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To the Graduate Council:

I am submitting herewith a thesis written by Raymundo Trejo entitled "Casein Micelles from Bovine Milk: Ethanol Induced Changes in Hydrophobicity and Interaction with Native Whey Proteins." I have examined the final electronic copy of this thesis for form and content and recommend that it be accepted in partial fulfillment of the requirements for the degree of Master of Science, with a major in Food Science and Technology.

Federico M. Harte, Major Professor

We have read this thesis and recommend its acceptance:

Juan Jurat-Fuentes, John R. Mount

Accepted for the Council:
Carolyn R. Hodges

Vice Provost and Dean of the Graduate School

(Original signatures are on file with official student records.)

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A Thesis Presented for the Master of Science Degree
The University of Tennessee, Knoxville

Raymundo Trejo
December 2009

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DEDICATION

This is dedicated to my parents, my grandfather (this one's for you, *buelito*!), and most importantly to my beautiful bride. I could not have done this without them.

"By perseverance the snail reached the ark."
Charles H. Spurgeon

"*It is a great nuisance that knowledge can be acquired only by hard work."*
W. Somerset Maugham

"Live like no one else will, so later you can live like no one else can."
Dave Ramsey

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ABSTRACT

Caseins, in the form of micelles, are the most abundant milk protein. The nature of these micelles is still not fully understood and several models have been proposed. The first chapter discusses this topic, along with the importance of milk proteins to the food industry, and their allergenic properties.

In the second chapter the changes in the hydrophobicity of dissociated casein micelles are explored. As new applications for milk proteins are discovered, it becomes more important to understand their physicochemical properties when subjected to different treatments. It has been reported that casein micelles disassociate when heated in the presence of ethanol. The changes to the hydrophobicity of milk proteins during that process were evaluated by utilizing the fluorescent hydrophobic probe 1-anilinonaphthalene-8-sulfonic acid (ANS). The results showed that the fluorescence intensity of ANS decreased when the samples were heated. Using the information obtained from the fluorescence spectroscopy it was possible to infer that the hydrophobicity of the milk proteins also decreased when the casein micelles disassociate in the presence of ethanol.

The third chapter deals with the association of native casein micelles and whey proteins. This association was thought to only be consequence of whey protein denaturation and interaction with k-casein through S-S linkages. Size exclusion chromatography (SEC) was used to isolate native casein micelles from raw skim milk samples with a pH value of 6.8, 6.0, and 5.5. In a separate experiment, casein micelles were precipitated by lowering the pH of milk to ~4.6, and the

whey was removed and substituted with protein free serum (PFS). Then, the casein micelles were re-suspended in PFS at a native pH. SDS-PAGE was conducted on the samples utilizing silver staining of total proteins for higher sensitivity of detection. Whey proteins were found in association with the casein micelles at all pH values. Samples of both native pH and re-suspended casein micelles were tested with gel electrophoresis under native and reducing conditions. The bands for the whey proteins were only visible under the reducing conditions. These findings demonstrate that whey proteins are also part of the native casein micelle structure.

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CHAPTER I
INTRODUCTION AND LITERATURE REVIEW

MILK PROTEINS AS FOOD INGREDIENTS

Milk has been regarded as the perfect food (Patton, 2004). As mammals, humans are able to survive exclusively on milk during early infancy. Milk serves as a medium for the mother to provide her infant with both nutrition and immunity. Later in life, during childhood and beyond, milk can provide high nutritional value to the daily diet. Therefore, bovine milk is widely accepted as a food source throughout the world. There are a large variety of dairy products; however, most of them are made using a relatively small number of processes. These include heating (at temperatures between 60 and 140 °C), concentration (by evaporation or ultrafiltration), homogenization, fermentation, and treatment with enzymes. These operations may be combined. In addition, milk may be separated, dried and recombined, and milk components may be used as ingredients in other foods (Dalgleish, 1993). Milk proteins are one of these components which are widely used as food ingredients. The popularity of milk proteins as food ingredients is due to several reasons. As well as being nutritious, milk proteins exhibit a range of important functional properties. These include gel, curd, emulsifying, foaming, ion-binding and flavor-binding; all of these are different for the various proteins (Dalgleish, 1993). Traditional milk protein products include: nonfat dry milk powder, whey powder, whey protein concentrate, whey protein isolates, caseins, and caseinates. As such, proteins make up a very small portion of the components in milk. Bovine milk contains generally between 3 and 3.5% by

weight of true protein, the amount depending on the breed and individual variation of the animal and, to a lesser extent, on the stage of lactation, the nutritional status and the health of the animal (Dalgleish, 1993). Nonfat dry skim milk is prepared by separating the milk fat from the milk, usually by centrifugation, and then spray-drying the skim milk. Therefore, this product contains all of the proteins found in milk. Among the milk proteins, casein is the most abundant protein. Caseins, unique milk-specific proteins, represent ≈ 80% of the total protein in the milk of cattle and other commercial dairying species (Fox and Brodkorb, 2008). Casein isolates are usually prepared by isoelectric precipitation at pH 4.6. This method was developed by Hammersten (1883) and improved by van Slyke and Barker (1918). Caseinates are obtained by treating the rennet or acid casein with an alkali (sodium or calcium), which is then dried. The remaining proteins found in milk are found in the milk serum or whey (i.e., the solution which is left when the casein micelles and the fat globules are removed from milk).Whey contains four major proteins, namely β-lactoglobulin (β-Lg), α-lactalbumin (α-La), bovine serum albumin (BSA) and immunoglobulins (Dalgleish, 1993). Whey powders are commonly used as ingredients in the food industry. They are prepared by separating the fat from the whey produced during cheese making and then spray drying the skimmed whey. The most common commercial method to produce whey protein concentrate from the skimmed whey solids uses ultrafiltration to separate the protein fractions. Then the fractions are condensed via evaporation and spray dried to produce a powder. These concentrates contain all of the whey proteins; however uses for the

individual proteins are currently being researched. α-lactalbumin is present at higher levels in human milk than in bovine milk and therefore the fortification of infant formula with respect to this protein is thought to improve its comparability to human milk. Hence, at present, the main interest of the food industry in this protein appears to be infant formula applications (Playne et al., 2003). The functional use of β-lactoglobulin has not been fully established, but there are certain possibilities. As a member of the lipocalin family, biological functions of β-lactoglobulin have been associated with the binding and carrier functions of small hydrophobic molecules in the hydrophobic cleft. Future opportunities for developing the useful functional properties of β-lactoglobulin as a functional food ingredient for humans may lie in the development of its carrier function in either aggregated or soluble forms (Playne et al., 2003)

Milk proteins can be also used as "functional" food ingredients. Functional foods (sometimes referred to as physiologically functional foods, nutraceuticals, designer foods or pharmafoods) can be defined as those that provide the consumer with an identified health benefit over and above basic nutritional value (Playne et al., 2003). One example of a milk protein serving as a functional food ingredient makes use of the Calcium carrier function of the caseins. This food ingredient has been developed by proteolysis and the extraction of the casein phosphopeptide (CPP) class of peptides. The product is commercially available as the food ingredient 'Recaldent™', which contains CPP. This product has been demonstrated to protect against the demineralization of tooth enamel and its application in various oral care and food products is in progress (Playne et al.,

2003). More possibilities exist since the β-lactoglobulin molecule contains a hydrophobic part which can bind vitamin A, vitamin D, calcium, and fatty acids, simplifying their absorption (Ebringer et al., 2008). However, β-lactoglobulin is responsible for milk allergies, which affect 2-3% of children. In the majority of cases these allergies disappear by the time the child reaches three years of age (Ebringer et al., 2008). In contrast to β-lactoglobulin, α-lactalbumin has low immunogenicity and thus also low allergy inducing potential, which make it a good candidate to become a valuable nutrient for children. Furthermore, α-lactalbumin hydrolysis produces peptides with immunomodulatory effects, and it also has stress reducing effects. It has been shown to raise brain serotonin, reduce cortisol concentration and improve mood under stress. It may also exert anti-ulcerative properties (Ebringer et al., 2008).

MILK ALLERGENS

Since milk proteins are an important food ingredient, it becomes vital to understand the role that they can play in food allergies. In order to differentiate between a food allergy and food intolerance it is important to understand the human allergic response. Most cases of food allergy are the “immediate” type in which symptoms develop within 2 hours after the causal food has been ingested. Food allergens absorbed through the digestive tract induce immediate reactions in the alimentary canal mucosa, since this is where they make direct contact with the body. This leads to cardinal symptoms such as vomiting, abdominal pain and diarrhea. In addition, the allergens absorbed through the digestive tract are

systematically delivered via the blood vessels to induce symptoms such as rhinitis in the respiratory tract or symptoms such as hives and angioedema on the skin. They also seem to be involved with the development of atopic dermatitis or anaphylactic shock, which is accompanied by systemic symptoms such as breathing difficulty and decrease in blood pressure, and damages to various organs (Shimada, 2005). These symptoms are the result of the inflammatory response induced by the reaction between the allergens and the body's response. This mechanism is illustrated in figure 1.1.

The mechanism works as follows: Allergens ingested and absorbed by the body are taken up by the antigen-presenting cells, which are then recognized by T-cells. The T-cells activate the B-Cells and convert them into the IgE antibody-forming cells that produce the IgE antibodies that bind to the mast cells. Allergenic stimulation induces degranulation, i.e., the release of chemotransmitters such as leukotriene and histamine from the mast cells, and these chemotransmitters trigger an inflammatory response, inducing the allergic reaction (Shimada, 2005).

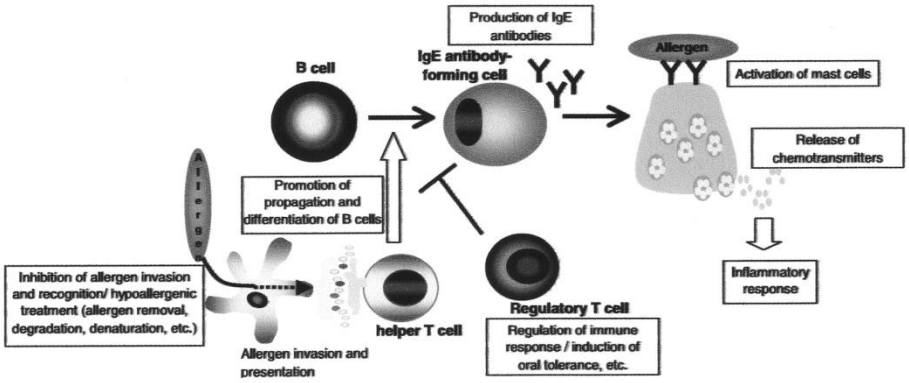

Figure 1.1. Immune mechanism underlying immediate allergy (From Shimada et al. 2005).

