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Casein Supramolecules: Structure and Coagulation Properties

Bonney S. Oommen

Utah State University

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CASEIN SUPRAMOLECULES: STRUCTURE
AND COAGULATION PROPERTIES

by

Bonney S. Oommen

A dissertation submitted in partial fulfillment
of the requirements for the degree
of
DOCTOR OF PHILOSOPHY

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UTAH STATE UNIVERSITY
Logan, Utah

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ABSTRACT

Structure and Coagulation Properties of Casein Supramolecules

by

Bonney S. Oommen, Doctor of Philosophy
Utah State University, 2004

Major Professor: Dr. Donald J. McMahon
Department: Nutrition and Food Sciences

The changes in quaternary structure of casein supramolecules with various physical and chemical treatments were studied using transmission electron microscopy, and a model to account for the changes is put forth. The effects of casein structure on coagulation properties were also studied. The sample preparation for transmission electron microscopy involved physical methods of fixation and flash freeing to preserve the structure of caseins in the sample.

The structure of caseins in sodium and calcium caseinate varied with sodium caseinate not exhibiting any spherical structure as opposed to the spherical structure seen in calcium caseinate, non-fat dried milk and native milk. This difference in structure was carried over to rennet coagulum made from those sources of casein. Addition of calcium and phosphate to sodium and calcium caseinate, respectively, improved their coagulation properties. Hydration parameters such as time and shear of hydration affected the extent of hydration. High shear (733 s⁻¹) or approximately 10 hr of hydration was required to disperse and hydrate the dried milk protein powders.
Acidification and treatment with excess EDTA resulted in dissociation of casein supramolecules into various sizes and shapes. Heat treatment of milk in the presence of ethanol also resulted in its dissociation. High heat treatment of milk at various pH levels induced different types of whey protein casein interactions.

All these changes can be explained using an irregular supramolecular structure of caseins based on a node and strand network of proteins and calcium phosphate nanoclusters. Such a filigreed sponge-like appearance is seen in native bovine milk and in milk of other species.
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Bonney S. Oommen
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LIST OF ABBREVIATIONS

MS4 = treatment with moderate shear (shear rate = 25 s\(^{-1}\)) and 4 h of hydration
MS10 = treatment with moderate shear (shear rate = 25 s\(^{-1}\)) and 10 h of hydration
HS1 = treatment with high shear (shear rate = 735 s\(^{-1}\)) and 1 h of hydration
NFDM = Non-fat dried milk
CaCN = Calcium caseinate
NaCN = Sodium caseinate.
RCT = Rennet coagulation time
A\(_{60}\) = Curd firmness after 60 min of renneting
TEM = Transmission electron microscopy
SE = Standard Error of Mean
SEM = Scanning electron microscopy
CHAPTER 1
GENERAL INTRODUCTION

The non-crystallizable nature of the caseins has resulted in conventional methods of structure determination, such as nuclear magnetic resonance spectroscopy and X-ray or neutron scattering in providing little information on the overall structure of casein micelles (Swaisgood, 1992). An alternative avenue for obtaining structural information is through electron microscopy. However, various techniques used in electron microscopy have yielded conflicting results on the structure of casein micelles (Heertje et al., 1985; McMahon and McManus, 1998). Some of this conflict arises from the introduction of structural artifacts during sample preparation. For example, metal coatings on the surface of casein micelles limits information on internal structure. Chemical fixatives and air-drying of samples can cause increased aggregation of proteins and shrinkage of the casein micelle from their native structure. Sectioning of the embedded samples also introduces dimensional compression.

Unveiling the native structure of casein micelles would provide better understanding of changes occurring during various processes involved in dairy technology. Examination of reconstituted casein micelles may not be representative of the native structure. The structure of native and reconstituted micelles can affect its functional performance. For example, the structure of the casein micelles may influence the coagulation properties of renneted milk and the resultant structural organization of rennet curd, which is an intermediate product in the manufacture of cheese.

A sample preparation method that introduces minimal artifacts and allows imaging of the internal structure of casein micelles at high magnification would permit
the native structure of casein micelles to be deciphered. In addition, differences in structure of native and reconstituted casein micelles could be compared, and changes in casein micelles that occur when their chemical environment or temperature are altered may be studied to further explain the casein micelle structure. The aim of this study is to image native and reconstituted casein micelles using transmission electron microscopy so as to determine their structure and coagulation properties.

**HYPOTHESIS AND OBJECTIVES**

The research hypothesis is that native casein micelles have a branched polymeric structure rather than a particulate submicellar structure. Testing of this hypothesis will involve comparison of native casein micelles and reconstituted casein micelles, and the influence of known casein micelle denaturation treatments on its structure. Based on this information, put forth a model for the quaternary, or supramolecular, structure for the colloidal casein particles in milk.

The specific objectives of the research were:

1. To view native casein micelles in milk and casein micelles depleted of their calcium content based on transmission electron microscopy method of McManus and McMahon (1997).
2. Compare structural differences between native casein micelles and casein micelles in reconstituted calcium caseinate, sodium caseinate, and non-fat dried milk systems.
3. Determine the effect of shear and time of hydration on the structure of reconstituted casein micelles.
4. Determine the effect of supplementing milk with reconstituted casein micelles on coagulation properties.

5. Determine the effect of supplementing milk with reconstituted casein micelles on the microstructure of rennet coagulum.

6. Compare the structural changes that occur in casein micelles during acidification of milk.

7. Determine differences in casein micelle structure when milk is heated at various levels of pH.

8. Compare the structure of renneted and native casein micelles.

**LITERATURE REVIEW**

**Casein Micelle Composition**

Proteins in milk may be broadly classified into caseins and whey proteins, out of which approximately 80% is casein. Linderstrøm Lang (1929) postulated that colloidal milk complex was composed of a mixture of calcium-insoluble proteins stabilized by calcium-soluble protein. The $\alpha_s$- and $\beta$-caseins are calcium insoluble, while $\kappa$-caseins are calcium soluble. Thus, the colloidal milk complex exists as a spherical aggregate of $\alpha_{s1}$-, $\alpha_{s2}$-, $\beta$-, $\kappa$-caseins, and calcium phosphate as its integral components. On a dry basis, the caseins contribute 92% of the micelles while the rest is contributed by inorganic salts which contain calcium, phosphate, citrate, magnesium, sodium, potassium, and zinc. These spherical aggregates were given the misnomer of casein “micelles,” before it was understood that they did not have a classical micellar structure, but the name still remains in use. The casein micelles vary in diameter from 20 to 600 nm with an average of 160
nm (Bloomfield and Morr, 1973). As its component proteins have a molecular weight of 22,000 to 24,000 Da, the molecular weight of the colloidal polymer is in the range of $10^8$ Da. Casein micelles are highly hydrated colloidal particles commonly recognized as containing about 3.7 g H$_2$O/g of protein (Korolczuk, 1981a, 1981b) with calculated values ranging between 1 to 8 g H$_2$O/g of protein depending on the method of measurement (Swaisgood, 1992; Mora-Gutierrez, et al., 1997; Farrell, et al., 2003).

**Casein Micelle Integrity: The Forces**

A variety of forces come into play in preserving the structural integrity of casein colloidal complex. They are:

*Hydrophobic Interactions.* When apolar amino acids are present in water, the entropy of the system decreases. If these apolar amino acids are oriented inwards in a protein molecule, so that they interact with other apolar groups, the stabilization energy of the solvent increases incrementally with the number of residues transferred (Thompson and Farrell, 1973). In caseins ($\alpha$-, $\beta$-, and $\kappa$-) apolar residues are clustered and thus contribute to protein hydrophobicity. Water molecules form ordered, low-entropy clusters around exposed hydrocarbon side chains, and when apolar side chains cluster together, water is released with consequent gain in entropy and lowering of free energy of the system. When hydrophobic interaction takes place between two protein groups, the number of water molecules in contact with the hydrophobic region is decreased even though it may still retain some water molecules around it (Nemethy and Scheraga, 1962).

At low temperatures, the entropy contribution to the free energy change becomes less important so that the complexes dissociate with migration of proteins out of the colloidal particles. Moreover, it is also known that $\beta$- and $\kappa$-caseins, and $\alpha_s$-caseins to
some extent, diffuse out of the micelles at low temperatures or high pressures. Thus, hydrophobic interactions are one of the forces holding these proteins together in the colloidal structure (Carroll et al., 1967) even though a single hydrophobic bond is weaker than most other side chain interactions in and among proteins. This is because of the large number of hydrophobic bonds formed in proteins with non-polar side chains (Nemethy and Scheraga, 1962). For example, in the case of $\alpha_s$ and $\kappa$-caseins, salting-out studies by Dosako et al. (1980) showed that the complex formation of $\alpha_s$- and $\kappa$-casein co-polymers was hydrophobic and that electrostatic interaction did not participate in complex formation.

At high pressures, the exposed non-polar side chains have a lower partial molar volume than do aggregated non-polar groups due to clathrate formation and thus get dissociated (Bloomfield and Mead, 1975). High pressure also disrupts the ionic bonding in water since the separated ions decrease the solvent volume by electrosuction.

Hydrophobic interactions are not as specific as other chemical interactions with respect to steric requirements of side chain orientation, or number and kind of side chains that can participate in their formation or strength of their interaction (Nemethy and Scheraga, 1962). The high hydrophobicity of caseins together with its high molecular weight prevents the formation of globular structure in which non-polar groups are completely buried in the protein interior (Schmidt and Payens, 1976). Thus, hydrophobic interactions are a favored method of protein association between caseins.

**Ionic and Electrostatic Bonds.** Many sites for ionic bonding exits within the different caseins that can play a role in micelle structure stabilization. This type of bonding is important for the close packing of the proteins. In the case of monomeric
proteins, ionic interactions between negatively charged carboxylic acid residues and positively charged groups contribute little to their stability (Thompson and Farrell, 1973). Exceptions occur when ionic pairs are formed in a hydrophobic environment (McMahon and Brown, 1984a). Electrostatic interactions between carboxylate residues and divalent metal ions can impart reasonable structural stability to a protein. In casein micelles, the ionic pairs of phosphate-calcium-phosphate bridges stabilize quaternary structure by the increased cross-linking between proteins.

Increase in calcium content decreases heat stability (Rose, 1968) possibly by affecting the solvation of the casein micelles. Thus, decrease in solvent interaction lowers the stability of the micelle. Therefore, the ionic residues of the individual casein monomers must be exposed to solvent rather than being totally buried. In proteins whose crystallographic structures are known, all the ionic side-chains are fully exposed to the solvent (Blow and Steitz, 1970; Klotz, 1970). The presence of calcium-phosphate bridges and the destabilization of the caseins at its isoelectric pH shows that ionic interactions are important for its stabilization. However, all of the charged groups of the casein monomers cannot occupy a surface position while a casein micelle is formed (Thompson and Farrell, 1973). Therefore, solvent-casein interaction has to occur inside the colloidal particle as well.

Hydrogen Bonds. Hydrogen bonding is unlikely to play an important role in the stabilization of tertiary or quaternary structure of caseins. Hydrogen bonding to solvent water is probably as strong as amino-carboxyl hydrogen bonds between amino acid residues. From X-ray analysis of crystalline globular proteins, hydrogen bonds may stabilize interactions between side chains of polar amino acids in the interior of the
molecule. Such interactions may occur in caseins at its intermolecular interfaces (Bloomfield and Mead, 1975). These hydrogen bonds are responsible for the different conformations such as $\alpha$-helix and $\beta$-sheets in individual casein monomers. Such a helix and sheet formation in the constituent casein monomers can influence the quaternary structure of colloidal casein complex.

**Disulfide Bonds.** Although cysteine residues often act to stabilize the fold of globular proteins through bridging disulfide bonds, in caseins, cystine bridges are rare. Of the four bovine caseins, cysteine residues are found only in $\alpha_{s2}$- and $\kappa$-caseins that can form disulfide bonds. Disulfide-linked homo-multimeric forms of caseins are formed in both $\alpha_{s2}$- and $\kappa$-caseins in cow, goat and pig milks resulting in the formation of inter-rather than intra-molecular bridges. In case of human milk, $\kappa$-casein and $\alpha_{s1}$-caseins form hetero-multimers (Rasmussen et al., 1999). Even though disulfide bridges of the casein micelle may contribute to the overall stability of the casein micelles, they are not the driving force nor a central feature of the formed colloidal particle (Thompson and Farrell, 1973). However, disulfide interactions become prominent during processing especially during heat treatment of milk in determining interactions between caseins and whey proteins.

**Charge and Hydrophobicity.** Caseins have a high concentration of charged, acidic groups such as phosphoserine and carboxylate moieties that are segregated from highly hydrophobic regions. This amphipolar structure enables the formation of small aggregates of some caseins (e.g. $\beta$-caseins) with an apparently classical micelle structure (Payens, 1966). In the glycomacropeptide of $\kappa$-casein, sialic acid residues contribute to the high charge. This electrostatic force may act as a barrier to aggregation of casein
micelles with κ-casein on its surface. Upon action of rennet, this electrostatic barrier is reduced as glycomacropeptide is cleaved off κ-casein. Amino acids with hydrocarbon side chains in αs- and β-caseins are clustered so as to impart hydrophobic bonding characteristics to those caseins.

Caseins are rich in proline, which acts as a helix breaker. In β-caseins, prolines are concentrated in the hydrophobic region and therefore most of the helical structures are in the polar region of the molecule. In αs1- and κ caseins, both prolines and potential helical regions are distributed evenly along the chain backbone. Monomeric caseins in aqueous solution are loose coils with little helix content (Bloomfield and Mead, 1975). The looseness of structure implies that solvent can penetrate well into the interior of the protein coil so that hydrophobic residues are not shielded from the solvent as in globular proteins. In terms of free energy, these hydrophobic residues will cluster with similar residues from other molecules resulting in aggregation.

Steric Stabilization. Steric stabilization is the phenomenon of stabilization of a lyophobic colloid by a surface layer of lyophilic substance, against aggregation. When two sterically stabilized particles approach one another, entropic and enthalpic interactions between the lyophilic molecules on the surface leads to a repulsion that is sufficiently strong to overcome the dispersion force of attraction (Horne, 1986; Walstra, 1990; Holt, 1992; Holt and Horne, 1996). When the lyophilic molecule is a polymer, stabilization results from a negative entropy change, due to a restriction in the configurational freedom of the polymer and a positive free energy change from the mixing of the polymer chains. Other factors that may be considered include polymer desorption and bridging. Steric stabilization is a short-range force and depends on the
magnitude of the solvation forces around the polymer segments. On the surface of casein micelles, the layer formed by the macropeptide region of κ-casein, is considered as a polyelectrolyte brush imparting steric stabilization against aggregation with other casein micelles (Holt and Horne, 1996).

Covalent Bonds. As no covalent bonds are involved in holding the different casein molecules together, along with the calcium phosphate in casein micelles, these aggregates of caseins may be better called as a colloidal casein supramolecule. A supramolecule is defined as “a system of two or more molecular entities held together and organized by means of intermolecular (noncovalent) binding interactions” (IUPAC, 1997).

Caseins

A rheomorphic structure has been assigned to caseins (Holt and Sawyer, 1993). A rheomorphic protein may be defined as a protein with an open conformation and therefore has a considerable degree of side chain and possibly backbone conformational flexibility. Unlike in random coils, the conformation of rheomorphic proteins is important to their biological function. Their functionalities include binding to surfaces and/or the formation of macroscopic networks. The rheomorphic nature of caseins can explain its nutritionally sub-optimal amino acid composition, ability of Ca-sensitive caseins to prevent pathological precipitation of calcium phosphate in the mammary gland, and high rates of mutational change in the homologous groups (de Kruif and Holt, 2003).

The calcium sensitive caseins (αs1-, αs2-, and β-caseins) are members of a single gene family as seen from the homologous gene sequences of different species while κ-casein gene is homologous to γ-fibrinogen (Swaisgood, 1992). Caseins undergo post-
translational phosphorylation to varying degrees at seryl and threonyl residues. Recently, Farrell et al. (2002), using a combination of circular dichroism, Fourier transform infra-red spectroscopy, electron microscopy and computer simulations on κ-casein and α-lactalbumin, proposed that caseins have a molten globule structure. The molten globule state has a compact structure with a higher degree of hydration and side chain flexibility. In this state, the proteins have native secondary structures with little tertiary folds. Both the rheomorphic and molten globule structure assigns conformational flexibility to the casein molecule that seems to be critical for the casein supramolecule to maintain its structure.

