteins as a whole. In milk, whey proteins are stable under acid conditions; however, they are sensitive to heat denaturation above 65°C. Heating above 70°C causes β-lactoglobulin to aggregate with itself and with other whey proteins. Furthermore, β-lactoglobulin can also interact with κ-casein at the micelle surface. These reactions modify properties of the milk system, such as heat stability and acid gelation (Oldfield and Singh, 2005).

α-Lactalbumin are present as a monomer and are nearly spherical based on their hydrodynamic properties. α-Lactalbumin has a capability of zinc binding and probably other metals. Removal of Ca²⁺ cause reduction in the heat stability of α-lactalbumin (Fox and Mcsweeney, 2003).

β-lactoglobulin can undergo limited self association and its structure is pH dependent. It occurs as a dimer at milk pH, below pH 3.5 behaves like a expanded monomer, between pH 3.5 and 5.2 dimer tetramerizes to result in octamer and above pH 7.5 the dimer dissociates and experience a conformational change to give an expanded monomer. The presence of sulphydryl group and disulphide bonds affects the functionality of β-lactoglobulin. β-
lactoglobulin takes part in sulphhydryl disulphide interchange reactions under appropriate conditions which also effects many of its characteristics such as solubility (Carr, 1999).

As the research problem of the project is related to the protein insolubility and it is found by Havea (2006) that whey proteins are completely soluble and caseins are responsible for this insolubility. The caseins have well defined quaternary structure which is important while considering the insolubility problem.

2.5 Types of Commercially Available Protein Powder

There is a wide range of dry dairy products produced all over the world. Some of these powders are listed in the Table 5 below.

Table 5. Range of Dried Dairy Products (Fox and McSweeney, 2003)

<table>
<thead>
<tr>
<th>Skim milk powder</th>
<th>Whey powders</th>
</tr>
</thead>
<tbody>
<tr>
<td>Instant</td>
<td>Normal</td>
</tr>
<tr>
<td>Regular</td>
<td>Demineralised</td>
</tr>
<tr>
<td>Low-, medium-, high-heat</td>
<td>Delactosed</td>
</tr>
<tr>
<td>Whole milk powder</td>
<td>Caseinates (Na, K, Ca)</td>
</tr>
<tr>
<td>Instant</td>
<td>Rennet casein</td>
</tr>
<tr>
<td>Regular</td>
<td>Acid casein</td>
</tr>
<tr>
<td>High free fat</td>
<td>Total milk proteinates</td>
</tr>
<tr>
<td>Filled milk powder</td>
<td>Casein coprecipitates</td>
</tr>
<tr>
<td>Infant formula</td>
<td></td>
</tr>
</tbody>
</table>

Out of these, the two principal commercial milk powders are skim milk powder and whole milk powder which are generally classified into regular (non-instant) or instant. The different food applications of these two powders are summarised in Table 6.
Table 6. Principal food applications of skim and whole milk powders (Fox and McSweeney, 2003)

<table>
<thead>
<tr>
<th>Skim milk powder</th>
<th>Whole milk powder</th>
</tr>
</thead>
<tbody>
<tr>
<td>Reconstitution</td>
<td>Reconstitution</td>
</tr>
<tr>
<td>Cheese-making (low heat SMP)</td>
<td>Convenience soups and sauces</td>
</tr>
<tr>
<td>Confectionery products</td>
<td>Milk chocolate (high free fat WMP)</td>
</tr>
<tr>
<td>Ice cream and other desserts</td>
<td></td>
</tr>
<tr>
<td>Hot and cold beverages</td>
<td></td>
</tr>
<tr>
<td>Recombined sweetened condensed milk</td>
<td></td>
</tr>
<tr>
<td>Bakery products</td>
<td></td>
</tr>
<tr>
<td>Calf milk replacers</td>
<td></td>
</tr>
<tr>
<td>Recombined milk production</td>
<td></td>
</tr>
<tr>
<td>Chocolate manufacture &amp; Meat products</td>
<td></td>
</tr>
</tbody>
</table>

Commercial SMP can be classified on the basis of the pre-heat treatment applied such as low-, medium, or high-heat powders. Fat-filled, or filled, milk powders are products with a fat content close to that of WMP, produced by drying a blend of non-milk fat and skim milk. Encapsulated milk fat powders (40-60% fat) may be prepared by using a blend of emulsifying salts, SMP and flour, starch or sucrose (Fox and McSweeney, 2003). Table 7 gives a summary of commercially available milk powders and their approximate composition which are used in protein bar manufacturing (Fonterra Co-operative Groups).

Infant formulae are powders having high microbiological and hygienic standards during manufacturing and must have good reconstitution in warm water to give a homogeneous liquid free of undissolved lumps. The composition of these powders may be altered to meet the specific nutritional needs of the new born babies during their manufacturing process. These processes include electrodialysis and ultrafiltration to reduce the ionic content of the milk (demineralisation). Milk constituents like specific sources of protein, fat, carbohydrates, minerals and vitamins may be added in these powders (Fox and McSweeney, 2003).
<table>
<thead>
<tr>
<th>Description</th>
<th>Moisture</th>
<th>Protein</th>
<th>Casein</th>
<th>Whey</th>
<th>Fat</th>
<th>CHO</th>
<th>Lactose</th>
<th>Ash</th>
<th>Ca (mg)</th>
<th>K (mg)</th>
<th>Na (mg)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Power protein 515</td>
<td>WPC</td>
<td>3.7 %</td>
<td>80.3%</td>
<td>0.0%</td>
<td>80%</td>
<td>6.2%</td>
<td>7.0%</td>
<td>7.0%</td>
<td>2.8%</td>
<td>-</td>
<td>340</td>
</tr>
<tr>
<td>NZMP 4861</td>
<td>MPC</td>
<td>4.5</td>
<td>83</td>
<td>66.4</td>
<td>16.6</td>
<td>1.5</td>
<td>4.0</td>
<td>4.0</td>
<td>7</td>
<td>1300</td>
<td>380</td>
</tr>
<tr>
<td>ALACEN 352</td>
<td>WPC</td>
<td>4.0</td>
<td>71.5</td>
<td>0</td>
<td>71.5</td>
<td>10</td>
<td>7.0</td>
<td>7.0</td>
<td>4.5</td>
<td>350</td>
<td>400</td>
</tr>
<tr>
<td>ALACEN 895</td>
<td>WPI</td>
<td>5.1</td>
<td>93.2</td>
<td>0</td>
<td>93.2</td>
<td>0.3</td>
<td>0.6</td>
<td>0.6</td>
<td>2.1</td>
<td>62</td>
<td>89</td>
</tr>
<tr>
<td>ALANATE 385</td>
<td>Calcium caseinate</td>
<td>4.3</td>
<td>92.4</td>
<td>92.4</td>
<td>0.0</td>
<td>1.5</td>
<td>0.1</td>
<td>0.1</td>
<td>4</td>
<td>1400</td>
<td>5</td>
</tr>
<tr>
<td>ALANATE 312</td>
<td>Ca Caseinate</td>
<td>4.2</td>
<td>91.3</td>
<td>0.0</td>
<td>0.0</td>
<td>0.8</td>
<td>0.1</td>
<td>0.1</td>
<td>3.6</td>
<td>1320</td>
<td>0.0</td>
</tr>
<tr>
<td>ALANATE 180</td>
<td>Na Caseinate</td>
<td>4.3</td>
<td>92.7</td>
<td>0.0</td>
<td>0.7</td>
<td>0.2</td>
<td>0.2</td>
<td>3.6</td>
<td>20</td>
<td>30</td>
<td>3</td>
</tr>
<tr>
<td>ALAPRO 4700</td>
<td>MPC</td>
<td>4.4</td>
<td>69.8</td>
<td>0.0</td>
<td>0.0</td>
<td>1.4</td>
<td>17.2</td>
<td>17.2</td>
<td>7.2</td>
<td>1900</td>
<td>800</td>
</tr>
<tr>
<td>ALAPRO 4850</td>
<td>MPC</td>
<td>4.5</td>
<td>81.2</td>
<td>0.0</td>
<td>0.0</td>
<td>1.5</td>
<td>5.8</td>
<td>4.3</td>
<td>7</td>
<td>2230</td>
<td>280</td>
</tr>
<tr>
<td>ALAPRO 4560</td>
<td>MPC</td>
<td>3.8</td>
<td>57.1</td>
<td>0.0</td>
<td>0.0</td>
<td>1.3</td>
<td>30.1</td>
<td>30.1</td>
<td>7.7</td>
<td>1760</td>
<td>0.0</td>
</tr>
<tr>
<td>TMP 1100</td>
<td>Total Milk Protein</td>
<td>4.2</td>
<td>91.3</td>
<td>0.0</td>
<td>0.0</td>
<td>0.7</td>
<td>0.1</td>
<td>0.1</td>
<td>3.7</td>
<td>23</td>
<td>0.0</td>
</tr>
</tbody>
</table>
Membrane processing techniques can be used as an alternative to evaporation for specific purposes. While processes such as ultrafiltration (UF) fractionate milk constituents, reverse osmosis (RO) or hyperfiltration or nanofiltration (NF) remove essentially only water, and thus can serve as a pre-concentration step. Reverse osmosis is limited to a low achievable solids level (<20% TS), a medium flow rate and is far less thermally intensive than evaporation. Concentration by UF presents advantages in that heat treatment during concentration is avoided and the levels of protein and lactose in powder may be standardized and controlled (Caric et al., 2009).

<table>
<thead>
<tr>
<th>Particle Size (μm)</th>
<th>0.0001</th>
<th>0.001</th>
<th>0.01</th>
<th>0.1</th>
<th>1.0</th>
<th>10</th>
<th>100</th>
</tr>
</thead>
<tbody>
<tr>
<td>Molecular wt., D</td>
<td>100</td>
<td>1000</td>
<td>10000</td>
<td>100000</td>
<td>500000</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Particle Characteristic</td>
<td>Ionic</td>
<td>Molecular</td>
<td>Macromolecular</td>
<td>Cellular + microparticulate</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>Ions</td>
<td>Whey Proteins</td>
<td>Fat globules</td>
<td>Yeast, moulds</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>Salts</td>
<td>Casein micelles</td>
<td>Vitamins</td>
<td>Bacteria</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Milk System Components</td>
<td>Lactose/derivate</td>
<td>Whey protein aggregates, cheese fines</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Separation Process</td>
<td>RO</td>
<td>UF</td>
<td>Traditional filtration</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>MF</td>
<td></td>
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</tr>
</tbody>
</table>

Figure 6. Spectrum of application of membrane separation processes in the dairy Industry (Caric et al., 2009)

Figure 6 shows the summary of application of membrane separation processes in the dairy Industry. Recently, by using the selective membrane technologies such as microfiltration, ultrafiltration and diafiltration a number of fractionated or semi-refined milk protein powders have been developed for specific functional properties (Fox and McSweeney, 2003).

2.5.1 Differences in composition and structure of different powders

Due to the advancements in the dairy science and technology it has been possible to manipulate the composition of the milk powders. As a result different milk powders meeting the market requirements have been developed. The different ingredients which have been manipulated are protein (Individual proteins like casein and whey as well as desired casein and whey protein ratio), fat, carbohydrate (lactose) and minerals (including addition of
specific ion) to achieve the desired functional properties for different food applications. The different types of milk powders are WMP, MPC, MPI, WPC, WPI, calcium caseinate and sodium caseinate. Milk powders with or without fat, with varying protein content (0-95%), with or without lactose and varying the calcium and sodium content have been developed.

The process of WPC manufacturing causes reduction in WPC solubility as a result of the processing treatment, excessive heat and pH extremes. WPC with increased protein concentration (up to 90%), minimal denaturation and high solubility is prepared by ultrafiltration and reverse osmosis. Undenatured proteins with good functionality were also prepared by combined reverse osmosis-ultrafiltration feasible method. Whereas solubility of WPC prepared by gel or membrane filtration techniques is very high. Methods of the protein extraction and preparation are limiting factors of protein solubility and are influencing the pH-solubility profile of WPC. The pH of the bulk solution was not changed by the solubility of WPC prepared by ultrafiltration and electrodialysis (Zayas, 1997).

Sodium caseinate is produced by solubilizing the acid casein with sodium hydroxide and the product contains caseins as individual casein molecules and casein complexes (Lucy et al., 2000). Calcium caseinate is prepared by mixing the acid casein with calcium hydroxide. The casein exists in a more aggregated state due to calcium binding (Srinivasan et al., 1999). The whey protein powders containing 70-85% protein are produced from sweet or acid whey using membrane processing techniques. The whey proteins in these products exist largely as individual molecules with compact globular structures while a small proportion may be aggregated or denatured (Singh, Ye and Havea, 2000).

2.6 Manufacturing of Milk Powder & Protein Interactions

The manufacturing process for milk powder consists of several stages which are described below. Figure 7 shows the general processes for a typical whole milk powder (WMP), skim milk powder (SMP) or milk protein concentrate (MPC). The steps involved in the manufacturing of milk powder are described below.

2.6.1 Milk Pre-Treatment

For powder manufacturing milk of very high microbiological quality is required. This is achieved by the application of the techniques of bactofugation or microfiltration and pasteurization resulting in the removal of the bacterial cells and spores from milk (Singh, 2007).
2.6.2 **Separation and pasteurization**

The standard process of milk powders production starts with the reception of the raw milk at the dairy factory and then pasteurization and separation of it into skim milk and cream using a centrifugal cream separator (Pearce, 2008), for obtaining the desired components for the final product specifications (Singh, 2007). Based on the need if WMP is to be manufactured a require amount of the cream is added back to the skim milk to acquire milk with a standardised fat content (typically 26-30% fat in the powder) (Pearce, 2008). Whole milk is generally standardized for the fat to solids non-fat ratio of 1:2.76, to control the fat content of the final powder (Singh, 2007).

