KAPPA -CARRAGEENAN INTERACTIONS IN SYSTEMS CONTAINING CASEIN MICELLES AND POLYSACCHARIDE STABILIZERS

A Thesis

Presented to

The Faculty of Graduate Studies

of

The University of Guelph

By

PAUL ANTHONY SPAGNUOLO

In partial fulfilment of requirements

for the degree of

Master of Science

September, 2004

Your file Votre référence
ISBN: 0-494-01861-5
Our file Notre référence
ISBN: 0-494-01861-5

ABSTRACT

KAPPA -CARRAGEENAN INTERACTIONS IN SYSTEMS CONTAINING CASEIN MICELLES AND POLYSACCHARIDE STABILIZERS

Paul Anthony Spagnuolo
University of Guelph, 2004

Advisor:
Dr. H. D. Goff

Phase separation, as a result of thermodynamic incompatibility between casein micelles and added polysaccharide, occurs when polysaccharide gums are added to milk based products. Addition of κ-carrageenan prevents the undesirable phenomenon, however, the mechanism by which this is achieved is not fully known. The existing theories, although reasonable, fail to fully explain κ-carrageenan's 'milk reactivity'. Therefore, the purpose of this research was to analyze the ability of κ-carrageeenan to prevent phase separation under a variety of conditions and to provide a viable explanation.

Field emission scanning electron micrographs, dynamic light scattering and micro differential scanning calorimetry results show that interaction occurs between casein micelles and κ-carrageenan. Dynamic light scattering and phase separation experiments also show that interaction is necessary but not sufficient to prevent phase separation and further suggest that aggregation of κ-carrgeenan helices is also required. These results, coupled with results from previous projects in our lab, lead to a mechanism that involves both casein-κ-carrageenan and κ-carrageenan-κ-carrageenan interactions, which prevents phase separation.

Acknowledgements

I would like to thank Dr. H. Douglas Goff not only for his guidance and patience during the last two years but also for opening many doors for me. He saw potential in a second year student with no experience or educational background and gave that student a chance. Without that foresight I would not be where I am today, I am and will be forever thankful. To Dr. Dalgleish, who has been like a second advisor, I thank you for the many talks and help along the way. Special thanks to Dr. Morris of U.C.C. and Dr. de Vries of Danisco for their input during the course of the research project. A note of gratitude is also extended to Dr. Corredig and Dr. Hill for their respective roles on my examination committee.

This project was made that much more fruitful with the microscopy knowledge of David Hoyle(from Hitachi Canada), Sandy Smith and Brenda Sun. Thank you to Danisco who funded a portion of the project and to all my lab mates, especially Mike Rogers who assisted me greatly particularly in Ireland, Liz Parker and Jen Harrington, whether from Cork or Guelph thank you for your help, humour and friendship.

Life is a journey made possible only with the love and support of family and friends. I am truly blessed by an immense web of family and friends through whose constant presence has made these two years possible. To my boys, Derek Spry and Joe VandenBerg, thank you for being like brothers to me through crazy ups and downs. To my habibi, Marwa Soliman, thank you for all the love and happiness you have given me and for the push when I needed it. And to my parents and sister: I am who I am because of you guys and I truly believe this world would be a better place if everyone had family like you. I thank you for your continued love, support and encouragement and I love you very much.

LIST OF TABLES

LIST OF FIGURES

1.0 INTRODUCTION

Polysaccharides, i.e. locust bean gum or guar gum, are commonly used in dairy products such as ice cream mix to impart solution viscosity and enhance the overall sensory attributes of the final product. Milk contains approximately 3.3 (wt) % protein (Langendorff et al., 1999), of which 80% are casein proteins organized into a supramolecular complex structure called the casein micelle (Bourriot et al., 1999). The functional properties of these proteins, including solubility, surface activity, conformational stability, gel-forming ability and emulsifying and foaming properties, are all affected by their interaction with polysaccharides (Tolstoguzov, 1997). An important consequence of the polysaccharide presence in milk systems is the undesirable phenomenon of phase separation: the formation of two distinct phases in which one is concentrated in protein and the other devoid of protein (Syrbe et al., 1998).

Phase separation can be prevented by the addition of sufficient amounts of κ-carrageenan, a polysaccharide extracted from the red seaweed of the species *Rhodophycea*. The ability of κ-carrageenan to prevent the occurrence of this phenomenon and improve the stability of dairy systems has been attributed to a specific interaction between the polysaccharide and the casein micelle. It was proposed by Snoeren et al. (1975) that an electrostatic interaction occurs specifically between κ-carrageenan and the κ-casein molecule of the casein micelle. κ-Carrageenan is negatively charged due to its degree of sulfation, and a positive "patch" is located between amino acid residues 97-112, of κ-casein.

However, since the micelle is negatively charged some authours have been sceptical of this proposed mechanism. Another theory proposed by Bourriot et al. (1999)

suggested that the mechanism of stability is by the formation of a weak κ-carrageenan gel that is able to trap the casein micelles and prevent their sedimentation and argues that no interaction occurs. Numerous works, most notably those from Dalgleish and Morris (1988) and Langendorff et al. (1997 and 1999), have attempted to substantiate the mechanism of this interaction and explain how phase separation is prevented by the addition of κ-carrageenan. Recent work by Thaiudom and Goff (2003) showed that microscopic phase separation, the creation of protein-enriched domains, occurs in casein micelle suspensions with LBG and κ-carrageenan even when the systems are macroscopically stable. Furthermore, Vega et al. (2004a) have shown rheologically that κ-carrageenan is associated with these protein-enriched domains, and have supported Thaiudom and Goff's explanation that the κ-carrageenan may act as an emulsifier to stabilize the protein-enriched droplets. Although all these works have contributed important information regarding the interaction substantiation of an actual mechanism still remains elusive. This research project is focused on the mechanism by which κ-carrageenan achieves macroscopic stability in dairy systems containing added polysaccharides.

2.0 OBJECTIVES

The objective of this research was focused primarily on the ability of κ-carrageenan to prevent visual macroscopic phase separation in soft serve ice cream mixes that contain polysaccharide. This work builds upon the preliminary research of Sukrit Thaiudom, Richard Andrew and Cesar Vega, who all worked generally in this area in our laboratory. We hoped to continue advancing knowledge as well as provide a working mechanism of the stabilizing ability of κ-carrageenan. With this aim field emission scanning electron microscopy, dynamic light scattering, differential scanning calorimetry, phase separation experiments and reverse phase high performance liquid chromatography were employed.

3.0 LITERATURE REVIEW

3.1. Bovine Milk

Milk is a very important substance from both a physiological and industrial standpoint. Physiologically the function of milk is to nourish and provide immunological protection for the mammalian young (Fox, 2003). Industrially there are a variety of dairy milk products that are commercially available and widely consumed. The total world production of milk is approximately 560 x 10^6 tonnes per annum, 85% of which is bovine milk (Fox, 2001).

All mammalian species produce milk for the purpose of providing nourishment to the neonate (Fox, 2001). Bovine milk is the most widespread and most thoroughly studied (Dalgleish, 1992) and is the focus of this study.

There is a variety of cow species that are used for the commercial mass production of milk and the composition varies depending on the producing species. The most common is the Holstein breed since it produces more milk per cow than any other species.

Fat (%)	3.90
Protein (%)	3.25
Lactose (%)	4.60
Ash (%)	0.95
Water (%)	87.3

Table 3.1: Typical composition of milk from a Holstein cow (Walstra and Jenness, 1984).

The composition, mostly the fat content, can vary depending on several factors including stage of lactation and diet, therefore market consistency is achieved by pooling and standardization of fat content (Jensen et al., 1991). Milk is a very complex fluid containing a number of different components, which are discussed below.

3.1.1 Fat

The importance of fat in milk is twofold. Primarily fat is a source of essential fatty acids, i.e. fatty acids that cannot be produced by the human body, and also fat-soluble vitamins. Secondly, fat is important in providing the overall flavour to milk (Fox and McSweeney, 1998).

Fat is present in milk in globular form and is surrounded by a thin membrane composed primarily of a mixture of protein and phospholipid (Walstra and Jenness, 1984). This fat globule membrane (FGM) protects the milk fat from degradative enzymes and most importantly maintains the integrity of the milk fat by preventing coalescence (Jensen et al., 1991).

The milk fat itself is composed of more than 98% triglycerides with the remaining small fraction including cholesterol, diglycerides, free fatty acids and phospholipids (Walstra and Jenness, 1984). The fatty acid composition of milk fat is variable and depends on season and feed however there are general conclusions that can be drawn. It contains a relatively high proportion of short chain fatty acids ($C_{4:0}$ – $C_{10:0}$) with butyric acid ($C_{4:0}$), specific to milk of ruminant species, being of particular importance as it is largely responsible for the characteristic flavour of milk.

Milk fat can change when processed. It is susceptible to lipolysis, which involves release of fatty acids and results in a rancid and soapy taste, as well as autooxidation, which involves the oxidation of the unsaturated fatty acids to produce aldehydes and ketones that have undesirable sensory properties (Walstra and Jenness, 1984). Crystallization of milk fat is also of importance in manufacturing as it largely determines the physical stability of the fat globules. The varying number of different fatty acids

results in a large melting range from –40°C to 72°C with a usual final melting point of roughly 37°C (Walstra and Jenness, 1984). This wide melting range has important implications in the production of ice cream and is discussed further in section 3.3.1.

3.1.2 Protein

Milk proteins are largely responsible for milk's functionality and versatility (Sawyer et al., 2002). Their role is to provide nutrition and other requirements, such as protective and growth factors as well as nutritional aids (calcium) to the neonate of the species. From a nutritional and physiological standpoint the milk proteins are the most important constituents of milk (Fox, 2001).

Milk contains 30-36 g/L of protein, which can be further divided into two categories: the caseins and the whey proteins (Swaisgood, 1996). The division is based primarily on their solubility at pH 4.6 (20°C) at which the caseins precipitate while the whey proteins remain in solution (Modler, 2000). The caseins comprise 80% of the total protein and are present, along with calcium, phosphate and small amounts of citrate, in roughly spherical colloidal unit called casein micelles (Dalgleish, 1992, Schorsch et al., 1999). More than 95% of the caseins are associated into the casein micelle (Fox and McSweeney, 1998), which functions primarily to carry calcium and phosphate to the mammalian neonate (Creamer and MacGibbon, 1996, Horne, 2002). The whey proteins, which comprise the remaining 20% of milk protein, are primarily globular proteins with well-defined structures. They have some important physiological functions with each protein specific in its biological role (Walstra and Jenness, 1984).

The milk proteins are synthesized in the endoplasmic reticulum and are further processed in the golgi apparatus of the female mammal (Walstra and Jenness, 1984,

Farrell et al., 2002). As a result they are slightly modified gene products, which can result in genetic variants among the protein classes (Allmere et al., 1997). The variants differ in the substitutions or deletion of different amino acids and phosphates in the case of the caseins (Allmere et. al., 1997) and does affect to some degree the property of the protein (Dalgleish, 1997). At present however there is little information on this topic (Dalgleish, 1997), but it is also known that there are specific variants more commonly found in Western Bovine milk (Swaisgood, 1992).

3.1.2.1 The Casein Protein

The caseins are small molecules with a molecular mass of 20-25 kDa (Fox, 2001). There are four types of caseins, which are divided, based on their composition and properties, into four classes αs_1:αs_2:β:κ with the approximate ratio of 4:1:4:1, respectively (Dalgleish, 1997). All of the casein contain phosphates and are phosphorylated at either the threonyl residues or more commonly the seryl residues (Swaisgood, 1996). The varying degree of phosphorylation between each casein class gives rise to their different properties, but in general the phosphate groups account for the ability of the caseins to bind large amounts of cations, most specifically calcium (Dalgleish, 1997).

A peculiarity of the caseins is that they contain high levels of proline, a structure-breaking amino acid (Farrell et al., 2002). Thus the caseins are classified as having an open structure and not a typical secondary structure, as the proline residues prevent the formation of α helices, β sheets and β turns (Fox, 2001). The lack of stable secondary and tertiary structures accounts for their heat stability and surface activity as well as their

susceptibility to proteolysis (Fox, 2001). These characteristics have led scientists to define the structure of the individual caseins as being rheomorphic, meaning a flexible, open conformation with a considerable degree of side chains and possibly a backbone (Holt and Sawyer, 1993, Fox, 2001, Farrell et al., 2003, De Kruif and Holt, 2003).

Standard techniques, such as X-ray crystallography and nuclear magnetic resonance spectrometry (NMR), have been unable to establish a three dimensional structure of the caseins, largely because it is not possible to crystallize them (Creamer and MacGibbon, 1996). Although experimentally a three-dimensional structure has not yet been obtained, this does not mean that one does not exist. It merely proves that the caseins are dynamic and very unique proteins and that current methods of measurement may not yet be adequate. Farrell et al. (2001) used computer modelling to predict what a three-dimensional structure would look like based on the primary sequence of the individual caseins. Although this approach may be insightful, it still does not provide useful evidence of secondary structure, as computer algorithms are not very practical in natural applications.

The following is a brief review of the individual casein proteins.

3.1.2.2 αs1 Casein (αs1-CN)

This group of caseins contains 199 residues, 17 of which are proline, and has an average molecular weight of 23 kDa (Fox and McSweeney, 1998). αs_1-CN contains 8-10 phosphate groups that tend to be clustered at residues 41-80 and this gives rise to a net negative charge of –20.0 to –22.6 at the pH of normal milk (6.7) (Modler, 2000). Also, the high degree of phosphorylation accounts for the calcium sensitivity of αs_1-CN with precipitation occurring in relatively small amounts of calcium (<6mm) (Fox, 2001). The

hydrophobic areas and charges are not evenly distributed along the casein chain, with residues 1-44, 90-113, and 132-199 exhibiting hydrophobicity (Swaisgood, 1996).

3.1.2.3 αs2 Casein (αs2-CN)

This group of caseins contains 207 residues, 10 of which are proline, and has an average molecular weight of 25 kDa (Fox and McSweeney, 1998). The structure of αs_2-CN has two distinct regions of high net charge including three phoshphoseryl clusters located at the 8-12, 56-63 and 129-133 residues and has a total of 10-13 phosphate groups (Swaisgood, 1992, Modler, 2000). The hydrophobic regions are located on the central 90-120 residues and the 160-207 residues of the C-terminal (Swaisgood, 1992). It has a large net positive charge on the C-terminal (+9.5 at pH 6.6) while the more hydrophilic first 68 residues on the N-terminal has a net charge of –21 at pH 6.6. As a consequence of its structure αs_2-CN is very sensitive to ionic strength and therefore is classified along with αs_1-CN as calcium sensitive (Swaisgood, 1992).

3.1.2.4 β Casein (β-CN)

This group of caseins contains 209 residues, 35 of which are proline and has an average molecular weight of 24kDa (Fox and McSweeney, 1998). A striking feature from the primary sequence is the high level of proline residues that are uniformly distributed throughout the molecule. The high degree of proline residues coupled with the uniformity of its distribution is evidence that this casein lacks any secondary structure (Fox, 2001). It also contains 5 phosphate groups, four of which are located between the 13-21 residues (Modler, 2000, Farrell et al., 2001). This gives rise to a strong negative charge (-11.5 at pH 6.6) on the N-terminal and also accounts for its calcium sensitivity

(Brunner, 1977, Swaisgood, 1992). The remaining portion of the molecule, which exhibits no charge, is strongly hydrophobic and gives rise to the amphiphillic properties of the molecule (Brunner, 1977, Modler, 2000). β-Casein, because of its large hydrophobic domain, is the most hydrophobic of the caseins and subsequently is the most temperature sensitive (Swaisgood, 1996), which has a consequence in its behaviour in the casein micelle at low temperatures.

3.1.2.5 κ-Casein (κ-CN)

This group of caseins contains 169 residues, 20 of which are prolines, and has an average molecular weight of 19 kDa (Fox and McSweeney, 1998). Comparing κ-CN to the other casein proteins there are two distinct differences. Firstly, κ-CN contains only one phosphate group and thus is the only casein protein that is not calcium sensitive (Modler, 2000). Secondly, another characteristic that adds to the calcium insensitivity is the glycosylation of the threonyl residues of the C-terminal (Fox, 2001, Swaisgood, 2003). The post translational modification, which occurs in the golgi apparatus of the lactating cow, results in the addition of tri- or tetrasaccharide moieties into the structure of κ-CN (Swaisgood, 1996). The presence of the oligosaccharides attached to the C-terminal results in an increased hydrophilicity of that region (Fox, 2001).

The N terminal, residues 1-105 (para-κ-casein) of κ-CN is very hydrophobic (Swaisgood, 2003) and the remaining 64 residues comprise the highly charged, polar glycomacropeptide (GMP), which contains no cationic residues and has a charge of -10 at pH 6.6 (Brunner, 1977, Swaisgood, 1992). Since the C-terminal GMP region is highly hydrophilic and the N-terminal para-κ-casein hydrophobic, the κ-CN is amphiphillic in

nature and it is vital, as will be discussed in the following section, in casein micelle stability (Swaisgood, 1992).

Most importantly in regards to the potential mechanism of milk reactivity κ-CN has a region of positively charged amino acid residues. This region, generally termed the positive patch, is located between residues 20-115 with the active region located between residues 97-112 where there are 1ARG, 3HIS, 2 LYS (Snoeren et al., 1975). This positive region is thus primarily located on the hydrophobic region of the κ-CN (Langendorff et al., 1997). Below are two figures that show the distribution of charge on the κ-CN as well as the other casein molecules.

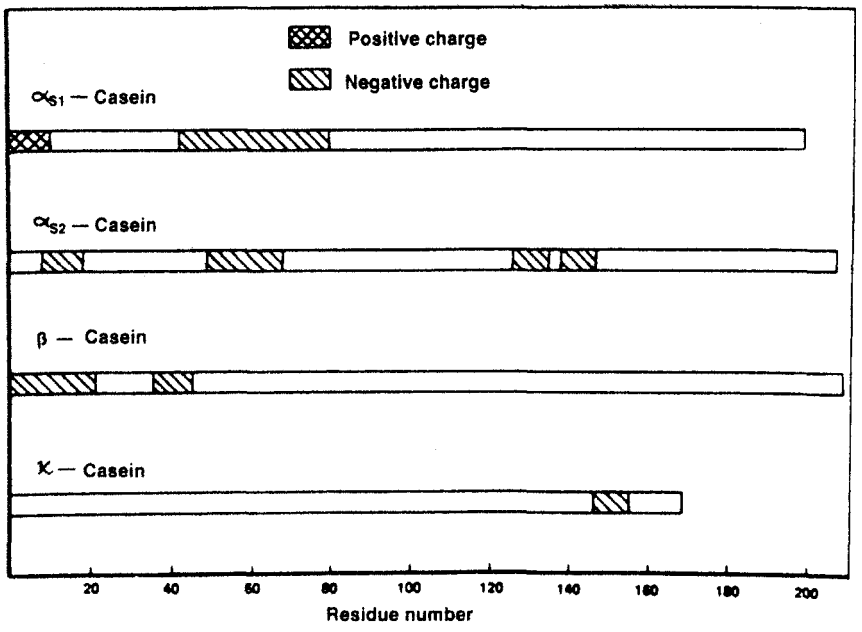


Figure 3.1: Residue sequences of the individual casein proteins with a charge density of 0.5 or greater at pH 6.7 (Swaisgood, 2003).

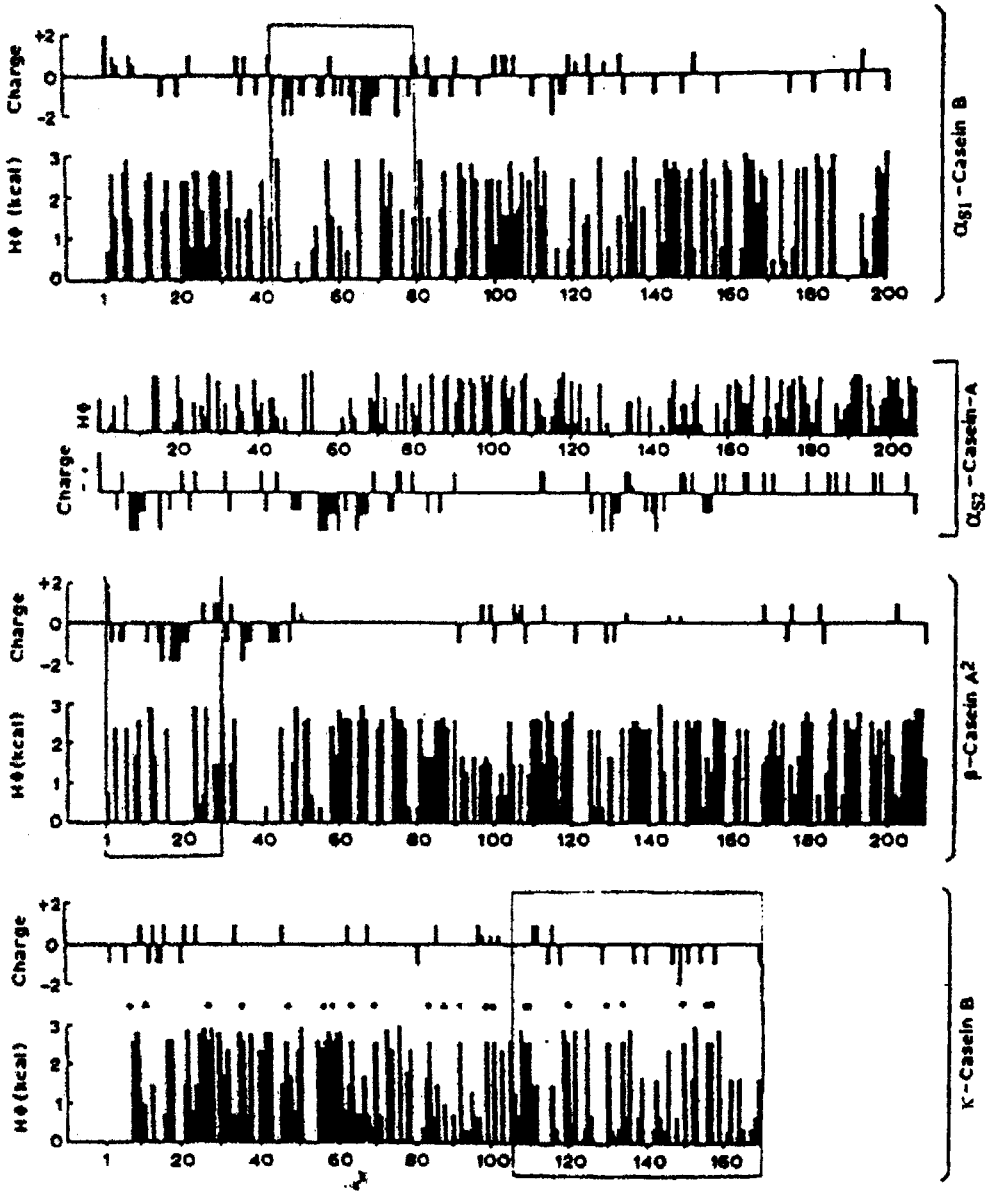


Figure 3.2: Schematic illustration of the distribution of hydrophobic and charged residues for the individual casein protein (Swaisgood, 2003).

3.1.3 Casein Micelles

As previously mentioned the individual casein proteins are organized in milk as colloidal structures called casein micelles. They range in size from 50-500nm, contain between 20 000 - 150 000 of the individual casein proteins and have a mass range between 10^6 to 3 x 10^9 Da (average ~10^8 Da) (Schorsch et al., 1999, Fox, 2003). They have a very hydrated (3.7 g H_2O per gram of protein), porous and open structure (Fox, 2001) with the proportion of individual caseins in the casein micelle approximately 3:0.8:3:1 by weight for αs_1, αs_2, β and κ-casein, respectively (Schorsh, 2000).

The casein micelle is comprised of 92% of the individual casein protein and 8% milk salts with the salts primarily being calcium, inorganic phosphate, magnesium and citrate (Horne and Davidson, 1986, Swaisgood, 1996). The principal salt is a mixture of calcium and phosphate organized into a colloidal structure called colloidal calcium phosphate (CCP) (Ca_3PO_4) (Fox and McSweeney, 1998). There has been much debate for decades as to the structure of the micelle and there still remains much controversy over the actual structure. There is however one feature that, fortunately for this discussion, is commonly agreed upon and that is the location of κ-casein on the surface. The following sections outline the proposed structures of the casein micelle and the nature of the surface and its stabilizing ability.

3.1.3.1 Casein Micelle Structure

The inconclusiveness of the structure of the casein micelle led to early working structural models, which have been continuously adapted and updated to include the most recent physico-chemical properties that are revealed with increasing technology. However, at present there is no one proposed structure that has gained complete

acceptance but there are two that have received the most attention; the sub-micellar model (Figure 3.3) and the open nanocluster model (Figure 3.4).

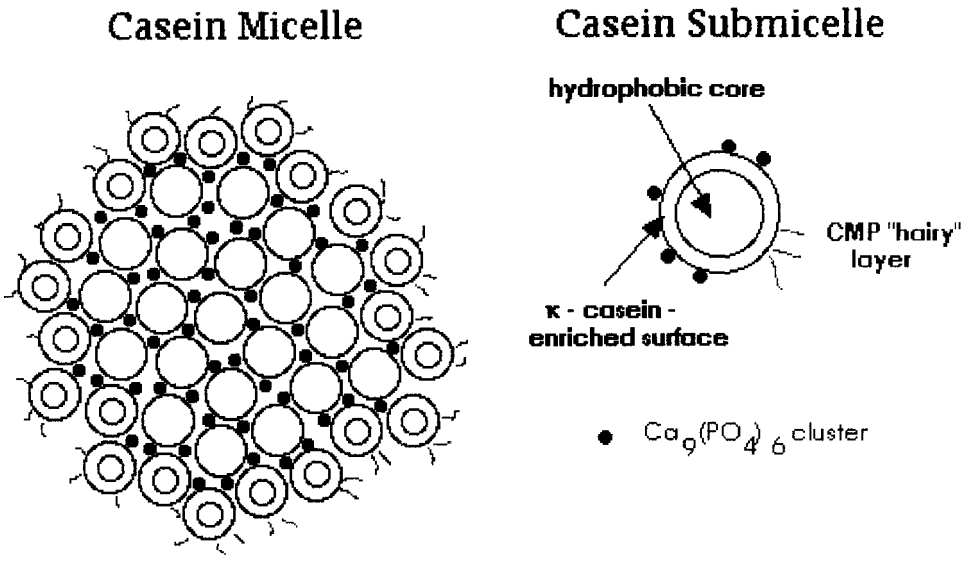


Figure 3.3: The sub-micellar model proposed by Walstra (Walstra, 1999)

Figure 3.4: The nanocluster model proposed by Holt (Fox and McSweeney, 1998).

Figure 3.3 depicts the sub-micelle model that is reviewed in detail by Walstra and Jenness (1984), Walstra (1990) and Walstra (1999). The model envisions the casein micelle to be composed of discrete subunits, called sub-micelles, which are of a mixed composition of the individual casein proteins (Walstra, 1990, Lucey, 2002). There are two types of sub-micelles, those with κ-casein and those without. The sub-micelles aggregate, via both hydrophobic and electrostatic (salt bridges) interactions, and are linked together by CCP until they have formed a micelle with the κ-casein containing sub-micelles located, almost entirely, on the outside of the micelle (Walstra, 1999).

The open nano-cluster model (Figure 3.4) suggests that the casein micelle structure is a mineralized, tangled or cross-linked web of the individual casein proteins (Lucey, 2002) forming a gel-like structure (Fox, 2003). This model suggests that the caseins are homogenously distributed through the micelle and the individual caseins are linked together by colloidal calcium phosphate interactions (De Kruif and Holt, 2003). The surface is believed to contain no such structure but rather a decrease in protein density with the C-terminal region of the κ-casein extending from the surface and acting as the hairy layer (Fox, 2003).