Farrell et al. (2003) hypothesized that concepts of tensegrity may be applied to explain the protein structural interplay that occurs for caseins. Structures which are tensegritic in nature stabilize themselves through a balance of compression and tension and resist torque. Tensegrity of caseins might also be an important factor in maintaining the integrity and stability of the supramolecular structure of casein micelles.

α_{s1}-Caseins. Five genetic variants of bovine α_{s1}-caseins have been identified. As in other caseins, the hydrophobic and charged residues are not uniformly distributed in the 199-residue long sequence of α_{s1}-casein. The noticeable hydrophobic regions are 1-44, 90-113, and 123-199 (Swaisgood, 1992). The calcium sensitive caseins also have a unique feature of phosphoseryl residue clustering. Its two phosphate centers (41-51 and 61-70) act as potential regions of association with calcium to form cross-links between proteins and calcium phosphate clusters (de Kruif and Holt, 2003). The polar domain of the casein (41-80) contributes to the net charge and segregates the protein into a polar hydrophilic region and neutral hydrophobic region.
Though $\alpha_{s1}$-casein has little $\alpha$-helix, it contains $\beta$-sheets as well as $\beta$-turns. Its C-terminal hydrophobic region has a conserved $\beta$-turn-$\beta$-strand-$\beta$-turn motif. The self-association of the peptide $\alpha_{s1}$-casein (136-196) is dependent on this local secondary structure. The $\beta$-turns and some aromatic dichroism of this peptide are conserved at temperatures as high as 70$^\circ$C, at pH 2 and in 6 M guanidine hydrochloride which suggest a heat stable, ‘molten-globule’, core structure of the peptide. This stability supports the role of hydrophobic proline-based turns as interaction sites of $\alpha_{s1}$-caseins in its self-association as well as with its association with other caseins in the formation of the casein micelle (Alaimo et al., 1999). Monomeric forms of $\alpha_{s1}$-casein exist at low ionic strength (0.003 to 0.01 M) and at neutral or alkaline pH. Self-association is determined by the ionic strength and pH rather than temperature (Rollema, 1992).

$\alpha_{s2}$-Caseins. Of all the caseins, $\alpha_{s2}$-casein is the most hydrophilic. Four bovine genetic variants have been identified containing 10-13 mol P/mol. It has three clusters of anionic phosphoseryl and glutamyl residues located at 8-12, 56-63, and 129-133 residues which contributes to a net charge of −13 to −18 at neutral pH (Swaisgood, 1992). The hydrophobic residues include 160-207 of the C-terminal and 90-120 of the central sequence. Both N and C terminals have opposing net charges resulting in its sensitivity to ionic strength and concentrations of cations such as protons and calcium (Swaisgood, 1992). The C-terminal region of $\alpha_{s2}$-casein has a compact structure containing some $\alpha$-helix and $\beta$-sheet while the N-terminal region forms a randomly structured hydrophilic tail. As in $\alpha_{s1}$-casein, the association of $\alpha_{s2}$-caseins depends on the ionic strength of the medium (Rollema, 1992). It has three phosphate centers (8-16, 56-63, 126-133)
corresponding to a functionality of three, considering those as the sites for calcium attachment.

**β-Caseins.** Of all the caseins, β-casein is the most hydrophobic and seven of its bovine genetic variants have been discovered. It has a highly charged N-terminal domain containing the anionic phosphoserine cluster and a very hydrophobic C-terminal region. The amphipathic nature of this molecule is evident from its 21-residue polar N-terminal domain and a large hydrophobic neutral C-terminal. Among different species, the genetic coding for the polar N-terminal, and hydrophobic C-terminal is conserved. The conservation is for the hydrophobicity rather than the actual sequence (Swaisgood, 1992).

Large amounts of prolyl content in β-casein have a major effect on its structure as it disrupts helix formation. It has been calculated that β-casein could have 10% of the residues in α-helices, 17% in β-sheets, and 70% in unordered structures. The hydrophobic domain of β-casein has conserved predicted α-helix and β-structure which may be responsible for the formation of structural motifs (Swaisgood, 1992). With its amphiphilic nature, β-casein associates to form soap-like micelles. This association is limited by the critical micelle concentration depending on temperature and ionic strength. The hydrophobic interactions between the C-terminal segments drive the soap-like micelle formation while the repulsion between the charged N-terminal groups limit the micellar size (Rollema, 1992).

It is known that β-casein dissociates from the casein supramolecule as temperature is lowered. At low temperatures an equilibrium between the colloidal and the monomeric states is reached which depends upon the total casein level and the portion of β-casein that is hydrophobically bonded. This portion of hydrophobically bonded β-
casein increases with demineralization of the casein micelle (Pierre and Brule, 1981). This suggest that β-casein that dissociates from the casein micelle interacts with other proteins primarily by hydrophobic interactions even though it has a phosphate center residue (14-21). Thus β-casein can interact with other caseins via its hydrophobic region while simultaneously interacting with colloidal calcium phosphate via its phosphate center.

κ-Caseins. In comparison with other caseins, κ-casein is unique in its absence of phosphoseryl residue clusters and the presence of glycosylated threonine. It is amphiphilic in nature with a hydrophobic N-terminal (1-105) and an inert polar C-terminal domain (106-169) which is insensitive to calcium. κ-Casein is one of the most highly ordered caseins with 23% of residues in α-helices, 31% in β-sheets, and 24% in β-turns. The association behavior of κ-casein can be described according to the monomer-polymer mechanism. Unlike β-casein, κ-casein forms polymers and micelles of fixed size irrespective of the temperature (Vreeman, 1979).

The micellar size may be limited by the entropic repulsion between the negatively charged C-terminal domains and special requirements. The calcium-sensitive caseins are stabilized by κ-casein and on action of chymosin, κ-casein is hydrolyzed at its Phe₁₀₅-Met₁₀₆ bond into para-κ-casein and glycomacropeptide resulting in destabilization of the casein micelle.

Colloidal Calcium-Phosphate

Approximately, 32 mM of calcium is present in milk in different forms and in two phases colloidal, and soluble (Bloomfield and Mead, 1975) that account for 22 and 10 mM of calcium respectively. Of the 10 mM of soluble calcium, only 3 mM is in the free
ion and chelation of colloidal calcium-phosphate by addition of EDTA to milk results in dissociation of the casein micelles and the milk become translucent. This results in an increase in serum casein content (Bloomfield and Morr, 1973). An increase in serum casein content and soluble Ca$^{2+}$ content is also noted when pH of milk is lowered to 5.4 (Rose, 1968). The calcium contained within the casein micelles may be directly bound to phosphate ester and carboxyl groups of the caseins, or bound to phosphate and citrate that in turn is associated with protein molecules within the casein micelle. The loop in the $\alpha$-helix-loop-$\alpha$-helix motif of calcium-sensitive caseins may be the center of phosphorylation that links the colloidal calcium phosphate with casein in native casein micelles.

McGannn et al. (1983), studied the ultra-structure of calcium phosphate in milk and complexes prepared from milk systems by re-precipitation and extensive proteolysis. Electron microscopic investigation of these artificial preparations of milk calcium phosphate revealed a very fine and uniform sub-structure which consisted of granules having an average true diameter of approximately 2.5 nm. This value was obtained after correcting the experimental value of 5 nm for the thickness of the deposited Pt/C layer. They also reported the transformation of granules in wet precipitates into thin platelets within 24 h. They attributed this metamorphism to the change in calcium phosphate structure resulting from the conversion of amorphous calcium phosphate to hydroxyapatite via a solution mediated autocatalytic mechanism.

Lyster et al. (1984), also reported that the calcium phosphate in the casein micelle was of the diffuse amorphous type and that there were no periodic lattice spacings greater
than 1.5 nm. Compositional analysis of milk calcium phosphate revealed that 56% of the total Zn found in milk is located with the calcium-phosphate-citrate complex rather than the conventional assumption of Zn being associated with the casein protein fraction and that Zn is an inhibitor of calcium phosphate crystal growth (Meyer and Angino, 1977).

Aoki et al. (1992) reported that at least three phosphate groups are needed for crosslinking of casein by colloidal calcium phosphate. In artificial casein micelles the exchange of calcium and phosphate between the diffusible and colloidal phases depends on temperature. At 16 to 18°C, approximately 50% of the colloidal calcium can exchange with serum in 1 min and an additional 20% in 24 h (Pierre and Brule, 1981; Pierre, et al., 1983). The exchange of calcium and phosphate between diffusible and colloidal phases proceed slowly and a portion of the calcium and phosphate in the artificial casein micelles is difficult to exchange (Zhang et al., 1996).

Colloidal calcium phosphate in casein micelles has been reported to have a chemical composition and physico-chemical properties which are consistent with it being a complex of the phosphate centers of casein with an acidic amorphous calcium phosphate (Holt et al., 1989). Brushite (CaHPO$_4$.2H$_2$O) is proposed as the crystalline model compound from which the short-range structure may be derived. Due to the similarity in X-ray absorption spectra of calcium phosphate in casein micelles to both brushite and other amorphous calcium phosphate, there exists a wide range of chemical composition that it can take.

Using slow-speed-spinning $^{31}$P magic-angle spinning solid-state nuclear magnetic resonance, Bak et al. (2001) determined inorganic hydroxyapatite type of phosphates
along with organic phosphates from \( \kappa \)-casein and phosphoserines from the calcium binding \( \alpha_{s1} \), \( \alpha_{s2} \), and \( \beta \)-caseins in native casein micelles. Holt et al. (1989) have proposed a core-shell model of calcium phosphate nanoclusters. They prepared calcium phosphate nanoclusters under standardized conditions using 10 mg/ml of the bovine \( \beta \)-casein (f 1-25) as a stabilizing agent. Using sedimentation equilibrium and small-angle X-ray and neutron-scattering measurements, they concluded that the nanoclusters were comprised of a spherical core of radius \( 2.30 \pm 0.05 \) nm consisting of \( 355 \pm 20 \) CaHPO\(_4\)2H\(_2\)O units and surrounded by \( 49 \pm 4 \) peptide chains forming a tightly packed shell with an outer radius of \( 4.04 \pm 0.15 \) nm.

**Hydration**

Considerable variation exists in the extent of hydration of casein micelles depending on the method of measurement. From the measurements of water content of casein micelle pellets, prepared by centrifuging skim milk at 67,000 \( \times \) g, at 37 °C for 35 min, the hydration was found to be 1.9 g H\(_2\)O/g of protein (Thompson et al., 1969). When hydration was determined from the voluminosity that was calculated from viscosity measurements, the hydration of casein micelles was estimated to be 3.67 g H\(_2\)O/g of protein (Bloomfield and Morr, 1973). They attributed this discrepancy to hydrostatic pressure which ‘squeezed’ the casein micelles of loosely bound water when pelleted using an ultracentrifuge. Of the 1 to 8 g water/g protein contained within the casein supramolecule only about 15% is bound to the protein with the remainder being occluded within the particle (Swaisgood, 1992; Mora-Gutierrez, et al., 1997; Farrell, et al., 2003)
Casein Micelle Dissociation

Various experiments for micelle dissociation using different agents such as low temperatures, high pressures, urea treatment, calcium phosphate removal by dialysis or gel filtration or treatment with oxalate, pyrophosphate, citrate or EDTA has been reported (Bloomfield and Morr, 1973; Holt, 1982; Chaplin, 1984; Britten et al., 1986; Holt et al., 1986; van Hooydonk et al., 1986; Griffin et al., 1988; Dalgleish and Law, 1989). Casein micelle dissociation has also been used as experimental evidence for the various structures proposed for casein micelles.

Calcium depletion from casein micelles in milk by dialysis against simulated milk ultrafiltrate decreases the high molecular weight light scattering material from milk, while the hydrodynamic radius of those particles remain constant up to a critical point of Ca\(^{2+}\) activity reduction (1.2 mM EDTA) (Bloomfield and Morr, 1973). Beyond this critical point there is disappearance of large mono-disperse scattering particles. It has also been reported that direct addition of EDTA into milk or a solution of casein micelles causes some of the casein micelles to dissociate completely while the rest of them still remain intact (Bloomfield and Morr, 1973). Depending on the extent of calcium dissociation from casein micelles, they can undergo either complete or partial structural disruption resulting in increase of soluble caseins.

Holt et al. (1986) dissociated the casein micelles by reducing the colloidal calcium phosphate content by dialysis against a phosphate-free buffer, and found that the soluble casein was predominantly β- and κ-caseins. Smaller quantities of α\(_{s1}\)-casein were also found with lesser amounts of α\(_{s2}\)-casein. When casein micelles were dialyzed against buffers saturated in colloidal calcium phosphate and reduced amounts of free
calcium, dissociation still occurred without dissolving the colloidal calcium phosphate if the serum free Ca\(^{2+}\) concentration was below 2 mM (Holt et al., 1986). This shows that the strength of binding of the proteins are in the order of \(\alpha_{s2} > \alpha_{s1} > \beta > \kappa\) which corresponds to their phosphoserine content.

Addition of small quantities of Ca\(^{2+}\) to micelles in SMUF by dialysis causes the micelles to sediment at a faster rate, along with the transfer of soluble casein to the micelle sedimentation peak without altering their hydrodynamic radii. Larger quantities of Ca\(^{2+}\) addition results in gross aggregation and precipitation of the micelles (Bloomfield and Morr, 1973). Thus it might be conceived that casein micelles can retain their overall structure even when proteins are added or separated from them at various levels of Ca\(^{2+}\) activity.

Rollema and Brinkhuis (1989) studied casein micelle structure using \(^1\)H-NMR, and reported that NMR spectrum of the particles generated by calcium removal from micelles resembled that of particles in a sodium caseinate solution. But this NMR spectrum was different from that of small micellar particles observed in preparations of small micelles, which showed a spectrum very similar to that of larger micelles. The structure of free casein submicelles generated by calcium depletion was different from the structure of casein when it forms part of the casein micelle. Therefore, they concluded that the interaction of casein with the colloidal calcium phosphate inside a micelle immobilized the flexible parts and induced a rigid structure. This observation does not support the submicellar model which portrays submicelles as separate entities only held together in the micelle by calcium phosphate clusters (de Kruiif and Holt, 2003).
Small-angle neutron-scattering experiments of casein micelles showed an inflection point that was attributed to inter-submicelle interference within the whole micelle and not due to calcium phosphate (Stothart and Cebula, 1982; Stothart, 1989). A contrast variation study by Hansen et al. (1996) showed a variation in the prominence of the shoulder with contrast that allowed for a polydisperse distribution of subunits whose radius varied from 9 to 13.4 nm rather than the presence of homogenous 18 nm subunits. This shows repeating subunits that are smaller than expected, and are varying in composition.

Holt (1998) dissociated casein micelles by selective removal of colloidal calcium phosphate while maintaining other sources of micelle integrity (such as pH, ionic strength, calcium concentration) by addition of urea to find no submicellar size distribution. Also, addition of κ-CN without altering the above parameters does not completely dissociate the casein micelles into its subunits. Thus dissociation of casein micelles does not occur as recurring homogeneous sub-units but as irregular, random protein aggregates or protein monomers.

**Casein Micelle Reformation**

Colloidal size aggregates similar in size to casein micelles can be synthesized from major caseins (α_{s1}-, α_{s2}-, β- and κ-caseins) and calcium, phosphate, and citrate as components (Schmidt et al., 1974, 1977; Slattery, 1979). Though they may seem to be similar to native casein micelles (Knoop et al., 1979), their structure may vary depending on the degree of casein hydration, pH, salt balance, and the forces contributing to its integrity. If caseinates or acid caseins are used to reform casein micelles, it may not be possible to bring back the native structure of the casein micelles even after adjusting the
mineral equilibrium as different chemical and physical agents would have changed the conformation of the component proteins during their manufacture.