Milk allergy is primarily observed in infants and young children. In a number of epidemiological studies, cow's milk protein allergy is reported with an incidence of 2-4% and it's therefore the most common allergy in early infancy. In the majority of infants with cow milk allergies, more than two organ systems are involved. The severity of symptoms varies from mild to life-threatening (anaphylactic shock) (Fritsche, 2003). Milk allergies tend to disappear after childhood and are rare in adults, as compared with other food allergies. However, the majority of adult patients (67%) reported severe symptoms (pulmonary and cardiovascular symptoms) and 27% even experienced and anaphylactic shock (Lam et al., 2008). Several milk proteins can be considered as allergens; however, classically it has been accepted that the major allergen in cow's milk is β-lactoglobulin. This protein is normally absent from human breast milk; however, it can be occasionally detected in human milk. The source of this β-lactoglobulin is probably from ingested cow's milk. The relative resistance of β-lactoglobulin to acid hydrolysis, as well as to proteases, allows some of the protein to remain intact after digestion. This increases the probability that intact β-lactoglobulin, as well as digested fragments, will be absorbed as antigens (Wal, 2002). The prescence of β-lactoglobulin in human milk may cause colic in breast-fed infants (Jakobsson et al., 1985). Although there is a great similarity between the α-Lactalbumin protein produced by cows and humans, it is also considered an allergen in cow milk. The complete amino-acid sequence of bovine α-Lactalbumin shows extensive homology with human α- Lactalbumin, since 74%

of the residues are identical and another 6% chemically similar (Findlay and Brew, 1972). Among the caseins, the major allergens are the calcium-sensitive α- and β-caseins (Wal, 2002).

MILK CASEIN MICELLE

As previously stated casein makes up the majority of milk proteins. Because of this fact, research involving caseins can be dated to the early nineteenth century. The term "casein micelle" was first utilized by Beau in 1921 (Fox and Brodkorb, 2008). Interest in the casein micelle has remained constant through the years, and research on the subject continues to be carried out throughout the world. Since many of the technologically important properties of milk, e.g., its white color, stability to heat or ethanol and coagulation by rennet, are due to the properties of the casein micelles, there has been an economic and technological incentive to characterize their properties and elucidate their structure (Fox and Brodkorb, 2008). The properties of the casein micelles are summarized in table 1.1.

By design, milk serves a medium for the mother to provide nutrients and minerals to their offspring. The principal mineral present in milk is calcium. The dry matter of bovine casein micelles is ~ 94% protein and ~ 6% mineral, referred to collectively as colloidal calcium phosphate (CCP). This CCP is regarded as the cement which holds the micelle together, since its removal by the EDTA sequestration of calcium or by dialysis against a calcium phosphate free buffer results in dissolution of the micelles at lower temperatures (Horne, 2006).

Table 1.1 Average characteristics of casein micelles. Fox et.al. 2008

Characteristic	Value
Diameter	120 nm (range: 50– 500 nm)
Surface area	8×10^{-10} cm^2
Volume	2.1×10^{-15} cm^3
Density (hydrated)	1.0632 g cm^3
Mass	2.2×10^{-15} g
Water content	63%
Hydration	3.7 g H_2O g^{-1} protein
Voluminosity	44 cm^3 g^{-1}
Molecular mass (hydrated)	1.3×10^9 Da
Molecular mass (dehydrated)	5×10^8 Da
No. of peptide chains	5×10^3
No. of particles per mL milk	$10^{14} – 10^{16}$
Surface of micelles per mL milk	5×10^4 cm^3
Mean free distance	240nm

Due to the large amount of calcium present within the casein micelle it is generally believed that it serves as a mechanism for calcium transfer in mammals (Patton, 2004).

In their review of the importance of the casein micelle to the food industry, Fox and Brodkorb further illustrate that in good quality milk, the casein micelles are stable to most processes to which milk it is normally subjected:

- Concentration by evaporation or ultrafiltration: However, stability decreases with the degree of concentration, due mainly to the closer packing of casein micelles, an increase in [Ca^{2+}] and a decrease in pH due to the precipitation of CaH_2PO_4 and $CaHPO_4$ as $Ca_3(PO_4)_2$ accompanied by the release of H+.
- Dehydration: In the absence of heat-induced changes, the micelles in milk powder reconstitute readily and their properties are changed little.

- Freezing: Freezing per se has little, if any, effect on the casein micelles but slow freezing and storage at a temperature in the range -10 to -20 ˚C cause destabilization due to an increase in [Ca^{2+}] and a decrease in pH, due to the precipitation of $Ca_3(PO_4)_2$; these effects are exacerbated by the crystallization of lactose, which lowers the freezing point of milk .
- Homogenization: Normal homogenization of milk, i.e., up to 20 MPa, has little or no effect on the casein micelles, but high-pressure homogenization (>200 MPa) or high-pressure treatment >200 MPa cause some dissociation.
- Heat treatment: HTST pasteurization (72 ˚C x 15s) has little or no effect on the casein micelles but heating at a higher temperature causes denaturation of the whey proteins and their interaction with the casein micelles via sulphydryl-disulphide interactions, especially between β-lactoglobulin and κ-casein. Severe heating, especially of concentrated milk, causes Maillard browning, a decrease in pH, dissociation of κ-casein from the micelles, and, eventually, coagulation.

The micelles can be destabilized by a number of factors, some of which are industrially important: hydrolysis of the κ-casein by selected proteinases (rennets), which is exploited in the manufacture of most cheese varieties; acidification to about pH 4.6, which is exploited in the manufacture of some cheeses, fermented milks and functional caseinate products; ethanol (or other alcohol); anionic detergents, e.g., SDS; high pressure (Fox and Brodkorb, 2008).

In spite of the large amount of research conducted, there is still not a definitive model for the casein micelle structure. Several models have been proposed based on the properties and behavior of the individual caseins and of the casein micelles. The most accepted structure models are the coated sphere model, the sub-micellar model, the Holt model and the Horne model.

The coated sphere models takes into consideration the properties of κ-casein, which is soluble at the calcium concentration in milk and which is about 12% of total casein, and it can stabilize about 10 times its mass of Ca-sensitive caseins (α_{s1}-, α_{s2}- and β-) (Fox and Brodkorb, 2008). In this model, the casein micelles are coated with a "hairy layer" of protein, mainly κ-casein. For this type of model the internal structure is not relevant, unless it changes in the course of processing. The coated sphere model is consistent with the observation that most of the κ-casein is on the micellar surface. It is also consistent with the fact that the micellar hydrodynamic diameter has been shown to decrease during renneting in dilute suspensions. Since the action of chymosin removes the protruding macropeptide portion of the κ-casein molecule, the decrease in micellar diameter is consistent with the loss of the hairy layer (Dalgleish et al., 2004). κ-casein also plays a role in the sub-micelle model, where the individual caseins come together in their appropriate proportions to form internal sub-micelles. An example of the sub-micelle model can be seen in figure 1.2. In the sub-micelle model, the micelle is held together mostly by the presence of κ-casein. However, if the casein micelle is depleted in κ-casein, or lacks external sub-units rich in κ-casein, then the colloidal calcium phosphate is regarded as the

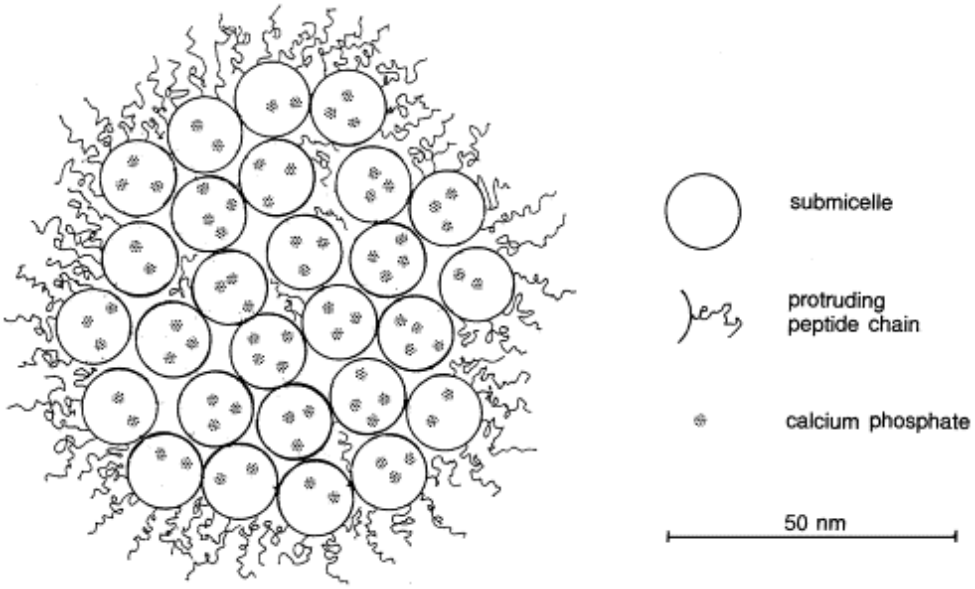

Figure 1.2 Sub-micelle Model (cross section) of a casein micelle (From Walstra 1999)

cement which links these sub-units together. In the Holt model, which is illustrated in figure 1.3, the micelle is regarded as a mineralized, cross-linked protein gel, the colloidal calcium phosphate nanoclusters are the agents responsible for cross-linking the proteins and holding the network together (Horne, 1998).

The formation of nanoclusters would drive micelle formation by randomly binding phosphoproteins causing an inverted micelle, then more proteins could coat this new hydrophobic surface and in turn, bind more calcium phosphate until a size limited colloid is formed (Farrell et al., 2006).

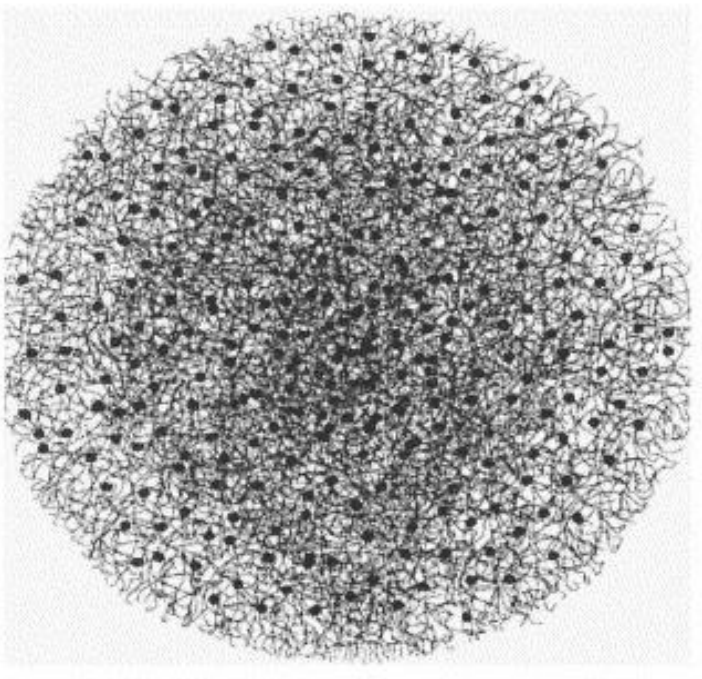

Figure 1.3. The Holt model of casein micelle structure (From Farrell et.al. 2006.

The Horne model, illustrated in figure 1.4, can be considered an extension of the Holt model. Horne, in contrast to Holt, considered the surface chemistry of the individual caseins and concluded that protein-protein interactions were indeed important, but in essence the model retains the gel concept. The amphiphilic nature of the caseins causes them to act more as block copolymers of alternating charge and hydrophobicity. Here again the growth of the calcium phosphate nanoclusters begins the process of micelle formation, but nanocluster growth is limited by binding to the phosphopeptide loop regions. Once bound to the amorphous inorganic matrix, further protein-protein interactions are related to the hydrophobic blocks and polymerization proceeds by repeating the entire process.