2.6.3 **Preheat treatment**

Heat stability depends on the type and sequence of the processing steps of the concentrates. In this regard pre-heat treatment of the milk prior to concentration plays a crucial role in comparison to other processing steps. High heat treatments will result in more heat stable concentrates (Deeth and Hartanto, 2009).
Preheating is carried out before evaporation to ensure the microbiological quality of concentrate and the final powder. It is also very important step in the control of the functional properties of the powder. In this step the highest temperature is applied as compared to all others during manufacturing and most of the whey protein denaturation occurs on this point (Singh, 2007). In this process the standardised milk is heated to temperatures between 75-120°C and held for a specified time which varies from few seconds up to several minutes (Pearce, 2008).

On the basis of heat application skim milk can be classified into three categories i.e., low heat (typically heated at 75°C for 15 sec), medium heat (typically heated for 75°C for 1-3 min) and high heat (heated at 80°C for 30 min or 120°C for 1 min). The standard preheating temperature in New Zealand is 72°C for 15 sec (Carr, 1999). Preheating may be either indirect (i.e., heat exchangers), or direct (i.e., via steam injection or infusion into the product), or a mixture of the two. Preheating results in a controlled denaturation of the whey proteins in the milk and destruction of bacteria, inactivation of enzymes, generation of natural antioxidants and also it imparts heat stability. The exact heating and holding period depends on the type of product and its intended end-use (Pearce, 2008).

Whole milk powder is not heat classified but is normally heated at 85-95°C for several minutes to ensure inactivation of indigenous lipase and to expose antioxidant sulphydryl groups (Singh, 2007). High temperature preheating of milk for whole milk powder results in improved keeping quality but reduced solubility (Pearce, 2008).

A lot of studies have been carried out on heat-induced interactions of milk proteins, but these have been performed on the laboratory scale conditions. Commercial heating equipment can produce quite different levels and types of protein interactions as compared with laboratory scale heat treatments.

Denaturation of whey proteins and their association with the casein micelles are the most important effects of preheat treatment on milk proteins formed largely through disulphide linkages between κ-casein and β-LG (Deeth and Hartanto, 2009). It is very interesting that αs2-casein which has one disulphide bridge does not interact with β-LG in milk systems (Singh, 2007). Aggregation reactions result in the interaction of denatured whey proteins not only with casein micelles (κ-casein) but also among themselves to form polymeric products. β-LG forms complexes with α-LA, which can subsequently interact with κ-casein. The minor whey proteins (serum albumin and immunoglobulin) start denaturation
at 65°C whereas the major whey proteins (β-LG and α-LA) show significant denaturation only at temperature range between 70-75°C. Heat-induced denaturation of various whey proteins follows the following order: immunoglobulins > serum albumin > β-LG A > α-LA (Singh, 2007). Aggregation reactions takes place after the denaturation of whey proteins as the conditions in milk are favourable.

In whey protein concentrate with the increase in protein denaturation the powder surface coverage of protein was decreased (Millqvist-Fureby et al., 2001). This results in more leakage of fat onto the powder surface.

The interactions of whey proteins with the casein micelles have been shown to be related to changes in casein micelle size during heat treatments. Moreover, it was reported that on heating the milk at temperatures up to 100°C, the change in the micelle size was dependent on the pH of milk at heating (Singh, 2007). The stability of concentrates is also dependent on the concentration of un-denatured β-LG and takes place over the entire pH range whereas addition of κ-casein can increase the heat stability of concentrates (Deeth and Hartanto, 2009).

2.6.4 Evaporation

Evaporation is used to remove maximum water without adversely affecting the quality of the powder (Pearce, 2008). With the increase in milk concentration, its viscosity also increases, making it hard to remove water. This step is carried out by multiple-effect evaporator to remove water. After the preheating, the milk is concentrated to 45-50% or 42-48% total solids (TS) for whole or skim milk, which is normally carried out between 50-70°C with having a residence time of 1 minute in each stage (Fox and Mcsweeney, 2003). More than 85% of the water in the milk may be removed in the evaporator (Pearce, 2008).

At this temperature range, whey proteins denaturation is considered as minimum. Recently, it has been found that evaporation of skim milk to 47-49% total solids in a multiple effect evaporator and subsequent heat treatment of the concentrate in the range of 64–74°C had no significant effect on the denaturation of β-LG, α-LA, serum albumin or immunoglobulin G. This was considered to be partly due to the high stability of whey proteins at increased total solids concentrations (Fox and Mcsweeney, 2003).

There was decrease in pH of milk during concentration from the average initial value of 6.7 to 6.3 at 45% total solids. This is due to variation in salt equilibrium as more calcium
phosphate is transferred from the soluble to the colloidal state, with a concomitant release of hydrogen ions. It has been reported that when the milk is concentrated about fivefold by evaporation, there is increase in soluble calcium and soluble phosphate by a factor of about two, the remainder of the soluble calcium and phosphate being transformed into the colloidal state. This results in the slight increase of calcium ions activity but there is marked increase in the ratio of monovalent to divalent cations. The increase in the size of casein micelles may be due to the increase in colloidal calcium phosphate or due to coalescence of the micelles (Singh, 2007).

Crystallization of lactose prior to drying is desirable for many products, particularly high-lactose products such as whey powders and consequently the concentrate may be held before drying under conditions which promote crystallization and may be seeded with finally-ground lactose crystals which promote crystallization by acting as nuclei (Fox and Mcsweeney, 2003).

2.6.5 **Homogenization**

This step is carried out in the manufacturing of whole milk powder where its function is to reduce the free fat content of powder and protect the fat globules from coalescence during the spray drying. High free fat content affects the rehydration badly due to poor wetting. Moreover, homogenization of whole milk concentrate results in the increase of the viscosity, which creates difficulties during spray drying. The increase in the homogenization pressure causes increase in the moisture content of the powder and decrease in the free fat content. The viscosity of the milk also increases with the increase in the concentration.

After concentration, milk may be homogenized. Conventionally, two-stage homogenization is used at a temperature in the range of 60-70°C; typical homogenization pressures are 15 MPa, followed by 5 MPa (Fox and Mcsweeney, 2003).

2.6.6 **Heat treatment**

The concentrate is given heat treatment to reduce the viscosity prior to its entry into the spray drier and to increase the energy available for drying (Pearce, 2008).

2.6.7 **Spray drying**

The concentrate is spray dried to remove most of the water. Spray drying involves atomising the milk concentrate from the evaporator into fine droplets. This is done inside a large drying chamber in a flow of hot air (up to 200°C) using either a spinning disk atomiser
or a series of high pressure nozzles. The milk droplets are cooled by evaporation and they never reach the temperature of the air (Pearce, 2008). The objective of atomisation is to convert the liquid feed to a spray of droplets 10-400 μm in diameter. Particle size and in particular the distribution of particle sizes is very important in determining the properties of milk powder. The selection of atomiser type is critical in determining the properties of the powder produced (Fox and Mcsweeney, 2003). Much of the remaining water is evaporated in the drying chamber, leaving a fine powder of around 6% moisture content with a mean particle size typically of < 0.1 mm diameter. Final or "secondary" drying takes place in a fluid bed, or in a series of such beds, in which hot air is blown through a layer of fluidised powder removing water to give product with a moisture content of 2-4% (Pearce, 2008).

During the manufacturing of skim milk powder, it is concentrated by ultrafiltration to increase the protein content and remove lactose and salts. Whereas, in the manufacturing of high protein powders (>70% protein in dry powder basis), diafiltration is normally applied. Generally there is no preheating step involved and after ultrafiltration and diafiltration of the skim milk the retentate is evaporated to remove more water followed by spray drying (Singh, 2007).

Separation of drying into two or three stages allows for improved control of powder properties, greater efficiency of the drying process and because the rate of heat introduction is adjusted to the rate of evaporation, the process is milder than single-stage processes (Fox and Mcsweeney, 2003).

The effects of heat exposure of milk during spray drying depends on the length of time the powder is held before cooling, on the design of the drier and the operating conditions. There is no substantial effect of moderate drying conditions on the native properties of the milk components. Reconstitution of spray-dried milk results in the recovery of normal size distribution of the casein micelles, their heat stabilities and renneting characteristics.

During spray drying, the factors responsible for the increase in protein denaturation and aggregation include the temperature of the air into which the milk is sprayed (inlet air temperature), the degree of concentration and the temperature of the concentrate prior to drying, the size of the drying droplets and the temperature of the air/powder mixture exiting from the drier (outlet air temperature). Drying is usually very fast and the temperature of the milk droplets does not exceed 70 °C until they have lost almost all their water. The temperature of the droplets approaches that of the outlet air as the drying
process nears completion. For this reason, the outlet air temperature is a critical parameter controlling heat damage to dry milk products. It has been reported that the denaturation of whey proteins during spray drying was minimal with no apparent loss of immunoglobulin G and only a small loss of serum albumin (3–7%). There was no significant effect found by changing the inlet and outlet air dryer temperature (200/100°C–160/89°C) on whey proteins denaturation (Singh, 2007).

Changes may occur in the salts equilibrium during spray drying. The process of drying will be expected to produce the same types of changes in salts equilibrium as by evaporation, i.e., a decrease in pH and an increase in colloidal calcium phosphate. It has been shown that the concentrations of soluble calcium and soluble phosphate in reconstituted skim milk are 20% lower than those in the original milk (Singh, 2007).

It has been reported that particle size distribution was affected by the viscosity of the concentrate. Particle size of the powdered milk (skim and whole) increases with increase in viscosity and this increase adversely affects the product solubility. Viscosity of the concentrate also influenced the characteristics of the spray dried milk powder. It was found that water evaporation from the drying droplet of a high viscous concentrate was difficult and protein denaturation could take place. Further research showed that particle size of powdered skim milk can be reduced by increasing atomisation pressure (to 25 Mpa) which is related to disruption of structural viscosity (Carr, 1999).

During the milk powder manufacturing process, drying and evaporation promote the migration of milk constituents particularly fat, protein and lactose toward the particle surface. Nijdam and Langrish (2006) reported that the surface fat coverage is much higher than the average fat component of the powder within the milk droplets and particles in a spray dryer. This shows accumulation of higher concentration of fat at the surface of the each milk particle than in the interior leading to a non-uniform distribution of fat throughout the solid matrix. For example, if the average fat content of milk powder is 15% then the surface fat coverage may reach up to 50%. The fat appearance on powder surface starts even at very low fat contents between 0-5% which increases up to 35%. It was found by Kim et al., (2002) that free fat was mostly present on the surface whereas fat globules protected by proteins were preferentially located below the surface fat. The next ingredient of milk powder present in excess on the surface is protein due to its surface active nature, then lactose (Kim et al., 2002, 2005; Nijdam and Langrish, 2006; Shrestha et al., 2007).
Shrestha et al., (2007) reported that the modification of protein content of skim milk powder by addition of lactose alters the surface composition, glass transition temperature and sorption behaviour of spray dried powder. Moreover, it is also found that lactose migrates to the surface slower than protein and fat.

2.6.8 **Instantization**

Production of milk powders that reconstitute well when dispersed in cold water (instant powders) has necessitated development of modified spray-drying processes.

For skim milk powder, instantization is achieved by agglomeration, i.e., the production of porous clusters of particles, 250 to 750 μm in diameter, with a high level of entrapped air. Instantization reduces the bulk density of milk powder (produces lighter particles) and enhances its wettability, sinkability and solubility (Fox and Mcsweeney, 2003).

With WMP, an extra step is required after agglomeration to make the product truly "instant" and overcome the hydrophobic nature of traces of free fat on the surface of the particles. This extra step consists of spraying minute quantities of the natural surfactant or wetting agent, soy lecithin, on to the powder in a fluid bed. Soy lecithin is extracted from soy bean oil. Lecithins are widespread in nature and they occur naturally in milk (Pearce, 2008).

In order to produce agglomerates with optimum reconstitution properties, it has been proposed that low- or medium-heat milk be used and that the powder particles should have a high particle density and a diameter in the range of 25 to 50 μm (Fox and Mcsweeney, 2003).

Standard powders, because of their fine dusty nature, do not reconstitute well in water. "Agglomerated" and "instant" powders were specifically developed to counter this. The manufacture of an agglomerated powder initially follows the standard process of evaporation and drying, described above. However, during spray drying small particles of powder leaving the drier (the "fines") are recovered in cyclones and returned to the drying chamber in the close proximity of the atomiser. The wet concentrate droplets collide with the fines and stick together, forming larger (0.1-0.3 mm), irregular shaped "agglomerates". Agglomerated powders disperse in water more rapidly and are less dusty and easier to handle than standard powders (Pearce, 2008).
To overcome the hydrophobic nature of milk fat, instantization of whole milk powder requires a combination of agglomeration and lecithinization. Agglomeration of WMP may be achieved by the same procedures as for SMP. Lecithin which aids instantization by virtue of its surfactant properties, is usually added to WMP at a level of approximately 0.2% between the spray-drying stage and the fluidised bed drying or alternatively, in a rewet process. When lecithin is added to WMP, the mixture should be held at ~50°C for 5 min to ensure complete coating of the particles with lecithin (Fox and Mcsweeney, 2003).