Ono et al. (1983) reported the formation of colloidal size aggregates from smaller casein aggregate at higher temperature (37°C) compared to their inability to coalesce at 5°C, showing the importance of hydrophobic bonding in the reformation of casein micelle-like aggregates. Variation in mineral contents of casein solution will result in changes in casein structure and casein micellar mineral composition that causes significant variations in casein hydration. When reformed micelles are added with Ca\(^{2+}\) at 37°C, a decrease in loop and helical structures with an increase in turns were detected showing an open hydrated structure using FTIR measurements of caseins in water (Curley et al., 1998). This shows the effect of Ca\(^{2+}\) and hydration of caseins on its structure.

When human \(\beta\)-caseins with two or four phosphorylated groups are added with bovine \(\kappa\)-casein in presence of calcium, \(\beta\)-CN-2P forms polymers of limited size and aggregates further into colloidal size aggregates with \(\beta\)-CN-4P (Sood et al., 2002). With the increased reactive sites in \(\beta\)-CN-4P, they have the ability to form crosslinks. The formation of reconstituted casein micelles under various conditions of pH, Ca\(^{2+}\) concentration, and \(\kappa\)-CN content indicates that both hydrophobic interactions and Ca\(^{2+}\) bridges or crosslinks may be responsible for protein aggregation and micellar stability.

**Casein Micelle Structures**

Numerous models describing casein micelles have been proposed and reviewed (Bloomfield and Morr, 1973; Garnier, 1973; Slattery, 1976; Schmidt, 1980; McMahon and Brown, 1984a; Rollema, 1992; Holt and Horne, 1996; Walstra, 1999). All these
models are based on the evidence of dissociation studies on casein micelles or scattering experiments or electron microscopy.

The core-coat model proposed by Waugh and Noble (1965) consisted of spherical particles of $\alpha_{s1}$- and $\beta$-caseins with $\kappa$-casein as the coat. The casein micelle model put forward by Payens (1966) showed considerable part of $\kappa$-casein located on the surface of the casein micelle and compactly folded $\alpha_s$-caseins attached to loose $\beta$-caseins as the core with calcium ions interacting with phosphate or carboxylic acid groups cohering the micelle. Existence of a framework of $\alpha_s$-casein and calcium phosphate core with $\beta$-casein hydrophobically bonded to it was also proposed. Bloomfield and Morr (1973) postulated the existence of a size determining micellar framework predominantly made of $\alpha_{s1}$-casein with $\beta$- and $\kappa$-caseins attached to the framework and filling its interstices through Ca$^{++}$ bridges based on experiments on limited calcium dissociation from the micelle. On limited depletion of calcium from the micelle, $\beta$- and $\kappa$-caseins dissociate leaving the framework which has the same frictional resistance as the original micelle.

Rose (1969) proposed a model in which end-to-end $\beta$-casein association is the primary phase of micelle formation to which $\alpha_s$- and $\kappa$-casein molecules are in turn bound to $\alpha_s$-caseins to form a protein aggregate. In the presence of calcium these are cross-linked by calcium phosphate to form the micelle. Garnier and Ribadeau-Dumas (1970) proposed a model based on the aggregation behavior of different caseins to give a three-dimensional porous structure. In this model, trimers of $\kappa$-casein acted as the branching nodes and $\alpha_s$ and $\beta$-casein as the branches.

On the basis of the results of sedimentation behavior of urea- and oxalate-treated casein micelles, Morr (1967) put forth the subunit model in which the subunit core was
made of β-αs-casein complex surrounded by αs-κ-casein complex and the subunits were held together by calcium and colloidal calcium phosphate linkages. Schmidt and Payens (1976) modified this model by proposing subunits of hydrophobic core surrounded by hydrophilic coat of carboxylic and phosphate groups. As proposed by Morr (1967), this model also suggested calcium, magnesium and colloidal calcium phosphate groups as the linkage between the subunits.

Slattery and Evard (1973), proposed that κ-casein is localized on particular regions of the submicellar surface, thus forming two distinct regions which are hydrophilic and hydrophobic. The aggregation of the subunits occurred by hydrophobic bonding until the whole micelle surface was covered with κ-casein. However, this model did not include any function of colloidal calcium phosphate in casein micelle stability. Schmidt (1980) and later Walstra (1990) improved on this model by postulating that those surface regions of the submicelles not covered with κ-casein consisted of the polar moieties of other caseins e.g., the phosphoserine residues. The subunits aggregated together via the colloidal calcium phosphate attached to the phosphoserine residues of αs1-, αs2-, and β-caseins. As in the model suggested by Slattery and Evard (1973), the casein micelle growth terminated when the micelle surface was covered with κ-casein.

Walstra (1999) modified this submicellar model of caseins to include calcium phosphate packages to be placed not only on the surface of submicelles as the bridging between them, but also inside the subunits. However, none of these models can explain the dissociation behavior of casein micelles on treatment with excess κ-casein and urea (Holt, 1998).
A polyelectrolye brush model of casein micelles in which polypeptides of different caseins form a random association to form an entangled structure within which nanoclusters of calcium phosphate are held has also been proposed (Holt 1992; Holt and Horne, 1996). The C-terminal region of the κ-casein on the periphery of the casein micelle forms the polyelectrolyte brush and imparts steric stabilization to the colloidal particle. The binding of casein molecules to the calcium-phosphate nanoclusters prevents their growth into crystals, and blocks calcification of the mammary tissue.

A polycondensation model proposed by Horne (1998) envisages cross-linking of individual caseins through hydrophobic regions of the caseins and bridging involving colloidal calcium phosphate for the assembly of the casein micelle. He postulated that the growth of the hydrophobically-bonded proteins is inhibited by electrostatic repulsive interactions, whereby total interaction energy can be considered to be the sum of electrostatic repulsion and hydrophobic interaction. According to this model, κ-casein is linked to the micelle by hydrophobic bonding of its N-terminal region and therefore, further growth beyond the κ-casein is not possible as it does not possess either a phosphoserine cluster for linkage via colloidal calcium phosphate or another hydrophobic anchor point to extend the chain. This can be considered as a variant of the subunit model as the strong complexes between caseins and colloidal calcium phosphate are linked together only by weak hydrophobic interactions.

Any model for the structure of casein micelles must account for the following experiment data:

1. A predominance of κ-casein on casein micelle surface
2. The importance of colloidal calcium phosphate in maintaining casein micelle integrity

3. The involvement of hydrogen bonding

4. The involvement of hydrophobic interactions

5. The inability to dissociate casein micelles by adding an excess of Κ-casein

6. A particulate structure as observed by electron microscopy

7. A heterogeneous internal substructure based on X-ray and neutron scattering experiments

8. Breakup of casein micelle structure by ion beam sputtering

In case of the electron microscopic evidence for the subunits, McMahon and McManus (1998) argued such structures did not represent the native casein micelle structure as sample preparation introduced many artifacts. They put forward a more representative image of casein micelles from samples prepared by rapid freezing, freeze drying, and uranyl staining to reduce the known artifacts and concluded from the stereoscopic images that the micellar structure was made of strands of electron dense regions not > 8 to 10 nm in length and it formed a hairy micelle rather than a raspberry like structure. In the same way, the variation in scattering intensities from x-ray and neutron scattering experiments does not imply an internal substructure but rather a heterogeneous internal structure of the casein micelle.

**Casein in Dried Milk Products**

Various sources of caseins are available in dried form. Non-fat dried milk, acid casein, rennet casein, sodium caseinate, calcium caseinate, and milk protein concentrates are examples. These products are usually rehydrated before use. The structure of casein
in these rehydrated dried products may vary and differ from the native casein structure as they undergo various methods of processing that can change the structure of the casein micelles (Schuck et al., 2002). Though calcium caseinate when reconstituted can form spherical aggregates, sodium caseinate forms linear aggregates (Farrer and Lips, 1999). Warm temperature (approx. 40°C) and high shear during rehydration optimizes solubility and dispersability of the dried milk proteins.

**Non-bovine Casein Micelles**

Considerable variation in protein content and ratios exist in milks from different mammals (Rollema, 1992). For example, human and goat milk caseins contain little αs-caseins (Ono and Creamer, 1986). However, there is unanimity in the surface location of κ-casein on casein micelles of all species. Considerable variation has also been reported (Ono et al., 1989) in the voluminosity of casein micelles (8 ml/g in human to 2.8 ml/g in sow milk caseins). The size distribution also varies, human and sheep milk having a narrow distribution whereas goat milk shows a broad distribution of size (Buchheim et al., 1989). However, various electron microscopic studies of casein micelles from different species show similar internal structure (Rollema, 1992).

**Rennet Coagulation of Milk**

The enzymatic coagulation of milk by chymosin may be divided into two overlapping phases of enzymatic hydrolysis and aggregation. During the primary stage of enzymatic hydrolysis, κ-casein forming the polyelectrolyte brush on the surface of casein micelles is cleaved into para-κ-casein and glycomacropeptide at its Phe105-Met106 bond. de Kruif et al. (1992) showed that the fraction of hairs left on the surface of casein
micelle at a time after addition of coagulant can be calculated from the volumetric concentration of coagulant added. This primary stage of hydrolysis results in a reduction in the size of casein micelles, net negative charge, and steric repulsion. These pave the way for the secondary phase of aggregation by increasing the attraction between micelles. This viscosity of the system undergoes an initial decline due to the reduction of volume fraction and then increases as micelles start aggregation (McMahon and Brown, 1984b).

Aggregation of casein micelles is minimal below a certain Ca\textsuperscript{2+} concentration and at low temperatures (< 15°C). Proteolysis of κ-casein occurs even at low temperatures even if coagulation does not occur. Coagulation does occur if the temperature is raised. The coagulation time is reduced by the addition of calcium chloride in low levels. At high levels of calcium chloride addition (0.4 M), coagulation time is retarded severely resulting in a weak curd (McMahon et al., 1983).

Changes in pH have a considerable effect on both the enzymatic phase and aggregation phase of caseins (McMahon and Brown, 1984b). These may be related to the effect of pH on colloidal calcium phosphate as reduction in pH by HCl decreases coagulation time while use of citric acid increases coagulation time. HCl reduces colloidal calcium phosphate and increases calcium activity whereas citric acid keeps the calcium activity a constant by forming calcium-citrate complexes as the colloidal calcium phosphate is dissolved. Phosphate also plays a major role in coagulation of caseins as seen from the absence of formation of a gel when milk with calcium and no phosphate is renneted (Zittle, 1970). The effect of temperature shows the role of hydrophobic interactions in aggregation, while the effect of Ca\textsuperscript{2+} and phosphate suggests that charge
neutralization or specific ion-binding effects are also responsible for the secondary phase of rennet coagulation.

References


the secondary structure of bovine casein by Fourier transform infrared

Dalgleish, D. G. and A. J. R. Law. 1989. pH-Induced dissociation of bovine casein
micelles II. Mineral solubilization and its relation to casein release. J. Dairy Res
56:727-735.


de Kruif, C. G., J. M. Jeurnink, and P. Zoon. 1992. The viscosity of milk during the

areas and net charge of as1-, K-casein and as1-casein:K-casein complex. J. Dairy
Res. 47:123-137.


Farrell, H. M., Jr., P. X. Qi, E. M. Brown, P. H. Cooke, M. H. Tunick, E. D. Wickham,


CHAPTER 2
CASEIN: AN IRREGULAR SUPRAMOLECULE

Abstract

A sample preparation protocol that used adsorption of proteins onto a poly-L-lysine and parlodion coated copper grid, followed by staining of the proteins by a uranyl oxalate solution, instantaneous freezing and then drying under a high vacuum, enabled high resolution, transmission electron microscopy stereo-images of the colloidal casein particles in milk to be obtained with minimal artifact formation. Based on these images, the supramolecular structure of casein micelles was observed to have the appearance of an interlocking ring and node structure rather than being a simple entanglement of proteins. The various proteins form linear and branched chains (typically 5 to 10 proteins long) that become interlocked together by calcium phosphate nanoclusters. Based on the known functionalities of the caseins and the calcium phosphate nanoclusters, individual electron dense areas within the casein micelles were assigned to be chain terminating units (κ-casein), linear polymer units (αs1- or β-casein), branching polymer units (αs1- or αs2-casein), or interlocking nodes with a functionality ≥ 4 (calcium phosphate nanoclusters). Thus, the binding of αs- and β-caseins via their phosphoserine clusters to calcium phosphate nanoclusters, and the homo- and hetero-polymerization tendencies of the caseins produces an open sponge-like colloidal supramolecule that would be very resistant to spatial changes. Calcium phosphate functions as nodes from which various sized strands and filigreed rings of protein extend, and become interlocked with other protein strands primarily at the calcium-phosphate nodes. It is this synergy between two concomitant aggregation processes that allows synthesis of casein supramolecules in the
mammary gland without causing pathological calcification. The supramolecule structure of casein micelles thus exhibits the principles of self-aggregation, interdependence, and diversity so often observed in nature

1. Introduction

Mammary gland cells have the biological function to provide nutrition for the growth and energy requirements of the neonate. The challenge in doing so, is providing a low viscosity, high protein, high calcium fluid while avoiding calcification of cells involved in synthesizing and secreting milk. Nature’s solution was to package protein with calcium phosphate as a colloidal supramolecule. This supramolecular structure has been investigated for 50 years (e.g., Swaisgood, 1973; McMahon & Brown, 1984; Holt, 1992; Rollema, 1992; de Kruif & Holt, 2003) and was incorrectly given the name “casein micelle” before it was understood that it did not have a classical micellar structure.

Casein supramolecules do not inherently require a high degree of ordered structure (McMahon & Brown, 1984), and wide variation in genetic variants of caseins results from the imperative of the mammary gland as a calcium transporter organ to avoid pathological calcification over the need for optimal amino acid nutrition of the neonate (de Kruif & Holt, 2003). That is, genetic changes in amino acid sequences are allowed provided they do not impede functioning of the casein supramolecule in sequestering calcium phosphate during its synthesis and transport through Golgi vesicles of the mammary gland. While secondary structure of caseins has been referred to as random coil, they can also be considered as rheomorphic proteins that have an open conformation and considerable conformational flexibility (Holt & Sawyer, 1993). Such rheomorphic
character allows caseins to react very rapidly to sequester small clusters of calcium phosphate before they precipitate and calcify mammary cells (de Kruif & Holt, 2003).

Casein supramolecules are highly hydrated and sponge-like colloidal particles. Their composition (and probably structure) varies between species, and in bovine milk the major components are the four caseins (\(\alpha_s\), \(\alpha_c\), \(\beta\), and \(\kappa\), with their numerous genetic and post-translational variants), calcium, and phosphate. Of the 1 to 8 g of water per gram of protein contained within the casein supramolecule, only about 15% is bound to the protein with the remainder being occluded within the particle (Farrell, Brown, Hoagland, & Malin, 2003; Mora-Gutierrez, Kumosinski, & Farrell, 1997; Swaisgood, 1982). Their size distribution varies from 20 to 600 nm diameter, with an average size between 100 to 200 nm (de Kruif, 1998). It is estimated that an average size casein supramolecule contains \(10^4\) individual protein molecules (Bloomfield & Mead, 1975; Kirchmeier, 1973), and that there are approximately \(10^{14}\) casein supramolecules per milliliter of milk (Schmidt, van der Spek, Buchheim, & Hinz, 1974).

Numerous structural models for the casein supramolecule have been proposed, with until recently the most widely accepted being the “submicelle” model in which casein molecules aggregate into 20-nm diameter particles, and then these “submicelles” further aggregate to produce the casein supramolecule (Rollema, 1992). However, electron micrographs that support the “submicelle” model do not exclude spatial artifacts that may have been introduced during sample preparation (Buchheim & Welsch, 1973; Calapaj, 1968; Kalab, Phipps-Todd, & Allan-Wojtas, 1982).

Some of the problems in preparing casein supramolecules for examination by electron microscopy are that metal coatings obscure fine details of the supramolecule
surface, while air drying and chemical dehydration can collapse outer surface layers.

Integrity of casein supramolecules depends on a combination of factors including strong electrostatic linkages of caseins to calcium phosphate, protein-to-protein interactions such as H-bonding, salt bridging (via calcium ions), and hydrophobic interactions (McMahon & Brown, 1984). Thus, casein supramolecules respond to environmental changes in various ways and subunit structure observed in dissociation (and association) experiments can be considered a function of the self-aggregation nature of caseins.