Worth mentioning is also the dual binding (polymer condensation) model proposed by Horne (1998). It is similar to the open nano-cluster model but includes that hydrophobic interactions between the individual casein proteins are also involved in micellar integrity as well as the nature of the linking of CCP is to neutralize the negative charge of the highly phosphorylated caseins (Horne, 1998). The model further suggests that assembly and growth of the micelle occurs by a polymerization process, which is driven by hydrophobic interactions between the individual caseins. The polymerization

process is terminated by κ-casein, which has only one hydrophobic region. Once attached to the other caseins via its hydrophobic end, the hydrophilic C-terminal, which is unable to interact further, terminates the growth and is thus found on the exterior of the micelle (Horne, 1998).

Electron microscopy has been a technique at the forefront that has attempted to elucidate on the structure of the casein micelle. Sample preparations and micrographs of both scanning electron and transmission electron microscopy were carefully re-examined by McMahon and McManus (1998). They concluded that micrographs showed no evidence of sub-micellar structure and that some TEM images showed great correspondence to the open nano-cluster model. Although a promising review, the authours also concluded that substantiation and confirmation remained elusive.

3.1.3.2 Hairy Layer Stability and Destabilization

Although the possibility for there to be consensus in micellar structure seems unlikely there is agreement on the nature of the surface. It is commonly agreed that the calcium sensitive caseins are located within the structure protected from precipitation by the calcium insensitive κ-casein (Calvo, 1995, Creamer et al., 1998, Rasmussen et al., 1999, Horne, 2002). This results in κ-casein being located primarily, if not entirely, on the surface of the casein micelle, the important feature as it is believed that the surface presence of this molecule is responsible for stabilizing the colloid from self-aggregation (Creamer et al., 1998). The negatively charged C-terminal (GMP) of the molecule protrudes, approximately 5-7nm, from the surface of the casein micelle and provides not only electrostatic but also steric stabilization (Holt and Horne 1996, Dalgleish, 1998). It thus prevents aggregation of micelles by both charge repulsion and volume exclusion

effects, as there is a considerable amount of water associated with the protruding C-terminal. Thus the casein micelles exist in solution as individual colloidal particles and aggregates. This portion of the glycomacropeptide, termed the hairy layer, is also hydrophilic and increases the solubility of the more predominantly hydrophobic caseins.

Interestingly, only one third of the micellar surface is covered by κ-CN with the remainder being possibly occupied by other casein proteins (Dalgleish, 1998). Furthermore, κ-casein is not necessarily uniformly distributed on the surface, which may result in higher concentrated regions of the protruding hairs but more importantly regions fully devoid of the stabilizing glycomacropeptide (Dalgleish, 1998). Unfortunately it is not clear as to which of the other individual caseins, if there is one at all, that shares the surface with κ-CN. It has been calculated that there is one κ-CN molecule per 10-50 nm^2 of micellar surface, which results in the conclusion that there are bare patches that slightly expose the surface (Dalgleish, 1998). This would explain how other protein molecules, such as whey proteins, and enzymes such as rennet, are able to penetrate the hairy layer as well as account for the ability of β-casein to migrate, at low temperatures, in and out of the micelle without disrupting the structure (Dalgleish, 1998). This may be crucial, coupled with the flexible nature of the hairy layer (Langendorff et al., 2000), in understanding the interaction with the large polymer κ-carrageenan.

The glycomacropeptide portion of the κ-CN is responsible for stabilization of the individual casein micelles and consequently, through manipulation, destabilization. By altering the surrounding environment instability is achieved and this gives rise to numerous dairy products commercially available. In cheese making rennet is used, which contains the enzyme chymosin that cleaves off the GMP from the κ-casein hairy layer

(De Kruif, 1999). The enzyme attacks the 105-106 (Phe-Met) bond resulting in the loss in steric stability and causing flocculation of micelles via hydrophobic, van der Waals forces and calcium bridging (Lucey, 2002). In acidified milk lowering the pH results in a neutralization of the charge on the micelle, which eliminates electrostatic repulsion and thus favours aggregation (Holt and Horne, 1996). Also, the colloidal calcium phosphate becomes soluble resulting in micellar dissociation and precipitation occurs (Holt and Horne, 1996, Dalgleish, 1997) at the isoelectric point of pH 4.6 (Lucey and Singh, 2003). Sufficient concentrations of ethanol, greater than 35% (vol.), also causes the collapse of the hairy layer and results in aggregation because of the loss in steric stability (De Kruif, 1999). Therefore, knowledge of the micellar surface is of particular importance in understanding the nature of the casein micelle and can lead to further insight into its potential interaction with κ-carrageenan.

3.1.4 Whey Proteins

For many years the industrial importance of whey proteins was overlooked but knowledge of their role in biological function as well as low cost has led to their use in a variety of food products. The whey proteins, or serum proteins, can be further broken down into four main classes: β-lactoglobulin (β-lg), α-lactalbumin (α-la), immunoglobulins (Ig) and bovine serum albumin (BSA). β-lg and α-la are the main whey proteins comprising 50% and 20% of the whey portion, respectively (Fox, 2001).

In contrast to the caseins in term of structure, the whey proteins have been well characterized and are found to contain high levels of secondary, tertiary and in most cases

quaternary structure (Fox, 2001). They are globular proteins that contain intramolecular disulfide bonds that stabilize their structure and have a net negative charge at milk pH (Creamer and MacGibbon, 1996). At high temperatures (>70°C) whey proteins are known to denature (de Wit, 1998), which has great implications as this results in an interaction with the casein micelle. Unlike the caseins there is a rather uniform distribution of the polar, hydrophobic and charged regions, which allows for the formation of highly ordered structures (Swaisgood, 1996).

The whey proteins are very small compared to the casein micelle. Figure 5 shows the relative size comparison between the two major whey proteins and a small casein micelle of radius 50nm.

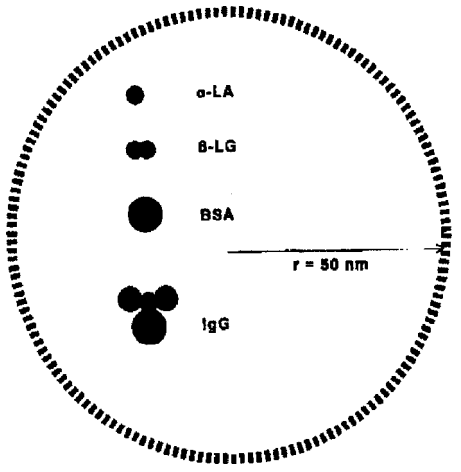


Figure 3.5: Size comparison of the four main whey proteins and a casein micelle of radius 50nm (de Wit 1998).

The following is a brief review of the main whey proteins β lactoglobulin and α lactalbumin.

3.1.4.1 β lactoglobulin

β Lactoglobulin is comprised of 162 residues with a calculated molecular weight of 18 kDa (Fox and McSweeney, 1998). At room temperature β-lg exists as a dimer but will dissociate above 30°C and will denature above 55°C (Gallagher and Mulvihill, 1997). It contains 5 cysteine residues, 1 of which is a free thiol group that is located within the structure of the protein. As will be discussed in section 3.2.4.3, the exposure of the buried free thiol group plays a significant role in the interaction with the casein micelle.

The physiological function of the whey proteins is to act as a carrier to ligands and other hydrophobic molecules. For example, β-lg is known to bind to retinol and carry it to the small intestine where it is then transferred to a retinol-binding protein (Fox and McSweeney, 1998).

3.1.4.2 α lactalbumin

α Lactalbumin contains 123 residues and has a molecular weight of 14 kDa (Fox and McSweeney, 1998). It is a very compact, almost spherical globular protein (Swaisgood, 1996) containing four disulfide linkages but has no free sulfhydryl groups (Dalgleish et al., 1997). The tertiary structure of α-la can unfold and refold reversibly, which accounts for the lower denaturation temperature as compared to β-lg (Swaisgood, 1996). Irreversible denaturation of α-la occurs at higher temperatures than β-lg but unlike β-lg, under most milk processing conditions α-la can refold to its native conformation (Swaisgood, 1996).

α-Lactalbumin has a very interesting physiological role in the synthesis of lactose as it combines with UDP-galactosyl transferase to form lactose synthetase, the catalyzing enzyme in lactose synthesis. The concentration of lactose is directly related to the concentration of α-la and therefore α-la acts as a regulator to the production of lactose (Fox and McSweeney, 1998).

3.1.4.3 Interaction between Casein and Whey

It has long been established that casein and whey proteins interact upon heating and that the interaction occurs between β-lactoglobulin and κ-CN (Smits and van Brouwershaven, 1980, Pearse et al., 1985, Noh and Richardson, 1989). The β-lg/κ-CN complex is formed via hydrophobic interactions and a thiol-disulfide exchange between two free sulfhydryl groups (Dalgleish et al., 1997, Anema and Li, 2003). Heating is a crucial precursor to the interaction as the hydrophobic groups and the free thiol group become exposed and available for the reaction as a result of denaturation and unfolding (Anema and Li, 2003). Once exposed the thiol group of β-lg becomes reactive and is free to react with the sulfhydryl group of κ-CN, located on the para-κ-casein portion of the molecule (Anema and Li, 2003). The interaction is initiated via hydrophobic interaction and stabilizing of the complex occurs via intermolecular disulfide bonding (Haque et al., 1989, Reddy and Kinsella, 1990).

The ability of α-la to interact with the casein micelle is affected by the presence of β-lg (Smits and van Brouwershaven, 1980). In short, α-la cannot bind to the micelle unless β-lg is present (Corredig and Dalgleish, 1999), as α-la must first react with β-lg to

form a complex, which then can attach to the surface of the micelle (Oldfield et al., 2000).

It has also been reported by Langendorff et al. (2000) that the complex formed between casein micelles and whey proteins has no effect on the interaction between casein micelles and κ-carrageenan.

3.1.5 Minor Milk Components

Milk contains a variety of minor components that are present in small amounts. Of the most important is the milk sugar lactose, a glucose-galactose disaccharide. It provides milk with a slightly sweet taste and is used as a fermentation substrate for the production of cultured milk products (Walstra and Jenness, 1984). Milk contains all 22 essential minerals that are present in milk predominantly as inorganic salts but also contain organic salts such as citrate (Walstra and Jenness, 1984). Vitamins are also present in milk, including all 4 fat soluble vitamins as well as the important dietary water soluble vitamins (B1 – thiamine, B2 – riboflavin, B6 – pyridoxine, B12 – cyanocobalamin, niacin and pantothenic acid) (Fox and McSweeney, 1998).

3.2 Soft Serve Ice Cream Mixes

Soft serve ice cream involves the production of the dairy dessert just prior to consumption, therefore, the ice cream mix is manufactured commercially at an off-site location and delivered to the soft serve retailers (Marshall et al., 2003). As a result a main concern to soft serve mix manufactures is stability of the mix since the required shelf life can range between 14-21 days (Vega et al., 2004a). This problem is not

encountered with usual ice cream production since the mix is manufactured 4-24 hours prior to freezing.

The composition of a typical soft serve ice cream mix is shown below.

Table 3.2: The typical composition of a soft serve ice cream mix

Composition	Average %
Milk Fat	4
Sweeteners	13
Milk Solids not fat	13
Emulsifier	0.1
Stabilizer	0.155
	Total Solids: 30.255%
	Balance Water: 69.745%

(Andrew, 2002).

Milk fat is extremely important to the overall flavour and structure of the final ice cream product. It provides stiffness, dryness and creaminess to the extruded product as well as producing the 3-dimensional network that gives ice cream its structure (Marshall et al., 2003). Milk fat provides an ideal mix of triglycerides with a wide melting range, which provides an ideal liquid to crystalline ratio that results in the presence of fat crystals at freezing temperatures. The crystalline fat is crucial for structural development as it is responsible for forming the bridging between the fat globules. The destabilization of the individual fat globules is known as partial coalescence and is depicted below.

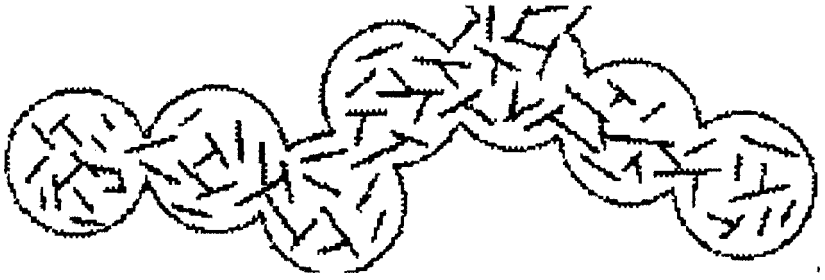


Figure 3.6: A schematic diagram of partial coalescence. (modified from Goff, 2004).

In order to promote partial coalescence emulsifiers are added. The emulsifiers, usually a blend of mono and di-glycerides (mdg) and sorbitan esters, are responsible for displacing the protein from the surface of the fat globule creating, on certain areas of the fat surface, a new thin membrane. This facilitates partial coalescence as this area is easily punctured by the fat crystals and allows for the bridging between neighbouring fat globules and the subsequent creation of the 3-dimensional structure (Goff & Jordan, 1989). The emulsifiers also enhance foam stability as they promote adsorption of fat globules to air bubbles. These product properties are important to soft serve formulations as they improve shape retention and foam stability as well as reduce meltdown of the final product.

A major component of the milk solids not fat (msnf) are the milk proteins, discussed in section 3.1.2. The proteins play important functional roles in both the mix and the final ice cream product as they are involved with aeration and foam stability as well as enhance solution properties such as viscosity enhancement (Goff, 1997, Vega and Goff, 2004b). In the mix the proteins can exist as free proteins or adsorbed proteins, either at the air or fat interface. Their presence in ice cream mixes cannot be avoided, as they are naturally present as a milk component.

Polysaccharide stabilizers are added in soft serve ice cream mixes to increase viscosity of the mix, which results in improved sensory attributes of the frozen product (Marshall et al., 2003). The stabilizers add to the overall palatability of ice cream by improving the smoothness of the body, providing uniformity to the product and also aiding in melting and handling properties (Regand and Goff, 2003). These advantageous properties are attributed to increasing the viscosity of the unfrozen phase in ice cream (Marshall et al., 2003).

Each polysaccharide provides a particular body, texture, meltdown and stability to the ice cream but rarely will there be one that performs all the desired functions. Therefore, blends of the individual polysaccharides may be used in order to achieve a balance of the desired properties with locust bean gum and guar gum being the most commonly added primary stabilizers. However, these polysaccharides are incompatible with the milk protein, particularly the casein micelles, and result in an undesirable process known as phase separation (Bourriot et al., 1999, Schorsch et al., 1999). Discussed further in section 3.4, phase separation is the creation of two visible distinct layers, one opaque containing the milk protein and the other clear containing no protein.

The incompatibility between the two required ingredients is a major problem to mix manufactures. The resultant phase separation is not only undesirable by an aesthetics stand point, which usually results in the rejection of the ice cream mix at the retail site, but also in the production of a poor final ice cream product. Therefore, phase separation must be prevented and this is achieved by the inclusion of a secondary stabilizer, κ-carrageenan (Marshall et al., 2003). The addition of κ-carrageenan to ice cream mixes results in the inhibition of phase separation and overcomes the problems associated with

the presence of the primary stabilizers in the mix. The role of κ-carrageenan in inhibiting phase separation is discussed further in section 3.5 and a closer look at this intriguing polysaccharide is discussed below in section 3.3.

3.3 Carrageenan

Carrageenan is a term given to a family of water soluble, high molecular weight, linear sulphated polysaccharides extracted from the cell walls of the red seaweed *Rhodophycea* (Yuguchi et al., 2002). It has a wide variety of uses, including both food and non-food applications as a gelling and thickening agent but is predominately used in food applications, particularly in the dairy sector for the production of milk gels and in the stabilization of milk fat emulsions (Langendorff et al., 2000).

There are three generic carrageenan families: kappa (κ), iota (ι) and lambda (λ) (Schorsch et al., 2000). They are classified based on their structure with the differences occurring in the number and location of the sulfate and 3,6 anhydrogalactose groups (van de Velde et al., 2001). Consequently, these structural differences account for the varying properties between the three- carrageenan fractions and determine its application. Since λ cannot form a gel, it is used solely for thickening purposes whereas κ and ι are gel forming and therefore used in gelling applications (Tziboula and Horne, 1999).

3.3.1 Carrageenan Structure

Kappa, iota, lambda, nu (ν) and mu (μ) carrageenan are the most common types of the carrageenan family with ν and μ-carrageenan being the biological precursors for ι

and κ, respectively. It is through manufacturing that these precursors are converted to their commercial form via alkaline treatment, which causes an internal arrangement that modifies the polysaccharide backbone (van de Velde et al., 2001). An example of this procedure is seen in figure 3.7, as κ-carrageenan is produced via the removal of a sulfate group and addition of an anhydrous bridge, from the precursor μ-carrageenan.

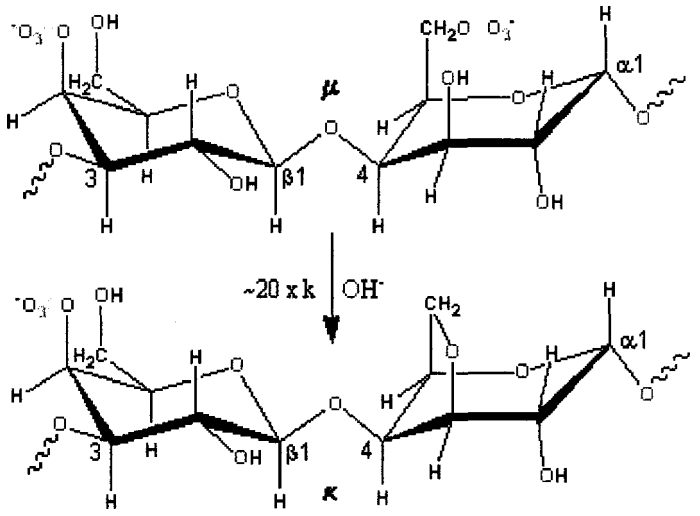
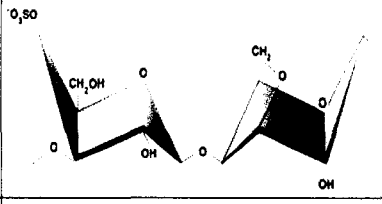
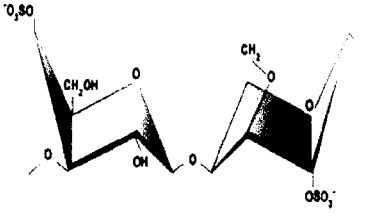
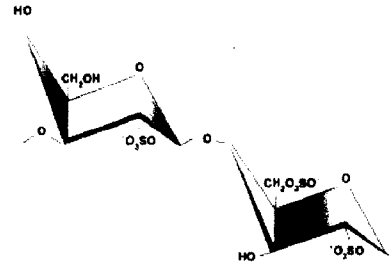


Figure 3.7: The production of κ-carrageenan via alkaline elimination from μ-carrageenan. (Chaplin, 2004)

A similar feature among the carrageenans is the backbone, which is comprised of a repeating disaccharide unit; a β-D-galactose glycosidically linked through positions 1 and 3 and α-D-galactose linked glycosidically through positions 1 and 4 (Piculell, 1995, Imeson, 2000). The most important structural features of the carrageenans, however, are the presence of sulfate groups and the presence or absence of the 3,6 anhydrous bridge. As mentioned these vary among the different fractions and these differences are summarized in the following table.

Table 3.3: Summary of the 3 major carrageenan fractions, including their sulfate and 3,6 anhydrogalactose content as well as their idealized structures. (van de Velde, 2001)

Carrageenan Type	Sulfates (%)	3,6 Anhydrogalactose (%)	Species	Idealized Repeating Unit
κ	25	34	*Kappaphycus alverezii (cottonii)*	
ι	32	30	*Euchema denticulatum (spinosum)*	
λ	35	0	*Chondrus crispis* (Irish Moss)	

3.3.2 Carrageenan Gelation

The formation of carrageenan gels occurs only with κ and ι and thus will be the focus of this section. A gel is mechanically defined as a soft material that has both solid and liquid characteristics. They are viscoelastic in nature, meaning that when a strain is applied the gel exhibits both solid and liquid like responses (Ikeda and Nishinari, 2001). Gels can be further broken down into two types of viscoelastic networks: true gels and weak gels. True gels are free standing and stable, have high binding energy between

molecular junction zones and exhibit predominately solid like responses. Weak gels have essentially zero binding energy and exhibit strong shear thinning behaviour, which makes the solid-like nature less clear (Ikeda and Nishinari, 2001). In a weak gel a three-dimensional network may exist but is easily broken with applied shear. Carrageenan gels can be either true or weak gels depending on the concentration and cation content. The minimum concentration required for κ-carrageenan to be considered a gel has been cited by Schorsch to be 0.03% (Schorsch et al., 2000).

3.3.3 Effect of temperature –Helix Formation

At elevated temperatures, roughly >50°C depending on the ionic environment, carrageenan exists in solution as random coils. Upon cooling below such a temperature there is a change in conformation in which the random coil adopts an ordered double helical conformation. This transition (termed coil-to-helix transition) is affected by temperature as well as ionic environment (Vibeke et al., 1994), but in general the temperature transition range is between 40-60°C (van de Velde, 2001). The gelation mechanism, as will be discussed in greater detail in the following section, requires the formation of double helices (Vibeke et al., 1994). Put simply, without the formation of the double helix there is no gelation.

X-ray diffraction imaging and optical rotation techniques have been used extensively in the study of double helix formation (Takemasa and Chiba, 2001). A decrease in temperature induces an ordered helical state in the random coil by the twisting of the anhydro-galactose sequence (Yuguchi et al., 2002). It is the flexible nature of the anhydrous bridge that allows for the twisting and the subsequent formation of the

double helix. Since λ-carrageenan is fully devoid of these anhydride bridges, it is unable to form a gel as their absence results in no double helix formation.

Morris et al. (1980) have suggested that the presence of certain cations may effect helix formation. The presence of these specific cations results in a higher coil-helix transition, meaning that gelation occurs at higher temperatures. The transitions from coil-to-helix and from helix to gel occur simultaneously for κ-carrageenan, which makes it very difficult to measure and accounts for the uncertainty behind the gelation mechanism and role of ions. It is, however, generally accepted that the lowering of the temperature causes the onset of helix formation and the role of the ions is to promote gelation (Morris et al., 1980).

3.3.4 Mechanism of Gelation

Once the ordered helical state is achieved junction zones can form between adjacent helices resulting in an aggregated network or a gel (Stanley, 1990). Gelation, however, can only be accomplished if there are structural irregularities or kinks along the polysaccharide molecule. In the structure of carrageenan the kink is seen in the occasional absence of the anhydride bridge in the 4-linked residue (Doyle et al., 2002) resulting in the presence of a chair conformation $^{4}C_{1}$, instead of the helix forming $^{1}C_{4}$ (Piculell, 1995). These irregularities occur in approximately 10% of the entire molecule and prevent that portion of the polymer from twisting into helical form (Morris et al., 1980). Without these kinks the aggregation of the helices would result in precipitation, as the polymers would form of a tight crystalline structure. Therefore, the kinks facilitate the

solubility of the carrageenan and allow for the creation of a network that can efficiently trap and hold water (Tombs and Harding, 1998).

The most widely accepted theoretical model for carrageenan gelation was proposed by Morris et al. (1980) and is depicted below (Figure 3.8).

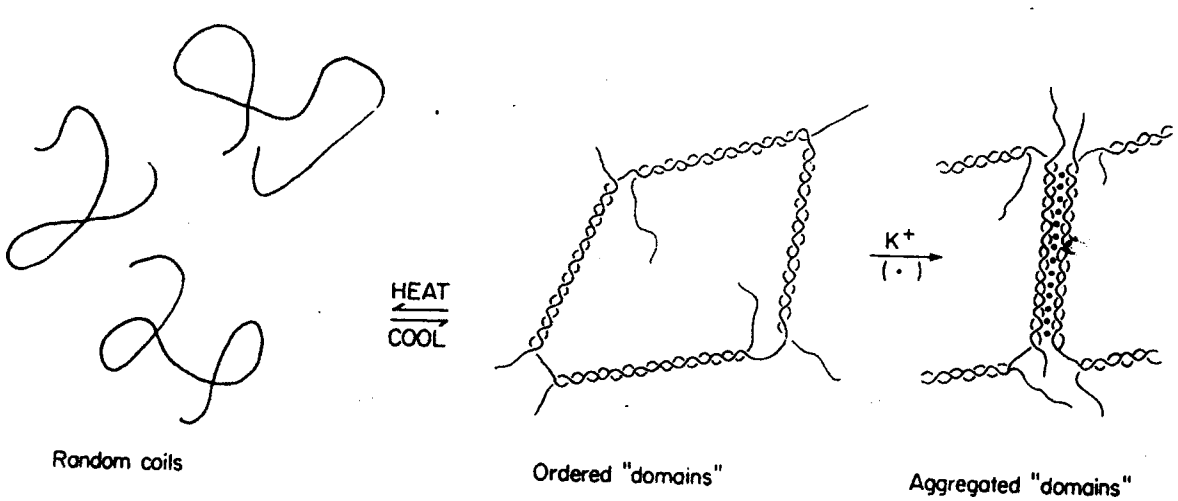


Figure 3.8: The domain model for κ - carrageenan gelation proposed by Morris et al. (1980).

The formation of a double helix via molecular association leads to the formation of small independent ordered domains (Figure 3.8b) that involve only a limited number of chains. Aggregation and cross linking of the domains leads to an aggregated matrix or a gel (Figure 3.8c) (Morris et al., 1980). The cross-linking of the double helices leads to gelation at the super helical level, whereas gelation as a result of branching and association via incomplete double helices in such a way that one polymer chain is able to join in double helices with more than one other chain is only of the helical level (van de Velde et al., 2001). The domain in figure 3.8b is an example of helical level association.

3.3.5 Effect of ions and degree of sulfation

It is well known and has been well documented that potassium ions and calcium ions play a major role in the gelation of κ and ι carrageenan, respectively (Doyle et al., 2002). ι carrageenan is less affected by ionic environment because the double helix is strengthened by hydrogen bonds formed between the O (2) and O (6) of the D-galactose residues on adjacent strands (Morris et al., 1980). κ-carrageenan however, relies on ions to stabilize the helix (Morris et al., 1980) and thus is much more ion dependent and is therefore the primary focus of this section.

The ions investigated can be divided into three categories as to their effects on κ-carrageenan gelation, the non-specific monovalent cations (Li^+ and Na^+),the divalent cations (Ca^{++} and Mg^{++}) and the specific monovalent cations (K^+, Cs^+, NH_4^+ and Rb^+) (Doyle et al., 2002) . The addition of these latter ions not only promotes gelation but also increases the strength and rigidity of the gel (Doyle et al., 2002).

Before exploring the role of ions any further a firm understanding of the role of the sulfate groups must first be examined. The sulfate esters give the carrageenan molecule an overall net negative charge, even under strongly acidic conditions, as these groups are always ionized (Bemiller and Whister, 1996). Once the temperature induced helix begins to form the charge density of the carrageenan increases as these charged groups come closer together (Langendorff et al., 2000). In κ-carrageenan the distance between neighbouring sulfate groups decreases from 1.0nm to 0.4nm upon helix formation (Langendorff et al., 2000). This decrease in distance and subsequent increase in charge density can lead to a greater association with the cations.

As seen from the domain model (Figure 3.8) ions play a major role in the aggregation of the helix. Although the exact mechanism is not fully understood it is believed that the cations affect the overall charge of the carrageenan by shielding the charged sulfate groups (Lai et al., 2000). The binding of the cations to the helix forms an ion-pair between itself and the sulfate resulting in a neutralization of the charge thus balancing the repulsive forces between polymers, which allows for further aggregation (Rochas and Rinaudo, 1980, Montero and Perez_Mateos 2002).

The reason as to why certain ions have a greater effect in promoting gelation and others not is still a major question. As seen by Morris et al. (1980), the extent of helical aggregation for ι- carrageenan is virtually independent of the nature of the cation whereas for κ-carrageenan it is not. For example, gelation of κ-carrageenan occurs in the presence of potassium but not sodium ions even though both are monovalent cations. The specificity of certain cations has been attributed to their size and the relative ease and efficiency of incorporation into the structure of the junction zones (Morris et al., 1980). Furthermore, specificity has also been attributed to the ability of the cation to reduce the helical solubility, as a decrease in the solubility of the helix would promote self-aggregation (Morris et al., 1980). Potassium ions must, therefore, be able to bind to the helix, balance the negative charge of the negative sulfate groups, incorporate into the junction zones and decrease the solubility of the helix whereas sodium ions cannot.