2.6.9 Summary of Processing Operations during Manufacturing of Powdered Milk Protein Products

All processing operations except preheat treatments and homogenization during the manufacture of powdered milk protein products are aimed to minimise the cost of production. Due to the increase in the apparent viscosity of the concentrate by evaporation, the level of concentration is not fully achieved. The factors affecting the basic viscosity include preheat treatments, concentration, temperature, and for whole milk, homogenization whereas the structural viscosity is influenced by concentration, length of holding and holding temperature. The affects of structural viscosity on the quality of the powder are dependent on the shear during spray drying. The pumping problems also cause increase in the apparent viscosity (Carr, 1999).

2.6.10 Milk Protein Interactions involved in the Manufacturing of MPC

In the manufacture of MPC, skim milk is concentrated by ultrafiltration prior to evaporation and spray drying. As there was no preheating (except pasteurization), whey protein are present largely in their native state. The ultrafiltration process retains casein micelles and whey proteins and allows water, lactose, non-protein nitrogen compounds and soluble salts to pass through the membrane. Some calcium, magnesium, phosphate and citrate are found associated with the casein micelles in milk, and hence are retained in the concentrate. The ratio of casein to whey proteins in the MPC is similar to that in the SMP.

Electron microscopy studies revealed that the size of the highest proportion of casein micelles (80–100 nm) in milk was reduced (to 60–80 nm) at a volume concentration factor of about 5. The volume distribution and the average diameter of casein micelles was reduced upon ultrafiltration of skim milk. These changes in micelle size were due to the changes in the levels of milk salts, particularly calcium and phosphate. In contrast, it was
also reported that there was little variation in particle size at the early stages of ultrafiltration, but at the end of the ultrafiltration process, and particularly during diafiltration there was significant increase in the average micelle size.

Progressive swelling and formation of non-micellar material of casein micelles were found by electron microscopy during the course of ultrafiltration and diafiltration. After ultrafiltration there appears to be no considerable change in casein micelle size, but more non-micellar material was found. After diafiltration, there was significant change in the structure of casein micelles. There is an increase in non-micellar material, and casein micelle appears to be swollen and less dense. After evaporation, the micelles appear to pack together, resulting in aggregation of some of the micelles which was due to the non-micellar material.

It is likely that the loss of serum calcium and phosphate during ultrafiltration and diafiltration causes the dissolution of colloidal calcium phosphate. Consequently this continual loss of colloidal calcium and phosphate results in loosening of casein micelle structure and is thus responsible for swelling of the micelles. During evaporation, there appears to be an increase in micelle aggregation, involving the non-micellar material or dissociated casein micelles. Spray drying probably induces further protein-protein interactions, but the nature of these interactions is not known.

MPC powders with very protein content (e.g. MPC85) are generally known to have poor solubility upon reconstitution in water at 20°C, but the solubility improves at higher reconstitution temperatures. The solubility of these powders decreases with storage time at elevated temperatures. The major factor affecting the solubility behaviour of MPC85 appears to be related to the rate of water transfer into the powder particle rather than to the thermal processes during manufacture. Various processes have been proposed for manufacturing MPC powders with improved solubility in cold water, involving the addition of a monovalent salt to the ultrafiltered retentate prior to drying (Carr, 2002) or partial replacement (30%) of calcium content of ultrafiltered retentate by sodium ions. The insoluble material found in the reconstituted MPC powders has been characterised. It was found that insoluble material in MPC85 consists of large particles (up to 100 μm) formed by fusion of casein micelles, involving some kind of protein-protein interactions. These fused casein micelles appear to form a skin-like structure on the outside of a powder particle,
inhibiting the movement of water into the particle. Upon reconstitution in water, large parts of these particles remain intact.

McKenna (2000) found that some protein-protein interactions are responsible for the fusion of casein micelles resulting in formation of relatively large (~100 μm) particles. It has been reported that the insoluble material consists predominantly of αs- and β-caseins, as revealed by PAGE analysis. This material was held together by weak non-covalent interactions that were easily disrupted under SDS-PAGE conditions. Although some disulfide-linked protein aggregates consisting of κ-casein and β-LG were present in MPC powders, these aggregates were not considered to play an important role in the formation of insoluble material (Singh, 2007).

There are two types of interactions which result in the formation of insoluble material based on covalent and non-covalent bonds. Covalent bonds consist of inter- and intramolecular disulphide bonds which are formed through sulphydryl-disulphide interchange or sulphydryl oxidation reactions. The non-covalent interactions included hydrophobic, hydrogen bonding, ionic and other weak interactions which are also responsible for formation of these insoluble materials (Havea, 2006).

2.7 Functional Properties of Dairy Protein

Functional properties of proteins are those characteristics that determine their satisfactory use in food products. Proteins as macromolecules perform important roles in functionality in foods and pharmaceuticals as well as in biological systems (Singh, 2002). Therefore, the growing demand for proteins as important ingredients in formulated food mixtures has created a necessity for proteins with specific and consistent functional properties. Proteins exhibit many functional properties as governed by their physicochemical activities in a bulk liquid phase.

These characteristics may include structure, appearance, texture, viscosity and mouthfeel. Milk proteins possess functional properties which provide desirable textural or other properties to the final product and for this reason have found various applications in traditional dairy products and other foods (Hui, 2006). Some important functional properties for which proteins are added to food products and the relevant food systems are shown in Table 8.
The functional properties of milk products may be considered to be the consequence of the molecular structures and the interactions of milk proteins with other food components such as fat, sugars, polysaccharides, salts, flavours and aroma compounds. These interactions take place at two levels. First, the processing of milk into milk protein products alters the native structures and induces interactions of proteins that can exert a negative or positive effect on functional properties. Second, the milk proteins interact with other food components during the manufacture of prepared food products (Singh, 2002). Table 9 represents important functional properties of milk protein products and milk powders in food systems.

Table 9. Important functional properties of milk protein products and milk powders in food systems (Singh, 2002)

<table>
<thead>
<tr>
<th>Caseinates</th>
<th>Whey proteins</th>
<th>Milk powders</th>
<th>Milk protein concentrate powders</th>
</tr>
</thead>
<tbody>
<tr>
<td>Emulsification</td>
<td>Gelation</td>
<td>Dispersibility</td>
<td>Hydration</td>
</tr>
<tr>
<td>Foaming</td>
<td>Heat stability</td>
<td>Wettability</td>
<td>Solubility</td>
</tr>
<tr>
<td>Viscosity</td>
<td>Clarity</td>
<td>Solubility</td>
<td>Heat stability</td>
</tr>
<tr>
<td>Solubility</td>
<td>Water-binding</td>
<td>Stability in hot coffee or tea</td>
<td>Rennet coagulation</td>
</tr>
<tr>
<td>Heat stability</td>
<td>Foaming</td>
<td>Heat stability</td>
<td></td>
</tr>
</tbody>
</table>

Proteins usually exert several interdependent functional properties simultaneously in each food application. The functional properties of proteins vary with pH, temperature, ionic strength and concentration of calcium and other polyvalent ions, sugars and hydrocolloids as well as with processing treatments (Johansen et al., 2002).
Milk powders are used in a wide range of foods from ice cream, nutrition bars, processed cheese products and baked goods. Milk powders are used as a source of dairy proteins in prepared foods. They add opacity to reduced-fat food formulations; their functional properties include improved viscosity, mouthfeel, emulsification, water binding and a favourable nutritional profile.

2.7.1 Solubility

The solubility profile is an excellent index of protein functionality and potential usefulness. Among the functional properties of milk powders solubility is of primary importance due to its significant influence on the other functional properties. In most cases the food powder must be able to provide good solubility to be useful and functional. Insoluble proteins have very limited uses in food. Solubility is considered as the key determinant of the overall reconstitution quality (Singh, 2002). In general, proteins used for functionality are required to have high solubility, in order to provide good emulsion, foam, gelation and whipping properties.

Solubility of proteins relates to surface hydrophobic (protein-protein) and hydrophilic (protein-solvent) interactions with water. The relative proportion of surface hydrophobic and hydrophilic groups dictates the degree of solvation by water (Hall, 1996). Therefore, the solubility of a protein under a given set of solution conditions depends on an at equilibrium between the hydrophilic and hydrophobic interactions that can be expressed as

\[
\text{Protein-Solvent} \rightleftharpoons \text{Protein-Protein + Solvent-Solvent}
\]

Generally, the dissolution rate is favoured by the presence of hydrophilic molecules of sugar on the surface of the solvent. The solubility of protein is based on the ability of soluble, polar residues to interact with water via hydrogen bonds, while the hydrophobic part of the protein folds to avoid contact with water (Fang et al., 2008). The major interactions that influence the solubility characteristics of proteins are hydrophobic and ionic in nature. Hydrophobic interactions promote protein-protein interactions and result in decreased solubility, whereas ionic interactions promote protein-water interactions and result in increased solubility. Ionic residues introduce two kinds of repulsive forces between protein molecules owing to a net positive or negative charge at any pH other than isoelectric pH; the second involves repulsion between hydration shells around ionic groups (Fennema et al., 2008).
Solubility of protein is dictated by hydrophilicity and hydrophobicity of the protein surface that contacts with the surrounding water, rather than the average hydrophobicity and charge of the molecule as a whole. Since a majority of residues are buried in the interior of the protein, only those nonpolar groups that are on the surface would affect the solubility. The fewer the number of surface hydrophobic patches, the greater the solubility.

Proteins are classified into four categories on the basis of solubility characteristics. Albumins which are soluble in water at pH 6.6 (e.g. serum albumins, ovalbumin and α-lactalbumin), globulins are those which are soluble in dilute salt solutions at pH 7.0 (e.g. glycumin, phaseolin, and β-lactoglobulin), glutelins are those that are soluble only in acid (pH 2) and alkaline (pH 12) solutions (e.g., wheat glutelins) and prolamines are those soluble in 70% ethanol (e.g., zein and gliadins). Both prolamines and glutelins are highly hydrophobic proteins (Fennema et al., 2008).

Increasing the storage time and temperature could reduce the solubility of milk powder, due to the formation of a network of cross-linked proteins at the powder-water interface. This cross-linked network could act as a barrier for water to penetrate, thus inhibiting the rehydration of the powder particles. The solubility of milk powder is likewise affected by the pH and temperature of the solution (Fang et al., 2008).

Commercial caseins after processing are insoluble in water and are made soluble by the addition of alkali before utilization. Sodium and potassium caseinates are more soluble than calcium caseinates. Rennet casein remained insoluble under most conditions and solubility markedly increased with the addition of phosphate (NaH₂PO₄) at pH 6.2. At pH 5.6 ≤ 6.2, all caseins and caseinates showed their greatest solubility when sodium phosphate was added. The dephosphorylated caseins were completely soluble at pH ≥ 5.5 but almost insoluble (over 90% of the protein precipitated) below its isoelectric point. Addition of salts (NaCl, CaCl₂, NaH₂PO₄), increase in temperature and pH resulted in increased solubility of caseins and caseinates (Zayas, 1997).

These studies are carried out in excess moisture environments so there is no information whether the same behavior of these powders will be observed in limited water environment.
2.8 Rehydration Process

Rehydration is the most critical step in imparting desired functional properties to proteins in a food system. When a dry powder is exposed to water vapor, water molecules will be absorbed onto all available surface polar sites, thus forming monolayer coverage. Upon further water absorption, additional layers of water are formed as multilayers followed by water-water interaction to result in liquid-water condensation. At this state, the protein particles become swollen and if the protein is soluble, swelling continues until the individual protein molecules are surrounded by sufficient water molecules to solubilize them. This is a continuous over-lapping process with no clear-cut boundary between the individual stages (Chou and Morr, 1979). Figure 8 shows the sequence of protein-water interaction for dry protein.

![Diagram of protein-water interaction](https://via.placeholder.com/150)

Figure 8. Sequence of protein-water interaction for dry protein (Chou and Morr, 1979).

The rehydration process generally follows the sequence indicated in figure 6. However, different phases often overlap, such that observing each phase independently of the others would be a challenge. Four important rehydration properties of food powder are created by these identified phases; namely wettability, sinkability, dispersibility and solubility (Fang et al., 2008).

- Wetting of powder particles
- Sinking
- Dispersing
- Complete dissolution of particles in solution

Surface composition of powders plays important role in different properties of milk powder like dispersibility, wettability, flowability and oxidative stability (Hindmarsh et al.,...
2007). Each individual component of milk powder present on the powder surface affects the rehydration properties. Fat makes the powder surface hydrophobic which decreases its wettability and sinkability.

Flowability is another surface-related property which is controlled by the powder surface composition. Fat affects the powder flowability to a greater extent and makes the powder sticky and cause adherence between the particles which decrease the flowability. In skim milk powder flowability is higher due to presence of lactose and protein on the surface (Kim et al., 2005). The increase in the surface fat content decreased the rehydration rate of the milk powder (Millqvist-Fureby et al., 2001).

Figure 9 shows the schematic of dissolution timeline for different types of powder showing the overlaps between different phases with time but the sinking step is not shown in the figure.