Caseins are strongly interacting proteins and do not exist as monomers at physiological conditions (Swaisgood, 1992). They form both homo- and hetero-aggregates, via calcium-induced electrostatic interactions, and entropy-driven hydrophobic interactions. They can form tetramers (αs1-casein) and linear polymers (αs1- and β-casein) and soap-like micelles (β- and κ-casein) (Garnier, Yon, & Mocquot, 1964; Payens, 1966, 1968; Payens & Markwijk, 1963; Payens & Vreeman, 1982) (see Appendix A, Fig. A.1a and A.1b). They can interact and polymerize to different degrees under various conditions of pH, ionic strength, and temperature. Except for κ-casein, they precipitate in the presence of calcium.

Individual caseins and their aggregates are known for their non-crystallizing nature that limits the use of X-ray crystallography and NMR to determine structure. Thus, electron microscopy becomes important in deciphering native quaternary structure of the casein supramolecule, provided artifacts are not introduced during sample preparation.
2. Materials and Methods

A new technique of sample preparation of casein supramolecules for transmission electron microscopy, based upon freeze drying (Nermut, 1973) enables viewing of its structure with minimal variation from its native form (McMahon & McManus, 1998). Milk was diluted with water, and casein supramolecules were immediately adsorbed onto poly-L-lysine-treated, parlodion-coated copper grids. The grid with adsorbed proteins was rinsed to remove secondary adsorbed material, then placed on top of a drop of 12 mM solution of uranyl oxalate (50:50 uranyl acetate and oxalic acid), rinsed with water, then flash frozen in liquid nitrogen-cooled, liquefied Freon 22, and freeze dried. They were viewed with a Zeiss 902 (Thornwood, NY) transmission electron microscope at 80 kV. To produce stereopairs of images, multiple images were captured at varying tilt angles.

3. Results and Discussion

We have observed that uranyl oxalate acts as a stain for both caseins and calcium phosphate. In the stereoscopic image of the casein supramolecule, individual electron-dense locations (dark pixels in positive images, and light pixels in negative images) ranging from 1 nm x 1.5 nm to 4 nm x 4 nm in size were discernable. Each electron dense location represents a single particle that could be either a protein molecule or a calcium phosphate nanocluster. There is probably a slight underestimation of particle size (especially the proteins) as scattering of the electron beam is primarily by uranyl atom attachment at negatively-charged areas of the proteins and accessible areas of calcium-phosphate nanoclusters. A negative-image stereoscopic view of casein supramolecule captured at 85,000× is shown in Fig. 2.1.
On closer examination of electron-dense locations on the supramolecule periphery (see Fig. 2.2a), planes of interconnected particles were observed when the images were viewed stereoscopically. Classifying these particles based on the number of other particles to which they were physically associated, allowed functionality values ($f$) to be assigned. Chain-terminating particles ($f = 1$) were colored red {triangles}, particles associated with two other particles ($f = 2$) were colored green, those with $f = 3$ were colored blue, and those with $f \geq 4$ were colored black (Fig. 2.2b). Towards the center of the supramolecule, there were too many overlapping planes of electron-dense locations to visually isolate individual planes, however, no differences in arrangement of particles was apparent between the supramolecule interior and periphery.

Functionality of casein molecules can also be considered on a chemical basis on their possible interactions with other entities that are present in the casein supramolecule.
Fig. 2.2. Image of casein supramolecule (a) with electron-dense locations in the periphery color-coded (red \( f=1 \), green \( f=2 \), blue \( f=3 \), black \( f\geq4 \)) (a) and the background deleted to display an individual plane of the structure (b).

This includes their calcium-mediated interactions via clusters of phosphoserine groups (Dalgleish & Parker, 1979), their hydrophobic regions that allow interaction between proteins, and hydrophilic regions as sites for interaction with water (Yoshikawa, Sasaki, & Chiba, 1981). Horne, Parker and Dalgleish (1988) assigned chain-terminating \( f = 1 \), bifunctional \( f = 2 \), and trifunctional \( f = 3 \) roles to \( \kappa \)-, \( \beta \)-, and \( \alpha_5 \)-caseins respectively, although it may be more appropriate to assign \( \alpha_{s1} \)-casein as \( f = 2 \), and \( \alpha_{s2} \)-casein as \( f = 3 \). In addition, calcium phosphate nanoclusters can bind multiple phosphoproteins (i.e., \( \alpha_{s1} \)-, \( \alpha_{s2} \)-, and \( \beta \)-casein) such that they have a functionality \( \geq4 \) (Holt, Wahlgren, & Drakenberg, 1996).

From the digitally-magnified, colorized and background-deleted image of the fine structure of the casein supramolecule (Fig. 2.2b), it is evident that the electron dense locations are proteins and calcium phosphate nanoclusters while the space around them is
electron transparent. This outer region of the supramolecule has been described as a diffuse hairy layer and compared to a polymeric brush extending into the solvent (de Kruif & Holt, 2003). However, it is apparent that the surface structure is not different to the rest of the supramolecule as suggested by the term “hairy layer”, nor is the outer surface of uniform size. Instead, surface tendrils should be considered a natural extension of the internal structure and a result of chain-termination during synthesis of casein supramolecules. The imperfect spherical nature of the supramolecule surface and uneven extension of poly-protein strands on the supramolecule periphery can lead to various problems if it is assumed to be a hydrodynamic hard-shelled sphere when determining particle size or in assigning a thickness to the outer layer of proteins.

Based upon the potential functionalities of the caseins (and the calcium phosphate nanoclusters), a model of the colloidal casein particle can be constructed as an irregular supramolecule (Fig. 2.3). The flexibility of the caseins permits various structures, such as clumps, loops and linear strands, to be formed based upon random associations during

Figure 2.3. Schematic model of the casein supramolecule (a) based on interconnected protein strands between calcium phosphate nanoclusters (b). Grey-colored shapes indicate particles off the cross-sectional plane.
cellular synthesis of the casein supramolecule. This results in an irregular structure being developed rather than the repeated structures that form the basis for the formation of regular supramolecules. In Fig. 2.3, chain-terminating molecules (primarily \( \kappa \)-casein) are represented by triangles, chain-extending molecules (primarily \( \alpha_{s1} \)- and \( \beta \)-casein) are represented by squares, chain-branching molecules (primarily \( \alpha_{s1} \)- and \( \alpha_{s2} \)-casein) are represent by pentagons, and the calcium phosphate nanoclusters are represented by circles. Stabilization of the calcium phosphate nanoclusters occurs through binding of \( \alpha_{s1} \)-, \( \alpha_{s2} \)- or \( \beta \)-casein to the growing nanoclusters.

Interaction among proteins and between proteins and calcium phosphate in casein supramolecules has been described in various ways. In submicelle models, interactions between submicelles were proposed to be between calcium-sensitive caseins, unless there was sufficient covering of \( \kappa \)-casein (Horne, 1998; Walstra, 1999). A similar polyfunctional condensation model of casein supramolecules having an open structure with a network of proteins crosslinked by hydrophobic bonds with salt-bridging between calcium-sensitive caseins had also been proposed (Horne, 1998). In both types of models, \( \kappa \)-casein terminates growth as it has only one site for hydrophobic interactions and does not participate in calcium bridging. A further similarity is that within the supramolecule there are polymerizing units that are held together through complexing of phosphoserine clusters of \( \alpha_{s1} \)-, \( \alpha_{s2} \)-, and \( \beta \)-casein with calcium phosphate nanoclusters, or hydrophobic interactions between proteins. Thus, any actions that affect calcium phosphate electrostatic interactions (such as lowering pH) or hydrophobic interactions (such as lowering temperature) will disrupt the integrity of native casein supramolecules.
It may be hypothesized, that as caseins attach to the developing nanoclusters of calcium phosphate, chains of proteins concomitantly form via hydrophobic and electrostatic interactions. Because of the flexibility and three-dimensional functionality of the proteins these chains join together to produce a spherical-type structure with interlocked strands of protein throughout the entire casein supramolecule. When observed under the electron micrograph, the internal structure has the appearance of interconnected and filigreed rings. Such a structure is also observed in milks of other species (see Figs. A.2a, A.2b, and A.2c in Appendix A).

Interactions between caseins (and calcium phosphate) are random by nature and can cause considerable variation in the arrangement of proteins within the casein supramolecule (Fig. 2.4). It was observed that electron density increases from the periphery to the center as expected because of sample thickness. Except for the peripheral portions it appeared that the segment density on a volume basis was constant as would be expected with polymerization occurring in all directions as the supramolecule was synthesized until all available/allowable cross-linking sites were filled. Addition of κ-casein to growing protein strands would cause termination of that strand whether it is on the periphery or interior of the supramolecule. This does not preclude strand development from alternative directions within the supramolecule, although complete cross-linking may not occur at those locations occupied by a κ-casein molecule that is anchored to another poly-protein strand.

It is this dual process of protein polymerization into chains, and binding of multiple proteins to calcium phosphate nanoclusters that results in a somewhat regular sponge-like appearance of the casein supramolecule. The distance between calcium
Fig. 2.4. Low magnification (7,000×) field showing the typical size variation observed for casein supramolecules (a). Three different casein micelles captured at high magnification (140,000×) showing inherent variation in protein arrangement within the supramolecule (b, c, d).
phosphate crosslinking sites is relatively small, and typically there appeared to be three to six protein molecules between crosslinking sites. Thus, a structure is obtained that is very resistant to spatial changes. It also helps explain the high level of water occluded inside the casein supramolecule, and the permeation of large molecules (such as proteinases) into the supramolecule interior.

4. Conclusions

The irregular supramolecular structure (Fig. 2.3) for the colloidal casein particles in milk, supports an open structure in which different caseins can attach to calcium phosphate nanoclusters preventing calcium phosphate crystallization in the mammary gland. Chains of proteins can then grow until they encounter a chain terminating protein, bond with another chain, or become attached to another calcium phosphate nanocluster. Also, the different dissociation and aggregation behavior of casein supramolecules may be explained using this model. Overall, this study has put forth a molecular model for the casein supramolecule that satisfies the principles of self aggregation, interdependence, and diversity that are often observed in nature (Swimme & Berry, 1992).

Synthesis of casein supramolecules in the mammary gland rely on a controlled synergy between two concomitant aggregation processes. Calcium phosphate is formed into clusters because of its low solubility, and caseins are simultaneously undergoing polymerization because of their calcium sensitivity and hydrophobic nature. Precipitation of calcium phosphate is limited to formation of nanoclusters by binding of caseins via their phosphoserine side chains, and the polymerization of the caseins is limited to colloidal size by the chain-terminating influence of κ-casein.
References


Abstract

Microstructure of caseins in native raw milk, non-fat dried milk, calcium caseinate, and sodium caseinate were studied using transmission electron microscopy. Solutions of all the dried products in water were made to a casein concentration of 2.4% and pH of the resulting solution adjusted to 6.7 before samples were drawn at 4 and 10 h of hydration with moderate shear (shear rate = 25 s⁻¹). In another set, samples were mixed at high shear (shear rate = 735 s⁻¹) for 5 min and allowed to stabilize for 1 h before sampling. After 4 h of hydration and moderate mechanical stirring, small casein micelles in non-fat dried milk were seen as agglomerates, calcium caseinate micelles were seen interconnected with proteins and sodium caseinate appeared as a mesh of proteins. After longer hydration time of 10 h or with high shear the agglomeration of the micelles were not seen. In the case of sodium caseinate, where micellar structure is minimal, the proteins formed strands, or small aggregates of strands, when hydrated longer or sheared at a higher rate. We have demonstrated the use of physical methods of fixation and flash freezing in sample preparation for viewing protein aggregates using transmission electron microscopy with minimal changes in protein structure.

Coagulation properties such as rennet coagulation time and curd firmness of skim milk (2.91% protein) fortified with non-fat dried milk, calcium caseinate, and sodium caseinate to a protein concentration of 2.99%, 3.17%, and 3.35% were measured using a
Formagaph. At higher levels of added calcium caseinate and sodium caseinate, the milk exhibited longer rennet coagulation time and the resultant gel formed was weaker. Addition of potassium dihydrogen phosphate to milk supplemented with calcium caseinate and calcium chloride to milk supplemented with sodium caseinate, prior to the addition of rennet restored the coagulation properties and coagulum structure to that of the original milk.

1. Introduction

Dried milk products are increasingly being used as ingredients in different food products for their functionality and nutritional properties. Caseins in these dried products impart unique functional characteristics to the end product that can be attributed, at least partly, to its structure. Various processes involved before its end use could attribute to the change in structure of caseins. In most cases, these dried milk products are rehydrated for use as ingredients. The extent of rehydration of caseins may depend on the method and duration of rehydration. Furthermore, the salt balance of the solution especially, calcium, citrate, and phosphate contents are known to affect the structure and supramolecular mineral composition of casein (Fox & Nash, 1979). The salt balance of the casein solution being dried will also affect the water transfer properties of the dried casein during rehydration and thus its structure.

Four different types of caseins viz., $\alpha_{\text{s1}}$, $\alpha_{\text{s2}}$, $\beta$, and $\kappa$-caseins and calcium phosphate are held together in a supramolecule by non-covalent forces of attraction such as hydrophobic, ionic, and electrostatic interactions. Caseins are strongly interacting proteins and do not exist as monomers at physiological conditions (Swaisgood, 1992). They form both homo- and hetero-aggregates, via calcium-induced electrostatic
interactions, and entropy-driven hydrophobic interactions. Both \(\beta\)- and \(\kappa\)-caseins form soap-like micelles with a degree of association of 23 and 30, respectively (Payens & Vreeman, 1982), \(\alpha_{s1}\)-Casein forms tetramers and subsequent linear polymers (Payens, 1966), while \(\beta\)-casein forms linear polymers of indefinite size (Payens & Markwijk, 1963). Both \(\alpha_{s1}\) and \(\beta\)-casein form mixed complexes with \(\kappa\)-casein (Garnier, Yon, & Mocquot, 1964; Payens, 1968), and they can interact and polymerize to different degrees under various conditions of pH, ionic strength, and temperature. In the presence of calcium, \(\alpha_{s1}\), \(\alpha_{s2}\), and \(\beta\)-caseins will precipitate. Thus, in presence of monovalent and divalent ions, the aggregation behavior of caseins differ. Therefore, comparison of structures of sodium and calcium caseinate will help in determining the changes that occur in casein structure when excess \(\text{Na}^+\) and \(\text{Ca}^{2+}\) is present.

Calcium and sodium caseinates are manufactured from a dispersion of good quality, fresh acid-casein curd (or alternatively from dry acid-casein) by neutralization with calcium and sodium hydroxide respectively (Southward, 1985). Various manufacturing processes affect the properties of the end product. It is known that calcium caseinate dispersions appear opaque and white, while sodium caseinate solutions are translucent and non-milky. For the same solids concentration, calcium caseinate has a lower viscosity than sodium caseinate solutions. These differences may be attributed to the difference in structure of the aggregates formed in solution.

These differences in structure would consequently affect the properties of the products in which they are used. Usually, these dried proteins are hydrated before use and therefore, the structural changes during hydration are of importance in determining the end use of the product. In this experiment we have investigated the use of a sample
preparation method for transmission electron microscopy (TEM) and its use in monitoring the structural changes in caseins during its rehydration. Microstructure of caseins in rehydrated nonfat dried milk (NFDM), calcium caseinate (CaCN), and sodium caseinate (NaCN) were compared and the effect of time and method of their hydration on their microstructure investigated. We also examined the changes that occur in rennet coagulum structure when skim milk is supplemented with these dried milk proteins.

2. Materials and Methods

2.1. Samples and Hydration

Solutions of NaCN (Alanate® 180, New Zealand Milk Products, Santa Rosa, CA) and low viscosity CaCN (Alanate® 380, New Zealand Milk Products), and NFDM were made by dissolving appropriate quantities of the dried product in water at 40°C to form a 2.4% casein solution. They were hydrated for 10 h at a pH of 6.65 ± 0.05 using a magnetic stir bar imparting moderate shear (shear rate = approx. 25 s⁻¹). Samples were drawn for electron microscopy after 4 (MS4) and 10 h (MS10) of hydration.

In another treatment, these dried powders were suspended using high shear (shear rate = approx. 735 s⁻¹) for 10 min (HS1) and subsequent hydration for 1 h. A hand held homogenizer (Omni 5,000) at approximately 7,000 rpm was used.