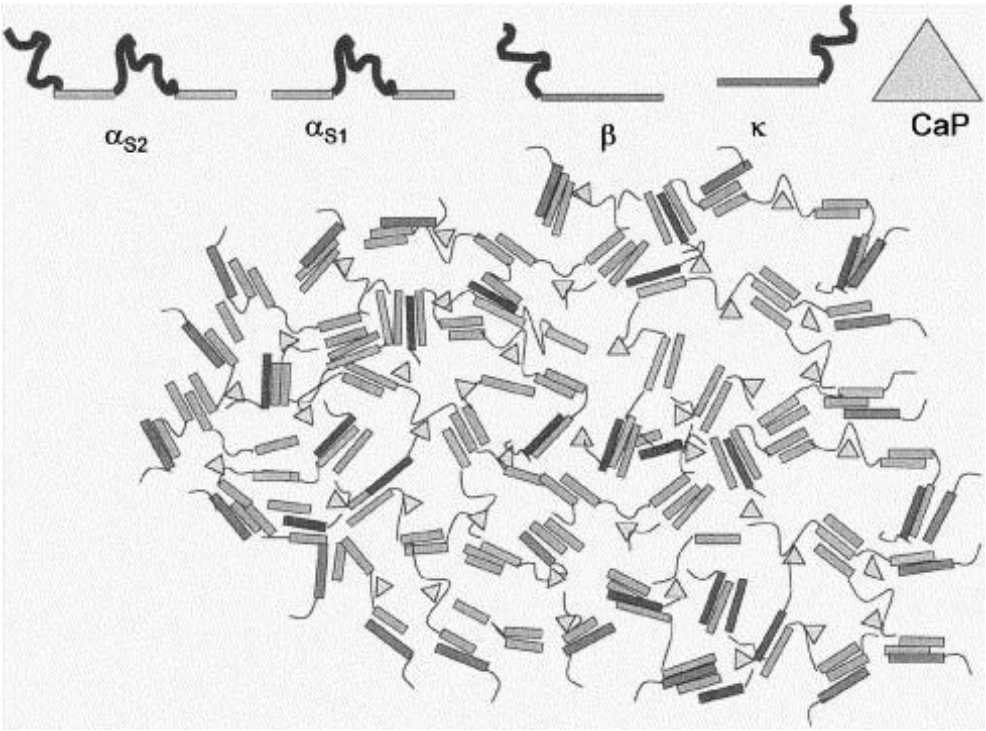

Figure 1.4. The Horne model of the casein micelle (From Farrell et.al. 2006)

Micelle formation leads to an internal gel-like structure with embedded nanoclusters of calcium and phosphate, and the reaction of κ-casein which contains only one phosphoserine residue limits micellar growth by acting as a dead end capping unit in analogy with the growth of synthetic polymers (Farrell et al., 2006).

The controversy and debate over the correct model of the milk casein micelle is continuously evolving, as new research uncovers further clues into the nature of the casein micelle. One such development involves the use of electron microscopy studies, from which Dalgleish et al. have proposed a possible new model for the milk casein micelle. Their results seem to show that the micelle consists of

tubules, presumably of caseins, the ends of which protrude from the bulk of the micelle structure. These tubules are about 20nm in diameter, which is consistent with the dimensions of the calcium phosphate/casein nanoclusters proposed by Holt (Holt et al., 1998). However, the micelle is still protected from close approach of large particles (such as other micelles) by the protruding tubules. On the other hand, we can see that there is a large amount of space, which is available for the approach of individual protein molecules, or even small aggregates of proteins (Dalgleish et al., 2004). It is generally known that whey proteins, primarily β-lactoglobulin, interact with the casein micelles as a result of heat treatments. β-lactoglobulin attaches to the micellar κ-casein through disulphide links. Apparently, even polymerized β-lactoglobulin can bind to the casein micelles in this way, so that the casein micelles may acquire new surfaces and therefore develop new properties, the change being dependent on the severity of the heat treatment (Dalgleish, 1993).

OUTLINE AND OBJECTIVES

The milk casein micelle is a fundamental component of milk. Therefore, it is of great importance to understand the behavior of the casein micelle when subjected to different treatments and conditions. The second chapter of this thesis deals with the changes to the properties of the casein micelle when subjected to heat treatment in the presence of ethanol.

It is also important to understand the interactions that the casein micelles have with other milk proteins. Chapter three of this thesis presents the association of whey proteins with casein micelles in native raw skim milk. This is a controversial new discovery, since it was widely accepted that whey proteins only interacted with the casein micelles as a result of heat or pH induced denaturation.

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CHAPTER II
HYDROPHOBICITY OF ETHANOL INDUCED DISSOCIATED CASEIN MICELLES

This chapter is a lightly revised version of a paper by the same title to be submitted to the *Journal of Dairy Science* by Raymundo Trejo and Federico M. Harte. The use of "our" in this chapter refers to my co-author and me. My primary contributions to this paper include (1) the experimental work, (2) the collection and analysis of data, (3) the gathering and interpretation of literature, and (4) the writing.

ABSTRACT

Milk proteins are a very important ingredient to the food industry. As new uses and applications for these proteins are discovered, it becomes more important to understand their physicochemical properties when submitted to different treatments. It has been reported that casein micelles disassociate when heated in the presence of ethanol. The changes to the hydrophobicity of milk proteins during that process were evaluated by utilizing the fluorescent hydrophobic probe 1-anilinonaphthalene-8-sulfonic acid (ANS). The results showed that the fluorescence intensity of ANS decreased when the samples were heated. Using the information obtained from the fluorescence spectroscopy it was possible to infer that the hydrophobicity of the milk protein also decreased when the casein micelles disassociate.

INTRODUCTION

Milk continues to be a fundamental element of the human diet, and the use of milk proteins in the food industry continues to increase as more applications are developed. Numerous studies have been performed to understand the physicochemical properties of milk proteins. Hydrophobicity appears to be an important property of proteins, highly related to their stability, conformation, and functionality (Kato and Nakai, 1980). The surface (or effective) hydrophobicity is considered to be one of the most important properties determining protein functionality (Gatti et al., 1995). The net hydrophobicity of the proteins can vary due to conformational changes induced by the physicochemical stimuli to which milk is subjected during processing and storage (Bonomi and Iametti, 1991, Bonomi et al., 1988, Gatti et al., 1995). Heat treatment can lead to conformational changes in the proteins, such as denaturation and structural rearrangement, which in turn may increase exposure of hydrophobic amino acid residues, altering the functional properties of the protein (Nakai, 1998).

It has been reported that the milk casein micelle undergoes a semi-reversible disassociation when heated up to 60 °C in the presence of ethanol (Dunkerley et al., 1993, O'Connell et al., 2001, Zadow, 1993). When heated, a naturally opaque solution containing milk and at least 30% ethanol (v/v) becomes translucent. The change in color is due to the disassociation of the casein micelles into its single casein constituents. However, this change is reversible and the solution will

regain its opaque color upon cooling. The objective of this study was to determine the changes in hydrophobicity of the casein micelles during disassociation. Fluorescent markers have been used effectively to monitor hydrophobicity changes in proteins (Alizadeh-Pasdar and Li-Chan, 2000, Gatti et al., 1995, Hayakawa and Nakai, 1985, Risso et al., 2008). The fluorescent marker 1-anilinonaphthalene-8-sulfonic acid (ANS) has been utilized on hydrophobicity studies due to its affinity for hydrophobic sites on proteins (Bonomi et al., 1988). We monitored the changes in fluorescence intensity of ANS to effectively track the variations in the surface hydrophobicity of casein micelles during the heat and ethanol induced disassociation.

MATERIALS AND METHODS

MILK SOURCE & SAMPLE PREPARATION

Commercially available pasteurized skim milk was obtained from a local grocery store, and stored at 4 °C. Raw milk was obtained from a local dairy farm and skimmed by centrifugation (6414 X*g* for 20 min). A solution of whey protein isolate (BiPro, Davisco Foods International, Inc., Eden Prairie, MN) was prepared by mixing the isolate with de-ionized water (w/v) to obtain a protein concentration similar to the concentration of whey proteins found in milk (0.8%). Samples containing milk, ethanol and de-ionized water were prepared using the following procedure: all of the samples contained 4% milk (v/v) for fluorescence studies and 10% (v/v) for absorbance studies, in one of the following solutions:

0%, 10%, 20%, 30%, 40%, 50%, and 60% v/v ethanol (99 proof, Fisher Scientific, Fair Lawn, NJ) in de-ionized water. The temperature of the samples was maintained by using a temperature controlled water bath (Fisher Scientific, Fair Lawn, NJ). Samples containing raw milk and samples containing whey protein isolates (WIP) were also prepared as controls.

SPECTROPHOTOMETRY AND SPECTROPHOTOFLUROMETRY

Absorbance values for the samples were measured using a spectrophotometer (Genesys2, Thermo Spectronic, Madison, WI) at a wavelength of 500 nm. The samples were measured at 10 °C intervals from 20 °C to 60 °C.

Fluorescence spectroscopy was done following the methodology previously used by Bonomi and Alturner with modifications (Altuner et al., 2006, Bonomi et al., 1988). Briefly, six stock solutions of 1-anilinonaphthalene-8-sulfonic acid (ANS; Fisher Scientific, Fair Lawn, NJ) were prepared to insure a final ANS concentration in the measurement cuvette of 2, 4, 6, 8, 10, and 12 x 10^{-9} M when 50 µl of the stock solution were added to a 2 ml volume of the sample. The stock solutions were prepared from an initial 0.1 M ANS in de-ionized water solution. The samples containing ANS were constantly stirred inside the cuvette to insure proper mixing, and controlled temperature water was constantly circulated through the cuvette holder to maintain the sample temperature. Fluorescence measurements were done using a RF-1501 spectrophotofluorometer (Shimadzu, Columbia, MD) set at λ_{EX} = 390 and λ_{EM} = 400 – 500. The intrinsic fluorescence values of all the samples without ANS were measured as controls. These

measurements were taken at 10 °C intervals from 20 °C to 60 °C. After measuring the intrinsic fluorescence, a set volume of 50 µl of stock ANS solution was added to the cuvette and the relative fluorescence intensity value was recorded. This procedure was repeated for all of the ANS concentrations to obtain a concentration gradient. Separate cuvettes were used for each of the stock solutions.

The change of fluorescence intensity ($\Delta I_{f\,(ANS)}$) was calculated by using the following formula: $\Delta I_{f\,(ANS)} = I_{f\,(sample+ANS)} - I_{f\,(sample)}$. This measured the amount of the fluorescence intensity that is due to the ANS interaction with hydrophobic zones.

HYDROPHOBICITY CALCULATIONS

Hydrophobicity of the milk proteins was calculated based on the method described by (Kato and Nakai, 1980) with modifications. For λ_{EM} = 390 nm, peak fluorescence was found at λ_{EM} = ~ 450 nm for all treatments and this emission wavelength was used for hydrophobicity calculations. The fluorescence intensity peak of ANS when interacting with hydrophobic zones has been recorded at λ_{EM} = 480 nm (Bonomi et al., 1988). However, these experiments did not involve ethanol, which can account for the shift on the peak wavelength (Lakowicz, 2006). Hydrophobicity was calculated as the slope of the line between relative fluorescence at 2.0 x 10^{-9} M and 4.0 x 10^{-9} M ANS. Higher ANS concentrations (> 4.0 X 10^{-9} M) resulted in saturation of the fluorescence intensity readings for some of the samples and were not used for hydrophobicity calculations.