Anions are also known to greatly affect the gelation of κ-carrageenan. It has been well proven by Grasdalen and Smidsrod (1981) that iodide prevents the gelation of κ-carrageenan. As with most carrageenan mechanisms, it is not understood but it is hypothesized that the anion binds to the helix of κ-carrageenan and causes structural

stability, which does not favour aggregation (Grasdalen and Smidsrod, 1981). Even in the presence of equal amounts of potassium, the iodide anion has been shown to prevent gel formation. This may be as a result of the repulsion between helices caused by the overall negative charge exhibited by the complex of κ-carrageenan and iodide.

3.4 Protein – Polysaccharide Incompatibility

When creating a ternary solution, a mixture of biopolymers of differing compositions, there are generally three outcomes: incompatibility, complex coacervation or miscibility (Syrbe et al., 1998). Incompatibility results in the creation of two immiscible aqueous phases. Complex coacervation is incompatibility with the creation of two immiscible phases resulting in one phase containing both polymers and the other depleted in polymer. Miscibility is when there is a homogenous distribution of the polymers (Syrbe et al., 1998). Miscibility is exhibited in few solutions because mutual interpretation of macromolecules is an entropically unfavourable process. Therefore, incompatibility is the rule rather than the exception (Tolstoguzov, 1997).

The problem associated with soft serve ice cream mixes is related to a phase separation phenomenon that is as a result of thermodynamic incompatibility between the casein micelles and the added polysaccharide, predominantly locust bean gum (Marshall et al., 2003). It is widely accepted that the mechanism by which phase separation, the creation of two distinct layers one rich in protein and the other devoid of protein, occurs is via depletion flocculation (Syrbe et al., 1998).

Depletion flocculation is a demixing phenomenon, which tends to be exhibited by mixtures containing spherical colloidal particles (casein micelles) and a non-adsorbing

polymer (locust bean gum) (De Kruif and Holt, 2003). In solution, colloidal particles are surrounded by a solvent layer (depletion layer) that is equivalent to the radius of the particle droplet away from the surface (Walstra, 2003). This depletion layer is volume associated with the colloid and thus when a non-adsorbing polymer comes into close proximity of the depletion layer the result is exclusion of the polysaccharide because of the restriction of volume (de Kruif and Holt, 2003). The difference in osmotic pressure between the bulk solvent and the space between the colloidal particles (where the polymer was just excluded from) creates an osmotic gradient that results in the outward movement of water and the overlapping of the depletion layers of two similar colloids (Figure 3.9).

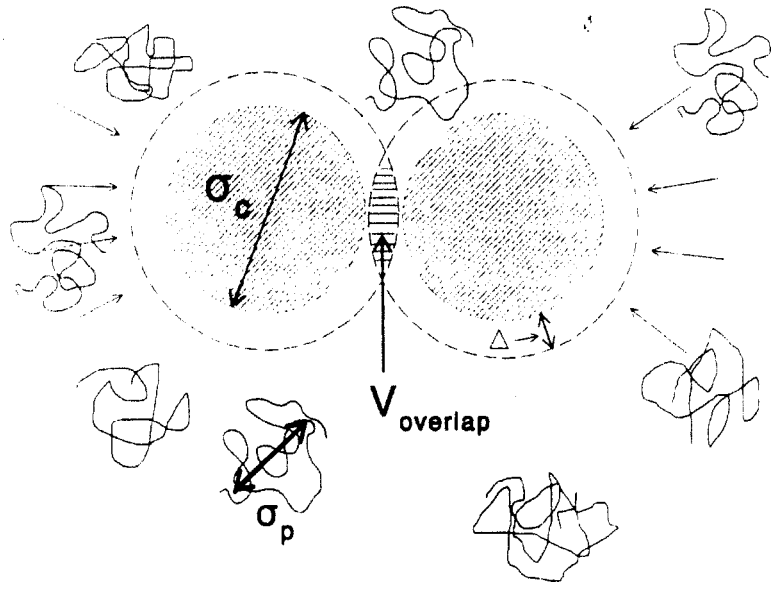


Figure 3.9: A schematic diagram of depletion flocculation (de Kruif and Holt, 2003).

The result of this process is the formation two phases, one rich in the colloidal particles and the other devoid of the colloidal particles. In the case of milk systems the formation

of two phases is evident as the white protein enriched phase is clearly divisible from the clear, yellowish serum phase.

3.5 κ-Carrageenan – Casein micelle Interaction

'Milk reactivity' is the ability of κ-carrageenan to gel in milk at concentrations much lower than any other gelling agent (de Vries, 2002). One important aspect of milk reactivity is the ability of κ-carrageenan to inhibit visual phase separation at very low concentrations, 0.015-0.02% in 4% milk protein systems (70% casein/30% whey) (Vega et al., 2004a). Although an active area of research for nearly 30 years an explanation of the mechanism of milk reactivity has remained elusive.

Historically the ability of κ-carrageenan to exhibit milk reactivity has been attributed to a specific electrostatic interaction between the negatively charged κ-carrageenan and the positive patch region on the κ-casein molecule of the surface of the casein micelle (Snoeren et al., 1975). The conclusions from Snoeren et al. (1975) were based on isolated individual caseins as they found that κ-casein was the sole casein protein that interacted with κ-carrageenan. Electron micrographs from Snoeren et al. (1976) showed an interaction between κ-casein and κ-carrageenan. The authours describe the resultant structure created by the two biopolymers as 'necklace-like' and envisioned the κ-casein incorporated as fillers within a carrageenan network.

There have been numerous works throughout the later 1970's and 80's that have increased our understanding on dairy systems containing κ-carrageenan. The research, however, focused primarily on isolated casein fractions and not entirely on systems containing casein micelles. The general conclusions were that κ-casein was the only

casein fraction to interact with κ-carrageenan in the absence of calcium, however αs_1 and β-casein were shown to interact in the presence of calcium (Snoeren et al., 1975, Skura and Nakai, 1980 & 1981). Furthermore, κ-casein was the only isolated individual casein to be found to increase the rigidity of the gel while simultaneously reducing the gel elasticity (Lynch and Mulvihill, 1994)

The most relevant research is that involving casein micelles. Hood and Allen (1977) used electron microscopy to show that aggregation of casein micelles occurred only in the presence of κ-carrageenan and not with λ-carrageenan. Dalgleish and Morris (1988) measured electrophoretic mobilities and diffusion coefficients to show that all types of carrageenan adsorb onto the micelle. They supported the electrostatic interaction theory and also proposed that the conformation of the carrageenan did not have a dominant effect on interaction since they found that the helical κ-carrageenan as well as random coil λ- carrageenan interacted with the casein micelles. Dalgleish and Morris (1988) also concluded that κ-carrageenan absorbs strongly but does not completely cover the micelles whereas ι and λ absorb to the point of complete coverage.

Recent research from Langendorff et al. (2000) using dynamic light scattering has also shown that all carrageenans, depending on their conformation, have the ability to interact with casein micelles. They showed an increase in casein micelle diameter at all temperatures with added λ-carrageenan as well as a temperature dependent interaction with κ-carrageenan. The increase in casein micelle diameter for κ-carrageenan was only visualized at temperatures below the coil-to-helix transition where the polymer is in the ordered helical conformation. The ability of κ-carrageenan to interact with the surface of the micelle while only in the helical form was attributed to the increase in charge

density as the sulfate groups are brought into closer vicinity. The distance between sulfate groups in λ-carrageenan is 0.3nm while in κ-carrageenan in coil form the distance is 1.0 nm and decreases to 0.4nm when helix formation occurs. These authours also believe that this increase in charge density is responsible for the electrostatic interaction between the two biopolymers.

At first glance the results from Langendorff et al. (2000) and Dalgleish and Morris (1988) may seem contradictory as the latter claims that conformation has no effect and the former does. However, it should be noted that Langendorff et al. are speaking in terms of the conformation of an individual carrageenan type whereas Dalgleish and Morris are concluding based on the entire carrageenan family. Therefore, both sets of authours are correct in their conclusions, but it should be clarified that the helical form of κ-carrageenan interacts with the casein micelle not the random coil form and that λ-carrageenan, which only exists in coil form, also interacts with the casein micelle.

Schorsch et al. (2000) have agreed that temperature is involved in the ability of κ-carrageenan to prevent visual phase separation of protein-polysaccharide mixed systems. Interestingly, coupled with the conformation of the polysaccharide, the concentration of κ-carrageenan also has a large effect on the stability of the system. At high carrageenan concentrations (>0.3%w/w) and at elevated temperatures such that the polymer is in the coil form there can exist depletion flocculation and thus precipitation of the micelles. Conversely, at low temperatures the onset of gelation prevents the phase separation phenomenon from becoming prevalent even though depletion flocculation is still occurring. At concentrations above 0.05% (w/w) the system gels due to carrageenan-carrageenan interactions trapping any dispersed milk protein and thus preventing their

separation. Drohan et al. (1997) using rheological techniques made the same conclusion as at high levels of carrageenan concentrations, milk proteins had little effect on gelation. Drohan et al. (1997) also point out that gel formation is a result of carrageenan-carrageenan interactions and not carrageenan-casein or casein-casein interactions, although they do not rule out the possibility of interaction between the two biopolymers.

It is at lower concentrations at which polymer-polymer interactions are not present that attracts the most interest. At low carrageenan levels (<0.03% w/w) Schorsch et al. (2000) believe that there is competition for the carrageenan to bind to either itself or to the casein micelles. They believe that instability arises from the inability of the carrageenan to connect enough small aggregates of casein micelles and that a real competition and equilibrium exists between carrageenan self association and interaction with casein micelles. Their most interesting conclusion is that coupled with the interaction between the casein micelles and the κ-carrageenan, gelation of the polysaccharide is also an important aspect of milk reactivity. Drohan et al. (1997) also concluded that at low κ-carrageenan concentrations the contribution of carrageenan-carrageenan interactions is weak and thus attribute the changes in mechanical properties of the gel to the presence of the milk proteins.

Bourriot et al. (1999) found that phase separation between κ-carrageenan and casein micelles was initiated at temperatures above the coil to helix transition and was subsequently inhibited upon cooling below the transition temperature. Bourriot et al. (1999) do not believe that interaction between casein micelles and κ-carrageenan is responsible for prevention of phase separation for steric and electrostatic reasons. Since the casein micelles are overall negatively charged and surrounded by a protruding hairy

layer (57 amino acid residues) the penetration of a large, net negatively charged κ-carrageenan polymer does seem unlikely. Thus they attribute the prevention of phase separation to the ability of carrageenan to gel and are the proponents of the weak gel theory. It is interesting to note however, that the κ-carrageenan concentration used in their experiments was high (0.1%w/w) and thus, the behaviour of the systems would be similar to that of Schorsch et al. (2000).

It is important to note that the systems tested by both Schorsch et al. (2000) and Bourriot et al. (1999) were free of any non-interacting polymer, which would induce drastic microscopic molecular differences. In these studies the depletion flocculation mechanism is induced by the carrageenan coil and is subdued by the lowering of the temperature and the subsequent formation of the helical conformation. However, in the constant presence of a non-interacting polymer the phase separation driving force is continually present, hence altering the microstructure.

There is one mechanism envisioned in the absence of a non-interacting polymer, which attempts to describe what is happening at the molecular level. Xu et al. (1992) envisioned an interaction between the casein micelles and the κ-carrageenan occurring upon cooling caused by bridging with calcium ions when the latter is in coil conformation (Figure 3.10). Furthermore, only part of the carrageenan molecule is involved with the interaction and thus the majority of the polymer is free in solution as loops and tails (Figure 3.10b). Upon further cooling below the transition temperature the free carrageenan chains form junction points and subsequently gel (Figure 3.10c).

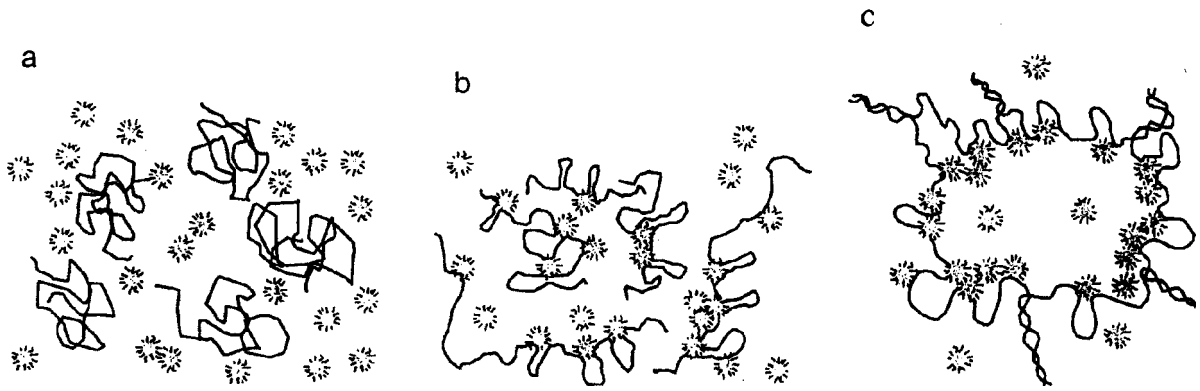


Figure 3.10: Mechanism of interaction between κ-carrageenan and casein micelles

These systems tested had an excess of carrageenan and the conclusions are consistent with most studies in that the predominant factor in carrageenan gelation is carrageenan-carrageenan interactions whereby the casein micelles act as fillers within the 3-dimensional gel.

It seems to be generally accepted that at high carrageenan concentrations the presence of casein micelles is as a filler trapped within a continuous carrageenan network. The predominant interaction is carrageenan-carrageenan with the carrageenan-casein interaction being secondary. This is in the presence of large amounts of carrageenan well above the minimum range of stability. Furthermore, these additional studies, similar to those of Bourriot et al. (1999) and Schorsch et al. (2000), did not include the addition of a second polymer that would constantly induce depletion flocculation. The studies performed by Thaiudom and Goff (2003), Vega et al. (2004a, 2004b) and Vega and Goff (2004a, 2004b) all included a second non-absorbing polymer.

Although visual macroscopic phase separation is prevented by the addition of κ-carrageenan, Thaiudom and Goff (2003) using transmission electron microscopy showed that microscopic phase separation is still evident. They described the systems as

emulsion like, with a discrete phase composed of micro domains enriched in casein micelles surrounded by a continuous phase devoid of casein micelles and attributed visual separation to sedimentation of the protein domains. Although at the time the exact location of the κ-carrageenan, either associated with the protein domain or within the continuous phase, was not known they postulated that the κ-carrageenan was behaving like a surfactant and stabilized the protein domains by interacting with the surface of the casein micelles and also providing structure to the continuous phase in such a manner that prevented the coalescence of the domains.

Vega et al. (2004a) determined that the minimum κ-carrageenan concentration required to achieve macroscopic stability was 0.015%. They also showed that stability was not merely an enhanced viscosity effect of the added κ-carrageenan, as no difference in apparent viscosities was observed at constant LBG levels for varying amounts of carrageenan. Furthermore, they confirmed that the casein micelles were responsible for driving phase separation as the addition of whey proteins did not affect the overall stability of the system.

Vega and Goff (2004a) using confocal microscopy further visualized the microscopic phase separation and added that increasing the carrageenan concentration decreased the size of the micro-domains thus furthering the belief that the carrageenan acts like as an emulsifier. In an attempt to determine which phase contained the κ-carrageenan they used rheological techniques and concluded that it was present in the protein phase. This led to the conclusion that the κ-carrageenan was indeed interacting with the micelles and in such a manner that it formed an aggregated or flocculated state with the protein so that it cannot separate from the serum phase. In essence they

concluded that the κ-carrageenan was adhering to the protein domains and forming a sort of hairy layer, which prevented coalescence and eventual sedimentation of the protein domains.

Vega et al. (2004b) further demonstrated that casein micelles are crucial to macroscopic stability of dairy systems containing polysaccharide. Replacing casein micelles with sodium caseinate resulted in no stabilizing power of the κ-carrageenan. Addition of calcium did aid in the stabilization power of the carrageenan by promoting aggregation of the caseins as well as bridging with the carrageenan. However, the research presented was further evidence that the phenomenon of 'milk reactivity' does indeed involve casein micelles and not solely casein protein.

Vega and Goff (2004b) studied the effect of low molecular weight emulsifiers on kappa carrageenan functionality and obtained interesting results. The increased concentration of emulsifiers led to an increase in the amount of non-adsorbed or free casein and resulted in a decrease in the stabilizing ability of κ-carrageenan. They concluded however that the presence of free, non-adsorbed casein was not necessarily the cause of the decreased stabilizing ability of the κ-carrageenan since they observed a reduction in serum separation with decreased adsorbed protein only in the presence of κ-carrageenan. This led them to suggest that a ratio of non-adsorbed casein micelle/carrageenan is crucial in the understanding of the functionality of κ-carrageenan.

Even with the recent findings and understanding of the behaviour of κ-carrageenan in these systems to date a working mechanism as to how κ-carrageenan inhibits visual phase separation at low (0.015-0.02%) concentrations in the presence of a non-adsorbing polymer has not yet been established. The mentioned works from our

laboratories have built a solid foundation of knowledge, which we hope to build upon and develop into a working mechanism of κ-carrageenan functionality in dairy systems containing added polysaccharide.

4.0 MATERIALS AND METHODS

4.1 Sample Preparation

Model solutions of skim milk and κ-carrageenan were formulated to emulate soft-serve ice cream mixes of composition found in table 3.2. In these model systems the casein micelle:κ-carrageenan weight ratio was the determining parameter and for a typical soft serve ice cream mix (13% msnf) containing 0.015% κ-carrageenan the ratio is 243. When it was desired to alter the κ-carrageenan content in the model solutions it was done so ensuring the proper ratio was maintained, so that the model solution emulated a soft serve ice cream mix of that κ-carrageenan composition. The microscopy experiments, however, do not follow this rule as these solutions were prepared to emulate an ice cream mix of 11% msnf. Although this is not consistent with the other experiments it does not prove detrimental to the interpretation of the results because the EM work is merely a technique used to visualize the potential interaction between the two biopolymers.

All samples were prepared by reconstituting low heat skim milk powder (purchased from Gay Lea Foods, Guelph Ontario)(containing approximately 35% protein and 5% moisture) at room temperature in deionized water. After 30 minutes of stirring κ-carrageenan, in powdered form, was slowly added and stirred for an additional 30

minutes followed by subsequent storage overnight at 4°C. The following morning 3 mL of the solutions were transferred to smaller vials and then heated to 63°C for 30 minutes to mimic pasteurization conditions. The solutions were then immediately quench cooled in ice and allowed to equilibrate back to room temperature prior to analysis.

For the experiments involving the inclusion of other polysaccharides they were solubilized similarly to the skim milk and in the cases where locust bean gum was also added it was added prior to κ-carrageenan.

The κ-carrageenan, extracted from *Kappaphycus alverezii* (formerly termed *Euchemia cottonii*), was supplied by Danisco (Braband, Denmark) and was used without further purification. NMR analysis, performed by Steve Ablett at Unilever R&D Colworth, determined that the κ-carrageenan content was 87% with the remaining 13% being composed of ι or other impurities. λ-Carrageenan, locust bean gum and guar gum were also courteously supplied by Danisco and the agarose used was purchased from Sigma Chemical Co. (St. Louis, MO, USA).

4.2 Scanning Electron Microscopy

Scanning electron microscopy (SEM) is a powerful tool used to examine topographical structures (Slayter and Slayter, 1992). The use of electrons, compared to light, allows for the observation of much smaller molecules as well as greater resolution. SEM was used to visualize any change in the structure of the casein micelle by the addition of κ-carrageenan as well as looking at the structures of the individual biopolymers.

4.2.1 SEM Theory

Electrons, emitted from a tungsten filament, are focused through a series of magnets onto the surface of the sample and as a consequence electrons from the sample, called backscattered or secondary electrons, are ejected and collected by detectors. The electrons are converted into a voltage that is then amplified using a cathode ray tube and a topographical image is created (Slayter and Slayter, 1992).

SEM is conducted under vacuum for several reasons including increasing the life of the filament and greater electron beam stability. Presence of air molecules can lead to collisions with electrons that result in poor images as well as fouling of the column. Since there is a requirement for a vacuum, samples to be imaged must not contain volatile matter and must be dehydrated (Schmidt, 1982). Therefore, biological samples must undergo a degree of fixation. The fixation procedure used in our experiments is highlighted in the following section.

4.2.1 FESEM

Field emission scanning electron microscopy is similar in theory to SEM and differs only in a few main aspects: the type of filament used and the temperature of operation. In conventional SEM the filament is heated to emit electrons whereas the FESEM emits the electrons by applying a high voltage without the use of heat. Also, the electron source for FESEM is a sharp and pointed needle, compared to the tungsten hairpin of conventional SEM, which results in a much more focused electron beam (Rochow and Tucker, 1994). These modifications have resulted in greater resolution with the FESEM.

4.3 SEM and FESEM Preparation

Two different substrates were used for the sample preparation. The purpose of the substrate is to adhere the molecules of interest to allow for sample fixation and imaging. Ideally the surface of the substrate should be smooth, so initially a glass substrate was used. The glass as well as the colloidal particles of interest are negatively charged so the glass substrate is first coated with poly-L-lysine (PLL). PLL adheres to the negative surface of the glass and because of its polycationic nature gives an overall positive charge to the substrate (Tst rsui et al., 1976). Once coated the substrate now has the ability to adhere to the negatively charged biopolymers.

A carbon substrate, in the form of a carbon planchet, was also used. The carbon is negatively charged and thus eliminates the need for poly-L-lysine. The surface of the carbon is rough, however, and does require some degree of preparation. In order to obtain a relatively smooth surface the carbon was polished with increasingly fine sand paper. When a glossy surface was obtained the polishing procedure was terminated.

4.3.1 Fixation Procedure

In order to preserve the structures of the biopolymers so that when they are dried they are representative of what they are like when hydrated in solution, physical methods of fixing are applied. The fixation procedure is carried out directly on the substrate and requires the use of primary and secondary fixatives. The initial step is placing the solution directly onto the finished substrate (either polished carbon planchet or poly L-lysine treated glass cover slip) for approximately five minutes. It was removed carefully with a Pasteur pipette and the substrate was rinsed with a phosphate buffer (pH =7). After two minutes the buffer was removed and the primary fixative, a 2% glutaraldehyde

solution prepared in Sorensen's buffer, was added. The glutaraldehyde was removed after one hour and the substrate was again rinsed with buffer. The secondary fixative, a 1% osmium tetroxide in phosphate buffer, was then added for an additional hour. After removal of the secondary fixative the substrate was washed three times with the buffer and then subjected to the dehydration process. Dehydration was carried out using a graded ethanol series: 70%, 90%, 95% and 100% with the elapsed time per solution being five minutes and repeated twice at 95% and three times at 100%. Following dehydration the samples were critically point dried using carbon dioxide and mounted onto SEM stubs with colloidal carbon. When a glass substrate was used the samples were then sputter coated with gold particles (~2nm in diameter). The finished mounted samples were stored in a desiccator at room temperature until imaging.

Glutaraldehyde (GLUT) is a dialdehyde that preserves fine structures by rapidly cross-linking proteins (Gamliel, 1985). The fixation is irreversible and widely used in the fixation of biological specimens as it is the most effective and reliable of known fixatives as well as the most common (Hayat, 2000, Glauert, 1975). It preserves structures by reacting with the amino groups as well as N-terminal amino acids and sulfhydyrl groups. The reaction of GLUT with carbohydrates is less known and much more research is needed, however, it is common practice to use GLUT to preserve the structure of glycogen, a glucose polymer (Hayat, 2000). Whether or not it is as effective in preserving polymers of galactose is not known.

The use of glutaraldehyde to preserve the structure of casein micelles is not uncommon. It was concluded by Carroll et al. (1968) that glutaraldehyde was successful in providing a reproducible method for stabilizing casein micelles for electron

microscopy. Furthermore, they concluded that much more of the micellar structure could be visualized with GLUT compared to osmium and formaldehyde, two other common fixatives used for casein micelle preservation.

Osmium tetroxide (OsO_4) is a non-polar, tetrahedral, perfectly symmetrical molecule that is most commonly used as a secondary fixative in electron microscopy (Gamliel, 1985, Hayat, 2000). It penetrates the samples slowly and importantly imparts electron density to the preserved structure (Hayat, 2000). This is imperative for imaging soft biological samples as it facilitates interaction with the electron beam of the microscope and hence visualization. Also, perhaps importantly in terms of fixing casein micelles, the non-polar nature of the fixative facilitates its penetration into charged surfaces. Generally there is no reaction of this fixative with carbohydrates (Hayat, 2000)

In the preservation of biological materials the use of glutaraldehyde as the primary fixative and osmium tetroxide as the secondary fixative is common practice. A deciding factor in the order of fixative addition is the rate of reaction, and since OsO_4 is much slower at penetrating the sample there is a requirement for more rapid fixation to preserve the desired structure (Glauert, 1975). Thus the more rapid fixative, GLUT, is used first followed by OsO_4.

Dehydration involves the removal of water from within the sample by subjecting the sample to an alcohol. Ethanol is most commonly used because it is reliable and fast acting and is the best of the dehydrants. A graduated ethanol series is used to slowly dehydrate the sample, which decreases the shock of the dramatic environmental change and ensures only minimal shrinkage occurs (Carson, 1997).

Critical point drying (CPD) is the final crucial step to the dehydration process. This process involves the final removal of the liquid, in this case 100% EtOH, from the sample. CPD relies on the principle that when the temperature and pressure of a liquid are increased there exists a point at which the liquid and gas states will be indistinguishable. At this critical point the surface tension of the liquid is zero and therefore the liquid can be converted to its gaseous state without the necessary high surface forces required for jumping a phase boundary. Therefore, CPD is a gentle process that minimizes the potentially damaging effects that phase changing could have on the sample (Schmidt, 1982). Once removed from the critical point dryer the samples are free from any liquid and are ready for imaging. In the case of glass as a substrate there is one final step before imaging can occur and that is sputter coating. Unlike carbon, glass is a non-conductive surface and therefore must be made conductive in order to produce secondary electrons (Schmidt, 1982). Therefore by coating the glass with a metal the glass substrate will then be able to facilitate imaging.

4.3.2 Imaging

The fixed samples were imaged on three different microscopes. The majority of the images were obtained from the Hitachi S4800 FESEM at their facility in Rexdale, Ontario. The Hitachi S4500 FESEM, located at laboratory services (University of Guelph), and the Hitachi S-570 SEM, located at the department of Food Science (University of Guelph), were also used to obtain images. The images were obtained using solely the upper detector and were done using different accelerating voltages. Images were acquired digitally using Quartz PCI software.

4.4 Phase Separation Experiments

Phase separation experiments involve the creation of solutions of differing composition in hopes to gain further insight into the mechanisms of milk reactivity. It is through manipulation of certain parameters as well as composition that this will be obtained. The sample preparation and solubilization of the ingredients has been outlined in section 4.1.

The first experiment involved the manipulation of temperature. With knowledge that the conformation of κ-carrageenan is governed by temperature and is in coil conformation at elevated temperatures the effect of κ-carrageenan conformation on the stability of the systems was examined. Therefore, the temperature of these experiments was constantly maintained above 60°C, well above the coil to helix transition, to ensure that the κ-carrageenan did not form any helices. Thus the ability of the coil conformation to stabilize these systems was analyzed.

Experiments that involved the replacement of κ-carrageenan with either agarose, guar gum and λ-carrageenan were done by preparing the solution by simply following the solubilization procedure and replacing κ-carrageenan with the desired polysaccharide. After the solutions were solubilized and pasteurized the solutions were stored overnight at 4°C and inspected approximately 24 hours later for phase separation.