![Figure 9. Schematic of dissolution timeline for different types of powder showing the overlaps between different phases with time (Fang et al., 2008).](image)

2.8.1 **Wettability**

The proficiency of a bulk powder to be penetrated by a liquid due to capillary forces is called wettability (Hogekamp and Schubert, 2003). It is the ability which is expressed as time (seconds) required for given quantity of powder to penetrate the surface of water. It is the capacity of powder particles to overcome the surface tension between themselves and water. It is the power of a powder to absorb water on the surface and get wet. A powder particle that fails to wet sufficiently will lead to the formation of a substance called scum. The scums will gradually adhere together, forming a distinct layer on the walls of the container (Fang et al., 2008).
Milk powder having less wettability forms lumps after coming into contact with water. Small particle size and symmetrical shape of spray dried milk powder inhibits penetration of water as they enhance the close packing of particles whereas larger powder particles having irregular shape provide more space in the interstices for wetting. Wettability is affected by the amount and dispersion of fat in milk powder affects. Generally, factors affecting the wettability of powder particles include the surface activity of the particles, particle size, surface area, density, porosity, surface charge and the presence of moisture absorbing substances.

2.8.2 Sinkability

It is the ability of powder to sink into the water (Gaiani et al., 2008). It is the capability of powder particles to overcome the surface tension of water and sink into water after passing through the surface. This property is affected by the forces that tend to submerge a powder particle on the surface and is dependent on the density of the particles. Higher particle density and a lower quantity of occluded air affect this property positively (Fang et al., 2008).

Agglomeration has more effect on sinkability for skim milk powder than for whole milk powder. Foam spray dried milk has very low sinkability. During the initial stages of rehydration the density of the particles decreases and heavier components such as lactose and minerals dissolve faster than the other components. At the same time density of the solution increases which inhibits further sinking resulting from the decrease of the density difference between particles and medium, so that particles start to rise again after the initial sinking. The sinkability is defined as the falling of powder particles below the surface of an aqueous phase or liquid and is primarily dependent on the density of the particles.

2.8.3 Dispersibility

Once the agglomerated particles are wetted and have sunk, they should immediately start to disperse uniformly as individual particles, while the agglomerates cease to exist. The dispersibility of a milk protein powder is mainly dependent on the capability of casein to disperse in solution (Fang et al., 2008).

This property has been chosen to measure the instantization nature of the product. For good dispersibility, the effect of total heat treatment on casein during processing is very
critical. Increase in heat application and increase in total solids results in a higher degree of irreversible changes in casein, which tends to an unstable dispersion.

The dispersibility of milk powder can be increased by minimizing the heat treatment at preheating, keeping the holding time and temperature of the concentrate to minimum and increasing particle size of the powder by selecting the proper atomization technique.

2.8.4 **Solubility**

Solubility is the final step of powder dissolution and is considered the key determinant of the overall reconstitution quality. The term solubility usually indicates the complete phenomenon of milk powder rehydration, including soluble (lactose, undenatured whey protein, salts) and dispersible (casein) components. Consequently, it is not strictly used in chemical sense but more as the rate of rehydrating, to explain the combination of all described properties connected to milk powder dissolution (Fang et al., 2008).

The solubility of powder is one of the properties which is required for by qualitative standards. This means that reduced solubility is a serious defect, which can result in rejection of the product. During dissolution, the insoluble part of the powder settles and forms a sediment layer on the bottom. Since reduced powder solubility is mainly the result of milk protein denaturation so the degree of denaturation is used for the determination of powder solubility to a great extent. Proper preheat treatment of milk of high quality affects positively the stability of the final reconstituted product. The parameters of drying procedures need to be adjusted in such a way that the last phase of drying is short when the concentration of total solids is high. Ion balance including pH and added salts have a major effect on protein stability and powder solubility. Proteins undergo a high degree of denaturation during roller-drying due to direct contact between milk and the hot metal surface of the rollers.

The degree of protein solubility in aqueous medium is the result of electrostatic and hydrophobic interactions between the protein molecules. Solubility is increased if electrostatic repulsion between the molecules is higher than the hydrophobic interactions. Protein solubility is affected by a sensitive balance between repulsive and attractive intermolecular forces and proteins are soluble when electrostatic repulsion between proteins is greater than hydrophobic interactions (Zayas, 1997).
2.9 Types of Solvents

There are different types of solvents based on their hydrophobic and hydrophilic natures and will therefore effect hydration. Generally proteins are more soluble in strongly polar solvents like water, glycerol, formamide, dimethylformamide or formic acid. In a less polar solvent such as ethanol, proteins are rarely soluble as was used to measure the particle size distribution of milk powder in earlier PGDip project.

2.9.1 Ethanol

Ethanol is a type of alcohol and is formed by the fermentation of glucose by enzymes in yeast. Ethanol belongs to the primary group of alcohol and belongs to that group which are soluble in water. Ethanol is also used as a solvent in perfumes, drugs and vegetable essences. The solubility in organic solvents is not good due to the polar characteristics of the amino acids.

2.9.2 Oil/Fat

Oil is a non-polar substance. It is highly viscous and hydrophobic in nature. This means that it does not like water and doesn’t mix with water.

2.9.3 Water

The solubility of protein in water is dependent on pH and salt concentrations. At low ionic strengths the solubility increases with rise in ionic strength and minimum solubility is shifted from pH 5.4 to pH 5.2. This shift is due to preferential binding of anions to the protein.

2.10 Protein Hydration

Protein hydration refers to the water that is more or less immobilized by a protein (Damodaran, 1997). Casein micelles are hydrated (about 2.5 g water/g protein).

Water molecules bind to several groups in proteins. These include charged groups (ion-dipole interactions); backbone peptide groups; the amide groups of Asn and Gln; hydroxyl groups of Ser, Thr and Tyr residues (all dipole-dipole interactions); and nonpolar residues (dipole-induced dipole interaction and hydrophobic interaction and hydrophobic hydration).

The water binding capacity of proteins is defined as grams of water bound per gram of protein when a dry protein powder is equilibrated with water vapor at 90-95% relative humidity. Amino acid residues with charged groups bind about 6 moles of water per residue, the uncharged polar residues bind about 2 mol/residue, and the nonpolar groups
bind about 1 mol/residue. The hydration capacity of a protein therefore is related, in part to its amino acid composition - the greater the number of charged residues, the greater is the hydration capacity. Water binding capacity is the limiting factor in protein food applications.

On a macroscopic level, water binding to proteins occurs in a step-wise process. The high affinity ionic groups are solvated first at low water activity, followed by polar groups.

Several environmental factors, such as pH, ionic strength, temperature, type of salts and protein conformation influence the water-binding capacity of proteins. Proteins are least hydrated at their isoelectric pH, where enhanced protein-protein interactions results in minimal interaction with water. Above and below the isoelectric pH, because of the increase in the net charge and repulsive forces, proteins swell and bind more water. The water-binding capacity of most proteins is greater at pH 9-10 than at any other pH. This is due to ionization of sulfhydryl and tyrosine residues. Above pH 10, the loss of positively charged ε-amino groups of lysyl residues results in reduced water binding.

At low concentrations (<0.2 M), salts increase the water binding capacity of proteins. This is due to hydrated salt ions especially the anions bind (weakly) to charged groups on proteins. At this low concentration, binding of ions to proteins does not affect the hydration shell of the charged groups on the protein, and the increase in water binding essentially comes from water associated with the bound ions. However, at high salt concentrations much of the existing water is bound by salt ions, resulting in dehydration of the protein.

The water binding capacity of proteins generally decreases as the temperature is raised due to decreased hydrogen bonding and decreased hydration of ionic groups. The water binding capacity of the denatured protein is generally about 10% greater than that of the native protein. This is due to an increase in surface area to mass ratio with exposure of some previously buried hydrophobic groups. If denaturation leads to aggregation of the protein, then its water binding capacity may actually decrease due to displacement of water by enhanced protein-protein interactions. Denatured food proteins generally exhibit low solubility in water. Their water binding capacities, however, are not drastically different from those in the native state. Thus, water-binding capacity cannot be used to predict the solubility characteristics of proteins. The solubility of a protein is dependent not only on water-binding but also on other thermodynamic factors.
2.10.1 **Hydration of Casein and Whey protein**

Casein micelles are hydrated (about 2.5 g water/g protein), whey protein in globular state needs 0.2 g water/g of protein whereas heat-denatured whey protein requires 10 g water/g of protein for hydration. These data represents the excess water environment. There is no information how these proteins will behave in limited water environment.

In food applications, the water-holding capacity of a protein is more important than the water binding capacity. WHC is a physical property and is the ability of a food structure to prevent water from being released from the three dimensional structure of protein. Water-holding capacity refers to the ability of the protein to imbibe water and retain it against gravitational force within a protein matrix. This water refers to the sum of the bound water, hydrodynamic water and the physically entrapped water. The physically entrapped water contributes more to water-holding capacity than do the bound and hydrodynamic water. However, studies have shown that the water-holding capacity of proteins is positively correlated with water binding capacity. The level of protein hydration and the viscosity of liquid systems in food are interrelated.

2.10.2 **Factors Effecting Protein Hydration**

The main factors effecting the protein hydration are pH, ionic strength and temperature which are described below.

2.10.2.1 **pH**

For proteins to be soluble they need the ability to interact as much as possible with the solvent. At the surfaces of proteins are amino acid residues that interact with water. The amino acids are referred to as hydrophilic amino acids and include arginine, lysine, aspartic acid, and glutamic acid. At pH 7 the side chains of these amino acids carry charges—positive for arginine and lysine, negative for aspartic acid and glutamic acid. As the pH increases, lysine and arginine begin to lose their positive charge, and at pH greater than about 12 they are mainly neutral. In contrast, as pH decreases, aspartic acid and glutamic acid begin to lose their negative charges, and at pH less than 4 they are mainly neutral.

In general, the milk protein solubility increases with the increase in pH. In the pH range 6.5-8.0 there were negligible amounts of insoluble proteins or insoluble carbohydrate materials in nonfat dry milk, whey protein concentrate and sodium caseinate in excess water systems. Now here again question arises about the applicability of this data to limited
water environments. At pH near the isoelectric point the lowest protein solubility was observed. Although the sodium caseinate was having excellent solubility it was insoluble around the isoelectric pH (3 to 5) (Zayas, 1997).

Casein micelles undergo considerable changes in the pH range 5.5 to 5.0 including dissociation of caseins, dissolution of micellar calcium phosphate and hydration changes (Singh, Ye and Havea, 2000).

The solubility of sodium caseinate was influenced by pH with minimum at pH 4.0 to 4.5. Moreover, Increase in its solubility was observed at pH greater than 4.6 (Zayas, 1997). In the figure 10 below the solubility of sodium caseinate (A), αs-/k-casein-enriched caseinate (B) and β-casein-enriched caseinate (C) was observed at different pH.

A number of different studies like the one above are carried out but all these were in excess water system. Now the question arises about the same effect in limited water environment.

![Figure 10. Effect of pH on solubility of sodium caseinate (A), αs-/k-casein-enriched caseinate (B) and β-casein-enriched caseinate (C) (Murphy and Fox, 1990)](image)

### 2.10.2.2 Ionic Strength

The effect of ionic strength on protein solubility probably involves solvation, electrostatic and salting in and salting out phenomenon. As a rule, neutral salts have a two-fold effect on protein solubility. At low concentrations they increase the solubility by suppressing the electrostatic protein-protein interaction whereas protein solubility is decreased at higher salt concentrations due to the ion hydration tendency of the salts.

At low ionic strength (<0.5), ions neutralize charges at the surface of proteins. This charge screening affects solubility in two different ways, depending on the characteristics of the protein surface. Solubility decreases for those proteins that contain a high incidence of
nonpolar patches and it increases for those that do not. The former behavior is typical for soy proteins and the latter behavior is exhibited by β-lactoglobulin. While the decrease in solubility is caused by hydrophobic interactions, the decrease in solubility is caused by a decrease in the ionic activity of the protein macroion. At ionic strength greater than 1.0 M, salts have ion specific effects on protein solubility (Fennema et al., 2008).

At constant ionic strength, relative effectiveness of various ions on solubility follows the Hofmeister series with anions promoting solubility in the order \( \text{SO}_4^{2-} < F^- < \text{Cl}^- < \text{Br}^- < I^- < \text{ClO}_4^- < \text{SCN}^- \) and cations decreasing solubility in the order \( \text{NH}_4^+ < \text{K}^+ < \text{Na}^+ < \text{Li}^+ < \text{Mg}^{2+} < \text{Ca}^{2+} \). This behavior is analogous to the effects on the thermal denaturation temperature of proteins (Fennema et al., 2008). The replacement of calcium by sodium ions prior to ultrafiltration and drying resulted in markedly increased solubility of whey proteins at all pH values and especially in the pH range of 4.5 to 5.5 (Zayas, 1997).

2.10.2.3 Temperature

The variation in protein solubility during heat treatment is the evidence of the conformational changes in the structure of protein. The solubility of different proteins decreases differently with temperature and time of heating. Diverse and complicated precipitation reactions take place due to the conformational changes in protein structure (Zayas, 1997).

Moist heat has a more complex effect on the solubility of proteins than dry heat does and is strongly affected by pH and ionic strength. The molecular structure of most proteins in aqueous medium is susceptible to changes in temperature (Zayas, 1997). At constant pH and ionic strength, the solubility of most proteins generally increases with temperature between 0°C and 40°C. Exceptions occur with highly hydrophobic proteins, such as β-casein and some cereal proteins, which exhibit a negative relationship with temperature. Above 40°C, the increase in thermal kinetic energy causes protein unfolding (denaturation), exposure of nonpolar groups, aggregation and precipitation, that is, decreased solubility (Fennema et al., 2008).