RESULTS AND DISCUSSION

ABSORBANCE

The pasteurized skim milk samples with higher concentrations of ethanol, 40% to 60%, showed a temperature dependent decrease in the absorption value as shown in figure 2.1. The samples containing less than 30% ethanol showed little or no decrease in their absorbance values. The observations were consistent with previous reports using absorbance to measure changes mediated by heat and ethanol of reconstituted skim milk powder and raw skim milk (O'Connell et al., 2001, O'Connell et al., 2003).

When the temperature was above 45 ˚C the samples containing 50% ETOH became translucent, which is consistent with previous reports (Dunkerley et al., 1993, Zadow, 1993) that reported changes in L- values for milk and ethanol mixes. The decrease in the values can be utilized as a means to confirm the dissociation of the milk casein micelles when heated in the presence of ethanol.

INTRINSIC FLUORESCENCE

Due to the presence of natural fluorophores, the intrinsic fluorescence of raw skim milk, pasteurized skim milk, and WPI samples prior to the addition of ANS was measured (λ_{EX} 390, λ_{EM} 450). The properties of these fluorophores are sensitive to environmental conditions and changes in the three-dimensional structure of the proteins (Kulmyrzaev et al., 2005). Both skim milk samples showed intrinsic fluorescence at the excitation parameters utilized in the

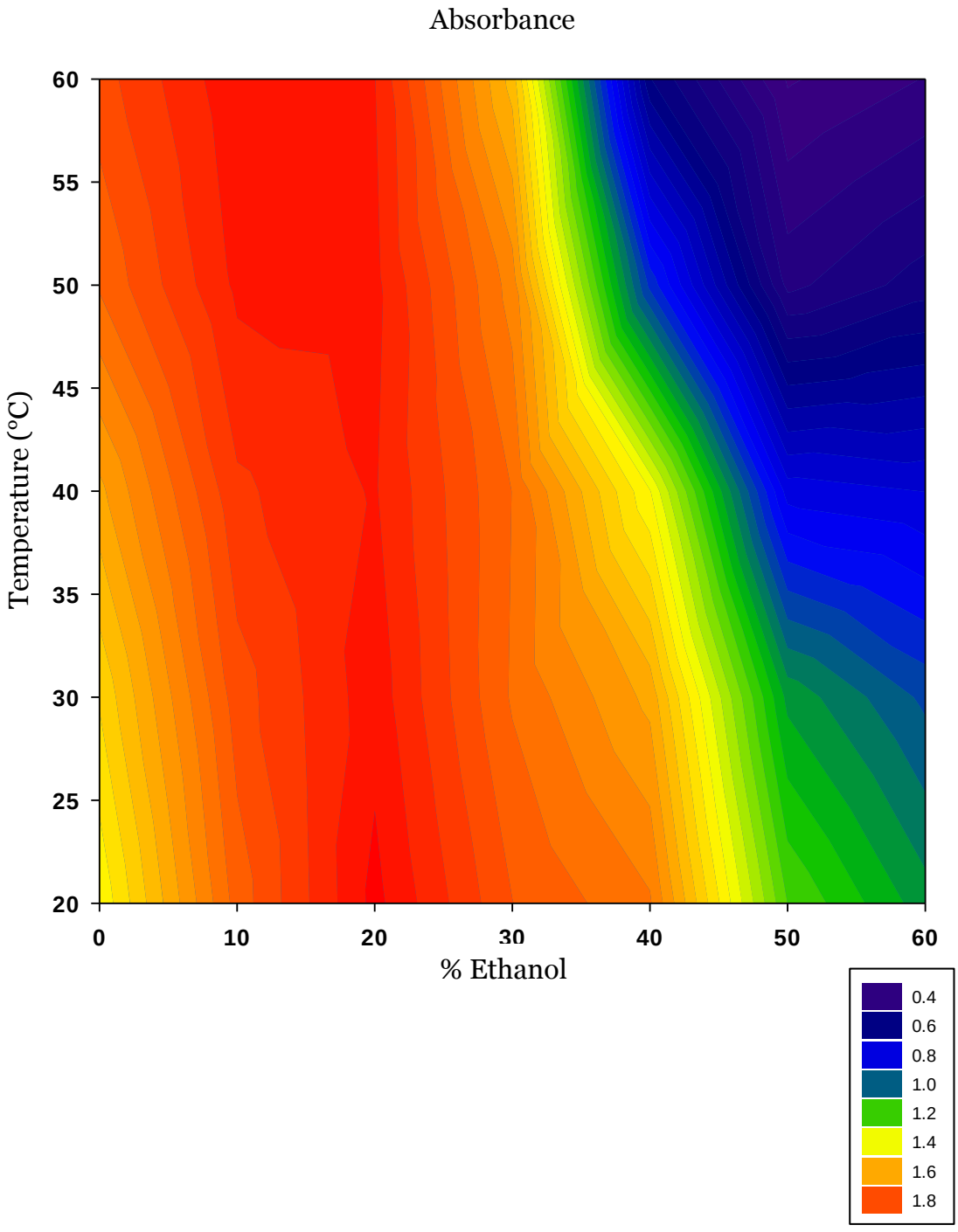

Figure 2.1. Absorbance of pasteurized skim milk (10% v/v) solution measured at 500 nm.

experiment. The intrinsic fluorescence was affected by the temperature change and by the ethanol concentration. The increase of temperature caused a decrease in the intrinsic fluorescence of the milk samples. In samples containing less than 30% ethanol there was no decrease in the intrinsic I_f due to the increase in temperature. However, for samples that contained more than 40% ethanol the increase in temperature resulted in a decrease in the intrinsic I_f of the sample. In order to confirm that the fluorescence could be attributed to the milk proteins, and especially to interactions within the casein micelles we recorded the fluorescence intensity of samples containing only whey protein isolates. As seen in figure 2.2, the skim and raw milk samples showed very similar patterns. The whey protein isolates showed very little intrinsic fluorescence intensity to the point to which any fluorescence produced by them in the sample was negligible.

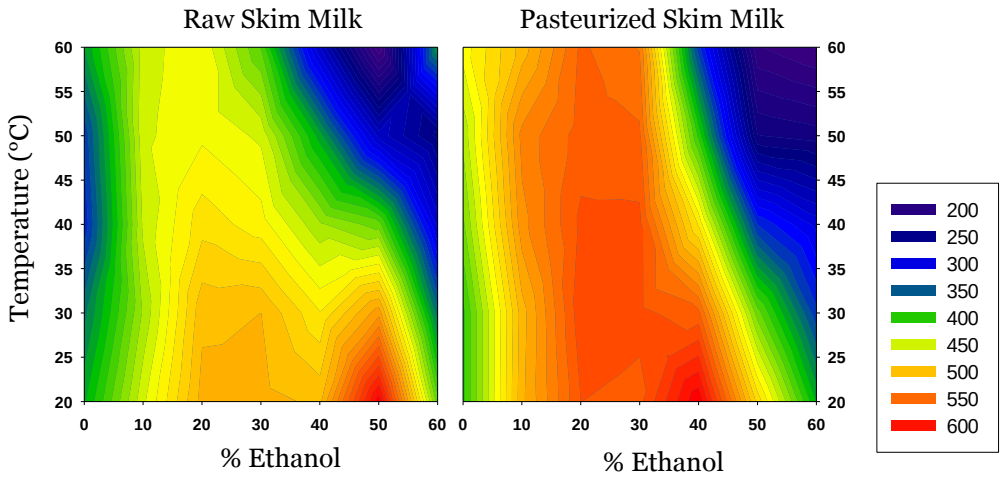

Figure 2.2. Intrinsic Fluorescence Intensity (λ_{EX} 390, λ_{EM} 450) of raw skim milk, pasteurized skim milk..

The intrinsic fluorescence of raw milk has been reported to decrease as heat is applied (Kulmyrzaev et al., 2005). Kulmyrzaev used front-face fluorescence to measure the effects of heat treatments on raw milk. It was observed that the intrinsic fluorescence of raw milk decreased after it was heated to 72 °C for 30 minutes. In our experiments, both the raw and pasteurized skim milk samples showed a behavior inconsistent with these reports. The intrinsic fluorescence of milk tends to remain constant when no ethanol was added, but shows a similar pattern of decrease when heat is applied in the presence of ethanol. Therefore, the intrinsic fluorescence of milk does change when it is subjected to more aggressive treatment. The observed behavior of the intrinsic fluorescence showed a correlation with the observed absorbance values with correlation values for the raw skim milk of .66 ($p < 0.0001$) and for pasteurized skim milk .92 ($p < 0.0001$).

CHANGE OF FLUORESCENCE INTENSITY

The fluorescence intensity of the raw skim milk and pasteurized skim milk samples produced with the addition of ANS ($I_{f\,(ANS)}$) at a concentration of $2x10^{-9}$ can be seen in figure 2.3. For all of the samples the $I_{f\,(ANS)}$ values decreased as both the temperature and ethanol concentration increased. The largest decrease was observed on the raw and pasteurized skim milk samples containing more than 50% ethanol. As seen in figure 2.4, it is interesting to note the positive ethanol dependent change of ΔI_f of the WPI samples due to the addition of ANS.

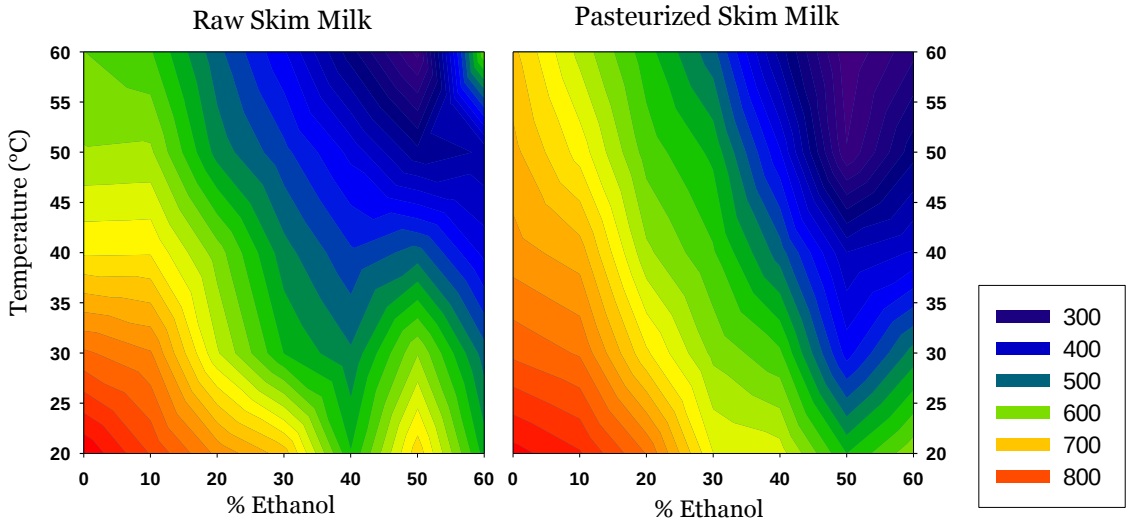

Figure 2.3. Fluorescent intensity (λ_{EX} 390, λ_{EM} 450) of raw skim milk, pasteurized skim milk, and whey protein isolates with the addition of ANS to achieve a concentration of $2x10^{-9}$ M.