The experiments in which glutaraldehyde treated milk was used involved more in-depth sample preparation. The skim milk powder was reconstituted by stirring in deionized water at room temperature for 30 minutes and mildly heated to ensure solubilization. One percent glutaraldehyde was added to the milk so that the final GLUT concentration of the milk was 0.04%. This was allowed to mix within the milk for

approximately one hour, while stirring, and then the milk was dialyzed against skim milk for 24 hours, changing the skim milk twice. This milk was then used to facilitate the solubilization of the κ-carrageenan but only a milk heat treatment was used since mock pasteurization temperatures resulted in a black colour of milk and curd formation. Therefore, only a mild heat treatment (40°C) for 10 minutes and prolonged stirring (1 hour) at room temperature was used.

The effect of sodium iodide on phase separation was also investigated. With knowledge that iodide has the ability to block helical aggregation and subsequently gelation, sodium iodide was used to see if the prevention of κ-carrageenan gelation would affect phase separation. The solutions were similarly prepared and 11.6 mM NaI (10 times the combined weight concentrations of calcium and potassium) was the final ingredient added to the model solution. The concentration of NaI used was determined by preparing a 0.1% κ-carrageenan solution in 10.2 mM calcium and 33.3 mM potassium with and without the addition of NaI. Without the addition of NaI the solution was a solid gel and at 11.6 mM NaI the solution was completely fluid, exhibiting no gel-like properties or visual increase in viscosity.

4.5 Dynamic Light Scattering (Photon Correlation Spectroscopy)

Light scattering techniques are widely employed to gain insight into particle size. Dynamic light scattering (DLS) or photon correlation spectroscopy (PCS) is a light scattering technique used to measure the size of small colloidal particles. It has been employed for many years in the study of how environmental factors affect the size of casein micelles (Dalgleish and Hallet, 1995). It was with the use of DLS that Walstra et

al. (1981) first determined, which was later confirmed by Holt and Dalgleish (1986), that the location of κ-casein was predominately at the surface of the micelle and that the inclusion of rennet into the system resulted in a decrease in particle size of 5nm as suggested by the former authors and 12 nm by the latter authors. In the study of the interaction between κ-carrageenan and casein micelles DLS has also been employed. Langendorff et al. (2000) also used DLS and observed an increase in casein micelle size at temperatures around the coil-helix transition concluding that this was as a result of the helical κ-carrageenan interacting with the surface of the casein micelle.

DLS has been employed in this research with the aim of visualizing any increase in casein micelle diameter by the addition of κ-carrageenan as well as λ-carrageenan, guar gum, agarose, κ-carrageenan in glutaraldehyde treated milk and κ-carrageenan in the presence of sodium iodide.

4.5.1 Theory

DLS uses a monochromatic beam of light to pass through a colloidal dispersion, which allows for the observation of time-dependent fluctuations in the scattered intensity via the use of a detector such as a photo multiplier. The fluctuations are as a result of Brownian motion and result in constructive and destructive interference of the scattered light and this gives rise to the fluctuations in intensity, captured by the detector. Upon analyzing the time dependence of the intensity fluctuation the diffusion coefficient of the particles is obtained. With this knowledge and other important parameters of the surrounding medium the diffusivity, the speed of movement of the particle, can be converted using Stokes-Einstein's equation (equation 4.1) to the hydrodynamic radius of the particle (Malvern, 1993, Dalgleish and Hallet, 1995).

$$D = KT/6\pi\eta R \qquad (4.1)$$

D = Diffusion Coefficient
K= Boltzmann's constant
R = Hydrodynamic radius
T = Temperature
η = Viscosity

(Ford, 1985)

The conversion to hydrodynamic radius using the Stokes-Einstein equation is pivotal in the use of the DLS as a measure of particle size. It must be noted, however, that the value obtained is indeed the hydrodynamic radius and not the absolute radius of the particle. Hydrodynamic radius is defined as the degree of occupancy of space or volume by the colloid and could include hydration layers as well as attached polymers or ions.

Critical to the particle size analysis of colloids in solution with DLS is their dilution. Because the diffusivity is measured as a result of changes in the scattering intensity it is important to ensure that the light is scattered only once. Therefore prior to analysis the sample solution is diluted in buffer or any other solvent that ensures the colloidal particle of measure remains stable.

Upon analysis of equation 4.1 it becomes evident that the indirect measurement of the hydrodynamic radius can be affected by the medium in which the colloid is diluted. Therefore, it is essential when using comparative techniques, for example measuring the effect of different polymer concentrations, that the difference in polymer concentration does not affect the viscosity of the solution. An increase in viscosity would result in a decrease in diffusivity that would translate into a miscalculated radius. Fortunately for these experiments the effect that the polymers have on the viscosity is negligible as the concentration range of polymers is extremely small.

4.5.2 Sample Preparation and Analysis

The model solutions were diluted in buffer directly into the cuvette just prior to analysis. The buffer used was a 5 mM $CaCl_2$ and 20mM imidazole buffer (pH 6.7) similar to that employed by Dalgleish and Morris (1988) and was filtered with a 22μm Millipore filter prior to sample addition. The samples, which were stored overnight at 4°C and then subjected to pasteurization conditions and allowed to equilibrate to room temperature, were diluted by adding 5μL to 3 ml of filtered buffer and then immediately placed into the sample chamber for measurement. Measurement was taken at 25°C and each sample was tested 3 times with the average of 7 measurements (each an average of 12 sub runs) for each run. Each solution was repeated in triplicate (different solution but same composition) for a total of 9 runs per model solution.

4.6 Micro Differential Scanning Calorimetry

Micro Differential Scanning Calorimetry (μDSC) is a thermal analysis technique that measures the energy uptake that occurs in a sample during a controlled increase in temperature (Cooper et al., 2000). The conformational transition of κ-carrageenan involves either exothermic or endothermic processes and knowledge of such transition was fundamental in the use of this technique. The effect of the presence of casein micelles on the conformation change of κ-carrageenan was studied using a DSC III micro calorimeter from Setaram in the laboratories at University College Cork, Cork, Ireland.

4.6.1 DSC Theory

μDSC measures the difference in heat capacity between two sample cells and not absolute heat capacity of the desired solution (Privalov, 1980). One cell contains the desired solution to be tested and the other is a reference cell containing a buffer or a solvent. The differences in heat energy uptake between the cells corresponds to differences in heat capacity and thus gives important insight into processes occurring within the sample.

μDSC is considered a continuous measurement, as opposed to conventional DSC measurement, meaning that the samples are continuously scanned for changes in heat capacity during heating, cooling and during an isothermal hold (Privalov and Potekhin, 1986). The differential changes are measured by area thermocouples that span and form a base where the sample cells are held. The thermocouples are connected in series and measure the differential heat flow. The results are plotted in a thermograph of heat flow versus temperature, which is a measure of the differential heat capacity. Subsequent integration of the area under the peak determines the calorimetric enthalpy (ΔH_{cal}) of the transition (Cooper et al., 2000).

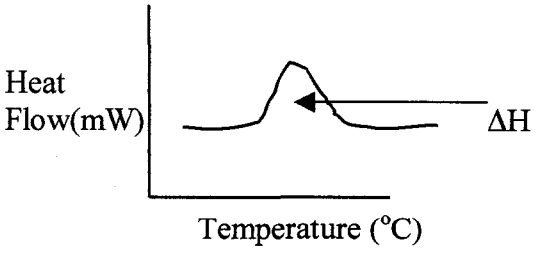


Figure 4.2: An example of the integration of a thermograph to obtain enthalpy (Cooper et al., 2000)

The sensitivity of μDSC to detect relatively small changes in heat flow is a major advantage to its use. A disadvantage is placing the sample in the cell as it is essential to ensure that the weights are similar. Furthermore, the placement of the samples into the cells is a very delicate procedure and weight inaccuracies are difficult to avoid.

4.6.2. Sample preparation and Measurement Procedure

Three solutions were effectively studied using the μDSC: a skim milk control, κ-carrageenan in the simulated skim milk ultrafiltrate (deionized water with similar molar concentration of potassium and calcium to that in milk) and mixture of the two. The κ-carrageenan control measured was a 0.030% solution instead of the usual 0.015% used in the other experiments and consequently the skim milk was adjusted to ensure the proper casein micelle/κ-carrageenan ratio was maintained. Doubling of the concentration was required because the measurements taken at the usual 0.015% concentrations were not reproducible and were sometimes missing the exothermic peak. This was attributed to the sensitivity of the machine being unable to detect such a small change in enthalpy difference between the reference sample and the sample cells and was overcome by doubling the concentration of the κ-carrageenan.

There were two different methods of sample preparation for analyzing the mixture of the two biopolymers. The first method involved solubilizing both biopolymers together and employed the same sample procedure as all other experiments. The second method involved solubilizing each biopolymer individually, similar to the preparation of the individual controls, storing separately overnight at 4°C and then mixing cold just prior to analysis. The second method ensures that the κ-carrageenan coil does not 'see' the casein micelles until it is under DSC analysis.

The samples were placed into the cells so that the difference between the cells was no greater than +/- 0.005 grams. The solutions were placed within the cells at room temperature and the reference cell for all experiments was filled with deionized water. The DSC procedure began at 25°C, heated to 63°C at a heating rate of 0.3 °C/min, held for 30 minutes and cooled to 10°C at the same ramping rate.

4.7 Reverse Phase High Performance Liquid Chromatography (HPLC)

Reverse phase high performance liquid chromatography (rpHPLC) is a separation technique based on the hydrophobicity of molecules (Rounds and Nielson, 1998). It, therefore, has the capability to separate the different individual caseins based on their differing degrees of hydrophobicity. It is known that β-casein migrates outside of the casein micelle when the temperature is lowered and it was postulated that if κ-carrageenan does adhere to the surface of the casein micelles then it might prevent or hinder the outward movement of the β-casein. A Waters 700 satellite WISP rpHPLC was used to detect the presence, not necessarily the quantity, of β-casein in the serum of centrifuged milk.

4.7.1 rpHPLC Theory

The separation of particles based on the different degree of hydrophobicity involves a non-polar stationary phase, in the case of our experiments a C-4 column, and a polar mobile phase. The sample is dissolved within the mobile phase and travels down the column interacting with the stationary phase. The nature of the molecule determines the strength of the interaction with the column and subsequently the residence time of the sample within the column. This results in the elution of the hydrophilic molecules first

followed by the more increasing hydrophobic molecules (Rounds and Nielson, 1998). It should be noted that in order to improve the degree of separation between the two similarly hydrophobic molecules a gradient is added to the mobile phase whereby the polarity is decreased. This results in greater capability of the mobile phase to hold the hydrophobic molecules at the later stages of the run and accounts for a greater degree of separation.

4.7.2 Sample and Buffer Preparation and Measurement Procedure

The sample procedure was modified by the methods used by Bordin et al. (2001). Three buffers were made for the use of rpHPLC: sample buffer, buffer A and buffer B. The sample buffer, maintained at pH 7.0, contained 6M urea (purchased from Fisher), 0.005M sodium citrate tribasic dihydrate (purchased from Sigma) and 0.03M (0.25mL in 100mL) of β-mercaptoethanol (filtered 0.22 μm). Buffer A contained 0.1%Triflouroacetic acid (TFA) (purchased from Sigma) and 10% Acetonitrile (purchased from Fisher) in Mili Q water and was filtered through a 0.22 μm filter. Buffer B contained 0.1% TFA, 90% Acetonitrile in Mili Q water and was also filtered through a 0.22 μm filter. From the composition of the buffers it is evident that buffer A is more hydrophilic while buffer B is more hydrophobic. The method gradient involved using a combination of both buffers as the mobile phase with buffer A being dominant at the start of the run followed by a slow transformation to a more predominant ratio in favour of buffer B. This allowed for the more hydrophilic molecules to elute first from the column followed by the more hydrophobic molecules.

The samples were prepared by centrifuging skim milk and skim milk containing 0.015% κ-carrageenan at both 5°C and 20°C. The pellets were discarded and the serums

were measured for the presence β-CN. Each measurement was measured in triplicate. The serums were dissolved in sample buffer and allowed to sit for one hour. The diluted samples were further diluted in buffer A 1:3 and filtered through a syringe driven Millipore filter 0.22μm and then injected into the column.

The method of detection of β-CN was at 40°C, since hydrophobic interactions are greater at elevated temperatures, and a detector at wavelength 214nm. The flow rate was 0.3 ml/min and the time per sample was approximately 2 hours. The column used was a Grace Vydac C-4 column, bead particle size 5 μm and a bead pore diameter of 300 Angstrom.

5.0 RESULTS AND DISCUSSION

5.1 Microscopy

Images obtained from SEM and FESEM are presented in Appendix I. The images obtained using the Hitachi S4800, when the sample was prepared on carbon substrates, did not require sputter coating, which allowed for more detailed surface features and avoided the possibility of artefacts caused by the application of a thin layer of metal. The switch to the carbon substrate coupled with the high-resolution power of the Hitachi S4800 was therefore advantageous and allowed for the acquisition of high quality images that where previously unattainable with older microscopes. The early microscopy results, with sample prepared on glass substrates, as well as images obtained using the Hitachi S570 are still presented and all images are found in Appendix I.

5.1.1 Casein Micelle Images

Images of the skim milk control show detailed structure of the casein micelle. Figures A-1 to A-3 are all images obtained using the S4800, prepared on carbon substrates. Images obtained with the other microscopes were unable to attain a resolution high enough to visualize any distinct surface structure. Analysis of these images shows detailed surface features that allows for some interpretation of the surface structure of this intriguing colloidal particle. The images show the casein micelles to be spherical with a diameter of roughly 250nm. The surface of the micelle, although no individual caseins (i.e. κ-casein) are visible or distinguishable, appears to be rather porous and comprised of cylindrical or tubular structures that protrude approximately 20nm from the surface.

In figure A-1 the casein micelle seems to be surrounded by smaller micelles roughly 75 nm in diameter. It is believed that these are smaller micelles that have been associated with the larger micelle most likely due to the nature of interaction with the substrate. Since the carbon substrate is hydrophobic it does not wet easily, evident from the behaviour of the solution when it is applied. The casein micelles, which are partly hydrophobic especially the interior, are known to interact with hydrophobic materials and will therefore tend to clump together upon addition to the substrate. This may account for the presence of the smaller surrounding micelles.

Along with the aforementioned hydrophobic interaction there is another force acting on the casein micelles and that is an electrostatic interaction of the negative κ-casein with the positive substrate. The combination of these forces could result in a pulling apart of the casein micelles and causing what would appear to be distorted micelles. This is evident from figure A-11, which shows an intact micelle (on the left)

and a flattened micelle with still some remaining distinguishable surface features (on the right). It must first be noted that figure A-11 is not solely of a skim milk solution but a solution also containing κ-carrageenan and is used in this section merely to illustrate the nature of the acting forces. The presence of these forces is important in that they could potentially alter the structure of the micelle. However, it seems like the imperfectly flat surface of the carbon as well as the presence of the smaller micelles seems to reduce the extent of the distortion. This is evident in figure A-1, in which the casein micelle seems to be unaffected by the external forces and appears to be a well-rounded unaltered particle, especially compared to the flattened micelle in figure A-11. Since the surface of the carbon substrate is not perfectly flat there exists regions in which there is certain elevation, which may be another advantage to using the carbon substrate. Attachment of the casein micelle to only the edge of a fragmented piece of substrate, which in 3-dimensional space at the nanometer scale is elevated from the bulk of the surface, would reduce the amount of external force. Also, the presence of the smaller micelles would inevitably act similarly and shield the larger micelle from the bulk of the external forces and therefore have minimal effect on is structure.

The micrographs presented in Appendix I suggest a great deal about the structure of the casein micelles. The surface features, which appear to be tubular, may suggest that this tubular structure is continuous throughout the micelle leading to a belief that the casein micelles are bi-continuous networks of the individual casein proteins. The exterior tubular nature also suggests that the casein micelles are not composed of sub-micelles. The classical sub-micelle model depicts the surface as 'raspberry' like and upon close examination of the images in Appendix I, most specifically figure A-3, the surface is

indeed tubular. The possible exclusion of the sub-micellar model does not necessarily indicate the acceptance of the nano-cluster model as acceptance or rejection of the theoretical models, or postulations of interior, is speculative as the conclusions are based solely on images.

The nature of the interior is truly indiscernible from these images and so conclusions are only speculative, however, the clarity and high resolution of these images can lead to some more in-depth conclusions about the surface structure. The surface appears to be porous, which indicates that the surface contains, as suggested by literature, bare regions. Evident from figure A-3 there seems to be regions where the protuberances, which may indeed constitute the hairy layer, results in areas, approximately 28-30nm in length, devoid of protein and thus areas where the interior may be accessible. If this is the nature of the surface then it may indeed be able to facilitate an interaction with κ-carrageenan.

5.1.2 κ-Carrageenan Images

Micrographs A-4 to A-6 show images of κ-carrageenan and show the polymer to be a long, thin stranded molecule roughly 20 nm in diameter. This corroborates well with literature that found similar structures of κ-carrageenan (Snoeren et al., 1976). The κ-carrageenan molecule, most apparent in figure A-4, also appears to be helical as the long polymer strand seems to be twisted, as evident from the charging, which shows areas of higher charge density, on the surface of the polymer. The degree of fixation on the polysaccharide is not well known or understood as both the primary and secondary fixatives are not specific for carbohydrates. The fixation procedure was specified more to

maintaining the structure of the more environmentally sensitive casein micelles. Furthermore, κ-carrageenan undergoes a degree of processing since it is usually, and in our case, used in the powdered form. The robust ability of the polysaccharide to survive the heat and dehydration treatment would lead to the belief that it can survive the dehydration and fixation treatment as well. The appearance of the κ-carrageenan in the micrographs does corroborate well with literature and with what was expected and therefore no artefacts are believed to be present in these images.

5.1.3 Casein Micelle - κ-Carrageenan mixtures

The micrographs of most interest are those presented in figures A-7 to A-10, which contain both casein micelles and κ-carrageenan. It appears that the κ-carrageenan is attached to the surface of the casein micelle and in some cases associated with groups of micelles and provides direct evidence that the κ-carrageenan interacts with the surface of the micelle.

Early microscopy of casein micelle - κ-carrageenan solutions by Snoeren et al. (1976) observed 'necklace-like' structures in which the casein micelles associated within a κ-carrageenan network. We have observed similar structures evident in figures A-8 and A-9. The lower resolution figure A-8, from the Hitachi S570, is similar to that observed by Snoeren, however, with the use of the high resolution S4800 further examination into the 'necklace-like' structure has determined that the 'beads' are indeed casein micelles. Whether or not one κ-carrageenan molecule is adhering to numerous casein micelles in series is not known, although these images do suggest that the micelles are involved in a κ-carrageenan network.

The most striking images are figures A-7 and A-11, which are high magnification images that clearly show κ-carrageenan interacting with the surface of the casein micelle. These images show the κ-carrageenan strand coming off the surface of the micelle. Also, it appears as if there is a group of micelles associated with the interacting micelle. It is not clear if the carrageenan is responsible for bringing together this group of micelles but it does appear that there is a clustering of casein micelles associated with the interacting biopolymer.

The sample preparation and fixation procedure requires the cross-linking of protein via glutaraldehyde treatment. This may result in bridging between micelles, which could result in the formation of strands between neighbouring micelles caused by the fixation. However, this potentially detrimental artefact was ruled out by dynamic light scattering studies, which show that the casein micelle diameter does not change with the addition of the primary and secondary fixatives (Figure 5.1).

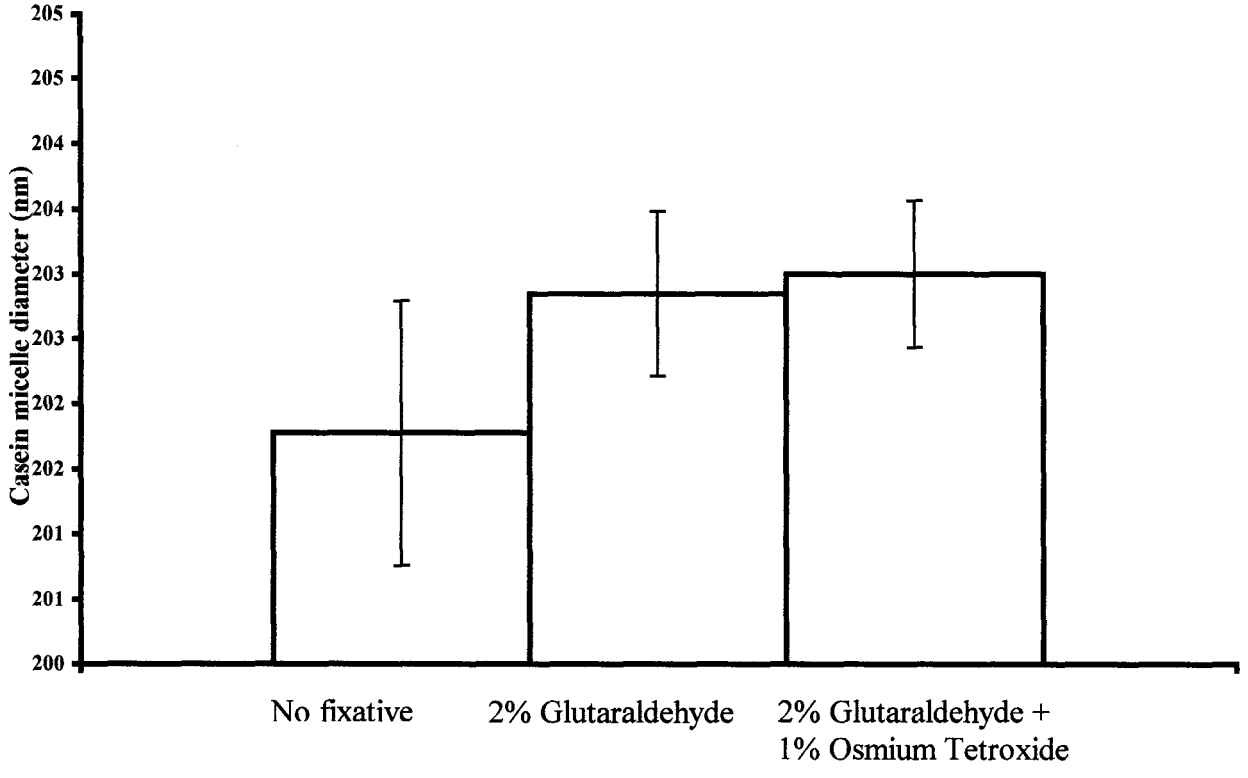


Figure 5.1: Effect of fixatives on casein micelle size as investigated by dynamic light scattering.

It is evident from figure 5.1 that the fixatives used do not affect the size of the micelle in solution, which has excluded the possibility that the fixative is responsible for the presence of these strands on the surface of the casein micelle. Therefore, it can be concluded that the fixative does not cross-link casein micelles but rather the casein protein within the micelle, which subsequently allows us to attribute the strands on the surface of the casein micelle to κ-carrageenan.

The micrographs show the κ-carrageenan absorbing onto the surface of the micelle and therefore can provide useful insight into a potential mechanism as to how this polymer can prevent phase separation in ice cream mix systems. When interpreting these images however it must be recalled that these are model solutions without the presence of a non-adsorbing, depletion flocculation promoting polymer. Furthermore, the isolation of

the two biopolymers onto a hydrophobic substrate has removed the biopolymers from their natural environment and has thus subjected them to uncharacteristic external forces. The purpose of the fixation procedure is to minimize these effects but it must not be ruled out that the initial placement of the sample onto the substrate may have somewhat altered the nature of the sample. With that said however it is clear, and consistent from sample to sample, that there is an interaction between the two biopolymers with the κ-carrageenan adhering to the surface of the casein micelle.

These findings however, in terms of relating it to a mechanism of κ-carrageenan ability to prevent phase separation, are solely that interaction occurs and the nature of the molecular mechanism (i.e. electrostatic interaction involving κ-casein) or the molecular orientation of the κ-carrageenan (i.e. that the κ-carrageenan bridges together neighbouring micelles or if it creates a second more stable hairy layer) is not discernable by this technique. Although some of the micrographs may suggest detail into the molecular orientation of the κ-carrageenan it must be remembered that these indeed are model solutions and that microscopy is a visual technique that leaves much to interpretation.

5.2 Phase Separation Experiments

Phase separation experiments with systems containing skim milk and locust bean gum and a third varied polysaccharide led to very interesting findings. The results from these experiments are succinctly outlined below in table 5.1.

Table 5.1: A summary of the phase separation results involving skim milk, locust bean gum and a varied third polysaccharide. Note: All phase experiments were performed at 4°C unless otherwise noted.

Variable	Separation
κ-Carrageenan	NO
κ-Carrageenan at 60°C	YES
κ-Carrageenan with added sodium iodide	YES
λ-Carrageenan	YES
Agarose	YES
Guar Gum	YES
κ-Carrageenan in glutaraldehyde treated milk	YES

In the presence of κ-carrageenan (0.015%) no phase separation was observed at 4°C, however, at 60°C, above the coil-to-helix transition, phase separation was evident indicating that the helix conformation is necessary for prevention of phase separation. These results corroborate well with literature that suggests a unique feature associated with the helical form of κ-carrageenan. This indeed could be the increase in charge density that occurs when the coil conformation twists into the helix and decreases the distance between sulfate groups. These results could also suggest, however, that gelation is required to stabilize the systems since the helical conformation is necessary to facilitate gel formation. Therefore, the only conclusion drawn from this experiment is that κ-carrageenan must be in the helical form in order to prevent phase separation.

When sodium iodide was added to these systems, which blocks κ-carrageenan gelation by preventing helical aggregation, phase separation was observed at 4°C. This would suggest that a gelation mechanism is necessary to inhibit visual macroscopic phase separation. There is however evidence that there is some dissociation of the casein micelle in the presence of such large amounts of sodium iodide. Electrophoresis results indicate the presence of some additional casein proteins in the serum of centrifuged milk

that contains equivalent iodide concentration used in the experiments compared to minimal casein proteins in milk without added iodide. The presence of free casein may also have an effect on the stability of the system as it was shown by Vega et al. (2004b) that κ-carrageenan has no stabilizing power in the presence of caseinate. Therefore, at first glance it could be stated that the appearance of the caseinate to the system would cause a driving force for, and eventually cause, instability. The work by Vega et al. (2004b), however, compared systems containing entirely casein micelles to systems containing entirely sodium caseinate. They concluded that casein micelles were required for prevention of phase separation by κ-carrageenan and that replacing them entirely with sodium caseinate resulted in instability. Therefore, the presence of the caseinate does not result in an increased driving force for instability merely that κ-carrageenan cannot prevent the caseinates from flocculating and phase separating. The inability of κ-carrageenan to prevent phase separation is most likely due to the structural difference between the caseinate and casein micelles. Even though interaction between the individual caseins and κ-carrageenan is known and well documented, the packing of large amounts of these proteins in a micellar structure, which in turn interacts, is what accounts for the stability. In relation to the systems tested in these experiments it is clear from figure 5.2 in the following section that the size of the micelle is not altered by the presence of sodium iodide. Therefore, even though some dissociated casein proteins are present the fact that intact casein micelles remain strengthens the belief that the presence of free casein was not responsible for promoting phase separation and that separation is indeed induced by the sodium iodide preventing helical aggregation.

A parallel experiment involved the replacement of κ-carrageenan with λ-carrageenan to see what effect the non-gelling, non-helical forming carrageenan would have on phase separation. λ-Carrageenan was unable to inhibit phase separation and therefore corroborates the findings of the NaI experiment, as both of these experiments suggest that a gelation mechanism is required for inhibition of phase separation. It is, however, believed that the gelation mechanism involved is not the formation of a conventional three-dimensional polymer gel as the concentration of κ-carrageenan required to stabilize casein micelle-locust bean gum systems from phase separating is extremely low, well below the critical gelling concentration (0.03%; Schorsch et al., 2000). Furthermore, earlier rheological results from our laboratory confirmed the absence of measurable weak gels as requirements for stability in similar systems (Thaiudom & Goff, 2003; Vega et al., 2004a). Therefore we believe the gelation mechanism to not necessarily involve the creation of a conventional gel but rather association of helices, which is a precursor for gelation, in order to prevent macroscopic phase separation.