Addition of salts like NaCl, CaCl_2, NaHPO_4, increase in temperature and pH resulted in increased solubility of caseins and caseinates. At pH 5.6 ≤ 6.2, all caseins and caseinates exhibited their greatest solubility after the sodium phosphate addition.
2.11 Water Activity

Water activity is an important factor which determines the quality, workability and texture of foods. Throughout history the importance of controlling water in food by drying, freezing or addition of sugar or salt was recognized for preserving and controlling food quality. Water activity is an equilibrium concept and should only be used for systems which are in either true equilibrium or where the activation energy barriers for the chemical transformations of species are sufficiently high relative to their thermal motions that kinetically transformed substances do not contribute to experimental properties.

Water activity is the ratio of the vapour pressure of water in equilibrium with a food to the saturation vapour pressure of water at the same temperature. The water activity of a food describes the degree to which the water is “bound” in the food and hence its availability to act as a solvent and participate in chemical/biochemical reactions and growth of microorganisms. It is an important property that can be used to predict the stability and safety of food with respect to microbial growth, rates of deteriorative reactions and chemical/physical properties.

It has been reported by Shrestha et al., (2007) that with the increase in lactose concentration in skim milk powder significantly increased the water adsorption in milk powders and also lowered the water activity range at which the crystallization occurred.

2.11.1 Moisture Sorption Isotherms

The migration of water from one ingredient within the food system to another is thought to be underlying cause of the bars hardening. Moisture sorption isotherms describe the equilibrium relationship between the relative humidity of the environment and the moisture content of the food system at a constant temperature (Foster, Bronlund and Paterson, 2005). These moisture sorption isotherms can also be used to determine water transfer between ingredients within a food system (Bell and Labuza, 2002).

By plotting the water content within the food against water activity ($p/p_o$) at constant temperature gives the moisture sorption isotherms (MSI). This plot can be used for multiple reasons such as for determination of moisture transfer between ingredients within food system as well as for the prediction of chemical and physical stability of food as a function of the water content (Bell and Labuza, 2002).
Sorption isotherms of proteins, that is, the amount of water bound per gram of protein as a function of relative humidity is invariably a sigmoidal curve. For most proteins, saturated monolayer coverage of water occurs at a water activity \((a_w)\) of about 0.7-0.8, and multi layers of water are formed at \(a_w > 0.8\). The saturated monolayer water is primarily associated with ionic, polar and non-polar groups on the surface the protein. Bound water is that water which is having hindered mobility.

At \(a_w = 0.9\), proteins bind about 0.3-0.5 g water/g protein. At \(a_w > 0.9\), liquid (bulk) water condenses into clefts and cervices of protein molecules, or in the capillaries of insoluble protein systems, such as myofibrils. The properties of this water are similar to those of bulk water. This water is known as hydrodynamic water, which moves with the protein molecule.

2.12 Experimental Techniques for Protein-Water Interactions

The interaction of water with other food components like macromolecules such as proteins and polysaccharides defines the rheological and textural properties of foods. Many functional properties of proteins such as dispersibility, wettability, swelling, solubility, thickening/viscosity, water-holding capacity, gelation, coagulation, emulsification and foaming depend on water-protein interactions.

There are different methods used for the studying the protein-water interactions and may be classified as follows.

2.12.1 Thermodynamic

These methods are based on the changes in enthalpy, entropy, free energy, activity, freezing point and boiling point of water which are used for studying the protein-water interaction mechanism under equilibrium conditions. The simplest example of these methods is water sorption-desorption isotherm which enables to estimate degree of protein hydration as a function of water activity and thermodynamic heat of sorption as a function of moisture content.

2.12.2 Kinetic

These methods involve the determination of the mobility of water in the vicinity of proteins or the mobility of the protein which is affected by its interaction with the water. One of the techniques is based on the association of water with a protein molecule changing its hydrodynamic properties which include density, volume and shape. Thus, viscosity and
frictional coefficient data can be used for the determination of the amount of water associated with the protein molecule. These techniques include nuclear magnetic resonance (NMR), dielectrics, laser light scattering and intrinsic viscosity which have been used to determine the changes in relaxation rates of protein molecules as a function of hydration.

2.12.3 **Spectroscopic**

Changes in the spectroscopic characteristics of water are used to reflect the hydrogen bonding environment of the water molecule. These techniques include Infrared (IR) and Raman spectroscopy used for the evaluation of the nature and strength of the protein-water hydrogen bonding.

2.12.4 **Diffraction**

These methods are used for providing information on the average position and orientation of the water molecules with respect to each other and to the protein molecule. Light scattering and small angle X-ray scattering techniques provide information about the density and concentration fluctuations of water molecules in solution and in the vicinity of the protein molecule. It should also be noted that each of these methods possesses inherent limitations and uncertainties. In many cases background contributions from protein and bulk water are difficult to separate from the reaction of interest and therefore only qualitative information is obtainable. However, each of the experimental techniques provides mutually supporting with respect to location, dynamics and energy of water molecules associated with proteins. By proper selection of the data from these different techniques, it is possible to gain a reasonably accurate description of the state of the water in the protein-water system.

2.13 **Techniques Used to measure the Protein Rehydration**

Different researchers investigated the rehydration of food powder using different methods such as the monitoring of turbidity (Gaiani, et al., 2006), ultrasonic spectrometry (Povey et al., 1999), viscosity (Kravtchenko et al., 1999; Gaiani et al., 2005), nuclear magnetic resonance spectroscopy (Schuck et al., 2002), and light microscopy (Gaiani et al., 2005).

In recent years, another approach to studying the kinetics of powder rehydration has been employed to examine spray-dried micellar casein powder (Schuck, et al., 2002; Gaiani et al., 2005). This technique has been applied to different dairy powders (Moughal et al., 2000; Gaiani et al., 2005) but not to MPC. This approach is based on the separation of the
overall “dissolution” process into individual processes (Schuck et al., 2007). All of these studies have been carried out in excess water environment so not clear about its validity in the limited water environments. Each of these techniques has its own advantages and limitations which are given below.

2.13.1 Monitoring of Turbidity

This technique involves dispersion of powder in the vessel which results to a quick increase of turbidity and the time taken to reach the first turbidity peak which is related to the wetting time. Indeed, as can be observed, the turbidity signal presented a peak or a shoulder when all the particles were wetted. Swelling time was defined as the time taken to reach the minimum of turbidity and was also related to the time taken to reach the maximal particle size. The process of swelling resulted in a disintegration of the wet particles and their progressive dispersion could explain the decrease and consequently the turbidity increase. This was named as the powder dispersion. The time for the powder to fully rehydrate was determined by the time needed to obtain turbidity stabilization (Gaiani, et al., 2006). It is very difficult to monitor the individual stages of the rehydration and the turbidity under the present resources.

2.13.2 Ultrasonic Spectrometry

This is a new technique which is used for studying particle size and other physical properties of materials dispersed in fluids. It can be used by measuring either or both of the attenuation of sound and its velocity over a range of frequencies and the data compared with a model of acoustic scattering. This technique has a number of features such as use in concentrated dispersions that are optically opaque without diluting the sample; use over a wide particle size range from 10 nm to 1 mm. Moreover, this technique provides information about particle size, the compressibility and state of aggregation of the dispersed phase. The measurements may be made rapidly and in-line, in a pipeline or tank (Povey et al., 1999).

2.13.3 Monitoring of Viscosity

In this technique the Stress Tech Rheometer was used which was equipped with a custom built paddle stirrer and a C25 cup (Giani et al., 2006). This paddle was made up of four blades specifically designed for large particles and the blades were perpendicular to each other to allow a good homogenization. Rehydration processes in the food industry
usually include stirring at constant speed, so constant shear rate (100 sec\(^{-1}\)) was used in these experiments and data was collected automatically every 20 seconds during 5000 seconds and then every 1000 seconds. The powder was dissolved in the rheometer cup, 50 seconds after starting the rheometer. This dissolution resulted in a series of increase in viscosity like quick increase of viscosity, followed by other increase of viscosity with a maximum; thirdly a decrease in viscosity took place. At the end of the profile, a low viscosity fluid is formed and the viscosity value is stabilized (Giani et al., 2006). If we monitor viscosity then there might be a problem of air entrapment then it’s very hard to monitor the individual wetting, sinking stages etc.

2.13.4 **Nuclear Magnetic Resonance Spectroscopy**

In this technique the major components of the bar material such as water, lipid, and polyhydroxy compounds (PHCs) could be analyzed separately with the help of their representative peaks in the proton NMR spectrum. The major PHCs, glucose and glycerol, could not be separated, due to their appearance at the –OH position in the proton spectrum (Schuck et al., 2002).

2.13.5 **Light Microscopy**

Different microscopic techniques have been used to monitor the hydration like light microscopy. The problem with the use of the microscopic techniques is the absence of the shear rate which is an important variable in the dissolution process.

While selecting any technique to be used to measure protein hydration the properties of each technique should be kept in mind. If a centrifugation based technique is used then the viscosity will affect sedimentation rates. So it will not be possible to study protein hydration in the presence of high concentrations of sugar as this will cause the viscosity to be high. A high viscosity retards the rate of settling and so there would be the risk of overestimating the amount of soluble material.

Solubility test will be used in this study for the rehydration of milk powders is based on the method used by Havea (2006) for rehydration of MPC85 in water. The problems like air entrapment etc can be overcome in this technique. The other advantage is that Electrical conductivity and ISE experiments can be carried out at the same time.
2.14 Summary, Conclusions and development of research objectives

The initial motivation for this thesis was to characterise the hydration of high protein milk powders to facilitate their use in low water food systems. This review has found that the majority of hydration studies have been conducted on systems where there is an excess of water. Within the work that has been done the only research that has been directed towards modification of the aqueous environment has been via pH, ionic strength and temperature. Little research was found that investigated the effect of food ingredients on hydration rates. The review of hydration methodologies showed that hydration studies in true water limited environments are complex and many of the specialist instruments required were not available for this study. Therefore the decision was made to study the impact on protein powder hydration of one of the most common ingredients in food systems: sugar. An additional feature of the solubility research reviewed here was that researchers focussed on total mass balances as an indicator of solubility. What is missing from the literature, is a more systematic study of the hydration and solubility of the individual components that make up the milk powder composition. Further to this while research has focussed on how much total mass has become colloidally stable there has been no research on how or if the remaining insoluble material has changed as a result of the reconstitution method. By choosing a dilute model system centrifugal-based solubility methods could be utilised that would allow for separation of the insoluble material and thus its study.

Therefore the new research objective that has developed through analysis of the literature is:

- Characterize the compositional hydration rate of high protein milk powders as a function of sugar concentration and temperature
- Characterize the effect of partial hydration on the morphology of the insoluble material following reconstitution under a range of conditions.

Specific research questions are:

1. What is the affect of sugar concentration on the solubility of different powders with respect to temperature?
2. How are the minerals affected during rehydration of dairy powders?
3. How is the insoluble material affected by changes in the rehydration environment?
2.15 Value of Research

Understanding the factors responsible for limiting the solubility of milk powders will enable modification of application processes that have milk powder as ingredient to increase the solubility. Arising from this study the project will investigate the manipulation of processing parameters to optimize the use of milk protein powders in products such as high protein bars in terms of processing, shelf life and sensory attributes.
3 Material and Methods

In order to achieve the objectives described in section 2.14, this section includes the details of raw material and different laboratory techniques used in this research.

1. To see the affect of sugar concentration on the solubility of different powders with respect to temperature, the powders will be rehydrated over a range of temperatures in water and 20% sugar solution. The solubility data will be helpful in estimating the solubility of these powders under different processing conditions.

2. To monitor the individual ingredient rehydration properties minerals were selected from the milk powder ingredients due to their importance in casein and other interactions and also to see the rehydration trend of minerals in relation to overall solubility.

3. Electrical conductivity gives the overall rehydration of the minerals and it is difficult to say about the individual contribution of individual ions like calcium and sodium. Moreover, by monitoring the individual ion rehydration we will be able to predict the water migration to different parts of the milk powder. Calcium and sodium ions were selected from the list of the minerals based on their importance in interactions with other ingredients. As sodium is widely spread throughout the milk powder and if it is so then it can be assumed that all sodium salts in milk powders are soluble then sodium may be used as an indicator of water ingress throughout a powder particle.

4. To observe the effect of changes in the rehydration environment on the insoluble material the ratio of sediment volume and mass of sediment and the solubility of the powders at different temperatures was determined.

5. To observe the morphological changes between the sediments rehydrated at different temperatures and to observe visually the morphological changes taking place over a range of temperature electron microscopy was used. From this it was tried to predict the mechanism of rehydration.

6. To observe the distance between the colloidal calcium phosphate centers within the casein micelles that are present in the sediments rehydrated at different temperatures Small Angle X-ray Scattering technique (SAXS) was used. The distance between colloidal calcium phosphate centers in the micelle is thought to be a proxy way of measuring micelle particle size in solid systems. If a micelle increases in size
for instance then one can expect that the CCP nanocluster will be further apart from each other regardless of the size of the micelle per se.