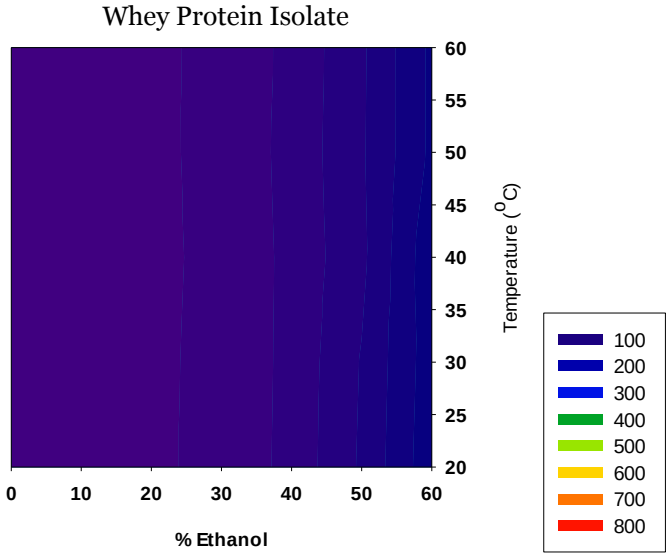

Figure 2.4. Fluorescent intensity (λ_{EX} 390, λ_{EM} 450) of whey protein isolates with the addition of ANS to achieve a concentration of $2x10^{-9}$ M.

While the WPI samples showed very little native intrinsic fluorescence, the addition of ANS caused an increase on the I_f for the samples with a higher ethanol concentration regardless of the temperature. This will then influence the $\Delta I_{f\,(ANS)}$ for the raw and pasteurized skim milk samples due to some of the fluorescence in the samples being produced by the whey proteins. In our study, we utilized native WPI, therefore we only adjusted the $I_{f\,(ANS)}$ for the raw skim milk sample. This adjusted $I_{f\,(ANS)}$ can be seen in figure 2.5. While the unadjusted $I_{f\,(ANS)}$ for the raw skim milk sample had a weak moderate correlation (.58, $p < 0.001$) with the absorbance results, the adjusted $I_{f\,(ANS)}$ values had a strong correlation values (.71, $p < 0.001$) with the absorbance. The $I_{f\,(ANS)}$ values for the pasteurized milk sample also showed a strong correlation (.82, $p < 0.001$) with the absorbance results. This further indicates that the decrease in I_f for the samples is related to the milk casein micelle dissociation.

Changes in the I_f of milk proteins interacting with ANS have been reported in previous fluorescent studies involving the heat treatment of milk proteins, yet without the presence ethanol. A decrease in I_f was reported in studies utilizing κ-casein and ANS solutions (λ_{EX} 350, λ_{EM} 480) in which as the temperature increased from 4 to 60 °C there was a decrease in relative fluorescent intensity (Clarke and Nakai, 1972).

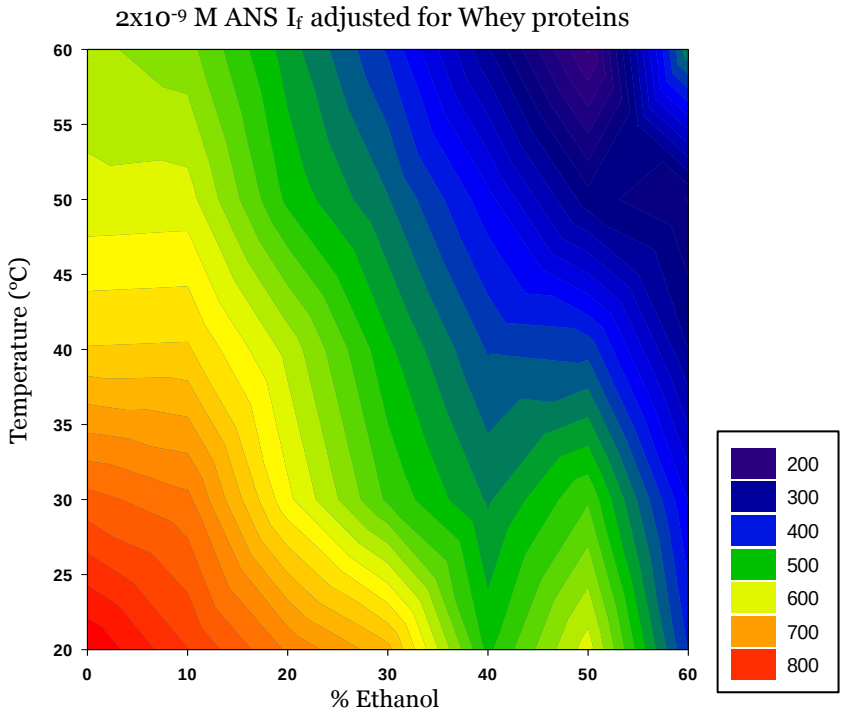

Figure 2.5. Fluorescence intensity (λ_{EX} 390, λ_{EM} 450) of casein and 2x10^{-9} M ANS obtained by removing the fluorescence intensity of whey protein isolates with 2x10^{-9} M ANS concentration from the fluorescence intensity of a raw skim milk + 2x10^{-9} M ANS concentration.

Clarke and Nakai proposed that the decrease must be the result of structural changes, involving hydrogen bonding. This behavior is consistent with the decrease of $I_{f\,(ANS)}$ of the raw and pasteurized skim milk samples observed in figure 2.3. Previous studies on the intrinsic fluorescence of α-Lactalbumin and ANS samples (λ_{EX} 390, λ_{EM} 480) when heated from 20 to 75 ˚C (Eynard et al., 1992) showed a behavior that differed from our results. In our observations, the I_f of the WIP increased as the ethanol concentration of the samples increased, but showed no changes due to the changes in temperature. However, in their study, the samples showed a slight yet constant decrease in fluorescence as the temperature increased. The greater decrease in the I_f was not observed until the

samples were heated past 60 ˚C which is a temperature higher than the one utilized in our studies.

The ΔI_f results are also consistent with the prior reports of the behavior of ANS as the hydrophobicity of milk proteins changes (Bonomi and Iametti, 1991, Bonomi et al., 1988, Risso et al., 2008). The original increase in fluorescence intensity is possibly due to ANS being able to permeate through the open casein micelle structure and therefore reaching hydrophobic patches on casein molecules located inside the micelles (Gatti et al., 1995).

In our original hypothesis, after the micelles disassociate the amount of hydrophobic sites available for ANS interaction should increase; therefore, resulting in an increase in fluorescence intensity. However, the opposite was observed. The change in intensity between intrinsic fluorescence and the fluorescence due to ANS interaction decreased as the temperature and the ethanol concentration of the sample increased. This indicates that during the ethanol-dependent heat-mediated dissociation of the casein micelle the I_f of milk proteins is reduced. This reduction is also observed when more ANS is added to the samples, which indicates that ethanol has a competitive inhibition of ANS. As the temperature increases, ethanol interferes with a greater number of hydrophobic zones and makes then unavailable for ANS interaction.

Figure 2.5 shows the change in the fluorescence intensity of casein due to the addition of ANS ($\Delta caseinI_{f(ANS)}$).

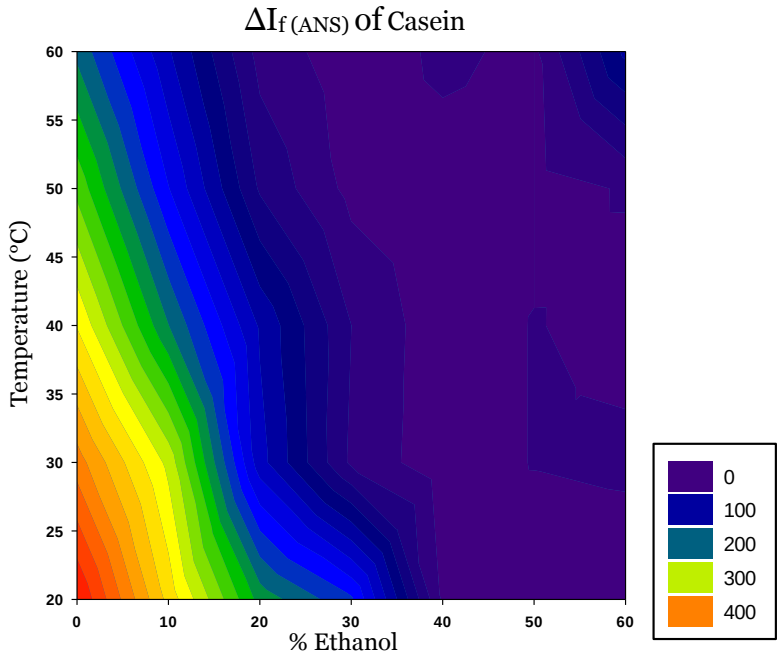

Figure 2.6. Change on the fluorescence intensity of casein due to the addition of ANS ($\Delta I_{f\,(ANS)}$) obtained by removing the fluorescence intensity of the whey proteins and the intrinsic fluorescence of raw skim milk.

In samples where the ethanol concentration was lower than 30%, the increase of temperature resulted in a slight reduction on the $\Delta caseinI_{f(ANS)}$ values. In samples where the ethanol concentration was higher than 30%, the addition of ANS had little effect on the $\Delta caseinI_{f(ANS)}$ values. Also, since the addition of ANS had no effect on the I_f of the caseins, it indicates that there were no available hydrophobic sites for ANS to interact with. Therefore, the ethanol reduces the functional hydrophobicity of the casein micelles, as it essentially blocks the hydrophobic sites from being able to interact with ANS.

The competitive inhibition of ANS binding to hydrophobic sites by ethanol has been proposed in the past (Avdulov et al., 1996). The samples with and without ANS showed a decrease of I_f as the temperature increased. This decrease of

intensity is an indicator that the hydrophobicity of the proteins also decreases. The decrease in the hydrophobicity of the proteins can be attributed to a combination of the following factors: whey protein restructuring and competition by ethanol for the diminishing number of hydrophobic sites.

HYDROPHOBICITY

The calculated values for the slope of the line of the raw and pasteurized skim milk samples can be seen in figure 2.5. This illustrates the effect that the increase in temperature has on the hydrophobicity of the milk casein micelles. At lower temperatures the changes are limited to samples containing from 30 to 50% ethanol. However, as the temperature increases, the range where the hydrophobicity is lowered grows larger to include all samples that contain at least 20% ethanol.