Since it appears that the stabilizing mechanism is κ-carrageenan helical aggregation, we wanted to determine whether casein micelle adsorption is even required to inhibit casein micelle-locust bean gum phase separation. This is what prompted the replacement of κ-carrageenan with agarose, since both polymers are structurally similar and exhibit similar gelation mechanisms via helix formation and subsequent helical association. Therefore it would be logical to expect that agarose could inhibit phase separation if indeed the helical aggregation was solely responsible for inhibition of phase separation. This was not the case as, when agarose was substituted for κ-carrageenan at

concentrations up to 0.05%, just below its gelling concentration, phase separation was evident. This would suggest that the gelation mechanism solely is not sufficient for prevention of separation and argues that interaction between the two biopolymers is necessary. Furthermore, it is evidence against the theory by Bourriot et al. (1999) that a weak gel is entirely responsible for the inhibition of phase separation.

Guar gum was also used to replace κ-carrageenan for the purpose of ensuring that the stabilizing power of κ-carrageenan was not solely a viscosity effect. The result was, as expected, phase separation and confirms that the ability of κ-carrageenan to prevent phase separation is not a viscosity effect and that this polysaccharide is indeed unique in its inclusion to milk.

Skim milk was treated with glutaraldehyde in hopes of locking the structure of the casein micelle. The belief that interaction occurs with κ-carrageenan at the surface of the casein micelle has long been disputed since the latter is sterically stabilized by the protruding hairy layer, the argument that led to the weak gel theory. However, as has been discussed throughout this work, the nature of the surface could possibly facilitate the interaction since the hairy layer is flexible and the surface contains bare regions. Therefore, decreasing the flexibility of the structure and removing one of the favourable possibilities for interaction was the motive behind using glutaraldehyde treated milk. The results were interesting as phase separation still occurred although it was less evident, not as a distinct barrier between the two phases, and with much slower kinetics compared to the other solutions. The other experiments showed visual phase separation after only a few hours whereas phase separation in these systems occurred after a few days. Furthermore, the visual barrier between the phases was less distinct and very minimal

separation occurred with only a thin clear layer visible. These results indicate that the hairy layer must be flexible in order for κ-carrageenan to completely stabilize these systems long term. Interestingly however the kinetics of separation were much slower and occurred over a few days implying that even though the flexible nature of the casein micelle was prevented or decreased by the presence of glutaraldehyde the κ-carrageenan molecule was still able to initially interact with the surface. This may seem contradictory to the requirement of a flexible hairy layer and poses an obvious question: does the hairy layer need to be flexible in order to facilitate κ-carrageenan interaction with the casein micelle surface? The answer to this question is no and is addressed in the following DLS section.

From the phase separation results a few important conclusions can be drawn. Firstly, that the κ-carrageenan helix is involved in preventing phase separation. Secondly, that the addition of polymers that, under whatever circumstances, cannot form helices or aggregates of helices results in phase separation implying helical aggregation is important to prevent phase separation. Thirdly, the addition of agarose, a polymer similar in structure and gelation mechanism to κ-carrageenan, resulted in phase separation further indicating that the stabilizing ability of κ-carrageenan is much more complex than simply a helical aggregation. Finally, that the flexible nature of the hairy layer also plays an important role in the overall stabilizing power of κ-carrageenan.

5.3 Dynamic Light Scattering

Dynamic light scattering results indicate that there is a direct linear increase (R^2=0.9779) in casein micelle diameter as the concentration of κ-carrageenan increases within the range of 0-0.03% (Figure 5.2).

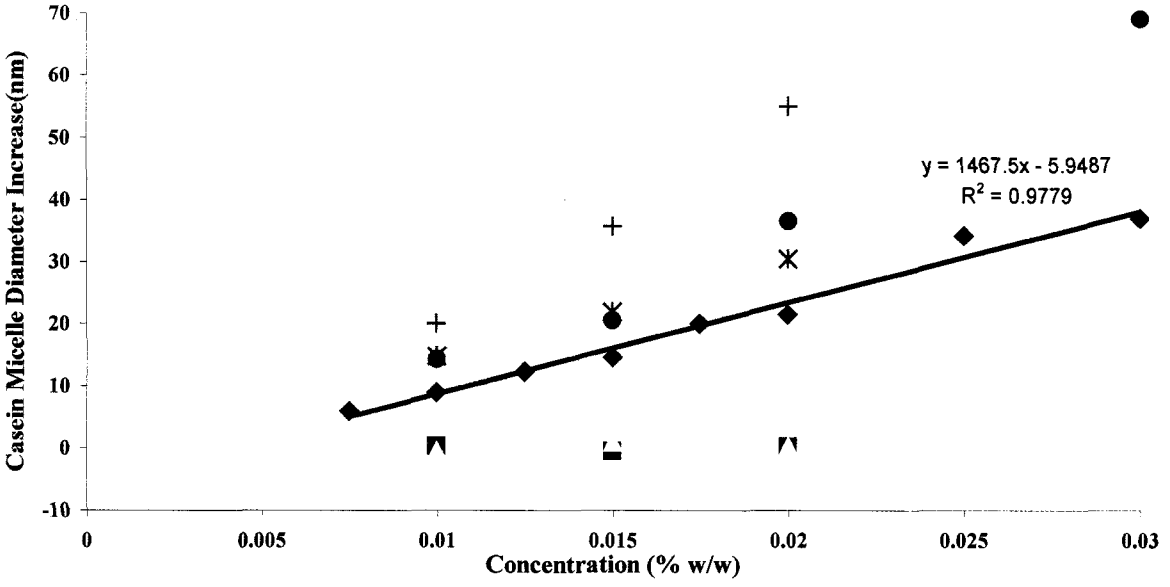


Figure 5.2: The effect of polymer concentration on casein micelle diameter as measured by dynamic light scattering. ◆ κ-carrageenan, ● κ-carrageenan with NaI, ■ Guar Gum, agarose, + λ-carrageenan, ✱ κ-carrageenan in glutaraldehyde treated skim milk

*Please recall that the analysis was done on model solutions and that the x-axis corresponds to the co-ordinating polymer concentration found in an ice cream mix with the proper adjustments being made to ensure the proper casein/carrageenan ratio has been maintained.

The values in this figure have been normalized, meaning that the diameter at the given κ-carrageenan concentration was subtracted by the diameter at zero concentration. This allows for the effect that κ-carrageenan has on the increase in casein micelle size to be

determined and also allows for comparisons to be made between subsequent experiments. (For individual non-normalized graphs see Appendix II).

The increase in casein micelle diameter is attributed to adsorption of the κ-carrageenan polymer to the surface of the casein micelle. The temperature of measurement was 25°C, below the coil to helix transition temperature, indicating that the κ-carrageenan is in the helical conformation when it interacts with the casein micelle. Furthermore, the low concentration range (0-0.03% w/w), is indicative of the specificity and sensitivity of the micelles to the presence of κ-carrageenan and the highly linear correlation implies a direct relationship between the amount of κ-carrageenan added and absorption (or interaction) of the polymer to the casein micelles.

κ-Carrageenan in the presence of sodium iodide also resulted in an increase in casein micelle diameter. The concern has already been mentioned over the affect of large amounts of NaI on the system. The diameter, however, of the micelles in the control samples (added NaI with no κ-carrageenan, Figure A-13) did not change. Furthermore, the ability of κ-carrageenan to increase the casein micelle size was not affected and implies that the surface of the casein micelle has not been significantly altered with the addition of the iodide. It would be assumed that if the micellar surface was affected by the presence of the iodide that interaction between the two biopolymers would also be effected but this was not the case. The electrophoretic experiments that indicated some degree of dissociation of the micelles, was done so in the absence of κ-carrageenan. It may be possible that a significant portion of the iodide could bind to the κ-carrageenan, as the nature of iodide in preventing helical aggregation is by interaction with κ-

carrageenan, and thus would not be free to interact with the casein micelles and unable to cause dissociation.

The degree of increase (linear regression slope) in the presence of NaI is greater than that in the absence of NaI (for slopes and r^2 values see Figures A-12 and A-13). The iodide in solution, which binds to the κ-carrageenan polymer, would increase its overall net negative charge. If the interaction between the biopolymers is indeed electrostatic as suggested by Snoeren et al., (1975) then the increase in charge density that results from the increased presence of iodide on the κ-carrageenan helix would result in a greater attraction to the positive region on the micellar surface. Thus, the greater rate of increase is attributed to a greater negative charge on the κ-carrageenan helix.

The casein micelle diameter was also shown to increase in systems containing λ-carrageenan. λ-Carrageenan clearly increases the size of the casein micelle diameter and interestingly enough more so than any other polymer analyzed (Figure 5.2 and Figure A-14). This may be attributed to a higher radius of gyration of the λ-carrageenan. The interaction of κ-carrageenan occurs in the helical conformation whereas the interaction involving λ-carrageenan occurs in the coil conformation. The more disordered coil conformation would have a higher radius of gyration and greater flexibility, allowing it to orient itself more easily for interaction, which could therefore account for the greater increase in casein micelle diameter. Furthermore, the charge density of λ-carrageenan is higher than that of the κ-carrageenan helix. The distance between the sulfate groups on a λ-carrageenan are 0.3 nm compared to 0.4nm on κ-carrageenan (Langendorff et al., 2000). This slight increase in charge density may lead to a higher affinity for λ-carrageenan on the surface compared to κ-carrageenan.

The λ-carrageenan results coupled with those from the κ-carrageenan in the presence of sodium iodide leads to interesting conclusions. As seen in section 5.2 both systems are unable to prevent visual macroscopic phase separation, however, both results indicate an increase in casein micelle diameter. This is striking evidence that suggests that although interaction between the two biopolymers occurs it is not sufficient to prevent phase separation. This further supports the belief that another mechanism discussed as helical aggregation is also necessary to inhibit phase separation.

In the presence of agarose no increase in casein micelle diameter was observed (Figure 5.2, Figure A-16) indicating no adsorption of this biopolymer onto the casein micelle surface. Since it appears that the stabilizing mechanism involves κ-carrageenan helical aggregation, we wanted to determine whether casein micelle adsorption is even required to inhibit casein micelle-locust bean gum phase separation. The DLS shows that there was no interaction between these two similarly constructed and gelling polymers. This is important evidence as it implies that helix aggregation alone is insufficient to inhibit phase separation and argues that interaction between the biopolymers is also necessary to stabilize the system from phase separating.

As was seen in section 5.2, agarose was unable to prevent separation and, as evident in this section, did not interact with the casein micelles. Since the main difference in structures between agarose and κ-carrageenan is the absence of sulfate groups in the former, this suggests that the presence of the sulfate groups on the κ-carrageenan are likely involved in the interaction with the casein micelles. Whether or not this implies electrostatic interaction is not possible to conclude with this technique but it

is viable to conclude that the interaction between the κ-carrageenan and the casein micelle surface does indeed involve the sulfate groups of the κ-carrageenan.

Since the DLS works on the principle of measuring the diffusivity of the colloid, increasing the concentration of polymer may affect the viscosity of the sample and could affect the colloids diffusivity. To ensure that the results obtained by the DLS were simply not as a result of a change in viscosity, guar gum was used to replace κ-carrageenan. No increase in casein micelle diameter was observed with the addition of guar gum (Figure 5.2, Figure A-17) and thus, as expected, the increase in casein micelle diameter seen by added biopolymer is not a viscosity effect and is attributed to the specific nature of the polysaccharide and its interaction with the casein micelles.

κ-Carrageenan in glutaraldehyde treated milk was also studied and shows that the casein micelle diameter increases with the increase in κ-carrageenan concentration. Interestingly, the increase is quite comparative to the other polymers. This is unexpected, as it would be believed that decreasing the flexible nature, most likely by affecting the hairy layer, of the surface would hinder the penetration of the κ-carrageenan polymer. However, the surface of the casein micelle does contain bare regions, evident from the FESEM results and the literature also suggests that the hairy layer does only cover approximately one-third of the surface (Dalgleish, 1998). Also, the action of the glutaraldehyde is not conclusive to include complete locking of the hairy layer, although it is assumed that it does decrease in flexibility. Therefore, the results imply that the hairy layer does not need to be entirely flexible in order to facilitate the interaction between the two biopolymers and that there seems to exist sufficiently enough of a bare region on the surface that would still facilitate the interaction between the two biopolymers.

5.4 Micro Differential Scanning Calorimetry (μDSC)

A thermograph is shown in figure 5.3 for a 0.036% κ-carrageenan solution in 408ppm calcium and 1334ppm potassium.

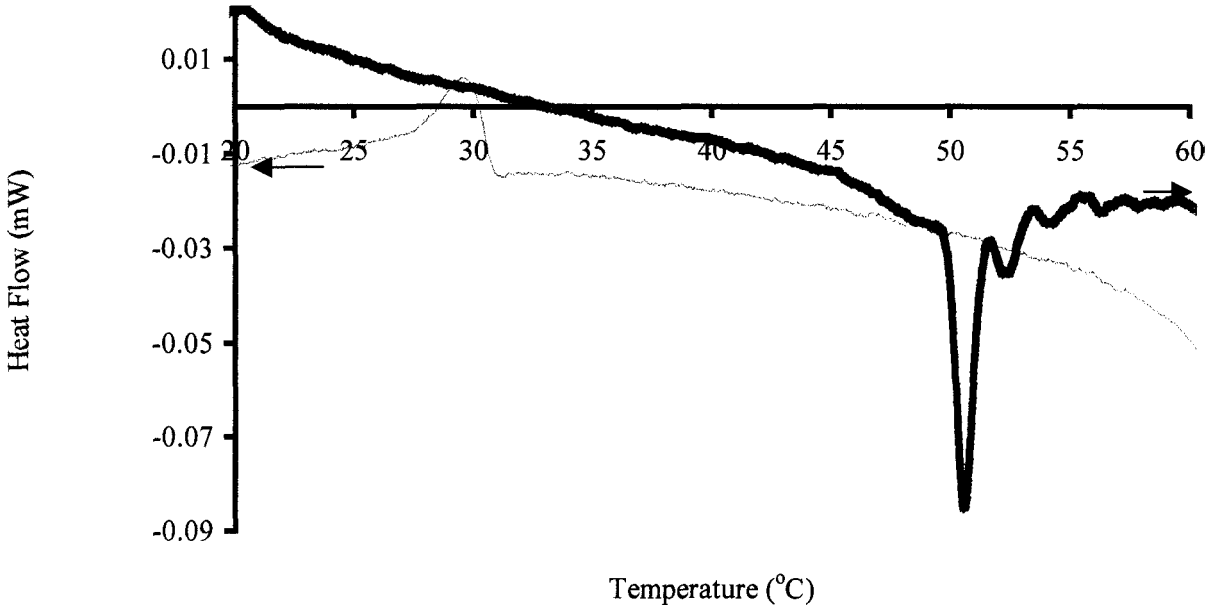


Figure 5.3: A thermograph of a 0.036% κ-carrageenan solution containing 408 ppm calcium and 1334ppm potassium.

The thermograph clearly shows both the helix to coil and coil to helix transition that occurs when a κ-carrageenan solution is heated and cooled, respectively. Figure 5.3 is a representative thermograph of many taken over a 6-month period and the enthalpies and peak temperatures are averages taken from several integrated thermographs. Upon heating the κ-carrageenan undergoes an endothermic reaction, visible in figure 5.3 by the thick line, in which the average calculated enthalpy was +0.0061 J/g +/- 0.0023 J/g. The peak temperature at which the transition occurs from a helix to a coil was recorded at 49.39°C +/- 1.23°C. On cooling the κ-carrageenan undergoes an exothermic reaction,

visible in figure 5.3 by the thin line, and has an average calculated enthalpy of -0.0075 J/g +/- 0.0023J/g. The peak temperature at which the transition occurs from a coil to a helix was 30.67°C +/- 2.93°C. These enthalpies correlate well with literature results presented by Doyle et al. (2002).

The skim milk control, as expected, showed no such transitions and the results were simply straight lines upon heating and cooling. The results from when the biopolymers were solubilized together, meaning that the casein micelle 'saw' the κ-carrageenan coil are presented below in figure 5.4.

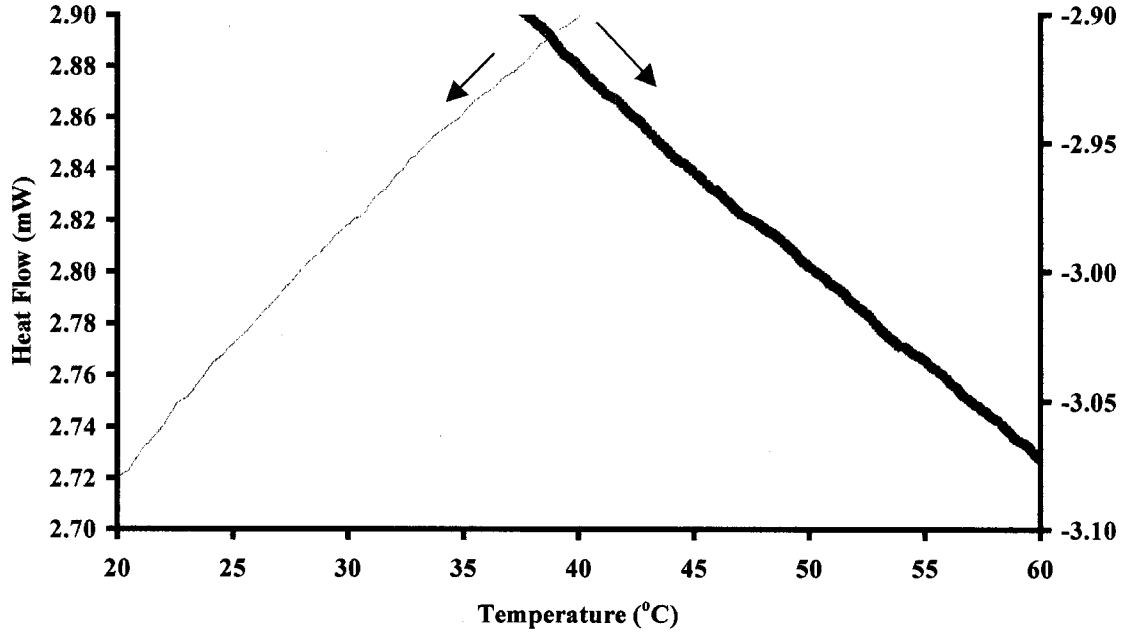


Figure 5.4: A thermograph of a skim milk and κ-carrageenan solution in which both biopolymers were solubilized together, stored overnight at 4°C and placed it the DSC chamber at 25°C.

The thermograph, which has been isolated to highlight the peak transition temperature region, shows that there is no evident transition, in either direction, when both biopolymers are added together. This is an interesting result in that neither transition was visible. Before drawing any conclusions a look at thermal behaviour when each polymer was individually solubilized is required (Figure 5.5).

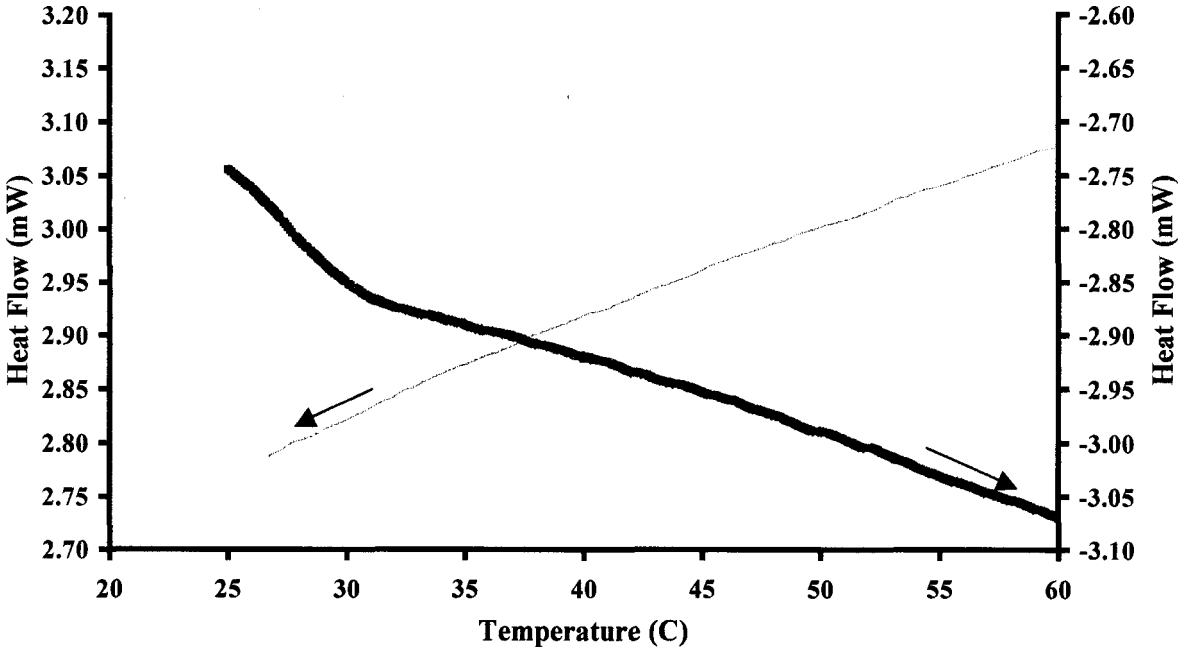


Figure 5.5: A thermograph of a skim milk and κ-carrageenan solution in which both biopolymers were solubilized individually, stored individually overnight at 4°C, mixed cold and placed in the DSC chamber at 10°C.

These results are similar to those presented in 5.4 where no visible transition is seen throughout the transition range.

The μDSC results indicate that there is an interaction between the casein micelle and the κ-carrageenan. The lack of transition also implies that the interaction is occurring

in such a manner that prevents the κ-carrageenan from twisting between conformations. These results further indicate that the conformation required for interaction is the ordered helical κ-carrageenan since no transition is evident upon heating when each biopolymer is solubilized individually. During this procedure, solubilizing each biopolymer individually and mixing together cold, the κ-carrageenan coil does not 'see' the casein micelles because at refrigeration temperatures the carrageenan is in the ordered helical conformation. Since there is no visible transition upon heating it is assumed that interaction between the two has already occurred and that this interaction is preventing it from adopting the coil conformation. It would be expected that if the coil was indeed the conformation upon which interaction occurred then a transition should have been seen upon heating as the interaction would not have yet happened, meaning that the κ-carrageenan would have been free in solution and able to undergo the transition from a helix to a coil. However, the absence of a transition does not necessarily indicate that all κ-carrageenan is bound. It is possible that interaction with the casein micelles is occurring but only with portions of the κ-carrageenan and is thus preventing only portions of the polysaccharide from twisting between conformations. Only small amounts of conformational changes would result in only minimal changes in heat flow, which may not be detectable by the μDSC and thus translate into no visible transitions.

To prove this theory, addition of κ-carrageenan to skim milk at temperatures above the coil to helix (>49.39°C) would have been performed to determine if a coil to helix transition was initially visible upon cooling followed by no transition upon heating. Unfortunately it was not mechanically possible to perform such experiments since

opening the instrument at the required elevated temperature is unsafe to the workings of the equipment.

The results corroborate the requirement for the helix and may also indicate that the sulfate groups are involved in the interaction. It has been established that the helix is required to interact with the casein micelles. The κ-carrageenan must therefore adopt a helical conformation in order to interact with the casein micelles and this seems likely attributed to the increase in charge density associated with the helical conformation. So if the increase in charge density between neighbouring sulfates is involved in interacting with the casein micelles then it is conceivable that the presence of the micelles adsorbed onto the κ-carrageenan could prevent the twisting of the helix to the coil when the temperature is raised. If two sulfates were necessary to interact with the casein micellar surface, their presence could in essence lock the κ-carrageenan in the helical conformation and prevent its ability to unwind into the coil.

DSC work confirms an interaction between the two biopolymers and that the interaction involves the κ-carrageenan helix, however, there is a potential limitation to the interpretation of these results. The measurement of differential heat capacity is a very sensitive measurement, especially in terms of measuring the temperature-associated transitions of κ-carrageenan at such low concentrations. This was evident by the necessity to double the concentration in order to visualize a transition. Therefore, in hindsight it would have been wise in the experiments in which the sample cell contained both biopolymers to replace the deionized water in the reference cell with a skim milk solution of equivalent concentration. This would have compensated for any related heat

capacity effects associated with any of the many milk components that may interfere with measuring the heat flow of the κ-carrageenan transition.

Despite this limitation, the results do corroborate well with the other techniques and confirm the well-proven requirement for the helical conformation for interaction and also that interaction does indeed occur.

5.5 Reverse Phase High Performance Liquid Chromatography

Reverse phase HPLC results indicated that no β-casein was present in the serum of centrifuged skim milk at 20°C, in the presence and absence of κ-carrageenan. This was expected since it is known that β-CN migrates outside of the micelles at low, refrigeration temperatures. When the centrifugation process was performed at 5°C the results indicate that β-CN can still migrate outside of the casein micelle even in the presence of adsorbed κ-carrageenan. Figure A-18 in is an example of a chromatograph containing β-CN compared to figure A-19, which does not contain β-casein. The data obtained from integrating the β-CN peaks, which determines the area under the peak and is in turn related to the concentration of β-CN, is summarized in Table 5.2.

Table 5.2: Summary of the reverse phase HPLC results that indicate the retention time and area values obtained for β-casein.

Number	Centrifuged Sample	Retention time (min)	Peak Area (microvolt-sec)	Area %
1	Skim milk (20°C) - Run 1	ND	ND	ND
2	Skim milk (20°C) - Run 2	ND	ND	ND
3	Skim milk (20°C) - Run 3	ND	ND	ND
4	Skim milk & 0.015% κ-carrageenan (20°C) -Run 1	ND	ND	ND
5	Skim milk & 0.015% κ-carrageenan (20°C) -Run 2	ND	ND	ND
6	Skim milk & 0.015% κ-carrageenan (20°C) -Run 3	ND	ND	ND
7	Skim milk (5°C) - Run 1	65.242	541809.4	18.24
8	Skim milk (5°C) - Run 2	65.033	4000242	11.5
9	Skim milk (5°C) - Run 3	65.842	15857288	43.83
10	Skim milk & 0.015% κ-carrageenan (5°C) -Run 1	64.758	4412672.3	12.4
11	Skim milk & 0.015% κ-carrageenan (5°C) -Run 2	66.767	53527.123	36.12
12	Skim milk & 0.015% κ-carrageenan (5°C) -Run 3	ND	ND	ND

The results not only show that the movement of β-casein can still occur in the presence of absorbed carrageenan but also that this movement is not at all hindered. There is no statistical difference (appendix III, table A-1) between the area values in the presence or absence of κ-carrageenan, which leads to the conclusion that the presence of the absorbed polymer does not impede in any way the movement of β outside the micelle. This suggests that the κ-carrageenan interacts with only a partial portion of the surface and that entire coverage of the micelle is not achieved by the polymer.

6.0 Proposed Casein micelle - κ-Carrageenan Interaction Mechanism

In order to amalgamate all the results presented herein and visualize the mechanism by which κ-carrageenan inhibits phase separation, it must first be recalled

that microscopic phase separation still occurs in these macroscopically stable systems and that the κ-carrageenan is associated with the protein phase. It is envisioned that κ-carrageenan helices align at the periphery of the micro-domains of the casein micelles and interacts with only the micelles at the periphery via surface interaction and that it is association of neighbouring helices (that are adhered to these domains) that kinetically stabilize the system from macroscopically phase separating (Figure 6.1). Without interaction of the polymer to the micro-domains of casein micelles, as seen with the agarose, and without association of neighbouring helices to stabilize these micro-domains, as seen with the λ-carrageenan and κ-carrageenan in the presence of NaI, the result is coalescence of the micro-domains and thus macroscopic phase separation. Therefore, interaction between the casein micelles and the κ-carrageenan as well as interaction between κ-carrageenan helices is therefore responsible for preventing phase separation.