Raw milk was obtained from Caine Dairy Research and Teaching Center (Utah State University, Logan, UT) and the fat was skimmed centrifugally to obtain raw skim milk. The effect of calcium depletion of casein micelles on their structure was studied by treating skim milk, and skim milk in which the proteins had been fixed by adding a 2%
glutaraldehyde solution, with 0.5g of disodium EDTA and 0.8g of tetrasodium EDTA per 100 ml of milk to chelate calcium.

2.2. *Standardization*

Protein solutions for fortification of milk were prepared by high-shear mixing (5 min) of NFDM, or CaCN, or NaCN at 40 °C. These were allowed to stabilize for 8 h with moderate stirring. Skim milk was fortified with 1% (NFDM1 or NaCN1 or CaCN1), 3% (NFDM3 or NaCN3 or CaCN3), or 5% (NFDM5 or NaCN5 or CaCN5) of the above 12%-protein solutions to increase the protein content in skim milk from 2.9% to 3.0, 3.2, and 3.4%, respectively.

2.3. *Formagraph Test*

The supplemented milks were stabilized for 1 h prior to Formagraph testing. As higher amounts of CaCN and NaCN caused undesirable coagulation properties (such as longer rennet coagulation time (*RCT*)), phosphate in the form of KH$_2$PO$_4$ was added to CaCN dispersions and calcium in the form of CaCl$_2$ was added to NaCN dispersions, 30 min prior to rennet addition in the Formagraph. Calcium and phosphate was added up to a concentration of 2.4 and 72 mM of Ca and PO$_4$, respectively.

Ten milliliters of these milks were brought to 35 °C and allowed to stand for 30 min at that temperature when 0.1 ml of double strength rennet diluted 1:100 was added. The milk was allowed to set in a Formagraph and time-firmness curves obtained (McMahon & Brown, 1982). Rennet coagulation time and curd firmness at 60 min (A$_{60}$) after rennet addition were calculated.
2.4. Electron Microscopy

Samples of protein or casein solutions were prepared for electron microscopy as described by McManus and McMahon (1997). The protein samples were diluted 100 fold with either water or skim milk ultrafiltrate to reduce protein concentration to less than 24 mg/100 ml. Copper grids (600 mesh) coated with parlodion (nitrocellulose) film was used as the support for adsorbing the protein particles and subsequent use in the TEM. Parlodion film was made by dissolving strips of parlodion in amyl acetate as the solvent. The parlodion film on the copper grids was coated with poly-L-lysine so as to give a positive charge to the film. This positive charge facilitates the adsorption of negatively charged protein particles. These grids were placed on the solution containing caseins for 60 s to allow protein adsorption. The grids were washed two times in water for 10 s before being stained with 12 mM solutions of uranyl acetate and oxalic acid for 60 s. The grids were washed again in water to remove the excess stain and flash frozen in liquid nitrogen-cooled, liquefied Freon 22 (−159°C; Mallinckrodt Inc., Paris, KY). They were then freeze dried to allow observation of the native structure of the protein particles. Samples were viewed in a Zeiss 902 (Zeiss, Thornwood, NY) transmission electron microscope and photographed at a magnification of 50,000×, 85,000× and 140,000× at 80 kV.

At the 3% level of casein supplementation, curd samples (approximately 1 mm × 1 mm × 10 mm) were obtained at twice their RCT for sample preparation for electron microscopy (SEM and TEM). Samples were prepared for SEM as described by McManus, McMahon and Oberg (1993) and for TEM as described by Paulson, McMahon, and Oberg (1998) and viewed using a S-4000T field emission scanning
electron microscope (Hitachi Scientific Instruments, Mountain View, CA) at an accelerating voltage of 3 kV and Zeiss 902 microscope at 80 kV. The figures shown for NaCN3 and CaCN3 with added calcium and phosphate represent an addition of 1.2 and 9 mM calcium and phosphate respectively.

Curd samples were fixed in 2% glutaraldehyde (Electron Microscopy Sciences, Fort Washington, PA) for 1 h at room temperature (22° C) and the solution changed and stored further for 2 d at 4 °C. Samples were frozen in liquefied Freon 22 (Mallinckrodt Inc.), transferred to liquid nitrogen, cryofractured perpendicular to their long axis, and thawed in 2% glutaraldehyde solution. They were then dehydrated in a graded ethanol series followed by fat extraction with Freon 113 (Mallinckrodt Inc.) and overnight storage in Freon 113 at 4 °C. The samples were rehydrated by reversing the graded ethanol series, and washed with 0.1 M sodium cacodylate buffer (Electron Microscopy Sciences), pH 7.2. The samples were then post-fixed for 2 h with a solution containing 1% OsO4 (Electron Microscopy Sciences) and 1.5% K4Fe(CN)6·3H2O (Fisher Scientific Co., Fair Lawn, NJ). Samples were treated with 2% tannic acid (Mallinckrodt Inc.) solution in cacodylate buffer for 3 h at room temperature. The tannic acid solution was then replaced with a solution of osmium tetroxide and potassium ferrocyanate, and samples left for 4 h after which they were left overnight in 1% hydroquinone (Mallinckrodt Inc.). The samples were washed with distilled water, dehydrated in a graded ethanol series, and critical point dried in a critical-point drier (Model 1200; Polaron, Waterford, United Kingdom) with CO2. Samples were viewed in the S-4000T field emission scanning electron microscope operated at 3 kV. Images from each sample, were recorded on Kodak TMX 120 film, and digitally using Spectrum 2.0 software (The
Dindima Group Pty. Ltd., Ringwood, Victoria, Australia). Fields were randomly selected from areas of the sample that showed good quality planes of fracture.

Curd samples fixed in 2% glutaraldehyde were placed into 1% OsO4 (Electron Microscopy Sciences, Fort Washington, PA) in 0.2 M cacodylate buffer for 1 h (Electron Microscopy Sciences, Fort Washington, PA). These were dehydration in a graded ethanol series, and the samples were infiltrated with Spurr’s epoxy overnight. Epoxy infiltrated samples were transferred to Beem capsules filled with Spurr’s epoxy, and incubated at 70°C for 24 h. Ultra-thin sections of 70 nm were cut on an Ultracut ultramicrotome (Leica Inc., Deerfield, IL), transferred to 300-hex mesh copper grids, and counterstained with uranyl acetate and lead citrate. They were then viewed on a TEM (Model 902; Carl Zeiss, Inc. Thornwood, NY) and photographed at a magnification of 85,000x at an accelerating voltage of 80 kV.

3. Results and Discussion

3.1. Electron Microscopy

According to McMahon and McManus (1998) the freeze-drying method allows for examining casein micelles and provides more structural information than other electron microscopic methods and minimizes formation of artifacts. As we used skim milk ultrafiltrate to dilute milk we have minimized potential structural changes that could be caused by altering ionic strength. In an experiment to determine the effect of ultrafiltrate and water as the diluting agents, we found that there were no noticeable differences between the two (see Appendix A, Fig. A.3.). This is probably because the time elapsed between diluting of samples and adsorption onto the parlodion-coated grids.
was minimal (<10 s) to such that no detectable change occurred in the quaternary structure of the proteins.

Once the proteins were adsorbed on to the film by electrostatic attraction, washing the grid with water removed any secondary layers of proteins that were held on to the film by surface tension (see Appendix A, Fig. A.4.). Staining with equimolar solutions of uranyl acetate and oxalic acid provides an acid medium for staining. Uranium is known to be a positive protein stain in acidic conditions. It has been shown that uranyl ions form reversible but stable complexes with phosphoryl and carboxyl ligands on the outer surface of membranes (Rothstein, 1970). Furthermore, it has also been demonstrated that uranyl ions can react with the phosphate groups in lecithin mono-layers (Shah, 1969). Studies of the infrared spectra of DNA-uranyl complex suggest that the uranyl ion forms a simple salt with the phosphate groups in DNA (Zobel & Beer, 1961). At higher pH, uranyl ions tends to complex, form larger species and exist as colloidal particles resulting in darker staining that prevented fine structural detail of casein micelles being observed (see Appendix A, Fig. A.5).

Free amino groups have also been shown to play an important role in binding uranyl ions (Lombardi, Prenna, Okolicsanyi, & Gautier, 1971). Therefore, it may be assumed that the uranyl ions can attach to all of the functional groups present in amino acids except the sulphydryl group. Changes in the structure of the protein at low pH could be expected, but no such changes were observed and the stained casein micelles had similar structure to unstained casein micelles (Figs. 3.1 and 3.2). The only difference between stained and unstained micelles were the differences in contrast. Thus, staining with uranium helps in obtaining an image with better contrast and therefore better clarity
without changing the structure. When a positively-stained image is viewed using TEM, the stained protein particle appears as a dark image against a light background (see Fig. 3.1b). This difference in contrast and brightness is a result of difference in electron scattering or electron impermeability of the positively-stained particle compared to the background material.

If the particle being viewed is thicker, or has heavy metal atoms attached, or is denser than the background material we are able to distinguish the image from the background. This implies that in order to detect the presence of a particle using a transmission electron microscope, the combined thickness and electron density of the particle must be greater than that of the support film (e.g., nitrocellulose) to which it is attached. Attachment of the heavy metal stain to the casein micelles imparted better contrast and clarity to the image.

Fig. 3.1. Micrograph of casein supramolecule: (a) unstained, and (b) stained with uranyl oxalate, bar = 100 nm.
Uranium has been suggested to replace calcium in casein micelles (Knoop, Knoop, & Wiechen, 1973). This raised the question of whether we were observing sites where calcium had been replaced by uranium, or protein that had been stained with uranium. When calcium-depleted casein micelles from EDTA-treated milk were examined (Fig. 3.2), the protein bound sufficient uranium to give adequate contrast and pictures with excellent clarity showing the proteins were obtained. Similarly staining was also obtained for sodium caseinate samples that contain little or no calcium attached to the proteins. We also imaged glutaraldehyde-fixed casein micelles which were treated with EDTA after fixation (Fig. 3.2b). These had a more compact appearance that has been observed to occur because of glutaraldehyde fixation (McMahon & McManus, 1998) and were heavily stained in comparison to the unfixed milk. Thus, it is evident that in acidic conditions the proteins in milk do bind uranium, and the uranyl oxide mixture acts as a positive heavy metal stain.

Fig. 3.2. Calcium-depleted casein micelles obtained by adding EDTA to (a) skim milk, and (b) skim milk in which the proteins had been fixed with glutaraldehyde.
It has been reported that colloidal calcium phosphate present within casein micelles has a long exchange time with calcium in solution, and by comparison, exchange of calcium with uranium may also be slow. However, we believe it is a reasonable assumption that using this electron microscopy protocol that both the proteins and the calcium phosphate nanoclusters in the casein micelles are being imaged as a result of the electron scattering from bound uranium.

3.2. Non-fat Dried Milk

After 4 h of dispersion in water, NFDM produced small particles (≤ 100 nm) as seen in Figs. 3.3a and 3.3b. While most protein particles were dissociated, agglomerates of some particles (casein micelles) were observed. Proteins that were not part of an aggregates of were observed as singular protein strands or spots dispersed throughout the micrographs. The colloidal aggregates formed from NFDM were not spherical as in milk (Fig. 3.1a) or as observed in rehydrated calcium caseinate. Increasing the hydration time to 10 h dispersed the agglomerates into individual particles of various sizes (Figs. 3.3c and 3.3d) that were more similar to the native casein micelles observed in milk. High shear for 10 min and hydration for 1 h also reduced the clumping of individual spherical casein colloids (Figs. 3.3e and 3.3f)

3.3. Sodium Caseinate

Sodium caseinate solution was transparent to translucent in appearance and was more viscous than the rehydrated NFDM and the calcium caseinate solution. The proteins that were adsorbed from the NaCN solution onto the parlodion-coated grids were observed to be present as a gel-like structure with the proteins forming a mesh across the
Fig. 3.3. Casein micelles obtained from nonfat dried milk after (a, b). 4 h of rehydration; (c, d). 10 h of rehydration; (e, f) high shear and 1 h rehydration in water.
grid surface (Fig. 3.4a). At the lower magnifications there was an absence of any spherical structure to the protein.

At higher magnification (140,000×), it was observed that the mesh structure was made up small protein particles that were approximately 2 to 5 nm in size (Fig. 3.4b). The absence of the cross-linking effect of calcium may explain the inability of sodium caseinate to form spherical aggregates. Hydration for 10 h (Figs. 3.4c and 3.4d) dispersed the caseins but not as effective as the HS1 treatment (Figs. 3.4c and 3.4f).

When hydrated at high shear, or for 10 h, the proteins appeared as strands or small agglomerates of proteins. It is likely that these small aggregates can take part in aggregation of caseins in the presence of calcium to form spherical colloidal casein particles. Consequently, such reconstituted casein micelles would be expected to exhibit a subunit structure even though such a structure was not apparent in native casein micelles from milk.

3.4. Calcium Caseinate

When calcium caseinate was rehydrated, it formed large spherical aggregates (100-600 nm in diameter) as seen in Figs. 3.5a and 3.5b. These particles were entrapped in a gel-like structure. Most of the particles were connected to each other in a mesh of proteins. The darker color (resulting from increased electron density) of the CaCN particles could result from a combination of higher concentration of protein in the particles or high calcium concentration, such that increased uranium-binding occurred during the heavy metal staining. Both the 10-h hydration at low shear and the high shear treatment were sufficient to separate calcium caseinate into individual particles. There
Fig. 3.4. Protein obtained from sodium caseinate powder after: (a, b) 4 h of hydration; (c, d) 10 h of hydration; (e, f) high shear and 1 h hydration.
Fig. 3.5. Casein micelles obtained from calcium caseinate powder after: (a, b) 4 h of hydration; (c, d) 10 h of hydration; (e, f) high shear and 1 h hydration
were both large colloidal particles (approximately 300 nm in diameter) and smaller particles of 10 to 20 nm diameter observed in the micrographs.

The differences in structure observed between the colloidal protein particles obtained by rehydrating NFDM, NaCN, and CaCN powders may be due to their compositional differences and the differences in processing used to manufacture the powders. The agglomerated casein particles seen in all three products after 4 h of hydration at low shear, indicate inadequate hydration or dispersion time to reach equilibrium. Others (Moughal, Munro, & Singh, 2000) have reported that it requires at least 4 to 6 h for dry caseins to disperse under moderate mechanical stirring to yield a typical size profile.

It has also been reported that destruction of the native structure of casein micelles would increase the rehydration rates (Schuck et al., 2002). They demonstrated this by studying the rehydration properties of native phosphocaseinate suspension with added citrate or phosphate. This would imply a higher rate of rehydration for sodium caseinate with no colloidal particle structure compared to calcium caseinate with a colloidal casein micelle-like structure. The water transfer rate of powders during rehydration also depends on the particle size of the powder (Baldwin, Baucke, & Sanderson, 1980; Bloore & Boag, 1982). The clumping of particles observed after 4 h hydration could also be a result of lack of dispensability of the powder particles. Once the powder particles are dispersed, then water transfer rate and rehydration of the individual proteins and colloidal aggregates would depend on the size and structure of caseins and their aggregates.
3.5. Coagulation Properties

Rennet coagulation time of skim milk fortified with NFDM decreased with higher amounts of fortification (Fig. 3.6). In contrast, skim milk supplemented with NaCN had longer RCT as the level of supplementation increased (Fig. 3.7). These differences diminished with calcium addition such that the RCT of skim milk supplemented with NaCN became comparable to that of the original milk when adequate calcium was added. At higher levels of protein fortification with NaCN, the more calcium was needed. A similar trend of increased RCT with when milk was protein fortified using CaCN was observed. Rennet coagulation time was resorted to that of the original milk by adding phosphate (Fig. 3.8).

Curd firmness, measured 60 minutes after adding rennet to the milk, increased as the milk was supplemented with NFDM (Fig. 3.9). This was expected based on the increase in protein concentration and the shorter RCT when NFDM was added.
Fig. 3.7. Rennet coagulation time of skim milk fortified with sodium caseinate to protein levels of 3.0% (diamond), 3.2% (square), 3.4% (triangle) as a function of added calcium chloride, compared to skim milk with 2.9% protein and no calcium addition (---). Error bar shows SE.