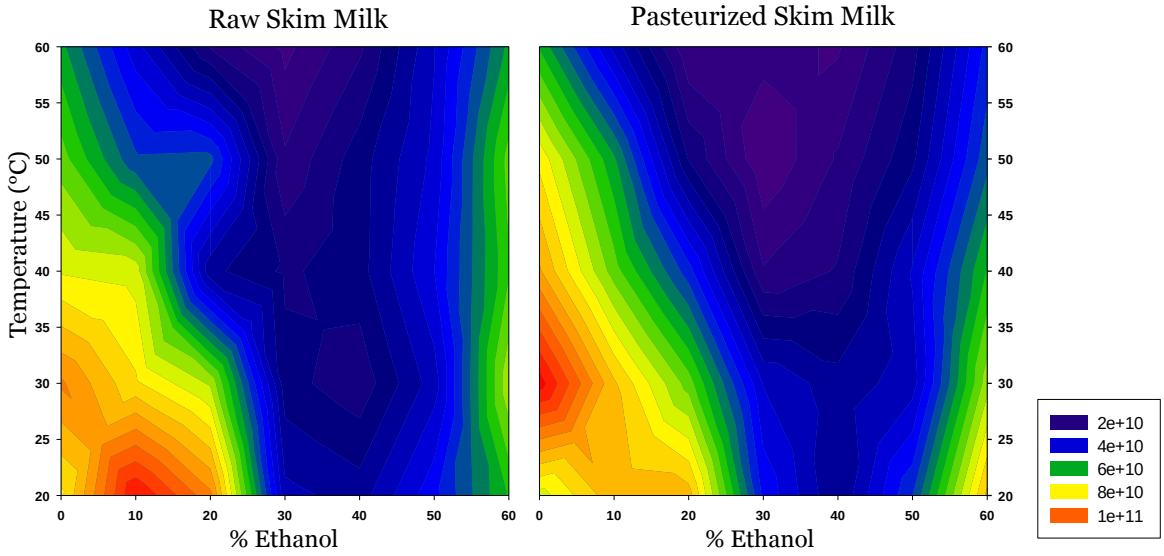

Figure 2.7. Differences in the value of the slope of the line calculated from the fluorescence intensity values of the samples when the concentration of ANS was increased from 2x10-9 M to 4x10-9 ANS.

CONCLUSION

By using ANS as a hydrophobic probe for fluorescence spectroscopy we were able to track the changes in the hydrophobicity of the milk proteins when the milk casein micelles undergo disassociation. We expected the hydrophobicity to increase due to ethanol-temperature induced disassociation, but the interaction of heat and ethanol caused an apparent reduction in the protein hydrophobicity. The heat and ethanol dissociation of casein micelles and exposure of hydrophobic sites could be used as a means to encapsulate low molecular weight hydrophobic compounds. However, our results indicate that ethanol exhibits competitive inhibition that can reduce that availability of hydrophobic sites . Further research concerning the nature of this inhibition may facilitate the use dissociated casein micelles as transport vehicles.

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CHAPTER III
PRESENCE OF WHEY PROTEINS WITHIN THE MILK CASEIN MICELLE AND ITS IMPLICATION TOWARDS CASEIN PROTEIN ISOLATES

This chapter is a lightly revised preliminary version of a paper by the same name to be submitted to the *Journal of Dairy Science* by Raymundo Trejo, Federico M. Harte, and Juan L. Jurat-Fuentes. The use of "our" in this chapter refers to my co-authors and I. My primary contributions to this paper include (1) the experimental work, (2) the collection and analysis of data, (3) the gathering and interpretation of literature, and (4) the writing.

ABSTRACT

Casein is the principal protein component in milk. In liquid milk casein is found in the form of casein micelles, which are large conglomerates of proteins. These micelles were believed to only have casein as protein components. It is known that casein micelles associate with whey proteins, but this was thought to be an artifact of heat treatment or pH manipulation. Size exclusion chromatography (SEC) was utilized as a method to isolate native casein micelles from raw skim milk samples at pH 6.8, 6.0, and 5.5. In a separate experiment, casein micelles were precipitated by lowering the pH of raw skim milk to 4.6, and then washed and rinsed using protein free serum to remove the whey proteins. Then, the casein micelles were re-suspended in protein free serum at native pH. SDS-PAGE was conducted on the samples utilizing a silver staining to achieve higher sensitivity of protein detection. Whey proteins were found in association with the casein micelles at all pH values. Samples of both native pH and re-suspended casein micelles were tested with gel electrophoresis under native and reducing conditions. The bands for the whey proteins were only visible under the reducing conditions. These findings are in direct contradiction of the current agreement that the casein micelle is composed solely of casein.

INTRODUCTION

Research concerning the isolation of casein micelles can be traced back to the late nineteenth century. The earliest method for isolating casein micelles involved the precipitation of the micelles by lowering the pH value of the milk (Hammarsten, 1883). This method of isolating casein micelles is still utilized today, with modifications. The pH manipulation method is one the most common commercial methods for casein preparation (Huffman and Harper, 1999). pH manipulation was the only method utilized for casein micelle isolation until 1955 when a new method utilizing high speed centrifugation was developed by von Hippel and Waugh. This method involved first separating casein micelles by centrifugation at 45,000X*g* for 90 minutes at 5 ˚C. The supernatant was discarded and the micelles were resuspended in a solution containing NaCl and $CaCl_2$. Then, the resuspended micelles were isolated by centrifugation at 90,000x*g* for 60 minutes at 5 ˚C. This process was repeated several times in order to "wash" the micelles (Vonhippel and Waugh, 1955). This new method proved to be less harsh on the micelle native structure than the acidification method. Modifications of this method are still utilized today. However, the centrifugation method worked on the assumption that the pellet, after centrifugation at speeds ranging from 25000 to 90000X*g* for 60 min contained only micellar material and the supernatant only non-micellar material (Martin et al., 2007). In fact, it has been shown that the method does separate all casein micelles from the supernatant (del Angel and Dalgleish, 2006), but not that the pellet contained only casein micelles. A previous report by Dalgleish et.al., where

resuspended casein micelles were analyzed by SDS PAGE after centrifugation, mentioned the presence of small amounts of whey proteins associated with the micelles. However, since the source material was skim milk powder, they attributed the presence of the whey proteins to the heat treatment utilized to produce the milk powder (Dalgleish, 2004).

Another method for casein micelle isolation involves the use of size exclusion chromatography. This is based on a method developed by Boulet et. al. in 1970 who demonstrated that the first elution peak obtained from a size exclusion chromatography (SEC) of a skim milk sample contained the casein micelles (Boulet et al., 1970). This was later verified by other researchers utilizing modifications of the Boulet method (Britten et al., 1986, Gupta, 1983). Furthermore, SEC has also been used successfully in the past to separate whey proteins (Hill et al., 1986). Further studies in the identification and quantification of milk proteins utilizing high pressure liquid chromatography have been conducted (Bordin et al., 2001). However, those studies utilized pasteurized skim milk instead of isolated casein micelles.

The interactions between casein micelles and whey proteins in milk have been reported in the past (Smits, 1980). Under heating conditions (~>75^{o}C), β-lactoglobulin denatures and forms disulphide bonds with the κ-casein layer of the casein micelle. Several authors suggested that such interaction was only due to effects of heat treatment, and that pH had an effect on the rate of attachment (Anema and Li, 2003, Corredig and Dalgleish, 1996, Oldfield et al., 2000). These

studies involved a method of micelle isolation utilizing centrifugation or precipitation via acidification.

In the past, our attempts to isolate casein micelles by ultra-filtration always encountered contamination by whey proteins. This was observed regardless of the pore size of the filter being used (from 3 KDa up to 0.45 μm) . The whey proteins were also found in commercial native casein isolates (see Figure 3.1). The possible presence of whey proteins in association with native casein micelles would prove to be a significant finding since it stands contrary to the previously thought composition of the casein micelles.

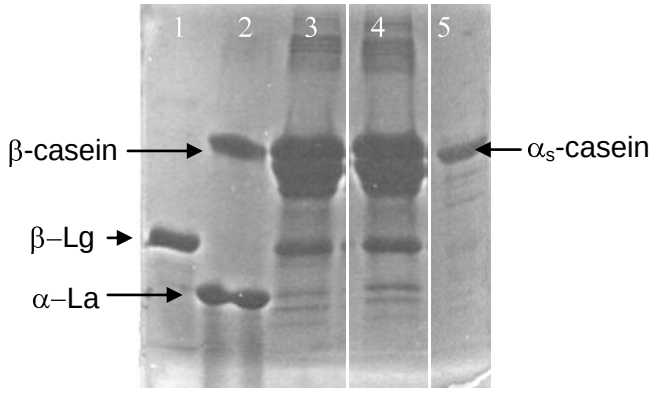

Figure 3.1. *SDS-PAGE of two commercial native casein isolates. β-Lg standard (Lane 1); α-casein and α-La standards (Lane 2); commercial casein isolate (Micellnor, Kerry Dairy Ingredients, Kerry, Ireland; Lane 3); commercial casein isolate (Micellar Casein, American Casein Company, Burlington, NJ; Lane 4); α_s-casein standard (Lane 5).(From Hernandez 2008)*

MATERIALS AND METHODS

MILK SOURCE & SAMPLE PREPARATION

Raw milk samples were collected in a local dairy farm. Fat was removed by centrifugation at 4 °C (6414X*g* for 20 min) and stored at 4 °C. The native pH of the raw skim milk sample was measured and recorded to be 6.8. Prior to testing, samples of the following pH values were prepared: 6.0 and 5.5. The pH of the samples was adjusted by strongly mixing using a 1.0 N HCl solution (6 N, Fisher Scientific, Fair Lawn, NJ). Commercially available pasteurized skim milk was obtained from a local grocery store and kept at 4 °C. Protein free serum (PFS) was prepared by tangential flow ultra-filtration using a 3KDa MWCO cellulose filter (PLBC Prep scale TFF Cartridge, Millipore, Billerica, MA). Prior to testing, the pH of the required volume of PFS was adjusted to match the pH of the milk sample being tested. As with the raw skim milk samples, the pH was adjusted using a 1.0 N HCl solution. The PFS was stored at 4 °C. Absence of proteins in PFS was confirmed using SDS-PAGE with silver staining.

SIZE EXCLUSION CHROMATOGRAPHY

A Superdex 200 prep grade SEC column (GE Life Sciences, GE Health Care, United Kingdom) connected to an AKTA (GE Life Sciences, GE Health Care, United Kingdom) fast performance liquid chromatography (FPLC) unit was used for size exclusion of milk samples. Raw skim milk samples of the desired pH were loaded into the FPLC and PFS of the same pH as the milk sample was used

for elution. Separation was achieved using a flow rate of 1.0 ml/min for 2 column volumes (400 ml total), collecting 2 ml fractions. The protein elution chromatograph was determined using absorbance set at 280nm. Collected fractions were stored at -40 °C .

RESUSPENDED MILK SAMPLES

The pH of raw skim milk was gradually lowered until casein micelles precipitated. The pH of the sample was measured throughout the process and recorded upon precipitation. The raw milk sample precipitated at a pH of 4.6. The pH of PFS was adjusted to 4.6. All of the whey was removed from the precipitate via decanting and pipetting. An equal volume of PFS pH 4.6 was used to replace the whey. The sample was then stirred for 2 minutes at low velocity, and then allowed to settle. The PFS was removed and replaced by an equal volume of fresh PFS pH 4.6. This process was repeated a total of 10 times. After the PFS was removed for the last time, the whey volume was replaced using native pH PFS. The pH of the sample was then measured and adjusted to the native pH 6.8 of milk.