We suggest that this macrostructure is established after pasteurization and during cooling of these systems (Figure 6.1). During pasteurization when the temperature is above the coil-helix transition, incompatibility exists between the casein micelles and the primary polysaccharide (e.g., locust bean gum) but also between casein micelles and κ-carrageenan (Schorsch et al., 2000), so that κ-carrageenan is forced from the inter-particle space of adjacent casein micelles. The incompatibility between the non-adsorbing polysaccharides (i.e. locust bean gum and κ-carrageenan in the coil conformation) would drive together, via a depletion flocculation mechanism, the casein micelles into micro-domains, excluding any polymer and resulting in the presence of the κ-carrageenan in the serum surrounding the micro-domains (Figure 6.1b). Once the temperature is lowered

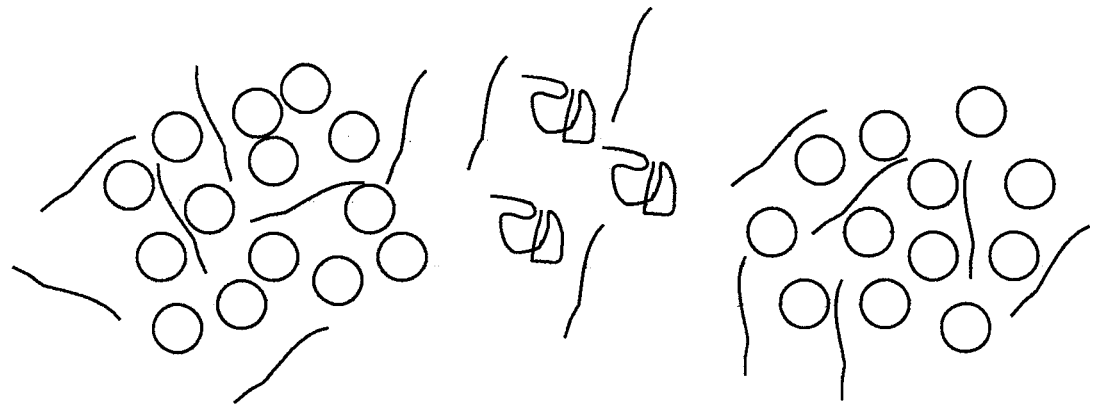
and the helix has been formed, interaction between κ-carrageenan helices and casein micelles, on the periphery of the micro-domains, occurs (Figure 6.1c). Subsequently, aggregation of helices stabilizes the domains and prevents them from coalescing, thus inhibiting phase separation (Figure 6.1d). Vega & Goff (2004) showed the size of the micro-domains of casein micelles to become increasingly smaller with increasing κ-carrageenan concentration, in effect "emulsifying" the water-in-water emulsion droplets, which are in essence the micro-protein domains. This is consistent with the mechanism suggested above, whereby the presence of more κ-carrageenan in the systems results in enhanced casein micelle interaction at the periphery of the discrete micro-domains.

Furthermore, these results also imply that the sulfate groups may also be involved in the interaction between the two biopolymers. The main structural difference between the agarose and κ-carrageenan is that the former is devoid of sulfate groups. The lack of interaction between the agarose and κ-carrageenan may thus be attributed to this structural difference. Also, the greater increase in casein micelle diameter visualized with λ-carrageenan, which has a higher degree of sulfation, is also evidence that the sulfates may be involved in the interaction. The effect of temperature on stability of the systems also suggests that the sulfates play a role in interaction. If the distance between the neighbouring sulfates is too great to induce attraction between the two biopolymers then interaction does not occur. However, upon lowering of the temperature resulting in a subsequent increase in charge density, as the coil twists into a helix bringing closer the sulfates, the interaction can thus be facilitated. The μDSC results also corroborate this conclusion as this technique asserts the requirement of helical conformation for interaction. Furthermore, the κ-carrageenan in the presence of NaI resulted in a greater

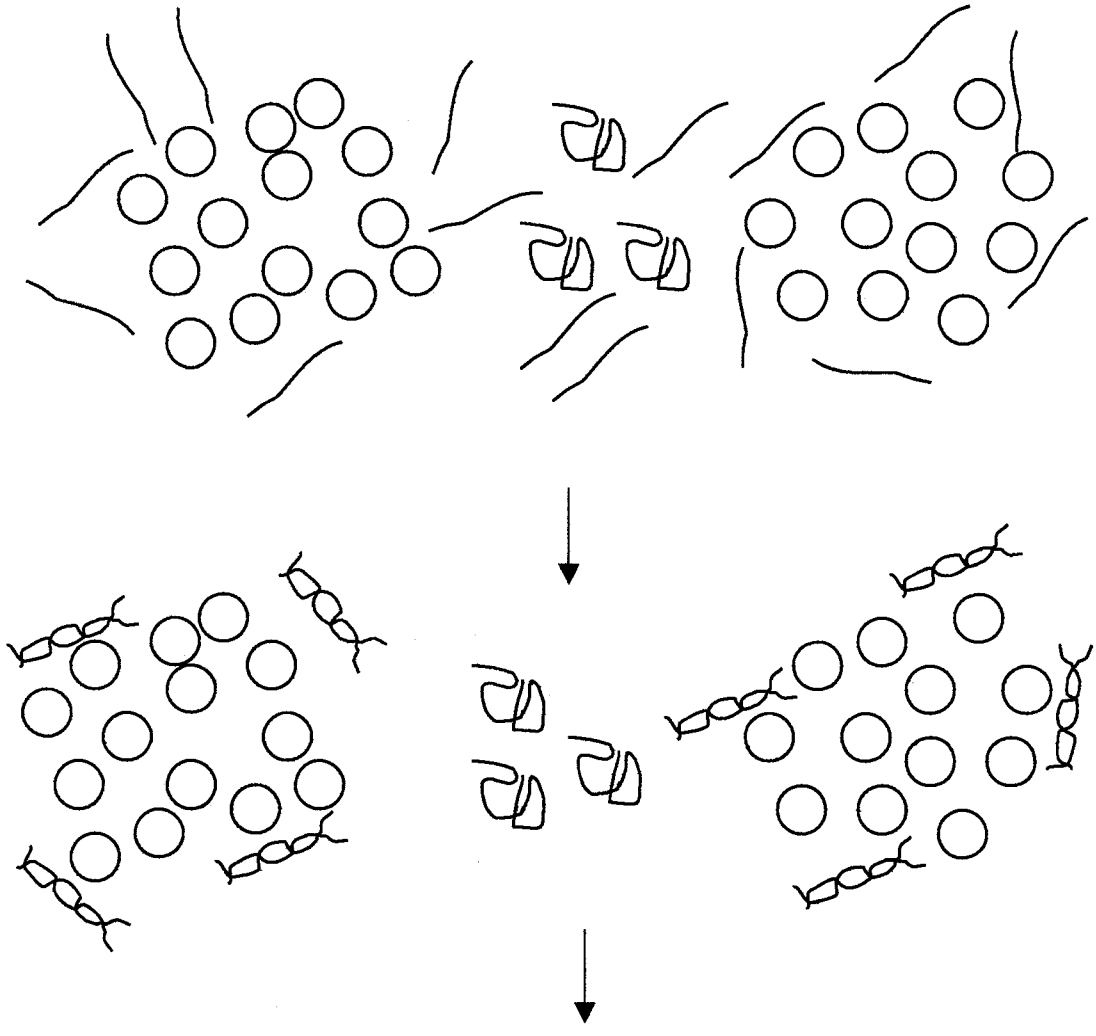
increase in casein micelle diameter, which may be as a result of the greater negativity of the polysaccharide caused by the absorption of iodide with the helix. This may imply that the nature of the interaction is indeed electrostatic as the increase in negativity of the polymer may explain the greater attraction between the two biopolymers.

(A)

(B)



(C)



(D)

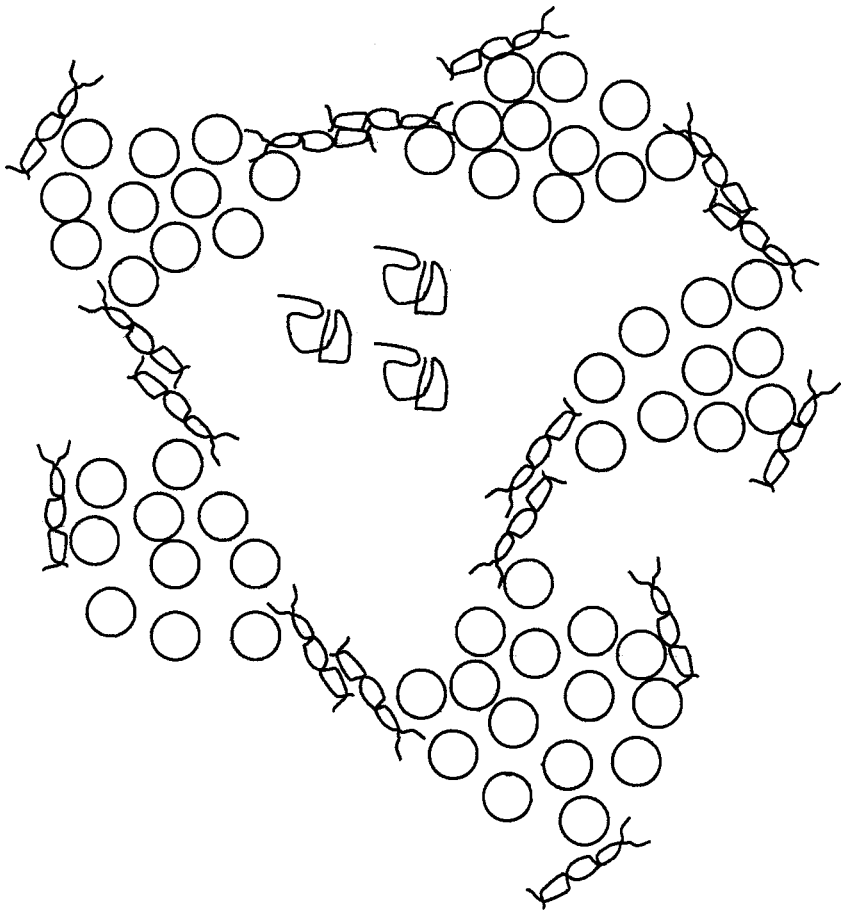
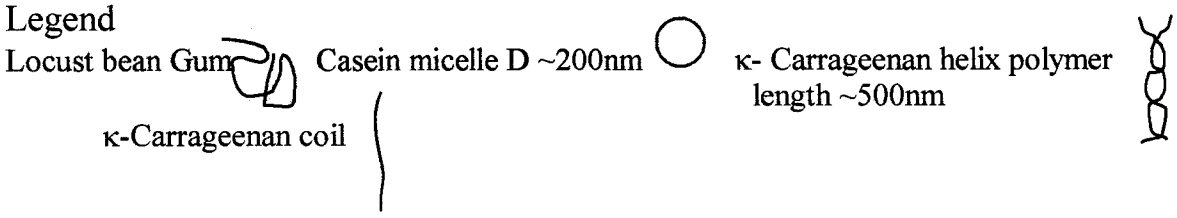


Figure 6.1: Schematic diagram outlining the mechanism of stability of κ-carrageenan.
A: at high temperatures (i.e. during pasteurization) the κ-carrageenan is in the coil conformation and is found distributed within solution. The casein micelles have been concentrated via incompatibility with locust bean gum. The carrageenan may be trapped within these domains.
B: When the ratio of casein micelles:κ-carrageenan coils within the domains becomes unfavourable there is an exclusion of κ-carrageenan via depletion flocculation and/or segregative interactions
C: Once the temperature is lowered the κ-carrageenan, now at the periphery of the domains, changes conformation to the helical form.
D: Interaction between the helical form of κ-carrageenan and the casein micelles at the periphery of the micro-domains occurs and subsequent interaction between neighbouring helices on adjacent domains stabilizes the domains against flocculation and coalescence and eventual separation.



With this proposed mechanism the following question may arise: how does the increase in casein micelle diameter visualized in the DLS relate to the proposed stabilizing mechanism? One may assume that if the κ-carrageenan is attached to the micro-domains of phase-separated casein micelles then a larger increase in casein micelle diameter should be seen in the DLS. However the solutions studied by DLS did not contain a non-adsorbing polymer (i.e., locust bean gum) that would induce depletion flocculation. Therefore, the casein micelles remained evenly distributed and free to interact with the κ-carrageenan. So the DLS results are taken to indicate that interaction occurs between the casein micelle and the κ-carrageenan but are not indicative of stabilization by 'homogeneous' adsorption, i.e. that every casein micelle is absorbed to a κ-carrageenan molecule.

All results presented herein support the suggested mechanism. The glutaraldehyde results although not entirely supportive, do agree with the mechanism but lead to a further conclusion that the system is rather dynamic. The DLS results indicate that the carrageenan can still interact with the casein micelles even though the structure has been locked. Assuming the glutaraldehyde does decrease the flexibility of the hairs the result would indicate that there is still sufficient bare regions on the surface to facilitate interaction. Since absorption and helical aggregation still occur, this results in the initial stability of the system. However, over a few days it appears that phase separation occurs, even though this separation is only minimal. It is therefore believed to be as a result of the dynamic nature of the system. It is assumed that the helical interaction between κ-carrageenan molecules, which are associated with neighbouring domains, stabilizes the domains by preventing them from coalescing. This by no means indicates that the system

becomes static, as it is possible that helical association between neighbouring domains can be readily broken and reformed. Furthermore, the casein micelles can readily exchange between the domains but do stay within a micro- domain structure since the driving force for separation, the locust bean gum, is still present. Therefore, if the flexibility of the casein micelles has been lost then association into the domains would be hindered, as close approach of the micelles would be affected. This would cause a decrease in the compactness of the domains and result in some exclusion of casein micelles leading to a rather small degree of phase separation. This is a rather slow process as the decrease in flexibility of the hairy layer is not a determinant factor in the overall stability, since κ-carrageenan can still interact with the casein micelle. This does however support the belief that the flexible nature of the hairy layer does play a role in the long-term stability of the system.

Earlier work by Vega et al. (2004a) suggested that the minimum weight ratio of casein micelles to κ-carrageenan in order to achieve macroscopic stability was 243. The work presented herein confirms this and also adds to the importance of that ratio. If the κ-carrageenan concentration was lowered (and the ratio subsequently increased) then the result would be insufficient κ-carrageenan to both adsorb to the periphery of the micro-domains of micelles and associate with neighbouring helices, resulting in coalescence of the micro-domains and macroscopic phase separation. Conversely, if the κ-carrageenan concentration were increased the formation of more finely dispersed protein domains would be created, analogous to the action of emulsifiers by increasing the number and decreasing the size of emulsion droplets, thus resulting in greater stability.

7.0 CONCLUSION

The objective of this project was to gain further insight into the mechanisms behind the ability of κ-carrageen to prevent macroscopic phase separation of milk based systems containing added polysaccharide. The two previous explanations into milk reactivity included a theory involving an electrostatic interaction between the negatively charged κ-carrageenan and a positive region on the surface of the casein micelle and a weak gel theory involving the formation of a κ-carrageenan gel capable of suspending the casein micelles. Both theories have supporting evidence as well as some shortcomings. It was the purpose of this research project to determine a viable mechanism that could explain the important interaction between the two biopolymers. With this aim many techniques were used that revealed interesting information that, coupled with knowledge of previous work on similar systems in our laboratory, has allowed for the development of a valid working mechanism.

FESEM micrographs suggest that an interaction between the two biopolymers occurs with the κ-carrageenan adsorbing onto the surface of the casein micelles. The micrographs of the individual biopolymers, most specifically the casein micelles, also reveal interesting features in relation to their structure and perhaps potential for interaction. The casein micelles appear to be rather porous and suggest that there are areas on the surface that could facilitate the interaction with the long stranded, helical κ-carrageenan.

Dynamic light scattering results also suggest that interaction is occurring between both biopolymers, as there is an increase in casein micelle diameter in the presence of κ-carrageenan, in the helical conformation, over the concentration range 0-0.03%.

Furthermore, as the κ-carrageenan concentration is increased the diameter of the casein micelle increases linearly with a highly correlated R^2 value of 0.9779 and this is indicative of the specificity and sensitivity of the micelles to the presence of κ-carrageenan. These results corroborate well with the micrographs and suggest that an interaction is occurring between the helical κ-carrageenan and the surface of the casein micelles.

Further DLS results coupled with phase separation experiments have led to great developments into the possible molecular orientation and molecular mechanisms of the ability of κ-carrageenan to stabilize these systems. κ-Carrageenan in the presence of sodium iodide, which stabilized the helical conformation and prevents helical association, and λ-carrageenan, which is the non-gelling, non-helical form, both were shown to interact with the micelle but were both unable to prevent macroscopic phase separation. This implies that helical association is necessary to the overall stability of the system. Agarose, which is a structurally similar and a similarly gelling polysaccharide to the carrageenans, showed no increase in casein micelle diameter and was also unable to prevent phase separation. This implies that even though a similar gelation pattern, i.e. helical aggregation, occurs the absence of an interaction between the two biopolymers results in phase separation. This indicates the necessity for interaction and is crucial to the development of a working mechanism.

Additional DLS and phase separation results involving the use of glutaradehyde treated milk and guar gum support the results. The addition of guar gum, which resulted in phase separation and no increase in casein micelle diameter, confidently excluded the possibility of a viscosity effect of added polysaccharide. κ-Carrageenan in glutaraldehyde

treated milk, which would decrease the flexibility of the hairy layer, resulted in the increase in casein micelle diameter but resulted in phase separation, although minimal and at much slower kinetics. These results initially may see contradictory as helical association would be unaffected and interaction still occurs. However, the minimal separation and slower kinetics is indicative of the dynamic nature of the system and its importance to overall stability, as decreasing the flexible nature of the hairy layer decreases the ability of the casein micelles to form and re-form micro-domains. This would, over-time, result in minimal phase separation.

The μDSC results further corroborate the obtained results and suggest that that interaction between the biopolymers occurs and that the helical, not coil, conformation is involved in the interaction. Furthermore, the μDSC results indicate that interaction between the two biopolymers occurs in such a matter that prevents the κ-carrageenan from twisting from the helical conformation to the coil conformation and is further evidence that the sulfates may be involved in the interaction since twisting between conformations is inhibited.

Reverse phase HPLC results suggest that κ-carrageenan does not absorb onto the surface of the casein micelles in such a manner that complete surface coverage is achieved, as it does not prevent the migration of β-casein out of the micelle. Therefore, these results imply that κ-carrageenan absorbs only onto a portion of the casein micellar surface.

The interaction between the κ-carrageenan and casein micelles is also suggested, by numerous experiments herein, to involve the sulfates groups. This interaction has been

shown to be further enhanced by the increase in the overall negativity of the polymer. These results would suggest the interaction to be electrostatic in nature.

With knowledge that microscopic phase separation occurs, in which micro-domains of proteins are formed, even though macroscopic stability is achieved and that the κ-carrageenan is associated with the protein phase, along with these results led to the development of a viable working mechanism. It is believed that κ-carrageenan functionality occurs in two steps. Initially the κ-carrageenan interacts with the casein micelles at the periphery of microscopically phase-separated discrete protein domains and that helical association between helices on neighbouring micro-domains stabilizes the micro-domains against coalescing and forming visual protein-enriched and protein-depleted phases. Therefore, a network is formed by which the micro-domains of casein micelles are stabilized against flocculation by the presence of the absorbed κ-carrageenan helices, which further interact with each other via helical association to prevent visual phase separation.

8.0 REFERENCES:

Allmere, T. Andren, A. and Bjorck, L. 1997. Interaction between different genetic variants of β lactoglobulin and κ casein A during heating of skim milk. *Journal of Agricultural Food Chemistry.* 45, 1564-1569.

Anema, S.G. and Li, Y. 2003. Association of denatured whey proteins with casein micelles in heated reconstituted skim milk and its effect on casein micelle size. *Journal of Dairy Research.* 70,73-83.

Andrew, R. A. 2002. Biopolymer Interactions: Implications in ice cream mix. University of Guelph MSc. Thesis.

Bemiller and Whistler. 1996. Carbohydrates In O.R. Fennema, *Food Chemistry Third Edition.* Marcel Dekker Inc. New York, New York. P. 211-213.

Bordin, G., Raposo, C. de lea Calle., Rodriguez, A.R. 2001. Identification and quantification of major bovine milk proteins by liquid chromatography. *Journal of Chromatography.* 928, 63-76.

Bourriot, S., Garnier, C. and Doublier, J.-L. 1999. Micellar-casein-κ-carrageenan mixtures. I. Phase separation and ultrastructure. *Carbohydrate Polymers.* 40, 145-157.

Brunner, J.R. 1977. In *Food Proteins:* Milk Proteins. AVI Publishing, Westport Connecticut, p. 175.

Calvo, M.M. 1995. Heat induced interaction between serum albumin, immunoglobulin, and κ casein inhibited the primary phase of renneting. *Journal of Agricultural Food Chemistry.* 43, 2823-2827

Carroll, R.J., Thompson, M.P. and Nutting, G.C. 1968. Glutaraldehyde fixation of casein micelles for electron microscopy. *Journal of Dairy Science.* 51, 1903-1908.

Carson, F. 1997. *Histology- A self-instructional text. Second Edition.* ASCP Press, Chicago. P. 2-27.

Chaplin, M. 2004 - http://www.martin.chaplin.btinternet.co.uk/hycar.html

Cooper, A., Nutley, M. A., and Wadood, A. 2000. Differential scanning microcalorimetry. In S.E. Harding and B.Z. Chowdhry, *Protein-Ligand Interactions: hydrodynamics and calorimetry.* Oxford University Press, Oxford New York, p. 287-318.

Corredig, M. and Dalgleish, D.G. 1999. The mechanism of heat induced interaction of whey proteins with casein micelles in milk. *International Dairy Journal.* 9, 233-236.

Creamer, L.K. and MacGibbon, A.K.H. 1996. Some recent advances in the basic chemistry of milk proteins and lipids. *International Dairy Journal.* 6, 539-568.
Creamer, L.K., Plowman, J.E., Liddell, M.J., Smith, M.H. and Hill, J.P. 1998. Micelle Stability: κ casein structure and function. *Journal of Dairy Science.* 81, 3004-3012.

Dalgleish, D.G. 1992. Bovine milk protein properties and the manufacturing quality of milk. *Livestock Production Science.* 35, 75-93.

Dalgleish, D.G. 1997. Structure and Function relationship of Caseins. In S. Damodaran and A.Paraf, *Food Proteins and Their Applications.* Marcel Dekker. New York, p.199.

Dalgleish, D.G. 1998. Casein micelles as colloids: Surface Structures and Stabilities. *Journal of Dairy Science.* 81, 3013-3018.

Dalgleish, D.G. and Hallet, F.R. 1995. Dynamic light scattering: applications to food systems. *Food Research International.* 28, 181-193.

Dalgleish, D. G., & Morris, E. R. 1988. Interactions between carrageenans and casein micelles: electrophoretic and hydrodynamic properties of the particles. *Food Hydrocolloids*, 2, 311-320.

Dalgleish, D. G, van Mourik, L. and Corredig, M. 1997. Heat Induced interaction of whey proteins and casein micelles with different concentrations of α lactalbumin and β lactoglobulin. *Journal of Agricultural of Food Chemistry.* 45, 4806-1813.

Drohan, D.D., Tziboula, A., McNulty, D. and Horne, D.S. 1997. Milk protein-carrageenan interactions. *Food Hydrocolloids.* 11, 101-107.

Doyle, J., Giannouli, P., Philip, K. and Morris, E. Effect of K+ and Ca2+ cations on gelation of κ-carrageenan. 2002. In P.A. Williams and G.O Phillips, *Gums and Stabilizers for the Food Industry 11.* p.158-164.

de Kruif, C.G. 1999. Casein micelle interactions. *International Dairy Journal.* 9, 183-188.

de Kruif, C. G. & Holt, C. 2003. Casien micelle structure, functions and interaction. In P. F. Fox and P. L. H. McSweeney, eds., *Advanced Dairy Chemistry –1. Proteins. 3rd edn.* (pp. 233-276). New York: Kluwer Academic/Plenum Publishers.

de Vries, J. 2002. Interaction of carrageenan with other ingredients in dairy dessert gels. In P. A. Williams & G. O. Phillips, Gums and stabilizers for the food industry (Vol. 11) (pp. 200-210). London: Royal Society of Chemistry.

De Wit, J.N. 1998. Nutritional and functional characteristics of whey proteins in food products. *Journal of Dairy Science.* 81, 597-608.

Farrell Jr., H.M., Wickham, E.D.,Unruh, J.J., Qi, P.X., and Hoagland, P.D. 2001. Secondary Structural studies of bovine caseins: temperature dependence of β casein structure as analyzed by circular dichroism and FTIR spectroscopy and correlation with micellization. *Journal of Dairy Science.* 85, 459-471.

Farrell Jr., H.M., Qi, P.X. Brown, E.M, Cooke, P.H., Tunick, M.H., Wickham, E.D., and Unruh, J.J. 2002. Molten globule structures in milk proteins: Implications for potential new structure function relationships. *Journal of Dairy Science.* 85, 459-471.

Farrell Jr., H.M., Brown, E.M., Hoagland, P.D. and Malin, E.L. 2003. In *Advanced Dairy Chemistry Volume 1: Proteins.* "Higher order structures of the caseins: A Paradox?".MORE

Ford, N.C. 1985. Light Scattering Apparatus. In R. Pecora, *Dynamic Light Scattering: Applications of Photon Correlation Spectroscopy*: Plenum Press, New York. p.1.

Fox, P.F. 2001. Milk Proteins as food ingredients. *International Journal of Dairy Technology.* 54, 41-55.

Fox, P.F. 2003. Milk proteins: general and historical aspects. In P. F. Fox and P. L. H. McSweeney, eds., *Advanced Dairy Chemistry –1. Proteins. 3rd edn.* (pp. 1-41). New York: Kluwer Academic/Plenum Publishers.

Fox, P.F. and McSweeney, P.L.H. 1998. *Dairy Chemistry and Biochemistry.* Blackie Academic and Professional.

Gallagher, D.P. and Mulvihill, D.M. 1997. Heat stability and renneting characteristics of milk systems containing bovine casein micelles and porcine or bovine β lactalbumin. *International Dairy Journal.* 7, 221-228.

Gamliel, H. 1985. Optimum fixation conditions may allow air drying of soft biological specimens with minimum cell shrinkage and maximum preservation of surface features. *Scanning Electron Microscopy*, 4, 1649-1664.

Glauert, A.M. 1975. *Fixation, Dehydration and Embedding of Biological Specimens: Practical Methods in Electron Microscopy.* North Holland Publishing Company, Cambridge.

Goff, H.D. 1997. Colloidal Aspect of Ice Cream – A Review. *International Dairy Journal.* 7, 363-373.

Goff, H.D. 2004. www.foodsci.uoguelph.ca/dairyedu/home.html

Goff, H.D., & Jordan, W.K. 1989. Action of emulsifiers in promoting fat destabilization during the manufacturing of ice cream. *Journal of Dairy Science* 72, 18-29.

Grasdalen, H. and Smidsrod, O. 1981 Iodide-Specific Formation of κ-carrageenan Singel Helices. ^{127}NMR Spectroscopic Evidence for Selective Site Binding of Iodide Anions in the Ordered Conformation. *Macromolecules*. 14, 1842-1845.

Hayat, M.A. 2000. *Principles and Techniques of Electron Microscopy for Biological Applications, 4th edition.* Cambridge University Press, New York.

Haque, Z., Kristjansson, M.M. and Kinsella, J.E. 1989. Interaction between κ casein and β lactoglobulin: Possible Mechanism. *Journal of Agricultural Food Chemistry.* 35: 644-649.

Holt, C. and Dalgleish, D.G. 1986. Electrophoretic and hydrodynamic properties of bovine casein micelles interpreted in terms of particles with an outer hairy layer. *Journal of Colloid and Interface Science, 114*, 513-524.

Holt, C. and Sawyer, L. 1993. Caseins as Rheomorphic proteins: Interpretation of Primary and Secondary Structures of the αs1, β and κ-caseins. *Journal of Chemical Society Faraday Trans??.* 89, 2683-2692.

Holt, C., and Horne, D.S. 1996. The hairy casein micelle: Evolution of the concept and implications for dairy technology. *Netherlands Milk and Dairy Journal.* 50, 85-111.