3.1 Selection of Milk Powders

There are many commercially available milk powders used in different food applications. Milk protein concentrates (MPC) and calcium caseinate were used for these experiments because of:

- Their wide use in protein bar, the model system in this research.
- Their flavor and texture stability.
- In milk protein concentrates the ratio of casein and whey protein is similar as to raw milk i.e., 80:20 which makes it close to natural milk composition.
- As described earlier caseins are responsible for the protein insolubility (Havea, 2006) and also based on their complex structure it was decided to select the powders having difference in their micellar microstructure which varies from native micellar casein (ALAPRO 4850), non-native micellar like (Ca Caseinate 380) and calcium depleted micellar structure (CSMPC 85).
- Milk powder is composed of different ingredients. In literature whey protein and casein have been monitored during rehydration but other components were not taken into account. So the variable is the mineral content while trying to keep all other ingredients same. ALAPRO 4850 (MPC85), NZMP 4861 (cold soluble MPC85) and Calcium caseinate 380 were used as have variation in the mineral content while keeping all other components approximately the same. Composition of these powders is shown in Table 10. Highlighted Ingredients show variation in the powder and are related to the project.

Table 10. Composition table of ALAPRO 4850, CSMPC 85 and CaCS 380

<table>
<thead>
<tr>
<th>Ingredients</th>
<th>ALAPRO 4850</th>
<th>CSMPC 85</th>
<th>Ca Caseinate 380</th>
</tr>
</thead>
<tbody>
<tr>
<td>Moisture (g)</td>
<td>4.5</td>
<td>4.5</td>
<td>4.9</td>
</tr>
<tr>
<td>Protein (g)</td>
<td>81.2</td>
<td>83</td>
<td>92.2</td>
</tr>
<tr>
<td>Fat (g)</td>
<td>1.5</td>
<td>1.5</td>
<td>1.0</td>
</tr>
<tr>
<td>Lactose (g)</td>
<td>4.3</td>
<td>4.0</td>
<td>0.1</td>
</tr>
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<tr>
<td>Sodium (mg)</td>
<td>70</td>
<td>1200</td>
<td>5</td>
</tr>
</tbody>
</table>
3.2 Modification of Hydration through Ingredient Addition

While most hydration data for proteins in the literature is gathered in pure water systems. The reality in the food industry is that typically powders are rehydrated in water containing other ingredients. In the literature most of the hydration data is at the fixed temperature whereas in the industry the processing is carried out at different temperatures for different applications. Thus it was important to characterize solubility over a range of typical processing temperatures for the wide application of the data. As the research is related to food system where sugar is mostly used that limits the water availability, preliminary experiments used different sugar solutions such as 10% and 20%. According to results, significant difference was found between water and 20% sugar solution (figure 11). Thus, these two solvents were selected to monitor the rehydration of different milk powders.

Figure 11. Temperature solubility curve of MPC 85 in water, 10% and 20% sugar solution.
3.3 Selection of Experimental Technique

The method used in collecting data for this study was laboratory experimentation. Determination of total solids was carried out in the Food Chemistry Laboratory, Massey University, Manawatu Campus. The microscopic analysis was carried out in Manawatu Microscopy and Imaging Center (MMIC), Massey University, Manawatu Campus. The Single Angle X-ray Scattering (SAXS) analysis was carried out in IRL branch Wellington.

3.4 Methodology

Determined variables included solubility determined using total solids, measurement of electrical conductivity and determination of ion selective electrodes measurements of calcium and sodium ions. Samples for the determination of total solids were collected after each 30 minutes of rehydration while the electrical conductivity and the ISE data was collected after each 5 seconds. For keeping the temperature of the water bath constant and to avoid the temperature fluctuations the water bath was kept at the required temperature with the cold finger turned on.

3.4.1 Rehydration Procedure

The procedure used in this study for the rehydration of milk powders is based on the method used by Havea (2006) for rehydration of MPC85 in water. In the present study, water and 20% sugar syrups were used as solvents. The sugar solution with required concentration i.e., 20% (w/w) was prepared for utilization in the experiment. Sodium azide was added to prevent microbial growth in sugar solution. Milk powder (5%) was weighed in a pre-weighed beaker. The stirrer was attached to an overhead motor and set to a speed which would create an appropriate vortex (1600 rpm) in the solvent and also to completely wet all the powder quickly. Subsequently, the sample was added to stainless steel beaker slowly to the vortex containing the solvent. The powder addition rate was kept constant (25 g) over a constant time period (per minute) to prevent lump formation or formation of layers. After adding all the powder the speed of the stirrer was adjusted to 800 rpm to eliminate a vortex. Elimination of the vortex minimized air entrapment and foam formation. During stirring the beaker was covered with aluminum foil to minimize evaporation loss. Firstly the rehydration was carried out in all milk powders for longer time periods such as 8 hours to see the effect and then rehydration time was finalized. Same method was used to
prepare all the rehydrated milk powder solutions related to this project. The overall preview of all the parameters to be studied in preliminary experiments is shown in table 11.

Table 11. Overall Preview of Experiments in ALAPRO 4850, CSMPC 85 and CaCS 380

<table>
<thead>
<tr>
<th>Temperatures (°C)</th>
<th>Solubility</th>
<th>Electrical Conductivity</th>
<th>ISE Data (Na)</th>
</tr>
</thead>
<tbody>
<tr>
<td>60</td>
<td>Yes</td>
<td>Yes</td>
<td>No</td>
</tr>
<tr>
<td>45</td>
<td>Yes</td>
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</tr>
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<td>30</td>
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</tr>
<tr>
<td>10</td>
<td>Yes</td>
<td>Yes</td>
<td>Yes</td>
</tr>
</tbody>
</table>

3.4.2 Determination of total solids in rehydrated milk powder Solution

3.4.2.1 Determination of Solubility

The principle of the solubility method is the stirring of the powder and solvent under a given set of conditions for a specific time, followed by measurement of the proportion of powder that has dissolved i.e. that will not sediment when sample is centrifuged.

After 30 minutes stirring, a 1-2 g aliquot of the sample of bulk rehydrated solution was taken by auto-pipette and placed in a pre-weighed moisture dishes for determination of total solids in the bulk solution. In this way potential changes in concentration due to evaporation at higher temperatures may be taken into account. The weight of the moisture dish containing the sample was recorded and the dish was placed in an oven set at 108°C to dry for 3 hours (trial experiments). The soluble material was determined by centrifuging a 50 ml sample of the rehydrated milk powder mixture at 700g for 10 minutes. After centrifugation samples of supernatant were taken by auto-pipette and total solids contents was determined by the method described above. This was carried out twice during that 60 minutes rehydration.

3.4.2.2 Method for Measuring Total Solids of the Rehydrated Solution

After drying, the moisture dishes were removed from the oven and placed in desiccators. The dishes were then weighed and total solids were calculated. The flow sheet diagram of the determination of total solids is shown in figure 12. The solubility was then calculated according to the equation 3.1.

\[
\text{Solubility} = \frac{\text{Total Solids of Supernatant}}{\text{Total Solids of Bulk Solution}} \times 100
\]  

(Equation 3.1)
3.4.2.3 Standardization of Rehydration Experimental Time

Change in solubility of ALAPRO 4850, CaCS 380 & CSMP 85 was calculated for 6 hours at 25±1 °C in 20% Sugar Solution to calculate the minimum time of rehydration experiment. The 25±1 °C temperature was selected randomly as it was near to room temperature and the interest was in the rehydration time irrespective of the temperature. If we take the solubility after 6 hours as 100% then we see that the after 30 minutes the powders have reached approximately 73-85% of the 100% solubility in all powders as shown in figure 13. Although there were changes in the solubility afterwards but by rehydration of the solution for 30 minutes will enable to predict the major changes in the solubility.

Theoretically the solubility should increase with the increase in temperature and once it is increased it cannot decrease from that level. In figure 13 there are some drifts in the data which are showing increase and decrease in the solubility data these are actually artifacts and it is very hard to believe on these data points which are forming these drifts.
3.4.3 Measuring the Electrical Conductivity

Electrical conductivity was used to see the effect on the minerals/ash during the powder rehydration and to measure the concentration of components that have hydrated to the extent they conduct electricity. To see the effect on the mineral components during powder rehydration electrical conductivity was measured at different temperatures. The maximum upper limit of temperature range of the conductivity meter was 80°C. Temperatures higher than 60°C were not used to avoid whey protein denaturation.

ProLab 4000 was used for measuring the electrical conductivity which recorded the reading after each 5 seconds through the entire time period (figure 14). The conductivity electrode was immersed in the rehydrated solution from the start till the end of the experiment.

Figure 13. Change in Solubility of ALAPRO 4850, CaCS 380 & CSMPC 85 in 6 hours at 25±1°C in 20% Sugar Solution
3.4.3.1 Calibration of ProLab 4000

The calibration of electrical conductivity electrode was carried out using a standard solution of 0.01 KCl whereas for ion selective electrodes (ISE) of Ca and Na different concentration of solutions of CaCl$_2$ and NaCl were used. To validate the results, an experiment was carried out to see the accuracy and precision in the data.

It was observed that the total conductivity and sodium ion dissolution followed a specified trend and were reproducible whereas there is much fluctuation in the calcium ion dissolution data (Figure 15). Therefore, the calcium ion dissolution was considered inconsistent. Calcium ion was omitted from the dissolution experiments due to the problems associated with the calcium ion selective electrode.
3.4.3.2 Standardization of Rehydration Experimental Time for Measuring Electrical Conductivity Data

The change in electrical conductivity in ALAPRO 4850, CaCS 380 & CSMPC 85 was recorded in 8 Hrs at 25±1°C as shown in figure 16.

If we take the electrical conductivity value after 8 hours as 100% then we see that the after five minutes the powders have reached more than 70% of the 100% electrical conductivity values in all powders as shown in figure 16. The electrical conductivity data for the first five minutes is shown in figure 17. The powder was added within the first two minutes of rehydration. Experimental conditions are pointed out on the graph.
3.4.4 Measuring the Ion Selective Electrode Data

The upper limit used in these experiments was 45°C at which the ISE data was collected. There is a limitation of the maximum temperature in the ISE of calcium and sodium which is 50°C so it is better to use these electrodes below 50°C. This data was used to compare the calcium and sodium ion dissolution at lower temperatures to see the trend. The ISE’s were immersed in the solution from the start of the experiment and data was automatically recorded after each 5 seconds through the entire time period of 8 hours. Care was taken by recording the known concentration of salt solutions before and after the experiment to calibrate and adjust the change in the ISE’s calibration. In general the ISE probe would measure about 10% higher after each measurement.

3.4.4.1 Standardization of Rehydration Experimental Time for Measuring Ion Selective Electrode Data

The change in Na ionic dissolution was recorded in ALAPRO 4850, CaCS 380 & CSMPC 85 was recorded in 8 Hrs at 25±1°C as shown in figure 18. It is evident that the major changes in the electrical conductivity have taken place in first thirty minutes.
When temperature data was observed during the rehydration time in all powders fluctuations in temperature data were observed. The reason due to which fluctuations were taking place was working of the heating motor of the water bath. When the water bath reached to a specific temperature the motor turned off and when the temperature dropped the motor of the heater turned on. The fluctuations in the data of all powders were caused by the turning on and off the heating motor of the water bath (Figure 18). To avoid these temperature fluctuations cold finger was used which kept the water bath temperature constant throughout the rehydration time. The temperature was monitored during 8 hours (Figure 19). The fluctuations trend corresponded with Figure 18.
Major changes in Ca and Na ion dissolution were noticed in the first five minutes (Figure 20) thus it was concluded that the ionic dissolution is not dependent on the overall solubility of milk powders.

Figure 20. Change in Ion Dissolution in ALAPRO 4850, CSMPC 85 & CaCS 380 in First 5 Min at 25±1°C (a) Ca (b) Na
Data Analysis

The samples collected for the determination of total solids were triplicated and data was analyzed using Generalised Linear Model (GLM). The total conductivity and ISE experiments were carried out once.

3.4.5 Change in sediment volume in comparison with the solubility during rehydration

The focus of the solubility studies in literature and experiment described in section 3.4.2 has been on the mass of solids which is colloidally stable and little attention is given to the characterization of the insoluble material- the sediment. The question arises about the sediment that what is the going to happen with the sediment during rehydration. For this reason an experiment was designed to see the effect on the ratio of sediment volume and sediment weight during rehydration of ALAPRO 4850 and CSMPC 85 in water and 20% sugar solution.

3.4.5.1 Determination of total solids in rehydrated milk powder Solution

3.4.5.1.1 Determination of Solubility

After 30 minutes stirring, a 1-2 g aliquot of the sample of bulk rehydrated solution was taken by auto-pipette and placed in a pre-weighed moisture dishes for determination of total solids in the bulk solution. The weight of the moisture dish containing the sample was recorded and the dish was placed in an oven set at 108°C to dry for 3 hours (trial experiments). The soluble material was determined by centrifuging a 50 ml sample of the rehydrated milk powder mixture at 700g for 10 minutes. Before centrifugation the samples in centrifugation tube were weighed. After centrifugation the samples were again re-weighed and the supernatant was removed after taking samples by auto-pipette for total solids for the determination of the total solids. The sediment was weighed and volume of sediment was also measured which was used to calculate the ratio between the sediment volume and mass of the solution.

3.4.5.1.2 Method for Measuring Total Solids of the Rehydrated Solution

After drying, the moisture dishes were removed from the oven and placed in a desiccators. The dishes were then weighed and total solids were calculated. The solubility was then calculated according to the equation 1.

\[
\text{Solubility} = \frac{\text{Total Solids of Supernatant}}{\text{Total Solids of Bulk Solution}} \times 100
\]  

(Equation 1)
3.4.6 Sample Preparation Methodology for Electron Microscopy

For the preparation of samples from the rehydrated sediments to view under the TEM following method was followed by the Manawatu Microscopy and Imaging Centre staff. Preparation was carried out in fume hood at all time.