SDS-PAGE and silver staining

Based on the FPLC chromatograph, the SEC fractions corresponding to elution peaks were selected for sample preparation. Samples for each of the pH values were prepared with the following procedure. For reducing conditions, a sample loading buffer was prepared using 0.5 M Tris-HCL pH 6.8, glycerol, 10% (w/v) SDS, β-mercaptoethanol, 0.5% (w/v) bromophenol blue in water (Fisher

Scientific, Fair Lawn, NJ). The samples were heat denatured for 5 minutes at 95 °C prior to electrophoresis. For identification purposes β-lactoglobulin and α-lactalbumin isolates (Sigma Aldrich, St. Louis, MO) were used to prepare standards using 1 mg of protein isolate for 1 ml of water (HPLC grade, Fisher Scientific, Fair Lawn, NJ). 15% TRIS-HCL ready gels were used for the electrophoresis (12well precast gel, Biorad, Hercules, CA). Sample volumes of 10 µl were loaded into wells and electrophoresis was run at 200 volts for 38 minutes. Gels were stained using manufacturer's protocol (Silver stain plus, Biorad, Hercules, CA). The gels were scanned and analyzed by densitometry using imaging software for the bands of casein, β-lactoglobulin, and α-lactalbumin. The data was then log transformed to obtain a normal distribution. An analysis of variance was conducted (SAS 2008) on the effects of pH on the protein concentrations. Native versus reducing conditions

These conditions were tested on the samples with pH 6.8 and 4.6. The loading buffer for these samples was prepared as above with the following exceptions: for native conditions, no β-mercaptoethanol was used in the loading buffer, replacing its volume with DI water. Also, the native samples were not heat denatured prior to gel electrophoresis. The reducing condition samples were prepared as mentioned above. Both samples were prepared by adding 50 µl of the SEC fraction to 450 µl of their corresponding loading buffer and vortexing. The gel electrophoresis, staining and analysis were conducted in the previously described manner.

RESULTS AND DISCUSSION

The protein content of the SEC peaks can be seen in figure 3.2, 3.3, and 3.4 for pH 6.8, 6.0, and 5.5, respectively. Whey proteins were found in all of the SEC casein micelles samples regardless of the pH of the milk. This is in disagreement with a previous report by Martin et al. (2007) who concluded that the native state of the raw milk contains soluble whey proteins and micelles consisting solely of casein (Martin et al., 2007). However, in their study, they conducted SDS-PAGE followed by Coomassie blue staining of the supernatant resulting from the centrifugation of raw milk samples and not on the pellet. Furthermore, the use of Coomassie blue, with its lower resolution, would not reveal the smaller concentrations of proteins present on the supernatant or the pellet. Whey proteins were also found in the precipitated (pH 4.6) and re-suspended casein micelles sample as seen in figure 3.5. This indicates that whey proteins are associated with the casein micelles even after they have come out of suspension via acidification. Furthermore, it indicates that the "washing" centrifugation method utilized by von Hippel and Waugh might not be sufficient to remove the whey proteins from the casein micelle. The small of amounts of whey proteins present could also be seen as a sign of non-sufficient centrifugation attributed to the sample treatment or processing (Dalgleish, 2004).

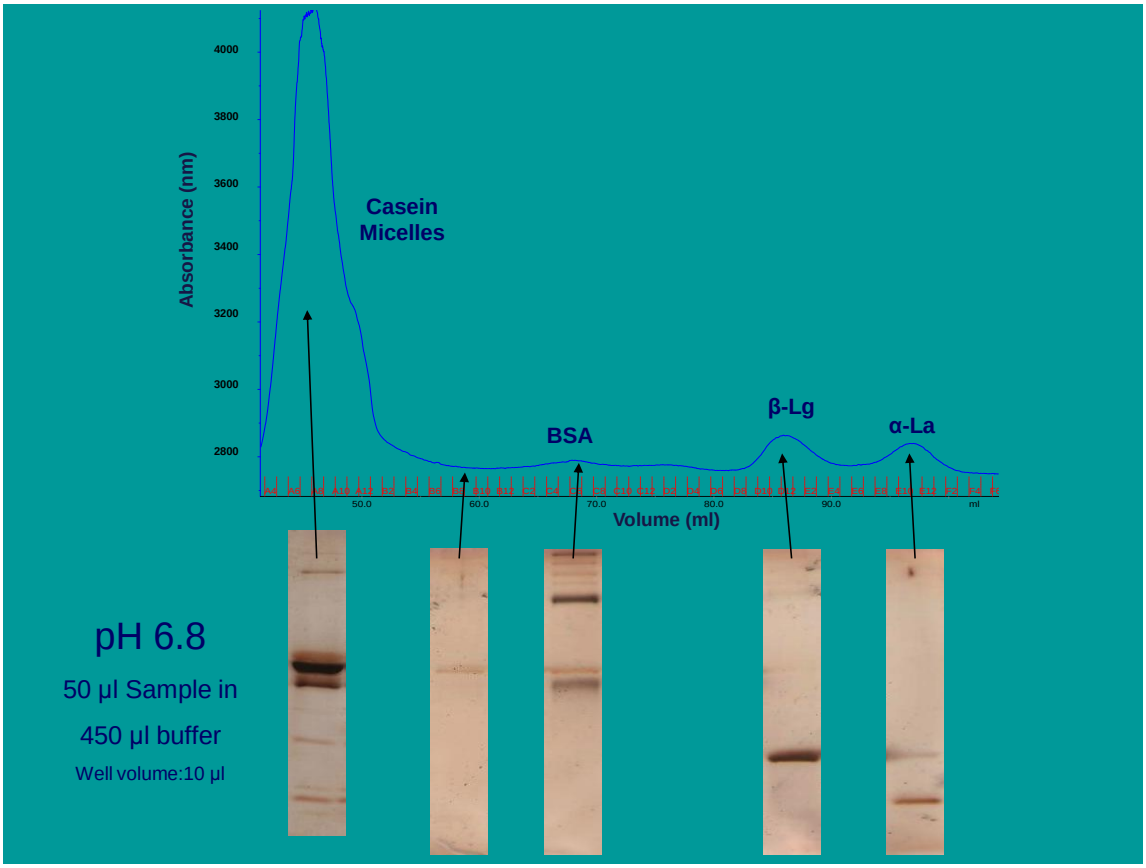

Figure 3.2. Results from gel electrophoresis of the different peaks of the size exclusion chromatograph of raw skim milk sample with a pH of 6.8. BSA = bovine serum albumin, β-lg = β-Lactoglobulin and α-La = α-lactalbumin.

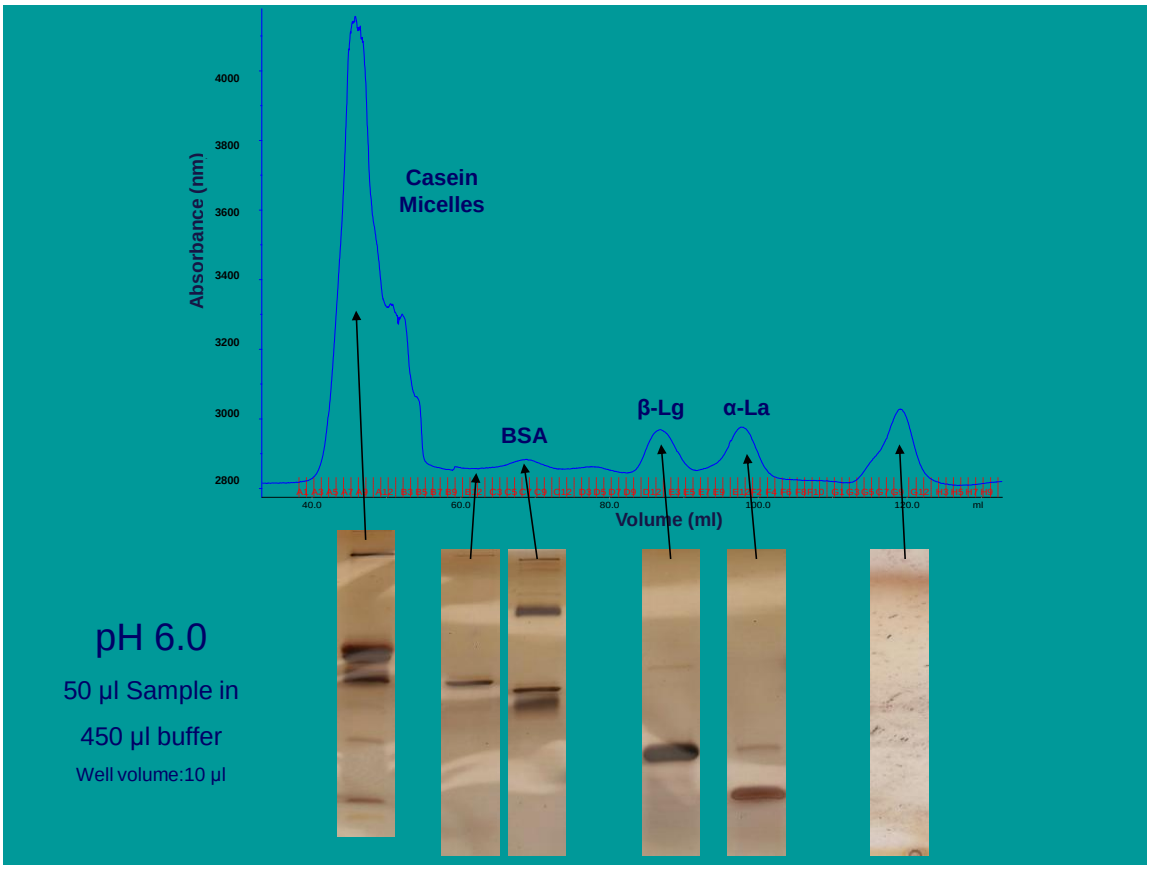

Figure 3.3. Results from gel electrophoresis of the different peaks of the size exclusion chromatograph of raw skim milk sample with a pH of 6.0. BSA = bovine serum albumin, β-lg = β-Lactoglobulin and α-La = α-lactalbumin.

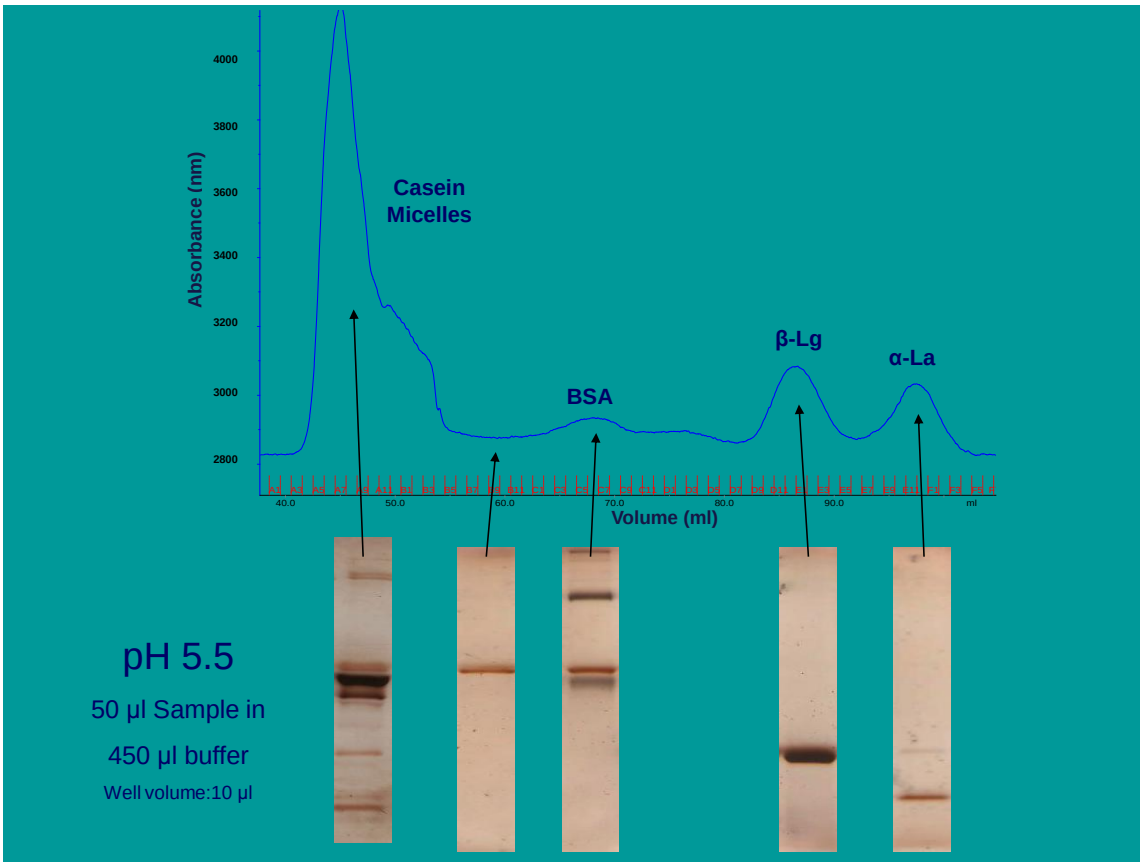

Figure 3.4. Results from gel electrophoresis of the different peaks of the size exclusion chromatograph of raw skim milk sample with a pH of 5.5. BSA = bovine serum albumin, β-lg = β-Lactoglobulin and α-La = α-lactalbumin.