Fig. 3.8. Rennet coagulation time of skim milk fortified with calcium caseinate to protein levels of 3.0% (diamond), 3.2% (square), 3.4% (square), as a function of added potassium phosphate, compared to skim milk with 2.9% protein and no phosphate addition (---). Error bar shows SE.
Fig. 3.9. Curd firmness (A60) measurement of skim milk fortified with nonfat dried milk as a function of protein content. Error bar shows SE.

However, when skim milk was supplemented with NaCN there was a reduction in curd firmness even though the casein content of the milk had been increased. This was most evident in sample NaCN5 in which the protein level had been increased to 3.4% (Fig. 3.10).

Addition of calcium to the NaCN-fortified milk increased their curd firmness just as it shortened the time for a coagulum to form (Fig. 3.7). In a similar manner, the effect of adding CaCN to skim milk on decreasing curd firmness (compared to the control skim milk with no fortification) was reversed by adding phosphate (Fig. 3.11). Similar results on RCT and curd firmness on reconstituted NFDM have been previously observed (Dalgleish, 1983; McMahon, Brown, Richardson, & Ernstrom, 1984). The decrease in RCT and increase in curd firmness resulting from protein supplementation using NFDM or ultrafiltration (McMahon & Brown, 1984; Oommen, Mistry, & Nair, 2000) may
Fig. 3.10. Curd firmness ($A_{60}$) measurement of skim milk fortified with sodium caseinate to casein levels of 3.0% (diamond), 3.2% (square), 3.4% (triangle) as a function of added calcium chloride, compared to skim milk with 2.9% protein and no calcium addition (---). Error bar shows SE.

Fig. 3.11. Curd firmness ($A_{60}$) measurement of skim milk fortified with calcium caseinate to protein levels of 3.0% (diamond), 3.2% (square), 3.4% (triangle) as a function of added potassium phosphate, compared to skim milk with 2.9% protein and no calcium addition (---). Error bar shows SE.
primarily result from an increase in number of effective collisions between casein micelles. Both of these processes would decrease the aqueous phase volume in comparison to the protein content. It could also result from increased secondary phase interactions brought about by an increase in calcium concentration as suggested by Lucey (1993).

In contrast, while the supplementation of skim milk with NaCN increases the casein content of the milk it results in a decrease in the calcium:protein ratio. Consequently, the calcium activity of the milk would decrease as calcium is bound to the added casein and is incorporated into existing casein micelles in the milk, or as new reconstituted casein micelles are generated. A new equilibrium position between soluble and colloidal calcium phosphate would be obtained and the calcium activity required for normal rennet coagulation would not be available for participation in the gelation process such that the RCT would be longer and the curd firmness weaker.

Addition of CaCl₂ to this mix of skim milk and NaCN would restore the calcium activity, and may induce the formation of more colloidal casein particles that could then take part in the coagulation process of and also. Calcium addition also reduces the pH by the exchange of Ca²⁺ for H⁺ which can also indirectly increase the rate of enzymatic reaction (Jen & Ashworth, 1970). Although the primary phase of coagulation is independent of calcium concentration, the secondary (non-enzymic) phase is calcium dependent (McMahon & Brown, 1984). Rennet coagulation time of reconstituted skim milk decreases when up to 50 mM calcium is added, and then increases to the original RCT at around 100 mM. At high calcium concentrations (400 mM), rennet coagulation is severely retarded (McMahon et al., 1984).
In a similar manner to the improvement in coagulability of NaCN-fortified milk when calcium was added to restore calcium:protein levels, it was necessary to add phosphate to the CaCN-fortified milk to restore its coagulability. Adding phosphate to milk prior to adding rennet will reduce its RCT (McMahon et al., 1984), with a minimum RCT in reconstituted NFDM occurring with 10 mM added phosphate. At higher level (40 mM added phosphate) there was an increase in RCT. Also, in the absence of phosphate, renneted caseins in presence of calcium forms a floc instead of a gel (Zittle, 1970). Thus, it could be envisaged that addition of phosphate to CaCN-fortified milk would be necessary to compensate for the increased calcium so as to reduce calcium activity to levels that are not detrimental to enzyme action on casein micelle aggregation and gelation.

The protein network structure of milk gels obtained by renneting skim milk, and CaCN3, NaCN3 milks are shown in Fig. 3.12. In the control milk and the CaCN-fortified milk, individual casein micelles were still observed as part of the gel structure at the time the proteins were fixed (i.e., twice RCT). The spherical nature of the casein micelles was particularly evident in the TEM images (Fig. 3.12b and d). In the SEM images the casein micelles were observed to form clusters and chains throughout the three-dimensional gel of network. In the TEM image there was less evidence of chain formation because only a 70-nm slice through the sample was being imaged. Both the control and the CaCN3 milks were similar in appearance.

Fortifying milk with NaCN, produced a gel in which the spherical nature of the casein micelles was less evident and there was considerable non-colloidal (or non-particulate) protein material attached to the casein micelles. Presumably, this resulted
Fig. 3.12. Structure of rennet-induced gels from (a, b) skim milk, and skim milk fortified to 3.2% protein using (c, d) calcium caseinate and (e, f) sodium caseinate, as shown by scanning (a, c, e) and transmission (b, d, f) electron microscopy.
from the coalescence of non-particulate caseins of the NaCN with the casein micelles of skim milk when the casein micelles were made reactive as a result of renneting.

When phosphate was added to the CaCN3 milk sample, the gel structure formed upon renneting contained regular-sized (i.e., 100 to 200 nm diameter) spherical particles as well as many smaller particles (Fig. 3.13). In the SEM image this was seen as non-particulate material attached to the casein micelles (Fig. 3.13c), while in the TEM image it was apparent that these were smaller particles (approximately 10 to 40 nm diameter) that were incorporated as part of the gel network. So even though the coagulability of the milk had been restored the structures of the phosphate-added, CaCN-supplemented milk was different from that of the control milk. The consequence of this upon subsequent syneresis of the curd is unknown.

Adding calcium to NaCN3 milk sample resulted in a gel structure (Fig. 3.13e, f) after renneting that similar to the control milk gel (Fig. 3.13a, b). The gel network was observed as individual spherical particles that were 100 to 200 nm diameter joined together into clusters and chains. The diffuse protein material that was observed in the NaCN3 milk sample without added calcium was not apparent. Rather, the casein micelles had gained a definite shape and were organized into a network structure. Thus, it appears that adding calcium to the milk supplemented with NaCN improved its coagulation properties by enhancing the incorporation of the added casein into existing casein micelles in the skim milk, or by their aggregation into new colloidal particles that then participated in gel formation upon renneting.
Fig. 3.13. Gel structure of skim milk fortified to 3.2% protein using non-fat dried milk (a, b), calcium caseinate with 9mM added phosphate (c, d) and sodium caseinate with 1.2 mM added calcium (e, f) as shown by scanning (a, c, e) and transmission (b, d, f) electron microscopy.
4. Conclusions

Complete dispersion and solubilization of milk protein powders requires long hydration times (e.g., 4 h) or high shear. The structure of the colloidal protein particles formed by hydrating sodium caseinate and calcium caseinate differ significantly from native casein micelles found in milk. Sodium caseinate formed smaller particles about 10 nm diameter and thin strands of protein. In contrast, the rehydrated calcium caseinate consisted of compact and highly electron dense spherical aggregates that were of a similar size to native casein micelles, although the heavy staining of the calcium caseinate particles suggests that they have a different internal structure.

When skim milk was supplemented with NFDM and then renneted, the coagulation time was decreased and curd firmness was increased. However, when protein powders that do not have the normal calcium and phosphate content of milk were used to increase milk protein content, the coagulation process was retarded and weaker gels were formed. Coagulation properties can be restored to that of the original milk by adjusting the calcium and phosphate levels in the milk. Thus, for milk supplemented with sodium caseinate it was necessary to add calcium, while phosphate had to be added to calcium caseinate supplemented milk.

References


CHAPTER 4

STRUCTURAL CHANGES IN CASEIN SUPRAMOLECULES
DURING ITS DISSOCIATION

Abstract

Dissociation behavior of casein supramolecules due to reduction in pH and calcium chelation were observed using transmission electron microscopy. A method of casein adsorption to a support film, uranyl oxalate staining, flash freezing, and freeze drying as a sample preparation method for transmission electron microscopy was used to minimize changes in the quaternary structure of proteins. Lowering pH induced changes with release of caseins from the native casein colloidal aggregate into solution and their rearrangement and subsequent association to form a reformed supramolecule which developed into networks and consequently formed a gel. Calcium chelation using EDTA also dissociated the casein colloidal aggregates into linear strands (1-3 nm in thickness) of proteins which rearranged themselves into various shapes and sizes. Such linear strands were also seen when milk serum was imaged for soluble caseins. These experiments provide supporting evidence for an irregular supramolecular structure of native casein based on a node and strand network of proteins and calcium phosphate nanoclusters resulting in a filigreed, open sponge-like appearance. The flexibility of the caseins permitted various structures, such as clumps, loops, and linear strands to be formed in different spatial dimensions within the casein supramolecule based upon random associations during cellular synthesis of the casein supramolecule. Calcium phosphate nanoclusters acting as nodes, calcium-mediated protein-protein linkages,
hydrophobic forces, and electrostatic bonding contributing to the integrity of casein supramolecules can explain its dynamic nature during assembly and dissociation.

1. Introduction

The quaternary casein structure varies with physico-chemical treatments such as pH, salt system, and ionic strength, or a combination thereof (Rollema, 1992; Holt & Horne, 1996; Lucey, 2002). These treatments are an integral part of the manufacture of various dairy products and so an understanding of the mechanism and the influence of these treatments on structural changes of casein is vital in deciphering the native structure of casein and tailoring the final characteristics and quality of dairy products.

Various structural studies have been performed on the changes occurring in casein supramolecular structure during chelation of calcium and removal of colloidal calcium phosphate from the supramolecule (Heertje, Visser, & Smits, 1985; Vreeman, van Markwijk, & Both, 1989; Gastaldi, Lagaude, & Tarodo De La Fuente, 1996). However, such studies have been limited by the introduction of artifacts during sample preparation protocols (e.g., fixing, freeze fracturing, and rotary shadowing) used for electron microscopy (McMahon & McManus, 1998). A sample preparation protocol involving protein surface adsorption, followed by flash freezing and freeze drying was used by McManus and McMahon (1997) to minimize structural changes to study how casein supramolecules react to various physico-chemical treatments.

Upon continued acidification of milk, it is known that casein supramolecules undergo changes based on charge neutralization (Davies, Shankar, & Brooker, 1977; Kalab, Emmons, & Sargant, 1976) and other factors (Heertje et al., 1985; Du, 1994; Holt & Horne, 1996; Lucey, 2002) that result in their aggregating into a network of chains and
clusters, thus forming a gel. Reducing the pH of milk solubilizes colloidal calcium phosphate which results in the dissociation of caseins from the casein supramolecule (Rollema, 1992). It has also been shown that calcium removal from native micelles initially dissociates the weakly bound β- and κ-caseins from the micelle, while the size determining micellar framework of αs-caseins remains intact (Bloomfield & Morr, 1972; Lin, Leong, Dewan & Bloomfield, 1972). Reduction in pH results in increased amounts of αs-, β-, and κ-caseins in solution. Among these three, β-casein is preferentially solubilized and so found in higher quantities in solution (Snoeren, Klok, van Hooydonk, & Dammam, 1984; van Hooydonk, Boerrigter, & Hagedoorn, 1986). However, Roefs, Walstra, Dalgleish and Horne (1985), Dalgleish and Law (1988), and Singh, Sharma, Taylor and Creamer (1996) reported equal dissociation of β- and κ-caseins and a lower percentage of αs-casein. It has also been demonstrated that among the calcium-sensitive caseins, those with higher levels of phosphorylation dissociate less readily upon removal of colloidal calcium phosphate (Aoki, Yamada, Kako, & Imamura, 1988; Aoki, 1991).

On further acidification to pH 5.1, dissociated caseins reassociate with the supramolecules (Du, 1994) before formation of a gel network.

Calcium chelation by sequestering agents such as EDTA also results in disintegration of the native casein supramolecules (Lin et al., 1972; Aoki, Kako, & Imamura, 1986; Holt, Davies, & Law, 1986). This disintegration into sub-units was considered as one of the supporting evidences for the submicelle model of casein micelle structure. However, caseins form a dynamic system and therefore can rearrange themselves after disintegration. Caseins are strongly interacting proteins and do not exist as monomers at physiological conditions (Swaisgood, 1992) so they would be expected
to remain as small aggregates upon disintegration of casein micelles. They form both homo- and hetero-aggregates, via calcium-induced electrostatic interactions, and entropy-driven hydrophobic interactions. In addition, β- and κ-caseins form soap-like micelles with a degree of association of 23 and 30, respectively (Payens & Vreeman, 1982). αs1-casein forms tetramers and subsequent linear polymers (Payens, 1966), while β-casein can form linear polymers of indefinite size (Payens & Markwijk, 1963).

Both αs1- and β-casein form mixed complexes with κ-casein (Garnier, Yon, & Mocquot, 1964; Payens, 1968; Garnier, 1973), and they can interact and polymerize to different degrees under various conditions of pH, ionic strength, and temperature. In the presence of calcium, αs1-, αs2-, and β-caseins will precipitate. In this study, we have elucidated various changes that casein supramolecules undergo when they are depleted of calcium.

2. Materials and Methods

2.1. Types of samples

Pasteurized skim milk was obtained from the Gary H. Richardson Dairy Products Laboratory (Utah State University, Logan, UT) and used to prepare calcium-depleted casein micelles by (1) acidifying milk at 40°C by adding 1% (wt/wt) glucono-δ-lactone (GDL), and (2) adding 0.5 g of disodium EDTA + 0.8 g of tetrasodium EDTA per 100 ml of milk at 5°C and 40°C. In addition to milk at pH 6.7, samples of acidified milk were drawn at pH 6.3, 5.9, 5.4, and 5.1. Gelation of the milk occurred at pH ≤ 5.05, which prevented sampling at lower pH values. The calcium-chelated milk was sampled 5 min after EDTA was added.
To study the structure of monomeric proteins and small polymers, milk serum was prepared by centrifuging raw milk at 27,500 g for 2 h and the serum separated from the pellet. A 2.4% (wt./wt.) sodium caseinate (Alanate 180; NZMP Inc., Santa Rosa, CA) solution was also prepared as described in Chapter 3. by dispersing it with high shear (shear rate = 735 s⁻¹) for 10 min at 40°C using a hand-held homogenizer (Omni 5000; Omni International, Gainesville, VA), and hydrated for 4 h before sampling.

2.2. Electron Microscopy

Samples were prepared for electron microscopy as described by McManus and McMahon (1997). Parlodion-coated copper grids were coated with poly-L-lysine to improve the adsorption of protein on to the parlodion film. Casein solutions were diluted 100 times with water so as to have a low protein content (<0.024 g/100 ml) and the grids were immediately immersed in the sample solution for 60 s. Grids were washed two times in water for 10 s before being stained with 12 mM solutions of uranyl acetate and oxalic acid for 60 s. They were washed again in water for 10 s to remove the excess stain and flash frozen in liquid nitrogen-cooled liquefied Freon 22. Images were photographed at various magnifications ranging from 7,000× to 140,000× at 80 keV using a Zeiss 902 (Zeiss, Thornwood, NY) transmission electron microscope.

3. Results

3.1. Native pH

Typical casein supramolecules in pasteurized milk at pH 6.7 are shown in Fig. 4.1. In the low magnification image (Fig. 4.1a), each electron dense (dark) spot represents a casein supramolecule and depicts the typical particle size distribution of
Fig. 4.1. Casein supramolecules from pasteurized milk at 40 °C and at pH 6.7 at (a) low (7,000×, bar = 1 µm), and (b) high (140,000×, bar = 100 nm) magnification.

casein supramolecules. In Fig. 4.1a the size varies between 30 and 350 nm. This is within the size range of 20 to 600 nm reported for casein micelles by Bloomfield and Morr (1973) and Holt (1992).