1. Fixation
   - Small amount of sediment was mixed with approximately 0.5 mL of propylene glycol, containing two drops of 25% glutaraldehyde in a tube;
   - Low-temperature gelling agarose was added at a concentration of 1:1 to the powder/propylene glycol/glutaraldehyde mixture and vortex. It was poured rapidly onto a glass slide to allow the sample to set;
   - Once set the agarose embedded samples were cut into 0.5x2x5 m³ cubes and placed into the primary fixative 3% glutaraldehyde in 0.1 M sodium Cacodylate buffer PH 7.2 for at least 24 h at 5 °C;
   - The samples were rinsed in 0.1 M sodium Cacodylate buffer for two hours (three changes);
   - The samples were placed in the secondary fixative 1% OsO4 in 0.1 M sodium Cacodylate buffer over night at 5°C;
   - The samples were rinsed in 0.1 M sodium Cacodylate buffer for two hours (three changes);
   - Dehydration in acetone series was carried out as follows:
     - 25% acetone 15 min
     - 50% acetone 30 min
     - 75% acetone 30 min
     - 95% acetone 30 min
     - 100% acetone 30 min x3
     1. 50/50 acetone/resin over night
     2. 100% resin on stirrer for 48 hours, replaced with fresh resin for 3 times
     3. Embedded in fresh 100% Procure 812 resin and curled at 60 °C for 48 h.

Sections 1 μm in thickness were cut from trimmed resin blocks using a glass knife and Ultramicrotome (Leica, Austria). They were heat-mounted onto a glass slide, stained with 0.05% Toluidine Blue and were viewed under a light microscope (Olympus BX51, Japan).
Digital images of the section were taken and areas of interest were chosen for examination using TEM.

Ultra thin sections (100 nm) were cut using a diamond knife and an Ultramicrotome. They were collected on a copper grid. The sections were stained with saturated uranyl acetate in 50% ethanol for 4 min and then by lead citrate for another 4 min. Examination of the specimens was conducted using a Philips CM 10 transmission electron microscope (Philips, The Netherlands).
4 Results and Discussion

4.1 Effect of different temperatures on the solubility of Milk Powders

The method used for the data collection aimed to see the effect of different temperatures on the solubility of milk powders is explained in section 3.4.2.

ALAPRO 4850

General Trend Found in Solubility Profile of ALAPRO 4850

The increase in rehydration temperature increased the solubility of ALAPRO 4850 in both water and 20% sucrose solution as shown in figure 21. A sharp increase was seen in the solubility at 20°C and 30°C in water and 20% sucrose solution respectively.

Discrepancy between the Solubility Profiles of ALAPRO 4850

Similar findings were obtained from preliminary experiments (Figure 22). The only difference between the two profiles is the solubility at lower temperatures i.e., 10°C and 20°C as in figure 21 the solubility in 20% sugar solution is higher compared to water which is opposite to previous results. The possible reason may be is the ageing effect of the milk powders as we do not have the manufacturing record of milk powder. A complete investigation of ageing as variable was outside the scope of this project. However, it is evident that the solubility of MPCs decreases in sugar solutions as the powder ages.

This data provides evidence that MPCs become more sensitive to solubility as the powder ages. Similar ageing studies of MPC in pure water have been reported by Havea (2006) and Carr (2002) and have shown a similar decrease in solubility profile with ageing but have not reported the increased sensitivity to solute addition. This is the most important
finding of this study as milk powder application involves a complex food system where there are other ingredients present. This should also be kept in mind while calculating the solubility of milk powders during the processing as other ingredients will interfere with them to affect solubility of milk powders.

**Figure 22. Temperature solubility curve (a) ALAPRO 4850 in Water, 10% and 20% Sugar Solution**

**CSMPC85**

Increase in solubility was also observed with the increase in temperature in both solvents in CSMPC85 (Figure 23). A linear increase was observed in 20% sugar solution from 10°C to 40°C and then it reached an equilibration. When water was used as solvent, there was increase in solubility from 10°C to 20°C and the equilibrium was reached faster compared to 20% sugar solution. Further, the difference in solubility between both solvents at lower temperatures was almost 25%.

**Figure 23. Temperature solubility curve after 30 Min in CSMPC 85 in Water and 20% Sugar Solution**
The solubility of calcium caseinate 380 is shown in figure 24. There was linear decrease in the solubility in water with the increase in temperature whereas there was not much change in the solubility in 20% sugar solution. The results are in agreement to decrease in solubility as observed in calcium phosphate solution (www.foodscience.uoguelph.ca/dairyedu/cheese.html).

![Temperature solubility curve after 30 Min in CaCS 380 in Water and 20% Sugar Solution](image)

Figure 24. Temperature solubility curve after 30 Min in CaCS 380 in Water and 20% Sugar Solution

It was also observed that solubility in ALAPRO 4850 and CSMPC85 decreased with the increase in the concentration of the solute (Figure 21 and 22). It was thought that this may be due to the increases in the viscosity of the sugar solution due to the presence of sugar molecules which lower the solubility rate. However, when solubility was plotted against viscosity there is not sufficient evidence to support this theory. The solubilities of 35% and 75% were obtained as shown in figure 25.

It was also observed that the solubility curve in different solvents shifted with the increase in temperature (Figure 21 and 23). This means that if 38% solubility in water was observed at 10°C then in 20% sugar solution the same solubility was observed at higher temperature than water (Figure 22). There was significant difference in solubility between the excess water and limited water environment (as p-value < 0.05).
These results are in agreement with Zwiggers (1992) who also found that the solubility of MPC could be improved by raising the temperature which results in water transfer towards the interior of powder particles. Singh (2007) also reported that solubility improves at higher reconstitution temperatures.

### 4.2 Effect of different temperatures on the Electrical Conductivity of Milk Powder Dispersions

The effect of temperature on the electrical conductivity was monitored in three powder dispersions ALAPRO 4850, CSMPC85 and CaCS 380 according to the method described in section 3.4.3.

The electrical conductivity increased with the increase in temperature in all the three powders (Figure 26). This means that the mineral content dissolution increases with the increase in temperature in all the powders. It was found that the electrical conductivity was higher in water as compared with the 20% sugar solution for all the powders. This suggests that the rate of mineral dissolution in water is faster as compared to 20% sugar solution. From the R-square value in all the samples it is evident that electrical conductivity followed a linear trend in water as compared to 20% sugar solution due to high R-square values in water in all powders as shown in figure 26 (except for CaCS 380).

Electrical conductivity is a useful indicator of total dissolved solids because the conduction of current in an electrolyte solution is primarily dependent on the concentration...
of ionic species. Besides the amount and composition of ionic species, EC is strongly dependent on temperature (Hayashi, 2004).

Further, the level of mineral content in all powders which was highest in CSMPC 85 followed by ALAPRO 4850 and CaCS 380 is based on their mineral composition (Table 10 section 3.1). The mineral content of ALAPRO 4850 and CSMPC 85 is same (Table 10 sec. 3.1).

However, the calcium and sodium content is different due to which the mineral level is higher in CSMPC 85 than ALAPRO 4850 (Figure 26). It has reported that increase in sodium content also increases the calcium solubility which may be the reason for high electrical conductivity in CSMPC 85.

![Figure 26. Temperature Electrical conductivity curve in Water and 20% Sugar Solution after 60 Min (a) ALAPRO 4850 (b) CSMPC85 (C) CaCS 380](image-url)
4.3 Effect of different temperatures on the Ionic Sodium Solubility of Milk Powders

The effect of different temperatures on the ionic sodium dissolution was also monitored. However, in CaCS due to lower sodium content the graphs do not show any trend as it was supposed to be 3.125 mg/l in water and in sugar solution it was in negative.

From the figure 27 (a) it is clear that the sodium ion dissolution in ALAPRO 4850 increased in sugar solution from 10°C to 20°C whereas it decreased in water at same temperature and then it is almost in equilibration in both solvents. The same decrease in sodium dissolution was observed in CSMPC 85 in both solvents as shown in figure 27 (b). The results do not seem to be true based on literature. The sodium dissolution should increase with the increase in temperature or should reach to equilibration. The increase in temperature should increase the rate of dissolution with the final value same.

Figure 27. Effect of temperature on Ionic Sodium in Water and 20% Sugar Solution after 30 Min
(a) ALAPRO 4850 (b) CSMPC 85 (c) CaCS 380
The discrepancies found between the current findings with the available literature, can be due to two reasons: firstly, it was found the instrument needed to be calibrated at each temperature which was not done previously, thus the results obtained from the temperature range of 10°C to 45° did not follow a trend. Further, the experiment was not carried out in three replicates. It was concluded that this section needs to be repeated.

4.4 The Solubility of Salts as a Function of Time

From figure 20 it is evident that ionic sodium reaches equilibration within 2-3 minutes of water addition. Note that all sodium salts are soluble and therefore sodium can be used as a marker for water ingress into the powder. Assuming that sodium salts are homogeneously dispersed throughout the milk powder including the centre of the milk powder particle. The ionic sodium dissolution reaches equilibration in the first five minutes. Thus, the water also is reaching all parts of the particle very quickly. From this it appears that the mechanism of water migration into the particle is not responsible for limiting the solubility. Rather it is individual ingredient solubility.

4.5 Change in sediment volume in comparison with the solubility during rehydration

The solubility of ALAPRO 4850 was determined at a range of temperatures with a rehydration time 30 minutes in water and 20% sugar solution. Moreover, the ratio between the sediment volume and the mass of sediment was also calculated. Figure 28 shows that the changes in the ratio of sediment volume and mass of sediment and the solubility of both the powders have taken place at different temperatures.

In figure 28 it was observed that initially there is increase in the ratio of sediment volume and mass of the sediment with the increase in the solubility in water and 20% sugar solution. After having increase in the ratio of sediment volume and mass of the sediment to certain point there is decrease in the ratio of sediment volume and mass of the sediment with the increase in the solubility in both the solvents. The possible reason for this increase and then decrease may be that initially there is increase in the volume of particle during rehydration and after reaching the maximum expansion in powder particle and then it started to break down which is shown by the decrease in the specific volume and with increase in temperature in water and 20% sugar solution. Upon collapse of the particles the
Figure 28. Change in the ratio of sediment volume and solubility in ALAPRO 4850 volume of water within the cavity will be lost thus the total solids of remaining sediment will increase.

The rehydrated solution is composed of large and small particle population. The increase in the solubility at as the rehydration temperature increases over the lower temperature range (i.e. from 10 to about 30°C) may be due to the dissolution of small particles as indicated by the Figure 28. As the solution was centrifuged at 700 g so it was thought that at that level of force the structural integrity of large particle population is maintained and therefore the overall volume that is observed is relatively constant. The rehydration behaviour of the different sized particles is shown schematically in figure 29. In figure 29a the sediment is composed of small and large particles. Whereas in Figure 29 (b) the overview of the volume of the sediment after the centrifugation is similar but the small
particles population that previously occupied the interstitial space between the large particles have become soluble and so are in the supernatant. The important point is the sediments have the same volume but with difference in particle size populations.

4.6 High Centrifugation Speed For Disruption of Sediment

To prove this another experiment was designed to increase the force during centrifugation and observe its effect on sediment height in the centrifugation tube. The sample was centrifuged from 700 g upto 21000 rpm (52356 rcf) but there was no decrease in the sediment height showing the structural rigidity of large particle population.

4.7 Textural analyzer

During rehydration at different temperatures the interaction between the particles varies. Textural analyzer was also used to attempt to calculate the difference in strength of sediment by calculating the force required for the breakdown of the sediment at different temperatures. From the results it was observed that the sediment was not so strong and the results were at the lower range of the instrument showing its low reliability. So this technique cannot be used for the sediment samples in question.

4.8 Effect of Concentration on Solubility

To facilitate the manufacture of sediment for the experiment described in sections 4.8 and 4.9 ALAPRO 4850 was rehydrated at a 15% concentration rather than 5% used previously at 25±1°C. Surprisingly the solubility was higher 60% cf. 28%.

![Figure 30. Effect of Concentration on Solubility of ALAPRO 4850 at 25±1°C](image)

It was thought that the 60% solubility could be an artefact due to higher viscosity of the 15% mixture restricting the settling of non soluble material. Thus non-soluble could potentially be incorrectly included in the total solids of the supernatant. To determine if this...
was any artefact as described a 15% rehydrated solution was then diluted to 5% for centrifugation. Note that the purpose of the experiment was to ensure that the centrifugation conditions were the same and thus only the concentration during rehydration was different.

The result of this experiment confirmed the data indicating that solubility improves with powder concentration. Following this an experiment was conducted to characterise the impact of powder concentration on solubility over a range of 5-15%. In all cases the solution were diluted to 5% immediately prior centrifugation at 700 g for 10 minutes. This data is presented in figure 30.

First it was thought to rehydrate the 15% ALAPRO 4850 for 1 minute and then use that supernatant for the rehydration of 5% ALAPRO 4850 to see the effect of 15% supernatant solution. But the results were not interesting and gave no information on that. Sodium phosphate is binding calcium from the micelle which is adding to the increase in the solubility. To prove this an experiment was designed to calculate the required amount of calcium needed for phosphate based on sodium data.