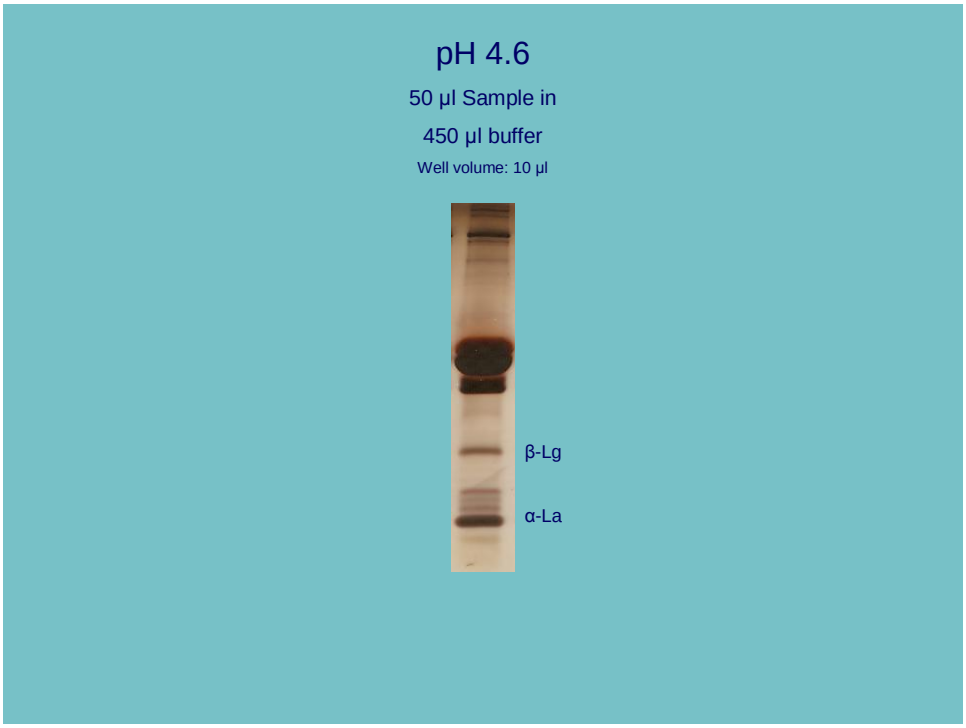

Figure 3.5. Gel electrophoresis results of a sample of casein micelles re-suspended in native pH protein free serum.

The SDS-PAGE of samples was performed under reducing and non-reducing/non denatured conditions in order to further explore the nature of the casein micelle-whey protein association. Also, in pH values around its isoelectric point of 5.1, β-lactoglobulin can make octamers (Wong, 1997). These would be larger than the single globular proteins, and could possibly exit alongside the much larger casein micelles. Since the samples were not heated, they would not be associated with the micelles via disulfide bonds. Therefore they should be visible in native / non-reducing samples. The results can be seen in figure 3.6. Whey proteins were detected only in the gel under reducing /denatured conditions indicating that the whey proteins were interacting with the casein micelles via disulphide bonds.

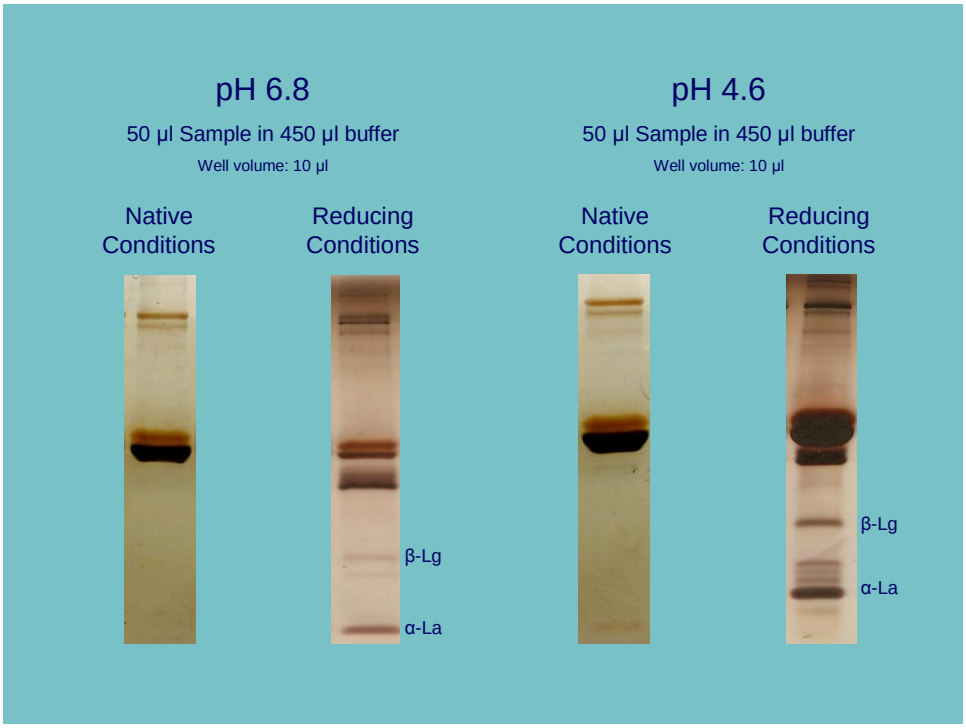

Figure 3.6. Comparison of Gel electrophoresis under native versus reducing conditions of a sample of native pH casein micelles obtained via size exclusion chromatography and casein micelles re-suspended in native pH protein free serum. β-lg = β-Lactoglobulin and α-La = α-lactalbumin.

The analysis of variance showed that pH had no effect on the amount of whey proteins or casein found in the samples ($p > 0.05$). Figure 3.7 shows that the integrated density of the bands did not exhibit any significant change for the pH range used in this study. This observation is in contradiction with a previous report by Smits and Vanbrouwershaven (1980) who found that a change in pH of milk samples prior to heating resulted in different amounts of β-lg associating with the casein micelle. In their study, a pH value of 7.3 reduced the association between β-lg and caseins while a pH of 5.8 had the opposite effect.

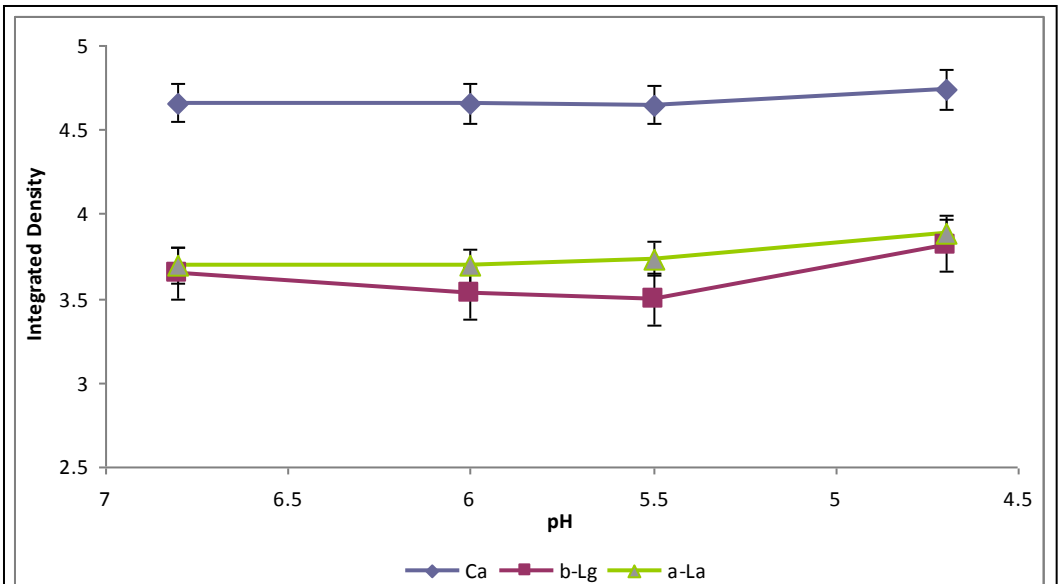

Figure 3.7. Integrated densities of the gel electrophoresis bands for different milk proteins throughout the pH range.

CONCLUSION

The presence of whey proteins in association with the native casein micelles at different pH values has been confirmed. The nature of the association has not been fully explained, but our results suggest that whey proteins are associated to casein micelles through disulfide bonds in raw milk. This association via disulfide bonds was thought to be a result of the thermal processing of milk. These findings are also in disagreement with the idea that casein micelles, regardless of the proposed structure model, are composed solely of the different caseins and contain no other proteins. Furthermore, the presence of whey proteins associated to casein micelle in the pH range used in our experiments, shows that changing pH is not an effective means to completely isolate the caseins.

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VITA

Raymundo “Ray” Trejo was born in Brownsville Texas. He grew up in Guanajuato and Matamoros, Mexico. After high school graduation, he returned to the United States to pursue a degree in Computer Science at the University of Texas at Brownsville (UTB). While at UTB he became involved, by mere chance, with the Rancho del Cielo Biological Field Station Program. His experiences there led him to change his major to Biology. After obtaining his Bachelor’s of Science in Biology from UTB and marrying his college sweetheart, he enjoyed a rewarding career as a middle school Science teacher in Harlingen, TX and later in Nashville, TN. Serendipity once again intervened in his life, and he moved on to a position as a food inspector with the Nashville Davidson County Metro Health Department and later with the Knox County Health Department in Tennessee. While working as an inspector, he was bitten by the higher education bug, and decided to pursue a graduate degree in Food Science & Technology from The University of Tennessee. As a graduate student he was awarded the Graduate Diversity Enhancement Fellowship (2007), and the Access & Diversity Scholarship (2008). He worked under the guidance and tutelage of Dr. Federico Harte. In the future, he plans to continue his education while pursuing a doctorate degree in Food Science at the University of Tennessee, and to further continue to enjoy his life to the fullest in the company of his family, friends and numerous pets.