Hood, L.F. and Allen, J.E. 1977. Ultrastructure of carrageenan-milk sols and gels. *Journal of Food Science*, 42: 1062-1065.

Horne, D.S. 1998. Casein Interactions: Casting light on the Black Boxes, the structure in Dairy Products. *International Dairy Journal*, 8, 171-177.

Horne, D.S. 2002. Casein structure, self-assembly and gelation. *Current Opinion in Colloidal and Interface Science*. 7, 456-461.

Horne, D.S. and Davidson, C.M. 1986. The effect of environmental conditions on the steric stabilization of casein micelles. *Colloid and Polymer Science.* 234, 727-734.

Imeson, A.P. 2000 Carrageenans. In G. O. Phillips & P. A. Williams, eds., *Handbook of Hydrocolloids* (pp. . 87-102). Cambridge: Woodhead Publishing.

Ikeda, S. and Nishinari, K. 2001. "Weak-Gel"-Type Rheological properties of aqueous dispersions of nonaggregated κ-carrageenan helices. *J. Agricultural Food Chemistry.* 49, 4436-4441.

Jensen, R.G., Ferris, A.M. and Lammi-Keefe, C.J. 1991. Symposium: Milk Fat-composition, function and potential for change: The composition of milk fat. *Journal of Dairy Science*. 74, 3228-3243.

Lai, V.M.F., Wong, P.A.-L, and Lii, C.-Y 2000. Effects of cation properties on sol-gel transition and gel properties of k-carrageenan. *Journal of Food Science*. 65, 1332-1337.

Langendorff, V., Cuvelier, G., Launay, B. and Parker, A. 1997. Gleation and flocculation of casein micelle/carrageenan mixtures. *Food Hydrocolloids*. 11, 35-40.

Langendorff, V., Cuvelier, G., Launay, B., Michon, C., Parker, A. and De Kruif, C.G. 1999. Casein micelle/iota carrageenan interacations in milk: influence of temperature. *Food Hydrocolloids*. 13, 211-218.

Langendorff, V., Cuvelier, G., Michon, C., Launay, B., Parker, A., and De Kruif, C.G. 2000. Effects of carrageenan type on the behaviour of carrageenan milk/mixtures. *Food Hydrocolloids*. 14, 273-280.

Lucey, J.A. 2002. Formation and physical properties of milk protein gels. *Journal of Dairy Science*. 85, 281-294

Lucey, J.A. and Singh, H. 2003. Acid Coagulation of Milk. In P. F. Fox and P. L. H. McSweeney, eds, Advanced Dairy Chemistry –1. Proteins. 3rd. *Advanced Dairy Chemistry Volume 1 Proteins:* P. 1001

Lynch, M. G. & Mulvihill, D. M. 1994. The influence of caseins on the rheology of k-carrageenan gels. In G. O. Phillips, D. J. Wedlock, & P. A. Williams, Gums and stabilisers for the food industry (Vol. 7) (pp. 323-332). Oxford, IRL Press.

Malvern, PCS Software Reference Manual, Dynamic Light Scattering. 1993.

Marshall, R., Goff, H.D., & Hartel, R.W. 2003. Ice Cream. 6th Ed. Kluwer Academic, New York.

Modler, H.W. 2000. Milk Processing. N. Shuryo and H.W. Modler, In *Food Proteins Processing Applications*. Wiley-VCH, Toronto. 2000, p.1.

Montero, P. and Perez-Mateos, M. 2002. Effects of Na+, K+ and Ca2+ on gels formed from fish mince containing a carrageenan or alginate. *Food Hydrocolloids*. 16, 375-385.

Morris, E. R., Rees, D. A. and Robinson, G. 1980. Cation-specific Aggregation of Carrageenan Helices: Domain model of Polymer Gel Structure. *J. Molecular Biology*. 138, 349-362.

McMahon, D.J. and McManus, W.R. 1998. Rethinking casein micelle structure using electron microscopy. *Journal of Dairy Science*. 81:2985-2993.

Noh, B. and Richardson, T. 1989. Incorporation of radiolabeled whey proteins into casein micelles by heat processing. *Journal of Dairy Science*. 72, 1724-1731.

Oldfield, D.J., Singh, H, Taylor, M.W. and Pearce, K.N. 2000. Heat-induced interactions of β lactoglobulin and α lactalbumin with the casein micelle in pH-adjusted skim milk. *International Dairy Journal*. 10, 509-518

Pearse, M.J., Linklater, P.M., Hall, R.J., and Mackinlay, A.G. 1985. Effect of heat induced interaction between β lactoglobulin and κ casein on syneresis. *Journal of Dairy Research*. 52, 159-165.

Piculell, L. 1995. In *Food Polysaccharides and their Applications*: Gelling Carrageenans. Marcel Dekker Inc: New York, p. 205.

Privalov, P.L. 1980. Scanning microcalorimeters for studying macromolecules. *Pure and Applied Chemistry*. 52, 479-497.

Privalov, P.L. Potekhin S.A. 1986. Scanning microcalorimetry in studying temperature-induced changes in proteins. In C.H.W. Hirs and S.N. Timasheff, eds., *Enzyme Structure Part L.* (pp.3-51). New York: Harcourt Brace Javanovich, Publishers.

Rasmussen, L.K., Johnsen, L.B., Tsiora, A., Sorensen, E.S., Thomsen, J.K., Nielsen, N.C., Jakobsen, H.J., and Peterson, T.E. 1999. *International Dairy Journal*. 9, 215-218.

Regand, A. and Goff, H.D. 2003. Structure and ice recrystallization in frozen stabilized ice cream model systems. *Food Hydrocolloids*. 17: 95-102.

Reddy, M. and Kinsella, J.E. 1990. Interaction of β lactoglobulin with κ casein in micelles as assessed by chymosin hydrolysis: Effect of temperature, heating time, β lactoglobulin concentration and pH. *Journal of Agricultural Food Chemistry*. 38, 50-58.

Rochas, C. and Rinaudo, M. 1980. Mechanism of Gel Formation in κ-carrageenan. *Biopolymers*. 23, 735-745.

Rochow, T.G. and Tucker, P.A. 1994. *Introduction to Microscopy by Means of Light, Electrons, X-Rays or Acoustics*. Plenum Press, New York. 1994. p. 329.

Rounds, M.A. and Nielson, S.S. 1998. In *Food Analysis: Second Edition*. "Basic Principles of Chromatography". Aspen Publishers, Inc. p. 485.

Sawyer, L., Barlow, P.N., Boland, M.J., Creamer, L.K, Denton, H., Edwards, P.J.B.E, Holt, C., Jameson, G.B., Kontopidis, G., Norris, G.E., Uhrinova, S. and Wu, S.Y. 2002. Milk protein structure: What can it tell the dairy industry? *International Dairy Journal*. 12, 299-310

Schmidt, D.G. 1982. Electron Microscopy of milk and milk products: Problems and Possibilities. Food Microstructure. Vol. 1, 151-165.

Schorsch, C., Jones, M.G. and Norton, I.T. 1999. Thermodynamic incompatibility and microstructure of milk protein/locust bean gum/sucrose systems. *Food Hydrocolloids.* 13, 89-99.

Schorsch, C., Jones, M.G., Norton, I.T. 2000. Phase behaviour of pure micellar casein/κ-carrageenan systems in milk salt ultrafiltrate. *Food Hydrocolloids.* 14, 347-358.

Skura, B.J. and Nakai, S. 1980. Pysicochemical verification of non-existence of αs_1-casein-κ-carrageenan interaction in calcium free systems. *Journal of Food Science.* 45, 582-591.

Skura, B.J. and Nakai, S. 1981. Stabilization of αs_1 by κ-carrageenan in the presence of calcium. *Canadian Institute of Food Science and Technology Journal.* 14, 59-63.

Slayter, E.M. and Slayter, H.S. 1992. *Light and Electron Microscopy.* Cambridge University Press. Cambridge UK.

Smits, P. and Van Brouwershaven, J.H. 1980. Heat Induced association of β lactoglobulin and casein micelles. *Journal of Dairy Research.* 47, 313-325.

Snoeren, T.H.M, Payens, T.A.J., Jeunink,J., and Both P. 1975. Electrostatic interaction between kappa-carrageenan and kappa-casein. *Milchwissenschaft,*30, 393-395

Snoeren, T. H. M., Both, P. & Schmidt, D.G. 1976. An electron-microscopic study of carrageenan and it's interaction with κ-casein. *Netherlands Milk Dairy Journal*, 30, 132-141.

Stanley, N.F. 1990. Carrageenans. In P. Harris, *Food Gels.* Elsevier Science Publishers. p. 79.

Swaisgood, H. 1996. Milk Proteins. In O.R. Fennema, *Food Chemistry Third Edition:.* Marcel Dekker Inc. New York, New York. p.63.

Swaisgood, H. 1992. Chemistry of Caseins. In P. F. Fox, *Advanced Dairy Chemistry 1. Proteins.* Elsevier Applied Science: London. P 63.

Swaisgood, H.E. 2003. Chemistry of Caseins. In P. F. Fox and P. L. H. McSweeney, eds., *Advanced Dairy Chemistry –1. Proteins. 3rd edn.* New York: Kluwer Academic/Plenum Publishers.

Syrbe, A., Bauer, W.J. and Klostermeyer, H. 1998. Polymer Science Concepts in Diary Systems - An overview of milk protein and food hydrocolloid interaction. *International Dairy Journal.* 8, 179-193.

Takemasa, M. and Chiba, A. 2001 Gelation mechanism of κ and ι carrageenan Investigated by Correlation between the Strain-Optical Coefficient and the Dynamic Shear Modulus. 2001. *Macromolecules* 34 7427-7434.

Thaiudom, S., & Goff, H. D. 2003. Effect of κ-carrageenan on milk protein polysaccharide mixtures. *International Dairy Journal*, 13, 763-771.

Tolstoguzov, V. B. 1997. Protein-Polysaccharide Interactions. In S. Damodaran and A.Paraf, *Food proteins and their applications*. Marcel Dekker., p. 171.

Tombs and Harding. 1998. In *An Introduction to Polysaccharide Biotechnology*. Carrageenans and red seaweed. Taylor and Francis: London, England, 134-141.
Tst rsui, K., Kumon, H., Ichikawa, H., Tawara, J. 1976. Preparative method for suspended biological materials for SEM by using of polycationic substance layer. *Journal of Electron Microscopy*. 25, 163-165.

Tziboula, A. and Horne. D.S. 1999. Influence of milk proteins on κ-carrageenan gelation. *International Dairy Journal,* 9, 359-364.

van de Velde, F., Peppelman, H.A., Rolleman, H.S. and Tromp, H. R. 2001. On the structure of κ/ι hybrid carrageenans. *Carbohydrate Research.* 331, 271-283.

Vega, C., Andrew, R. A., and Goff, H. D. 2004a. Serum separation in soft-serve ice cream mixes. *Milchwissenchaft*, 59, 284-287.

Vega, C., Dalgleish, D.G. and Goff, H.D. 2004b. Effect of κ-carrageenan addition to dairy emulsions containing sodium caseinate and locust bean gum. *Food Hydrocolloids.* In press

Vega, C. and Goff, H. D. 2004a. Phase separation in soft-serve ice cream mixes: rheology and microstructure. *International Dairy Journal*, Submitted for publication.

Vega, C. and Goff, H. D. 2004b. Kappa-carrageenan functionality in dairy emulsions as affected by low molecular weight emulsifiers. *International Dairy Journals*. In Press.

Viebke, C., Piculell, and Nilsson, S. 1994. On the mechanism of Gelation of Helix forming
biopolymers. *Macromolecules*. 27, 4160-4166.

Walstra, P and Jenness, R. 1984. *Dairy Chemistry and Physics*. John Wiley and Sons, New York.

Walstra, P. 1990. On the stability of casein micelles. *Journal of Dairy Science,* 73, 1965-

1979.

Walstra, P. 1999. Casein sub-micelles: do they exist? *International Dairy Journal.* 9:189-192.

Walstra, P. 2003. *Physical Chemistry of Foods.* Marcel Dekker, New York. p. 466.

Walstra, P., V.A., Bloomfield, G.J. Wei, and Jenness, R. 1981. Effect of chymosin action on the hydrodynamic diameter of casein micelles. Biochimica et Biophysica Acta 669: 258-259.

Xu, S.Y., Stanley, D.W., Goff, H.D., Davidson, V.J. and Le Maguer, M. 1992. Hydrocolloid/Milk Gel formation and Properties. *Journal of Food Science.* 57, 96-102.

Yuguchi, Y., Thuy, T.T.T., Urakawa, H. and Kajiwara, K. 2002. Structural characteristics of carrageenan gels: temperature and concentration dependence. *Food Hydrocolloids.* 16, 515-522.

I. Appendix I

1.0 Casein micelle Images

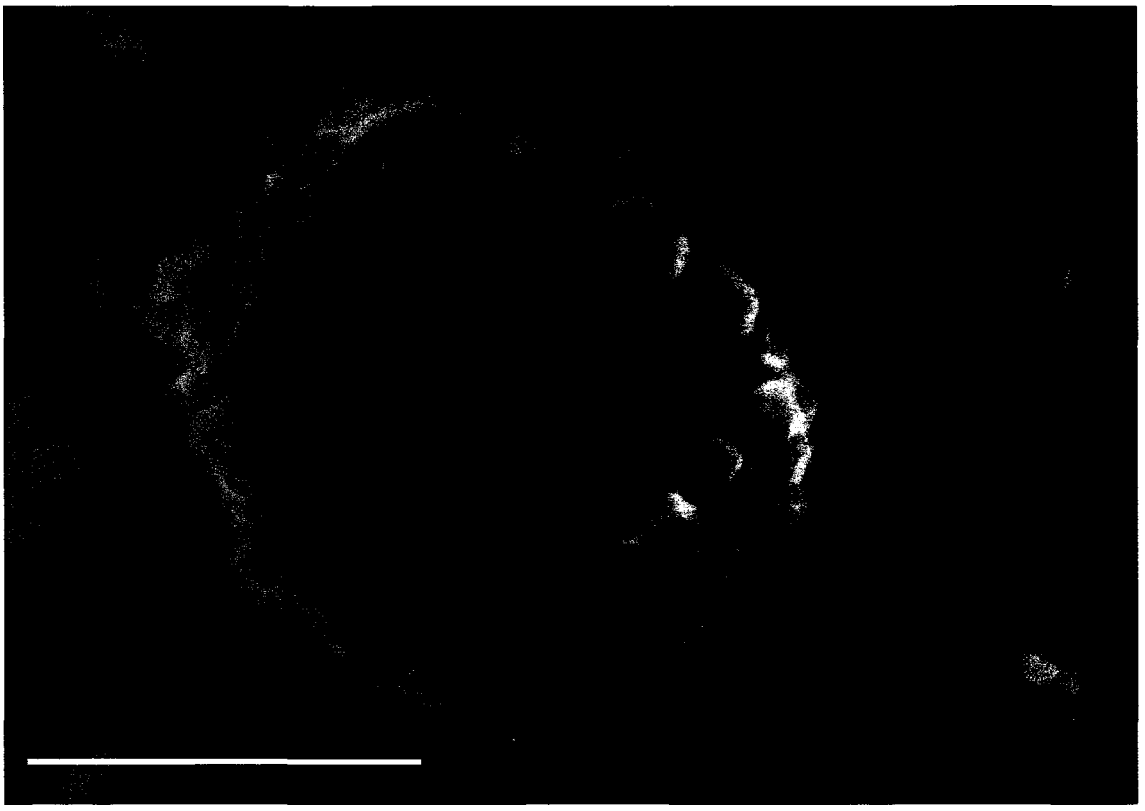


Figure A-1: An 11% w/w skim milk powder solution prepared on a carbon substrate imaged with the Hitachi S-4800. Bar = 200nm

Figure A-2: An 11% w/w skim milk powder solution prepared on a carbon substrate imaged with the Hitachi S-4800. Bar = 100nm

Figure A-3: An 11% w/w skim milk powder solution prepared on a carbon substrate imaged with the Hitachi S-4800. Bar = 100nm

2.0 κ- Carrageenan

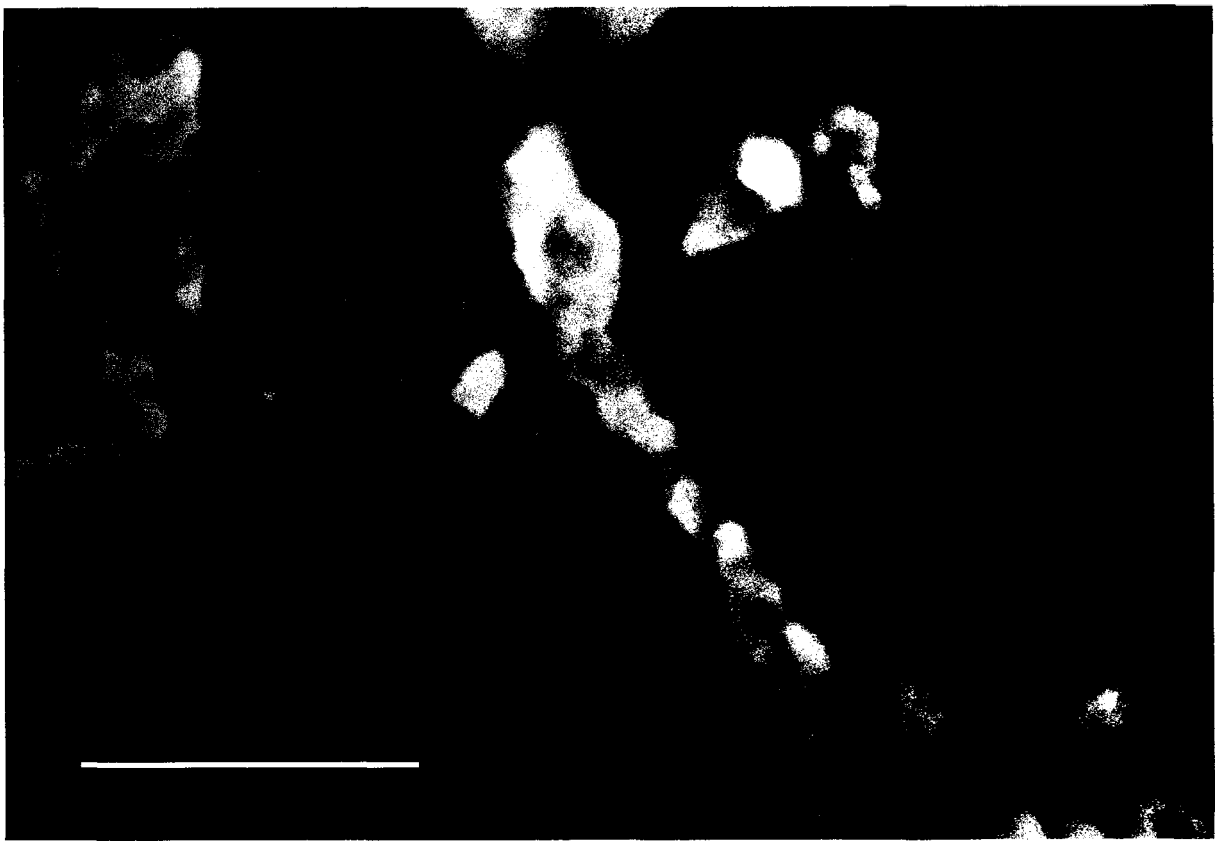


Figure A-4: κ-carrageenan, Hitachi s4800, 100nm

Figure A-5: κ-carrageenan, Hitachi s4800, 200nm

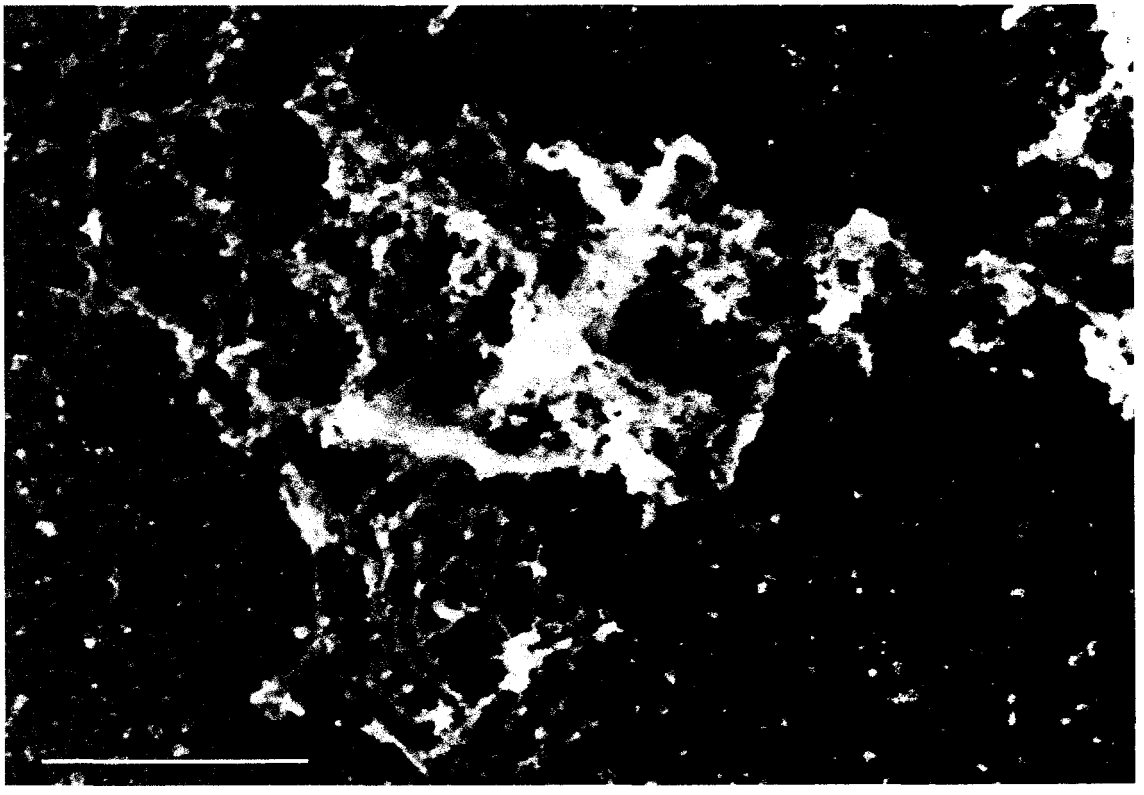


Figure A-6: A 0.015% w/w κ-carrageenan solution prepared on a glass substrate and imaged with the Hitachi S-4800. Scale bar = 500nm

3.0 Casein Micelle - κ-Carrageenan mixtures

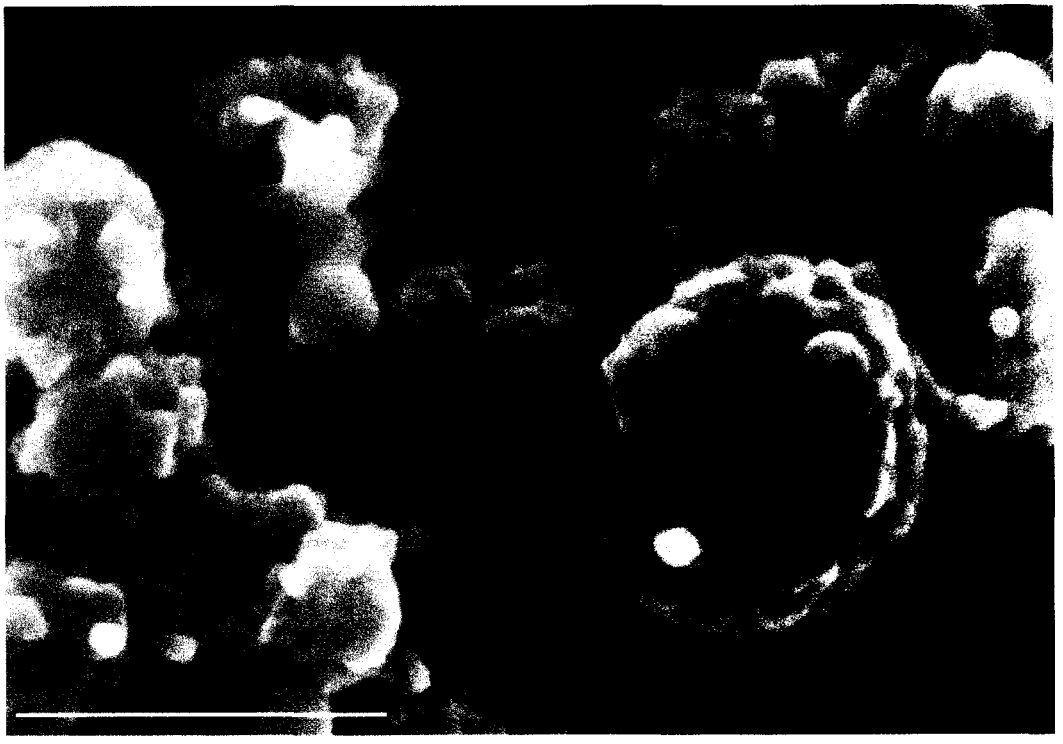


Figure A-7: An 11% w/w skim milk powder and 0.015% w/w κ-carrageenan solution imaged on a carbon substrate with the Hitachi S-4800. Scale bar = 200 nm

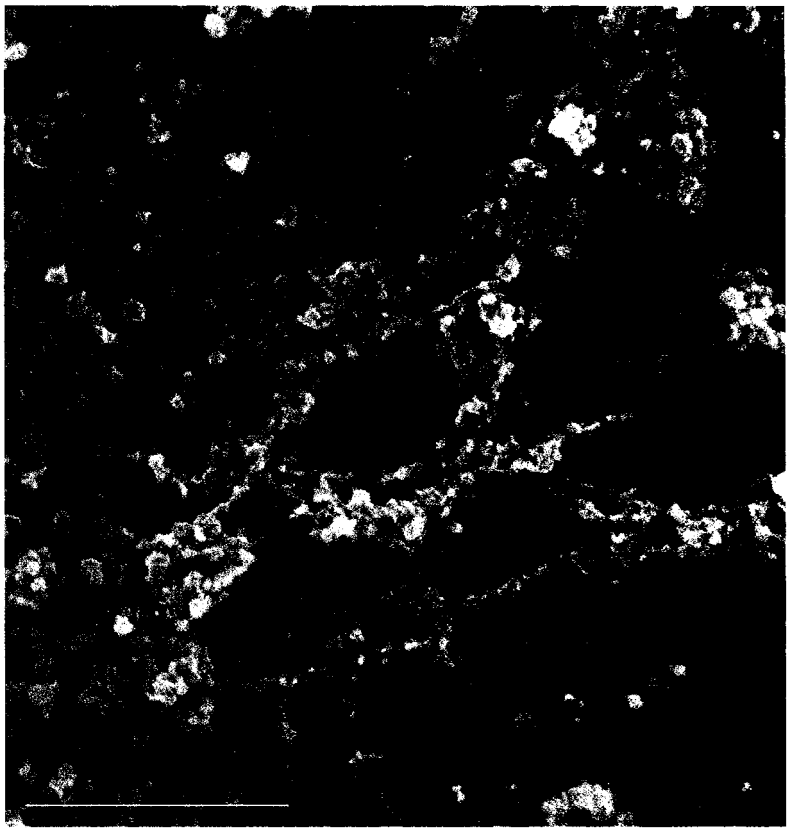


Figure A-8: An 11% w/w skim milk powder and 0.015% w/w κ-carrageenan solution imaged on a glass substrate with the Hitachi S-570. Scale bar = 1.2 um

Figure A-9: An 11% w/w skim milk powder and 0.015% w/w κ-carrageenan solution imaged on a carbon substrate with the Hitachi S-4800. Scale bar = 300 nm