4.9 Effect of Calcium Addition on the Solubility

A potential mechanism to explain the improved solubility might lie with the co-ions that dissolve with the sodium on water addition. If it is assumed that the co-ions are likely to be divalent and therefore act as calcium chelators (i.e., citrate or phosphate) then any advantage gained by having a high concentration in the solvent could potentially be negated by adding soluble calcium in the form of calcium chloride to the solvent. As the solvent dissolves the sodium salts the co-ions will immediately come in contact with calcium ions and precipitate effectively being removed from the system. To assess this possible mechanism ALAPRO 4850 was added to water containing calcium chloride at sufficient concentration to give a half the moles of calcium ions compounds to the moles of sodium that were expected (via ISE data) to dissolve. This would give a 1:1 ratio of calcium to sodium co-ions.

To this end 0.4675 g Calcium Chloride was added to 170 g water which was then used as a rehydrating medium for 15% ALAPRO 4850 for 30 minutes. The solubility was approximately same in the 5% ALAPRO4850 rehydrated solution and calcium chloride added ALAPRO4850 rehydrated solution in line with our hypothesis. Thus it appears that the
sodium co-ions may participate in solubilisation of MPC. It has been reported in the literature that calcium is responsible for restricting solubilisation and due to this cold soluble milk protein concentrates have been developed (Bhaskar et al., 2007) wherein the MPC has a portion of the native calcium removed via ion exchange prior to drying.

4.10 SAXS Analysis

From the results analysed it was found that the sediment samples at 10°C and 20°C were closely matching with the dry particle whereas the sediment samples rehydrated at 35°C and higher temperature were similar to fully rehydrated samples at 50°C and 60°C in water and 20% sugar solution respectively. Analysis of the data at IRL indicated that the distance between colloidal CaPO₄ centres decreases with the increase of temperature. Mechanistically one would expect such a decrease as the strength of hydrophobic bonds increases with temperature. Therefore one might expect the micelle to decrease in size and thus the CCP centres would become closer to each other.

4.11 Morphological Changes in Undissolved Material

The main reason of observing the morphological changes was to compare them with the solubility results and to observe visually the morphological changes taking place over a range of temperature in both solvents and try to predict the mechanism of rehydration of ALAPRO 4850.

4.11.1 Light Microscopy

The monitoring of morphological changes particle size changes of MPC85 was carried out by using a light microscopy technique. A compound light microscope with digital camera was used to monitor the morphological changes in ALAPRO 4850 dissolved in water and 20% sugar solution. The same procedure was carried out in sample preparation as was used for the determination of solubility data. After centrifugation at 700 g for 10 minutes, the sediment was dispersed in the same solvent in which it was initially dissolved. When this method was used the particles were overlapped and were not clearly observable. So some modifications in the method were made. After centrifugation at 700 g for 10 minutes, small portion of the sediment was taken in the tube and was diluted with same solvent in which it was prepared. The samples were observed at 20 and 30°C. The images were taken at 10x, 40x and 100x magnifications. At 10x it was observed that there were two populations of particles comprising of small and large particles at 20°C whereas at 100x individual particle
structure can be viewed. When the sediment of the solution rehydrated at 30°C was observed it was found that only large particle population is present. The difference shows that small particle population was dissolved at 30°C which was present at 20°C.

<table>
<thead>
<tr>
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<th>20% Sugar Solution @ 30°C (10x)</th>
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<table>
<thead>
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<th>20% Sugar Solution @ 20°C (40x)</th>
<th>20% Sugar Solution @ 30°C (40x)</th>
</tr>
</thead>
<tbody>
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<td><img src="image3.png" alt="Image" /></td>
<td><img src="image4.png" alt="Image" /></td>
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</table>

<table>
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<th>20% Sugar Solution @ 20°C (100x)</th>
<th>20% Sugar Solution @ 30°C (100x)</th>
</tr>
</thead>
<tbody>
<tr>
<td><img src="image5.png" alt="Image" /></td>
<td><img src="image6.png" alt="Image" /></td>
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</tbody>
</table>

Figure 31. ALAPRO 4850 rehydrated in 20% sugar solution at 20°C and 30°C observed under light microscope with different magnifications

4.11.2 **Transmission Electron Microscopy**

For further detailed examination the samples which were rehydrated in water at 10°C and 35°C were examined under transmission electron microscopy, which are shown in figure below.

<table>
<thead>
<tr>
<th>10°C in Water</th>
<th>35°C in Water</th>
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<tbody>
<tr>
<td><img src="image7.png" alt="Image" /></td>
<td><img src="image8.png" alt="Image" /></td>
</tr>
</tbody>
</table>
In figure 32a it is evident that the structural integrity is maintained at 10°C whereas in figure 32b the particles are separated which is rehydrated at 35°C. Vacuoles are formed inside the particle in both the samples which shows roughness. In figure 32b the individual casein micelles can be seen to separate during the rehydration at 35°C from the particle.

The vacuoles present in the powder form during the spray drying process and it is noteworthy that the outside surfaces of the particles are quite rough compared to the vacuole surface indicating that the micelles on the outer surface are relatively easier to hydrate as compared to micelles on the vacuole surface. This is in contrast to the work of McKenna (2000) who noted that a thick insoluble layer forms on the outside of MPC particles on storage.
In figure 33a the small particle seems to be going to rehydrate as seen by the individual casein micelles going to separate at 10°C. In figure 33b the caseins are separated from the particle also there is one small particle which has still maintained its structural integrity at 35°C with a sharp internal and external surface.

![10°C in Water](image1)
![35°C in Water](image2)

Figure 34. ALAPRO 4850 rehydrated in water at 10°C and 35°C observed under TEM with same magnifications

In figure 34a the structural integrity of the particle is maintained and the surface is much dense as compared to figure 34b. The individual casein micelles are going to separate from the outer surface which are visible in figure 34a. In the centre of figure 34b it seems to be the joint of two particles with one particle completely lost its half portion and caseins scattered and separating from the overall external surfaces of two particles. There is semi joint slightly visible between the two particles. This may be due to agglomeration of two primary particles during spray drying. From the figure 34a it appears as though the agglomerated surfaces are more readily hydrated indicating perhaps that the joints of micelles at this interface are not the same as true internal micelle-micelle interactions.

![10°C in Water](image3)
![35°C in Water](image4)

Figure 35. ALAPRO 4850 rehydrated in water at 10°C and 35°C observed under TEM with same magnifications
In the figure 35a small vacuoles which are likely to be the result of fat globules are present in different parts with a large vacuole in the right bottom of the particle rehydrated at 10°C and the individual casein micelles are intact in the interior whereas caseins are separating from the external surface of the particle. The individual casein micelles are scattered and are separating from the particle in 35°C as shown in figure 35b.

In the figure 36a the individual casein micelles can be seen to separate during the rehydration at 10°C from the particle whereas in figure 36b the casein seems to separate from the particle and the size of vacuole is increased which is representing the swelling of particle.

In the figure 37a small vacuoles are present in different parts of the particle rehydrated at 10°C and the individual casein micelles are intact in the interior whereas casein micelles are separating from the external surface of the particles. In figure 37b the casein micelles are separated from the particle also there is one small particle which has still
maintained its structural integrity at 35°C with a sharp internal and external surface. There is no separation of casein micelles from internal edges with some separation from the external surface of the particle.

<table>
<thead>
<tr>
<th>10°C in Water</th>
<th>35°C in Water</th>
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<tbody>
<tr>
<td><img src="image1.png" alt="Image" /></td>
<td><img src="image2.png" alt="Image" /></td>
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Figure 38. ALAPRO 4850 rehydrated in water at 10°C and 35°C observed under TEM with same magnifications

In the figure 38a small vacuoles are present in different parts with a large vacuole in the right bottom of the particle rehydrated at 10°C and the individual casein micelles are intact in the interior whereas casein micelles are separating from the external surface of the particle. The edges of the large vacuole are sharp showing no separation of casein micelles. In figure 38b small portion the particle is shown from which casein micelles are separating from external and internal surfaces of the particle at 35°C with few casein micelles separating from the internal surface of the particle.

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<thead>
<tr>
<th>10°C in Water</th>
<th>35°C in Water</th>
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<tbody>
<tr>
<td><img src="image3.png" alt="Image" /></td>
<td><img src="image4.png" alt="Image" /></td>
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Figure 39. ALAPRO 4850 rehydrated in water at 10°C and 35°C observed under TEM with same magnifications

In figure 39a it seems again to be the joint of two particles with having only half of the portion of the both particles visible and with small vacuoles scattered and few casein
micelles separating from the overall external surfaces of two particles at 10°C. There seems to be large vacuole present in the centre of both the particles. There is semi joint slightly visible between the two particles. In figure 39b representing 35°C rehydrated sample the individual micelles are more scattered as compared to 10°C sample. This shows that that the particle is more rehydrated at 35°C as compared to 10°C rehydrated sample.

<table>
<thead>
<tr>
<th>10°C in Water</th>
<th>35°C in Water</th>
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<td><img src="a" alt="Image" /></td>
<td><img src="b" alt="Image" /></td>
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Figure 40. ALAPRO 4850 rehydrated in water at 10°C and 35°C observed under TEM with same magnifications

Figure 40a gives the overview of the particles rehydrated at 10°C which clearly shows the maintenance of the particle structure. There are small vacuoles present all over the particle and casein micelles separating from some sides of the particles surface. In figure 40b the same process is observed with the sharp internal surface edge and casein scattering from the outside with the increased rate of casein separation in 35°C rehydrated sample. The size of vacuoles seems to increase which shows the swelling.

<table>
<thead>
<tr>
<th>10°C in Water</th>
<th>35°C in Water</th>
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<tbody>
<tr>
<td><img src="a" alt="Image" /></td>
<td><img src="b" alt="Image" /></td>
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Figure 41. ALAPRO 4850 rehydrated in water at 10°C and 35°C observed under TEM with same magnifications
In figure 41 the overall view of the rehydrated particles at 10°C (a) and 35°C (b) is shown. The 10°C rehydrated sample is denser than the 35°C sample.

Figure 42. ALAPRO 4850 rehydrated in water at 10°C observed under TEM with same magnifications

In the figure 42 different views of the particle rehydrated at 10°C are shown. It is clear that the internal edges are sharper than the external and casein micelles are separating from the external edges. In the top right figure the casein micelles are shown which are closely intact and bound together strongly.
In the figure 43 different views of the particle rehydrated at 35°C are shown.

Figure 43. ALAPRO 4850 rehydrated in water at 35°C observed under TEM with same magnifications
4.11.2.1 Conclusion drawn from Light and TE Microscopy

By studying carefully images from light and transmission electron microscopy the following important points can be concluded.

- Small particles tend to hydrate more quickly as compared to large particles as shown in figure 31.
- Particles tend to hydrate on external surfaces more readily than vacuole surfaces as shown in figures showing 35°C rehydrated images.
- It appears that the density of the particles within the sediment decreases at 35°C relative to 10°C. In part this could be explained by a temperature induced contraction of micelles. As temperature increases hydrophobic bonds increase and thus the internal structure of micelles become denser. Contraction of micelles would create cracks within particles resulting in the increased inter-micellar gaps evident in the 35°C images.

The key features to note in these images are that relative to the images of sediment from 10°C hydration the insoluble particles are less dense throughout the entire particle. In the 35°C sediment there are visible spaces between micelles. Additionally the distance between micelles appears to be the same throughout the particle. When interpreted with the SAXS, conductivity and ionic sodium data a potential mechanism unfolds.

The conductivity and ionic sodium data indicate that all soluble salts are hydrated within a few minutes of water addition. If the salts are evenly distributed throughout the particle then it can be assumed that water ingress into the particle is not the limiting factor in rehydration. There is significant difference in the distance between micelles in the 35°C Vs 10°C sediment images. This can arise either by water ingress which through osmotic pressure pushes micelles away from each other or by shrinkage of the micelles themselves. The SAXS data analysis performed at IRL suggests that the later is occurring. Chemically this mechanism for shrinkage could arise from the increase in temperature which would result in an increase in hydrophobic bonding within the micelle and thus a decrease in micelle particle size.
5 Conclusions

From the results it is found that there is significant difference in solubility, total conductivity and sodium data in water and 20% sugar solution. So based on these experiments the main conclusions derived from the results of the experiments are as follows.

- In all cases there was significant difference in measured parameters between the excess water and limited water environments.
- This data provides some indicative evidence that the solubility of MPCs becomes more sensitive to solutes as the powder ages. The decrease in solubility profile resulting from aging was noticeable in solutions with added sugar even though no difference was observed when the same powders were rehydrated in pure water. This finding is important to food formulators and as it implies that standard solubility quality control tests in water may not be indicative of behaviour in real world food manufacturing where solutes such as sugar are present. Similar ageing studies of MPC have been reported by Havea (2006) and Carr (2002) and have shown a similar decrease in solubility profile with ageing but have not reported the increased sensitivity to solute addition. This should also be kept in mind while calculating the solubility of milk powders during the processing as other ingredients will interfere with them to affect solubility of milk powders which in turn will affect the shelf life of the food products.
- There is critical solubility at which there is a sudden decrease in the ratio of sediment volume and mass of the sediment with the increase in the solubility and temperature in water and 20% sugar solution.
- The rate of change in the ratio of sediment volume and mass of the sediment with the increase in the solubility and temperature is also dependent on the solvent. This rate of change in water was higher than 20% sugar solution.
- A new mechanism for MPC powder rehydration was proposed wherein water ingress into the particles occurs over a very short time scale and that the influence of temperature on increasing the degree of solubility is the result, of shrinkage of the micelles in the particles due to increased hydrophobic bonding that in turn separates the micelles from each other allowing further ingress of water and solubilisation.
6 References


Milewski, S. (2001). Protein structure and physiochemical properties (Chap. 3). In chemical and functional properties of food proteins. CRC Press LLC, USA.