Average particle size and distribution of casein supramolecules in milk has been determined by light scattering, electron microscopy, and column chromatographic methods as reported by Holt (1992) and McMahon and Brown (1984). The smallest particles are not easily counted by electron microscopic methods and therefore the number fraction of caseins less than 50 nm is often underestimated. According to Schmidt, Walstra and Buchheim (1973), the number fraction of casein supramolecules less than 20 nm in diameter accounts for approximately 80% of particles, while their volume fraction comprises only approximately 3%.

A single casein supramolecule is shown in the high magnification image (Fig. 4.1b). This is similar to native casein supramolecules described earlier in Chapter 2 and
differs from the raspberry-like structure previously reported by Kalab et al. (1976), Heertje et al. (1985), and Du (1994). Within the supramolecule, variation in electron density were observed, and the individual electron-dense (dark) locations may represent either protein or calcium phosphate nanoclusters as those are the individual components that are stained with uranyl ions.

Individual particles ranging in size from 1 nm × 1.5 nm to 4 nm × 4 nm could be discerned at the periphery of the supramolecule. In the center areas there were many overlapping particles making identification of individual particles difficult, however, the same type of structure was observed throughout the particle. The peripheral region showed a characteristic interlocked chain structure formed by the proteins forming into linear and branched polymers that were linked together by nanoclusters of calcium phosphate as described in Chapter 2.

3.2. Acidification

When the pH of milk was reduced from 6.7 to 6.3 (Fig. 4.2), it was observed that there were increased amounts of electron dense dark particles around the supramolecule. The peripheral region of the particles tended to lose its integrity and shape as seen in Fig. 4.2b. There were also electron dense large tendril appendages originating from the supramolecule that extend into the surrounding area suggesting they were partially dissociated proteins.

As pH was further lowered to 5.9 (Fig. 4.3), more surface tendrils were present than at pH 6.3. These large conglomerates of electron dense material extending from the surface of the casein supramolecule are suggestive of a progressive breakup of casein supramolecules from its periphery towards the center. There were also more of the
Fig. 4.2. Casein supramolecules from pasteurized milk acidified to pH 6.3 at 40 °C at (a) low (7,000×, bar = 1 µm) and (b) high (140,000×, bar = 100 nm) magnification.

Fig. 4.3. Casein supramolecules and other adsorbed proteins from pasteurized milk acidified to pH 5.9 at (a) low (7,000×, bar = 1 µm) and (b) high (140,000×, bar = 100 nm) magnification.
smaller, electron-dense nonspherical dark objects in the background that would have been small protein aggregates that had adsorbed to the grid in addition to the casein supramolecules. These aggregates ranged in size from 10 to 100 nm.

At pH 5.4, no apparent dissociated material was observed around the casein supramolecule (Fig. 4.4). At low magnification, the casein supramolecules were observed to be well dispersed and in various sizes. On closer examination of the internal structure of the supramolecule, small clumps of electron dense spots (10-20 nm in diameter) were observed.

Visible gel or aggregates were observed during acidification at a pH of 5.05. This is in agreement with the observation that coagulation of pasteurized skim milk acidified at 35 to 50°C, starts at pH 5.1 to 5.2 (Kim & Kinsella, 1989). Thus, it was not unexpected that clusters of casein supramolecules were observed in the micrographs of the pH-5.1 milk sample (Fig. 4.5).

Fig. 4.4. Casein supramolecules from pasteurized milk acidified to pH 5.4 at 40 °C at (a) low (7,000x, bar = 1 μm) and (b) high (140,000x, bar = 100 nm) magnification.
Fig. 4.5. Casein supramolecules from pasteurized milk acidified at pH 40 °C to pH 5.1 at (a) low (7,000×, bar = 1 µm) and (b) high (50,000×, bar = 100 nm) magnification.

The ability to observe clusters of particles in multiple planes is an advantage of the TEM protocol used in this research because clusters of particles were adsorbed onto the grid even though the samples were still fluid. In contrast, when freeze etching or thin sectioning protocols are used, it is difficult to observe network structure because only a single plane of particles is imaged. In such cases, chains of particles or gel networks are only observed if they reside within the plane of the section (or the fracture surface). Chains of particles that cross the imaged plane may be observed as a single supramolecule or as a short chain depending on the angle at which they cross the plane and the thickness of the section (Kalab et al., 1976). In the adsorption-freeze drying method used to prepare samples in this work, the complete structure of clusters of supramolecules that adsorb onto the grid is imaged.
The casein supramolecules at pH 5.1 (Fig 4.5) were compact and electron dense such that we were unable to visualize the internal structure of casein supramolecules. Heertje et al. (1985) observed separate particles within the casein supramolecule at pH 5.2 using freeze-etch replicas, but was unable to definitively determine if this a result of a submicelle-type structure or an internal-chain structure because of the problem of chains of particles crossing the image plane.

3.4. Calcium Chelation

When calcium in milk was chelated using EDTA, the casein micelles were observed to have dissociated or disintegrated and clusters of thin tentacles and strands of proteins were observed (Fig. 4.6). When EDTA was added to warm milk (40 °C) there were many small filamentous aggregates observed that appeared to contain 3 to 6 filigreed rings of protein. In addition to clusters of particles (15 to 50 nm in size), linear

![Fig. 4.6. Calcium-depleted casein supramolecules after addition of excess EDTA to pasteurized skim milk at a) 5 °C, and b) 40 °C, examined at 140,000× magnification (bar = 100 nm).](image)
strands of protein were observed when EDTA was added to cold milk (Fig. 4.6a). These were approximately 1 to 2 nm in width and up to 30 nm long. These chains appeared to contain branching points or overlapped other chains. The remaining casein micelles lacked regularity in shape or size and were of low electron density.

When the serum obtained by ultracentrifuged (27,500 × g) raw milk at 5 °C was examined, it also consisted predominantly of linear chains and very small clusters of proteins (Fig. 4.7a). Similar protein structure was also observed in rehydrated sodium caseinate (Fig. 4.7b). Based upon electron micrograph work, Farrer and Lips (1999) had predicted the self-assembly of caseins into linear polymeric rods and weakly branched chains in sodium caseinate. It appears that this is the common structure of non-colloidal caseins, and especially when calcium is absent (as in sodium caseinate) or is chelated by, for example, EDTA.

Fig. 4.7. Proteins obtained from a) milk ultra-centrifugate serum at 5°C, and b) sodium caseinate solution hydrated for 4 h (140,000× magnification, bar = 100 nm).
4. Discussion

4.1. Transmission Electron Microscopy

In a positively-stained TEM image, the stained material appears dark against a lighter unstained background. This difference in contrast and brightness is caused by increased electron scattering (or electron impermeability) of the stained material compared to the background material. Therefore, any material that is thicker, is more dense, or has heavy metal atoms attached to it will scatter more electrons and produce a darker image. The parlodion film used to adsorb the caseins from milk will provide a uniform background and any caseins adsorbed onto the film will have an additive influence on scattering based on its thickness and uranyl staining.

Other methods of electron microscopy such as scanning electron microscopy (SEM), and methods of sample preparation such as freeze-fracturing, rotary shadowing, negative staining, and sectioning, have been used for imaging casein supramolecules (Alleyne, McMahon, Youssef, & Hekmat, 1993; Garnier, 1973; Heertje et al., 1985; Kalab et al., 1976; Kalab, Phipps-Todd, & Allan-Wojtas, 1982; McManus & McMahon, 1997). However, as discussed by McMahon & McManus (1998) such methods of sample preparation can result in artifact formation, especially when milk is the object of study.

Before proteins in a fluid material such as milk can be examined by TEM at the very high magnification needed to investigate the structure of casein supramolecules, it must first be converted into a solid object that is free of liquid water. Typically, this has been achieved by first converting the sample into a solid (by rapid freezing or by adding a gelling agent such as warm agarose). If freezing is used then the freezing rate must be
sufficiently rapid to freeze the water in an amorphous form to prevent ice crystal
damage to the sample. The adsorption method described in this work has the advantage
over other methods in that the sample thickness is only that of the film of water being
held onto the grid by surface tension, so freezing is almost instantaneous. Solidifying
milk by mixing it with an agar solution can generate artifacts if chemical interactions
between the proteins and the polysaccharide occur. Such interactions have been observed
in acidified milk (Du, 1994) unless the proteins are glutaraldehyde-fixed before the agar
is added.

When thin sectioning is used for TEM imaging of casein micelles, the internal
structure is visualized but it is necessary to glutaraldehyde fix the proteins in the casein
supramolecules (and typically dehydrate the sample) before it is embedded in resin and
sectioning. The extensive crosslinking that occurs when milk is fixed with
 glutaraldehyde can apparently cause some aggregation of proteins into clumps within the
casein micelle as shown by (McMahon & McManus, 1998). Thin metal coating has a
similar effect in that the fine strands of proteins on the casein supramolecule surface are
of a similar magnitude (or smaller if a metal sputtering is used) to the metal layer, so that
the surface can become compressed and take on a more globular appearance.
Furthermore, with SEM imaging, any conclusions regarding the internal structure of the
casein supramolecules has to be made by extrapolation of its surface appearance.
Similarly, with freeze fracturing and metal shadowing, it is a replica of the original
sample that is subsequently imaged by TEM.
4.2. Acidification

The dissociation and solubilization of proteins from the supramolecular structure can be attributed to the solubilization of colloidal calcium phosphate resulting from the reduction in pH (Pyne & McGann, 1960). Therefore, proteins which are primarily attached to the supramolecular structure by calcium phosphate nanoclusters may be preferentially solubilized simultaneously with those which are also held by weak secondary forces. In contrast, proteins attached to the supramolecule through calcium mediated salt bridges and hydrophobic interactions would remain as part of the supramolecular structure during acidification at 40 °C.

It has been reported (Dalgleish & Law, 1988; Singh et al., 1996) that lower amounts of caseins are dissociated when acidification is carried out at temperatures ≥ 20 °C. All these measurements on dissociated caseins were based on centrifugation experiments at forces > 60,000 × g, and therefore may not include small clusters of caseins which were released from the supramolecule during acidification as they could sediment along with other casein supramolecules. In our experiment where the acidification was carried out at 40 °C, the dissociated caseins were present as aggregates and linear polymers and not as monomeric caseins.

As the calcium phosphate nanoclusters that interlock the protein strands within the casein supramolecule (see Chapter 2) are solubilized, the structural integrity of the casein supramolecule is weakened which would promote rearrangement inside the supramolecule as well as dissociation. It could thus be expected that the casein supramolecules would undergo a transition to compensate for the loss of interactions with calcium phosphate nanoclusters by forming new protein-protein interactions. Such
protein-protein interactions would presumably involve hydrophobic interactions when acidification is conducted at higher temperatures (e.g., 40 °C) because of the increased importance of such interactions in lowering free energy of the system as temperature is increased.

As the pH is decreased to 5.4, soluble β-casein along with other proteins that dissociated outside the supramolecule may be precipitated at their combined isoelectric point (Heertje et al., 1985) resulting in formation of the compact aggregates of proteins observed within the casein supramolecules (see Fig. 4.4). However, at this pH there is still considerable non-colloidal protein that had dissociated from the supramolecules.

β-Casein has a theoretical pI of 5.26 at 20 °C (calculated from SWISSPROT database) and as pH is further reduced, such β-casein clusters would become positively charged. Thus, there would be an attractive force between these positively charged protein clusters and the residual casein supramolecules which are still negatively charged. This can lead to alteration of the surface structure of the casein supramolecule due to the attachment of this positively charged compact protein aggregates and as pH was further lowered all the protein polymers and aggregates would re-associate into the compact particles observed at pH 5. It may be construed that the structure of casein supramolecules at its gelation pH results from the combined influence of electrostatic interactions, hydrophobic interactions, and Ca salt bridging among the various caseins. This differs from native casein supramolecules in which the interactions between the caseins and calcium phosphate nanoclusters are a predominant contributor to its internal structure.
Vreeman et al. (1989) reported a decrease in the number of colloidal size casein supramolecules with reduction in pH while the total surface area of those particles remained a constant. This implies that either the casein supramolecules became small or they became more irregular in shape. There is little decrease in reported specific volume of the casein supramolecules between pH 6.7 and 6.0 but an increase below pH 6.0 was determined using electron microscopy (van Hooydonk, Hagedoorn, & Boerrigter, 1986; Vreeman et al., 1989). However, very small particles and small polymers of proteins, may not have been detected using the electron microscopy method that was used. When disassociation of the casein supramolecules was monitored using ultracentrifugation, their average size did not vary when skim milk was acidified down to pH 5.5 (Dalgleish & Law, 1988, 1989; Vreeman et al., 1989). This supports our finding of little visual change in size of casein supramolecules even though it was evident that protein dissociation had occurred (see Figs. 4.1, 4.2, 4.3, and 4.4).

The compact nature of the particles observed in skim milk at pH 5.1 (Fig. 4.5) suggests that the re-association of caseins with the residual calcium-depleted casein results in a colloidal particle that not only has a ζ-potential approaching zero, but whose surface lacks the tendrils of protein strands that extend into the solvent water. Such colloidal particles would lack the steric repulsion of native milk casein supramolecules (Tuiner & de Kruif, 2002) generated by such a surface “brush.” The decreased steric repulsion, and probably slight positive charge on parts of the supramolecule surface, would allow van der Walls attraction to overwhelm the repulsive forces preventing particle collision the casein supramolecules could undergo aggregation and subsequent gelation.
4.3. Calcium Chelation

Treatment of caseins with excess EDTA chelates calcium bound to the supramolecule, thus averting protein-protein Ca bridging interactions and protein-calcium phosphate nanocluster interactions. Therefore, assuming there are no crosslinking through calcium phosphate nanoclusters, the linear chains of proteins observed in Fig. 4.6 can be envisioned as linear chains of proteins that are polymers or co-polymers of αs, β, and κ-caseins as proposed by Garnier (1967); Garnier et al. (1964) and Payens (1968).

The aggregates formed were irregular non-spherical clusters demonstrating that the casein supramolecule does not consist of submicellar units held together by calcium phosphate linkages. The calcium-depleted aggregates also lack the characteristic filigreed ring-like structure observed in native casein supramolecules (see Chapter 2). Apparently, removal of the calcium phosphate nanoclusters caused a breakup on the supramolecule with many of the proteins being released as polymer chains. Such polymer chains may have been either linear or branch protein chains, with some entanglements occurring to form spherical aggregates. Alternatively, there may have been further rearrangements to form protein strands and aggregates. In various gel-filtration studies (Creamer & Berry, 1975; Aoki et al., 1986; Aoki, 1991; Aoki, Umeda, & Kako, 1992), it has been reported that the dissociated caseins on removal of colloidal calcium phosphate elute as a single peak thus making a case for disintegration of casein supramolecules into uniform sub-micelles. However, it is important to note that dissociated caseins are a part of a dynamic system of interacting casein aggregates (Creamer & Berry, 1975). The long chains of proteins that were present after calcium
chelation would also have a shorter elution time during gel filtration than would be expected from their molecular weight.

The protein strands seen in calcium-depleted supramolecules are thinner (1 to 2 nm in width, see Fig. 4.1) than those observed without EDTA addition (3 to 5 nm in width, see Fig. 4.6). Consequently, the interaction of protein strands is not only in a single plane but also in other spatial dimensions, and double strands of protein had been observed in some micrographs (data not shown). Another difference was that the protein chains observed after EDTA addition were more branched than the protein chains observed upon acidification.

When EDTA was added to milk at 40 °C, dissociation of casein supramolecules was apparently less complete resulting in small aggregates of proteins having the ring-like structure observed in native casein supramolecules. Incomplete dissociation of caseins after with direct addition of EDTA has also been reported by Bloomfield and Morr (1973). Even though it may be construed that the proteins rearranged themselves after calcium depletion to form such an entangled ring like structure, it seems to be very similar to the filigreed, or branched, rings seen in native casein supramolecules (see Chapter 2) and those at neutral pH (Fig. 4.1b). Also, in comparison to the low temperature EDTA treatment, the warm temperature treatment had shorter chains of proteins that were more irregular (having less end to end contact). The integrity of the filigreed rings composed of branched polymeric chain of various proteins may mostly be due to the non-disrupted hydrophobic interactions along with other electrostatic interactions or even calcium mediated interactions due to the incomplete calcium
Consequently, we can assume that hydrophobic forces that were not disrupted also contribute to the internal structure of casein supramolecule.