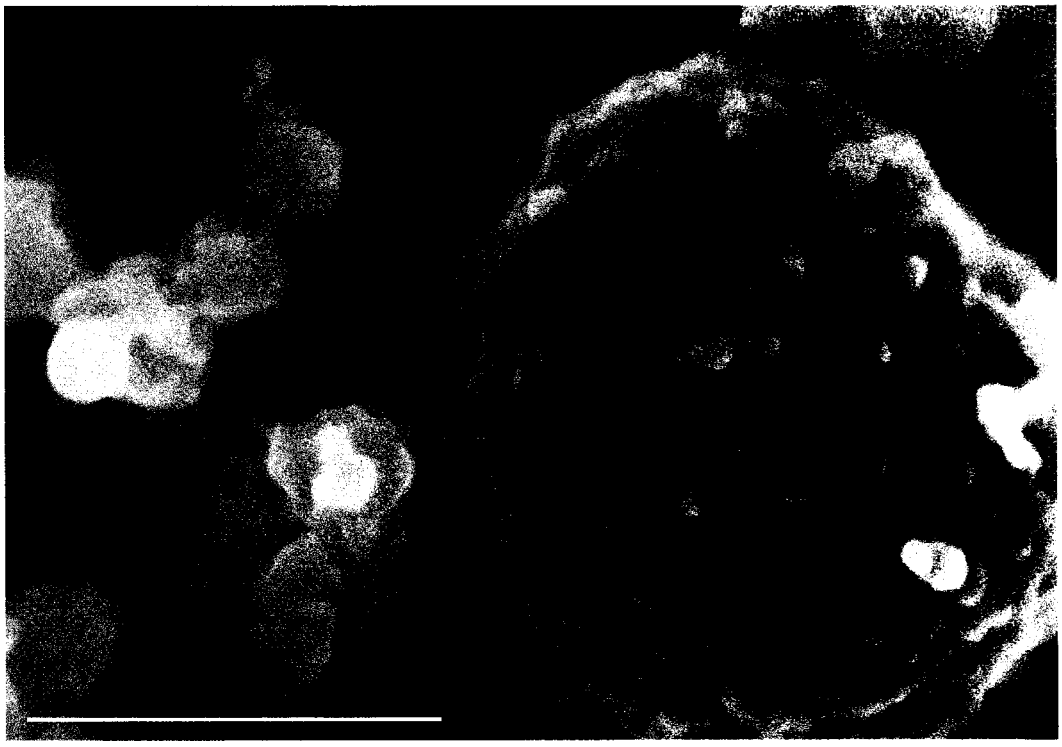


Figure A-10: An 11% w/w skim milk powder and 0.015% w/w κ-carrageenan solution imaged on a carbon substrate with the Hitachi S-4800. Scale bar = 200nm

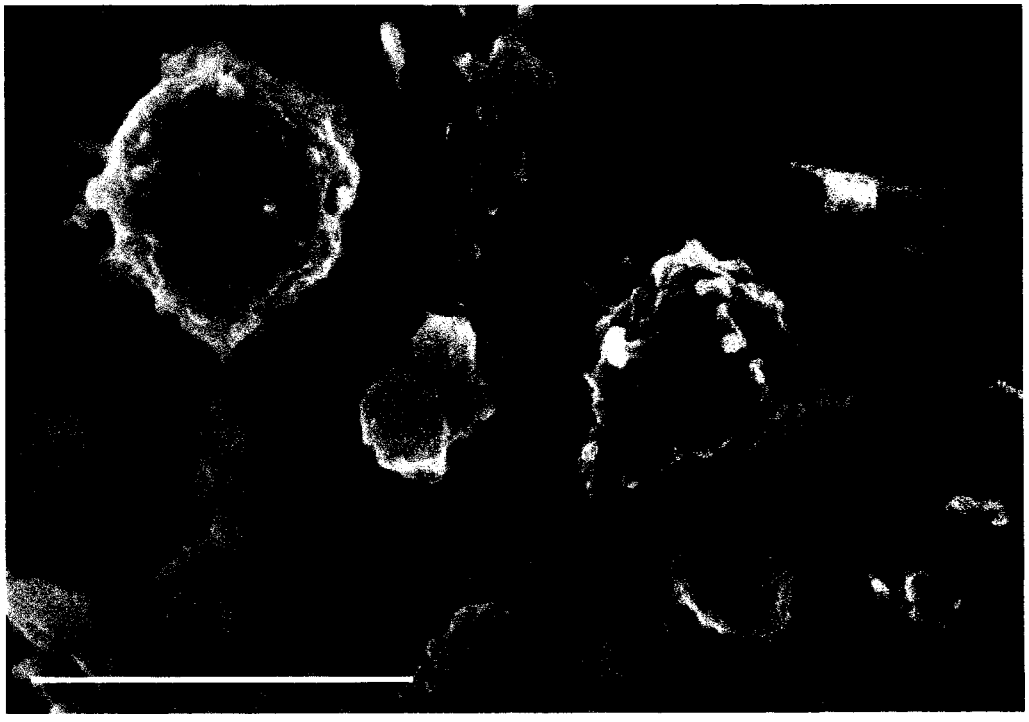


Figure A-11: An 11% w/w skim milk powder and 0.015% w/w κ-carrageenan solution imaged on a carbon substrate with the Hitachi S-4800. Scale bar = 200nm

II. Appendix II

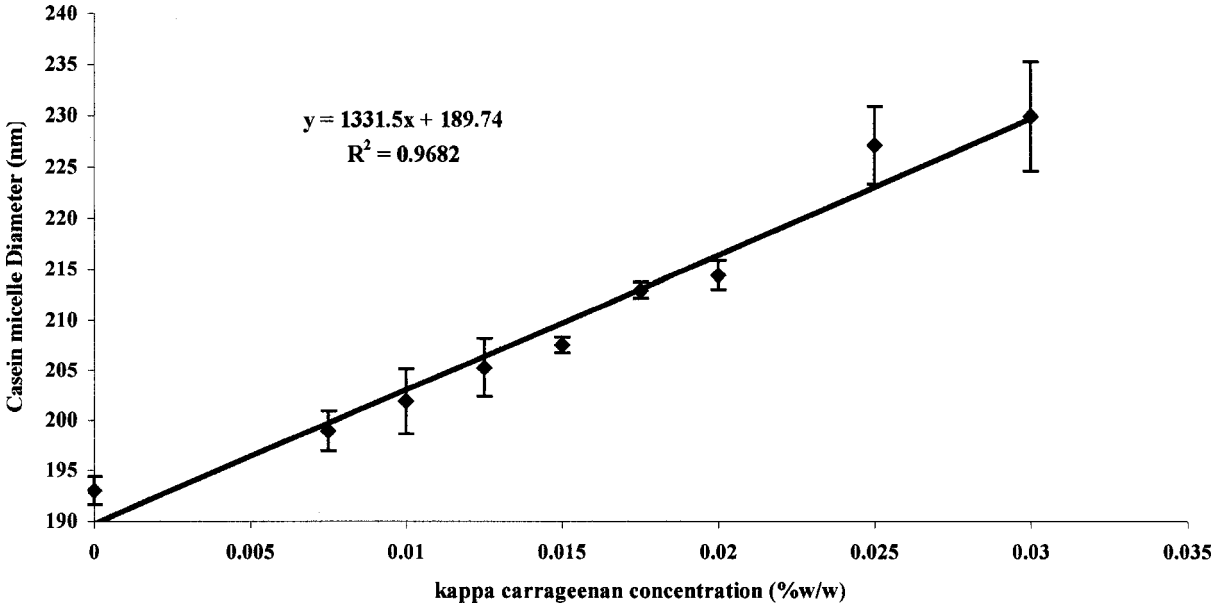


Figure A-12: The effect of κ-carrageenan on casein micelle diameter as measured by DLS.

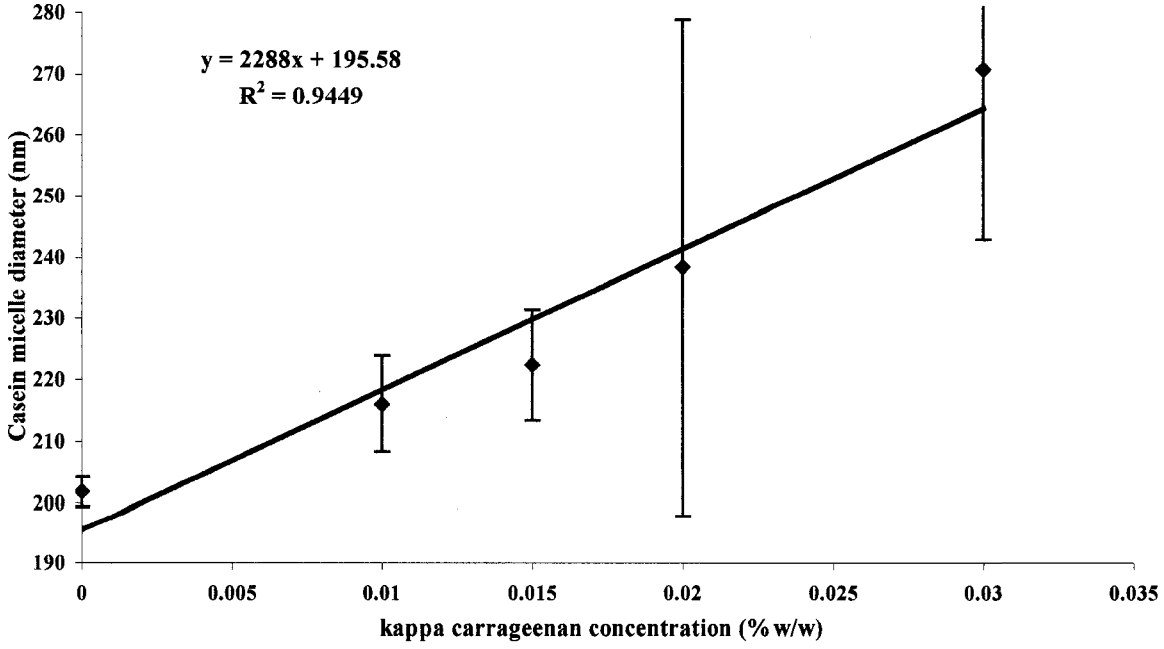


Figure A-13: Effect of κ-carrageenan and added sodium iodide on the size of casein micelle diameter as measured by DLS.

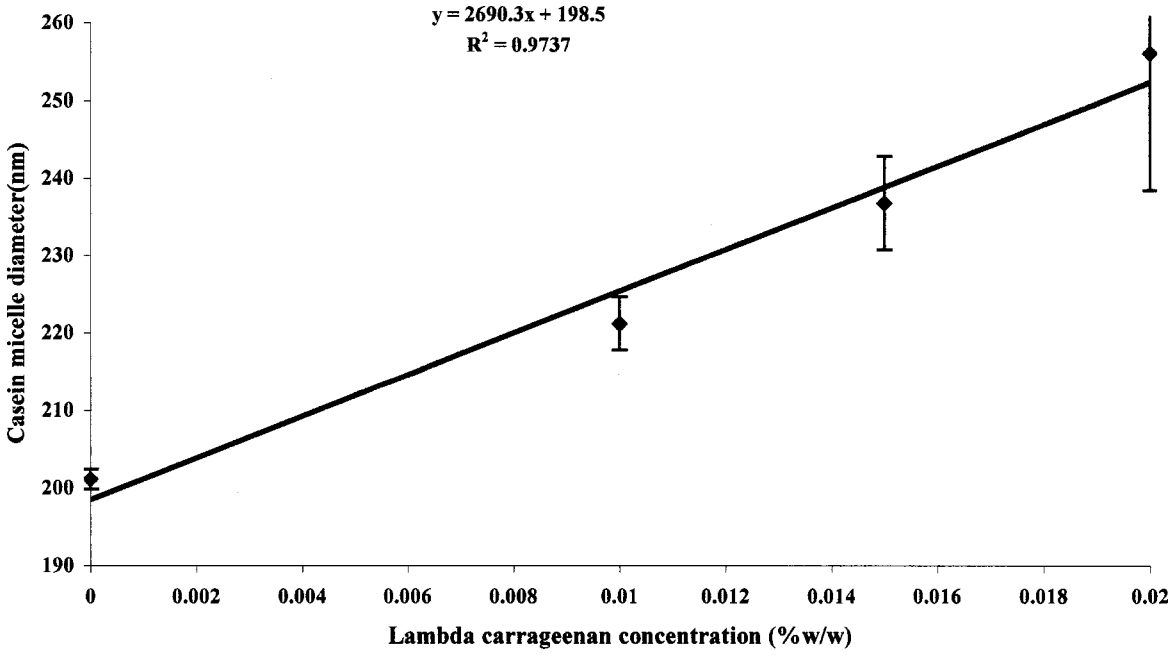


Figure A-14: The effect of λ-carrageenan on casein micelle diameter.

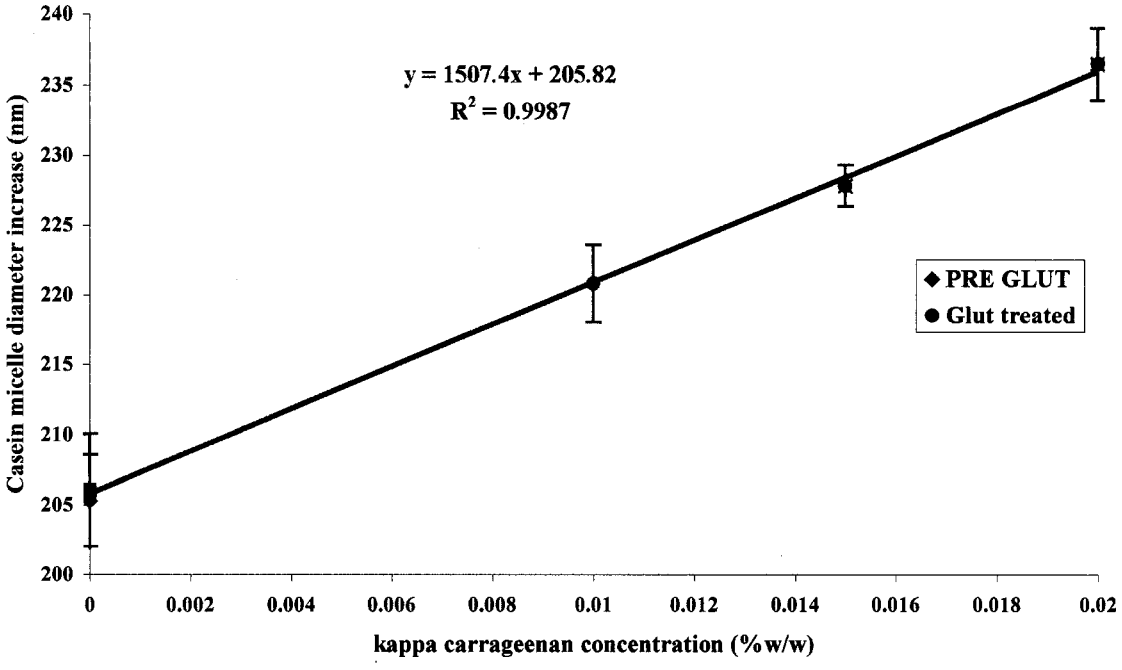


Figure A-15: The effect of κ-carrageenan on casein micelle diameter in glutaraldehyde treated milk measured by DLS.

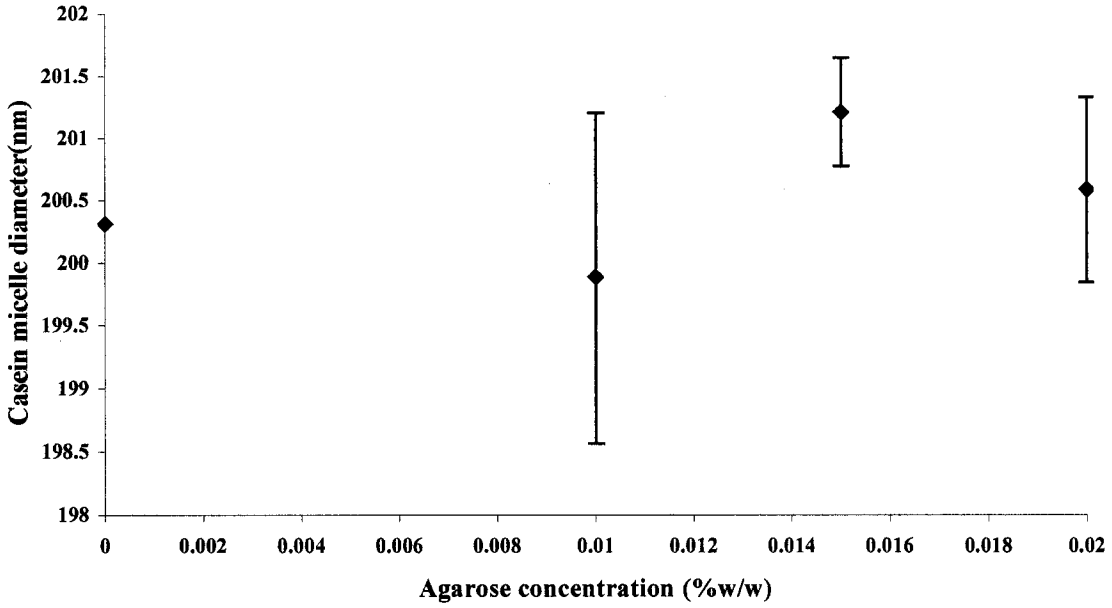


Figure A-16: Effect of agarose on casein micelle diameter as measured by DLS

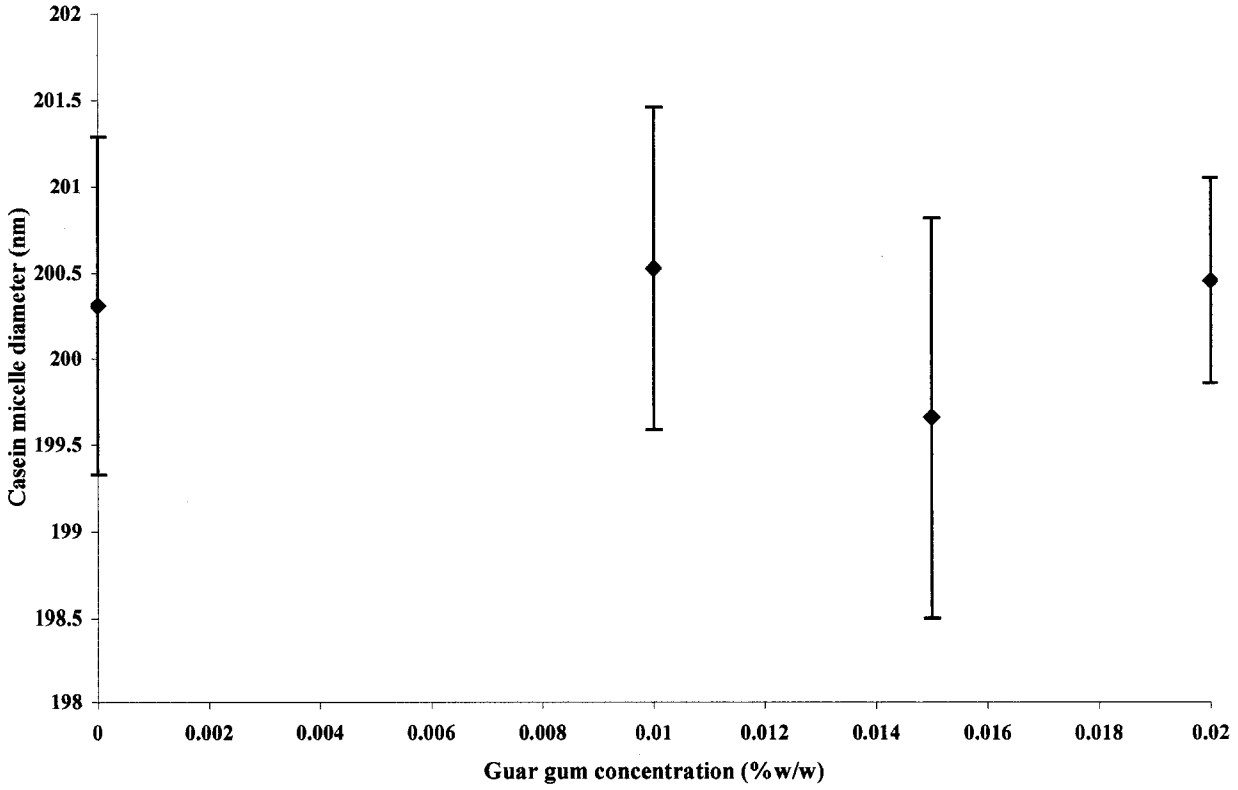


Figure A-17: The effect of guar gum on casein micelle diameter as measured by DLS.

III. Appendix III

Table A-1: Paired t-test analysis on the sample means that indicate that there is no statistical difference between the two sample means.

	Variable 1	*Variable 2*
Mean	2271025.7	2233099.712
Variance	5.98038E+12	9.50107E+12
Observations	2	2
Pearson Correlation	-1	
Hypothesized Mean Difference	0	
df	1	
t Stat	0.009702747	
P(T<=t) one-tail	0.496911617	
t Critical one-tail	6.313748599	
P(T<=t) two-tail	0.993823233	
t Critical two-tail	12.7061503	

- Please note that run 3 was excluded from the paired t test.

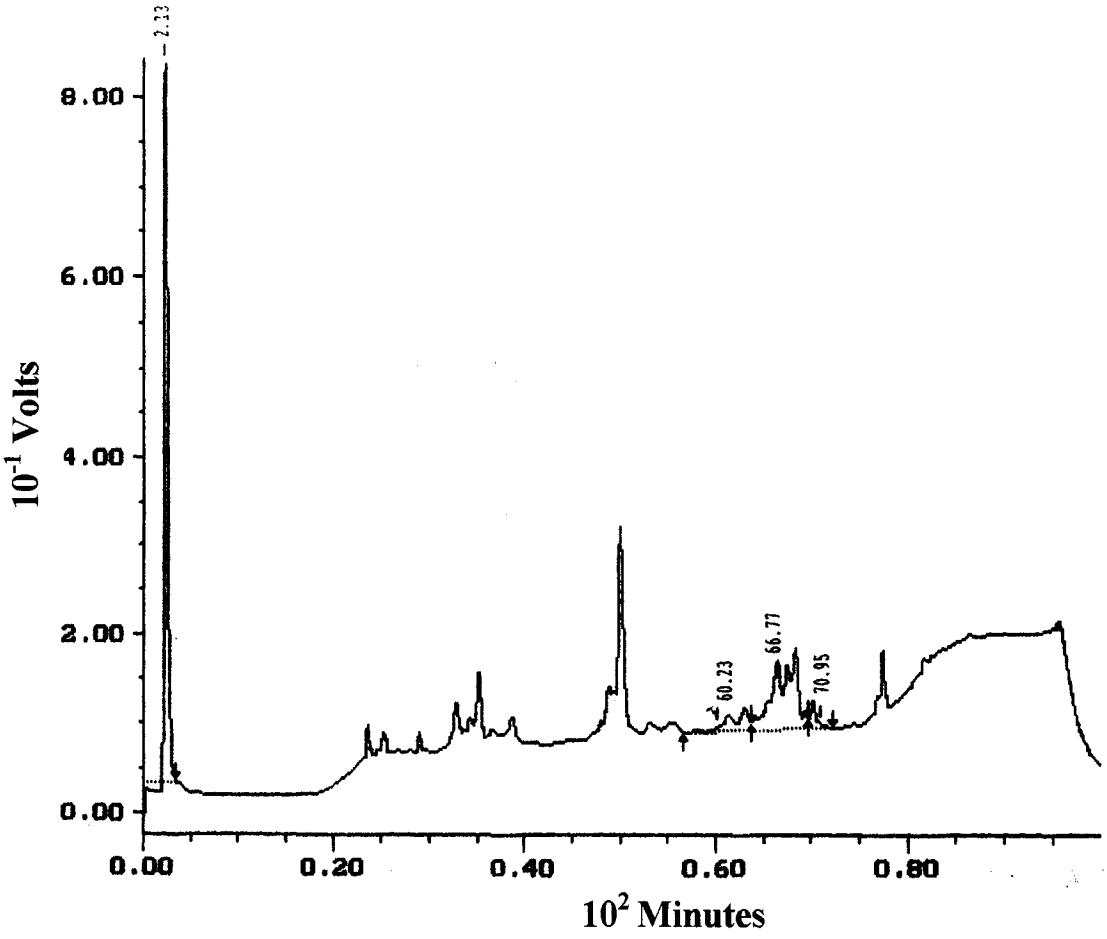


Figure A-18: Chromatograph of a skim milk centrifuged at 4°C that shows the presence of β-casein.

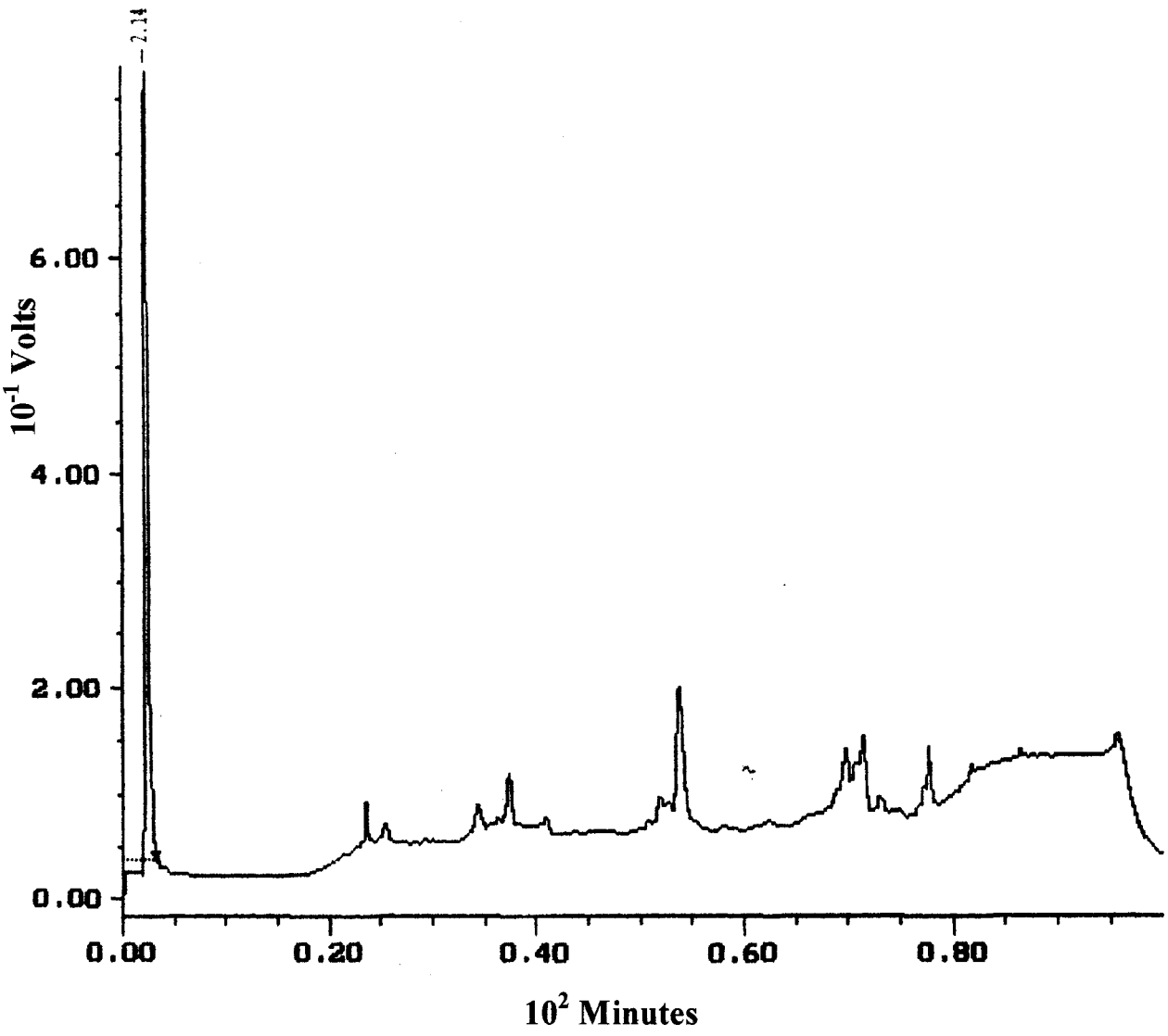


Figure A-19: Chromatograph of a skim milk centrifuged at 4°C that does not show the presence of β-casein