5. Conclusions

During acidification at 40 °C there was progressive partial breakup of the casein supramolecule from outside to inside that was visualized as tendrils extending from the periphery of casein supramolecule. These polymers formed small (<50 nm) loosely entangled aggregates outside the supramolecule until pH 5.9. Upon further acidification, changes in charge associated with the isoelectric points of the various caseins was probably the driving force behind the re-assemble of colloidal size supramolecules.

Calcium chelation using EDTA resulted in disruption of the casein supramolecules into small aggregates and chains of proteins. Dissociation of the supramolecules into individual chains of proteins was more prevalent at 5 °C, and the small particles formed contained long tendrils and loosely entangled proteins. At 40 °C, the resultant protein aggregates had similar appearance of native supramolecule with multiple ring-like structures.

Calcium is an essential constituent and integral factor in preserving the supramolecular structure of casein. Removal of calcium linkages in the supramolecule by either acidification or calcium chelation resulted in particles of various size, shape, and irregularity. This difference can be attributed to the more extensive removal of calcium mediated protein-protein interactions as well as colloidal calcium phosphate nanocluster mediated protein interactions when calcium was chelated by EDTA. By providing visual evidence on casein supramolecules dissociated by acidification and calcium chelation, we have substantiated the absence of any regular particulate subunits
while demonstrating the presence of branched copolymeric chains of caseins that are most likely held together by hydrophobic and electrostatic interactions, and by calcium bridging, with the chains interlocked together by the calcium phosphate nanoclusters.

References


CHAPTER 5

STRUCTURAL CHANGES IN CASEIN SUPRAMOLECULES
WITH TEMPERATURE

Abstract

Dissociation behavior of casein supramolecules due to temperature changes during renneting, heating, change in pH and treatment with alcohol were observed using transmission electron microscopy. A method of casein adsorption to a support film, uranyl oxalate staining, flash freezing and freeze drying as a sample preparation method for transmission electron microscopy was used. This method has the advantage of minimal change in quaternary structure of proteins as it involves physical fixation instead of chemical fixation and as it does not involve coating with heavy metals which can otherwise hide the interior details. Primary phase of rennet action on milk may be monitored by carrying out the reaction at lower temperatures. An increase in temperature would induce the secondary phase of rennet action. At higher temperatures, caseins fused into aggregates. Heating milk at different pH levels induced whey protein association with caseins. At lower pH, whey proteins interacted with caseins on the supramolecular surface while at higher pH the association of whey proteins and κ-caseins happened in the serum phase. Heat treatment of a mixture of milk and ethanol dissociated the casein supramolecules and were observed using electron microscopy. These experiments provide supporting evidence for an irregular supramolecular structure of native casein based on a node and strain interlocked network of proteins and calcium phosphate nanoclusters. The flexibility of the caseins permits various structures, such as clumps, loops and linear strands to be formed in different spatial dimensions to form a
sponge-like structure inside the casein supramolecule based upon random
associations during cellular synthesis of the casein supramolecule. Calcium phosphate
nanoclusters acting as nodes, calcium mediated protein-protein linkages, hydrophobic
forces and electrostatic bonding contributing to the integrity of casein supramolecules can
explain its dynamic nature during assembly and dissociation.

1. Introduction

Relatively severe milk temperature changes are involved in the manufacture of
many dairy products. Heating and cooling can cause changes in quaternary casein
structure and thus affect how casein supramolecules interact with other proteins such as
whey proteins. Factors such as pH, salt system, and ionic strength, presence of solvents
and other solutes can affect changes occurring during heating of milk. Understanding the
mechanism and the influence of these treatments on structural changes of casein is vital
in deciphering the native structure of casein and tailoring the characteristics and quality
of dairy products.

Rennet coagulation of milk is initiated when chymosin cleaves the Phe$_{105}$-Met$_{106}$
bond in κ-casein on the surface of the casein supramolecule, releasing the
glycomacropeptide portion and leaving the para-κ-casein portion attached to the casein
supramolecule (McMahon & Brown, 1984). This results in a decrease of particle
diameter of 4.5 to 6 nm (Niki, et al., 1994; Holt & Dalgleish, 1986; Van Hooydonk,
Hagedoorn, & Boerrigter, 1986). The secondary phase of aggregation involves formation
of aggregates from those casein supramolecules that have undergone sufficient
proteolysis so that they lose their colloidal stability.
Temperature dependence of the enzymic and aggregation phases of rennet coagulation of milk is different, with a temperature decrease of 10 °C decreasing the enzymic phase by 2×, and the aggregation phase by about 11 to 12× (McMahon & Brown, 1984). Therefore, changes in structure of the casein supramolecules may be monitored during the enzymatic phase by carrying out the reaction at low temperatures (<8 °C). Electron microscopy has been used to study milk gel formation and cheese making (Kalab, Emmons, & Sargant, 1976; Guthy, Auerswald, & Buchheim, 1989; Oberg, McManus and McMahon, 1993). A newer method (McManus & McMahon, 1997) that uses surface adsorption of protein, flash freezing and uranyl staining prior to examination by transmission electron microscopy (TEM) has potential for studying the changes that occur during the primary and secondary phase of rennet action at both low and high temperatures.

Heat treatment of milk above 70 °C results in denaturation of whey proteins (Parry, 1974) and their subsequent interaction with other denatured whey proteins and with caseins via disulfide, hydrophobic and ionic interactions (Sawyer, 1969; Hill, 1989). Concomitantly, the pH of the milk decreases as both soluble calcium and phosphate in serum phase gets associated with caseins in the colloidal state. Zittle, Thompson, Custer and Cerbulis (1962) showed that β-lactoglobulin and κ-casein were involved in heat-induced interactions by using purified protein solutions. Denaturation of whey proteins exposes the reactive amino acid side groups that can interact with other denatured whey proteins or κ-casein through disulfide linkages. The association of denatured whey proteins with caseins is pH dependent (Smits & van Brouwershaven, 1980; Corredig & Dalgleish, 1996; Anema & Klostermeyer, 1997). Anema and Li (2003) reported increase
in casein micelle size by 25 to 30 nm at pH 6.5 when heated at 90 °C for 30 min compared to an increase of 5 to 10 nm at pH 6.7. Electron microscopic investigation of this heat-induced complex formation using negative staining showed that at higher pH (pH ≥ 7.0), large aggregates formed that were not attached to the casein supramolecule, while at lower pH (pH ≤ 6.7) the aggregates were attached to the surface of the casein supramolecule (Heertje, Visser, & Smits, 1985).

Casein supramolecules dissociate when heated in the presence of ethanol or other moderately hydrophobic solvents such as propanol or trifluoroethanol (Horne & Davidson, 1987; Zadow, 1993; O’Connell, Kelly, Auty, Fox, & de Kruif, 2001a). O’Connell et al. (2001a) also reported the dissociation of β-casein micelles when heated in presence of ethanol. Light scattering and nuclear magnetic resonance studies have confirmed the presence of smaller particles after heating in presence of alcohol. It has been proposed that the dissociating effect of ethanol at elevated temperatures is related to a change in solvent quality as the temperature increases.

2. **Materials and Methods**

2.1. *Types of samples*

Pasteurized skim milk (Gary H. Richardson Dairy Products Laboratory, Utah State University) was used for all the experiments. Milk at 5 °C and 35 °C was separately renneted with double strength chymosin (0.1 mL/kg) and sampled for TEM at 15 and 30 min. Pasteurized skim milk in four batches at 20 °C was adjusted to pH 6.7, 6.4, 7.0, and 7.3 using 1 mol/L HCl or 1mol/L NaOH. After pH-adjustment, each was heated to 90 °C for 30 min, cooled to 20 °C and then sampled. A 1:1 (vol/vol) mixture of
pasteurized skim milk and aqueous ethanol (65 g/100 g) was heated to 75 °C and sampled before and after heating.

2.2. *Electron Microscopy*

Samples of casein solutions were prepared for electron microscopy as described by McManus and McMahon (1997) (see Chapter 3). Parlodion-coated copper grids were coated with poly-L-lysine to improve the adsorption of protein on to the parlodion film. Casein solutions were diluted 100 times with water so as to have a low protein content (<0.024 g/100 ml) and the casein particles were immediately adsorbed on to the grids by placing a grid into the solution for 60 s. These grids were washed two times in water for 10 s before being stained with 12 mmol/L solutions of uranyl acetate and oxalic acid for 60 s. They were washed again in water for 10 s to remove the excess stain and flash frozen in liquid nitrogen cooled liquefied Freon 22. Images were photographed at various magnifications ranging from 7,000× to 140,000× at 80 keV using a Zeiss 902 (Zeiss, Thornwood, NY) transmission electron microscope.

3. Results

3.1. Renneting

Images of casein supramolecules and noncolloidal proteins obtained from milk renneted 5 °C are shown in Figs. 5.1. At low magnification it is apparent that the casein supramolecules remained as a stable colloidal suspension with individual particles seen as small spherical electron dense areas either at 15 (Fig. 5.1a) or 30 min (Fig. 5.1c) of renneting. The less electron-dense material surrounding the casein supramolecules were soluble proteins that had presumably dissociated from the supramolecules because
Fig. 5.1. Casein supramolecules in milk renneted at 5 °C for (a, b) 15 min and (c, d) 30 min imaged at (a, c) low (7,000×) and (b, d) high (85,000×) magnification.
of the cold temperature, as well as serum proteins. This background material is pronounced in higher magnification images (Fig. 5.1b and 5.1d). The casein supramolecule depicted in Fig. 5.1b (15 min after renneting) was observed as a spherical electron dense dark image. After further renneting, the peripheral region of the supramolecules was more ragged with what may be described as spikes or tendrils extending out from the supramolecule (Fig. 5.1d).

When milk was renneted at 35 °C, the casein supramolecules were observed as clumps or chains of aggregated particles (Fig. 5.2). After 15 min of renneting, the casein supramolecules were observed as individual entities attached together at their surfaces, and their internal structure was still evident as described in Chapter 2. After, 30 min of renneting at 35 °C, the electron density of the supramolecules was greater (darker compared to the background) such that the internal structure could not be visualized. It was also difficult to differentiate individual casein supramolecules and the aggregates had the appearance of being made up of strands of material rather than spherical particles joined together through common contact areas on the periphery.

The effect of temperature on the rate of aggregation of renneted casein supramolecules was very apparent when milks at 5 and 35 °C after 15 min of renneting were compared at a medium magnification (Figs. 5.2e, 5.2f). At 5 °C, the individual casein supramolecules were evenly distributed against fairly dark background material (soluble proteins) while at 35 °C the casein supramolecules were clumped together with less soluble proteins being present as shown by the lighter background.
Fig. 5.2. Casein supramolecules in milk renneted at 35 °C for (a, b, f) 15 min and (c, d) 30 min compared to (e) milk renneted at 5 °C for 15 min, imaged at (a, c) low (7,000× and 12,000×), medium (e, f) and (b, d) high (85,000×) magnification.
Fig. 5.3. Electron micrographs of milk at pH 6.7 heated to 90 °C for 30 min and imaged at (a) 30,000× and (b) 85,000× magnification.

3.2. High Heat Treatment

At pH 6.7 after heat treatment to 90 °C, casein supramolecules were seen as dark electron dense particles with numerous appendages around their periphery (Fig. 5.3). These appendages were of various sizes and shapes and had the appearance of large, but less electron-dense, protein aggregates attached to proteins on the casein supramolecules. This is similar to what was observed by Heertje et al. (1985).

When pH-6.4 milk was heated to 90 °C for 30 min, the casein supramolecules were observed not as individual spherical particles but as clumps of protein material to which was attached a virtual web aggregated material (Fig. 5.4). Their electron density was less than that observed at pH 6.7 suggesting that some dissociation of the casein supramolecules had occurred. At high magnification, the casein supramolecules were seen to have some compact and electron-dense regions as well as numerous tendrils of attached protein chains and clusters of lower electron density.
Fig. 5.4. Electron micrograph of milk at pH 6.4 heated to 90 °C for 30 min and imaged at (a) 50,000× and (b) 140,000× magnification, bars = 100 nm.

When pH of milk was increased to 7.0 and then heated to 90 °C for 30 min, large (100 to 500 nm diameter) spherical electron dense particles with non-spherical particles of almost the same size attached to them were seen (Fig. 5.5a). At high magnification (Fig. 5.5b), casein supramolecules with large aggregates of lower electron density either attached to the casein supramolecules, or occupying the surrounding areas, could be observed in the background. The distinct internal filigreed ring-like internal structure of the casein supramolecule as described in Chapter 2, was observed. Similar association of caseins with whey proteins has been reported by Heertje et al. (1985).

Electron micrographs of commercial ultra-high temperature treated milk and evaporated milk are shown in Fig. 5.6a and 5.6b, respectively. In both figures, spherical electron dense casein supramolecules are attached to irregularly shaped less electron dense particles. These images are similar to those of casein supramolecules in milk at pH 6.7 after heating to 90 °C (Fig. 5.3) in preserving their spherical shape.
Fig. 5.5. Electron micrographs of milk at pH 7.0 heated to 90 °C for 30 min and imaged at (a) 20,000× (bar = 1 µm) and (b) 140,000× (bar = 100 nm) magnification.

Fig. 5.6. Electron micrograph of proteins in (a) ultra high temperature treated milk and (b) evaporated milk.
3.3 Ethanol Treatment

When milk and ethanol was mixed in equal proportions, few electron dense regions were observed in the lower magnification image (Fig. 5.7a). At higher magnification, tendrils and appendages were observed to surround be attached to the remaining casein supramolecules (Fig. 5.7b). Some ring-like internal structure was seen in both the central electron dense area as well as in the tendrils. When this mixture was heated to 75 °C, electron dense regions are scattered throughout the imaged field (Fig. 5.8). The particles were aggregated into various shapes and sizes. Compared to the unheated milk/ethanol mixture (Fig. 5.7a), the heated sample contained proteins that had dissociated from their aggregated form present at room temperature and rearranged so that no internal structure was visible. This dissociation of casein supramolecules in the presence of ethanol and after heating was accompanied by a loss of its opaque milky white color as reported by Zadow (1993).
Fig. 5.8. Electron micrographs of milk proteins obtained at 75 °C from a 1:1 milk and aqueous ethanol (65 g/100 g) mixture and imaged at (a) 20,000× (b) 85,000× magnification.

4. Discussion

4.1. Renneting

At 5 °C, no aggregation of casein supramolecules was observed until 30 min after renneting. This lag in the secondary aggregation phase of enzymatic coagulation at lower temperatures has been attributed to its high $Q_{10}$ value between 11 and 12 (McMahon & Brown, 1984). Pires, Gatti, Orellana and Pereyra (1997), attributed this to the ability of renneted casein supramolecules to retain steric stabilization. The entropic nature of steric stabilization is such that the interactions of water molecules with suitable polar stabilizing moieties, contributes to a positive free energy for flocculation.

The background material observed at high magnification of milk renneted at 5 °C (Fig. 5.1b and 5.1d) can be attributed to proteins that are not a part of the casein
supramolecule. These are characteristic of milk at lower temperatures as caseins especially β-casein, dissociate from the supramolecule when milk is cooled. The increase in size of background material after 30 min of renneting (Fig. 5.1d) may be attributed to the increasing amounts of κ-casein being cleaved.

At 35 °C, the caseins had started to aggregate after 15 min of renneting (Fig. 5.2) but the internal structure of the aggregating casein supramolecules and the contributing units of the aggregate was still discernable. After 30 min, the proteins had compacted and rearranged sufficiently that even the component supramolecules of the aggregate could not be differentiated and the aggregate no longer had spherical particles as its components monomeric units. This fusion of casein supramolecules has been reported to happen during cheese manufacture where individual casein supramolecules are not seen as components of the protein strands that make the cheese matrix (Niki et al., 1994; Oberg et al., 1993). In comparison, acid milk gels maintain spherical subunits inside the aggregate that forms the gel (see Fig. 4.5 in Chapter 4).

Walstra (1999) proposed that the fusion of casein supramolecules after renneting came about from placement of submicelles from milk serum in the space formed between two casein supramolecules in contact. However, such void space filling by external particles between two spherical supramolecules to form a strand was not explained. Based on the interlocked node and strand network of proteins constituting the casein supramolecule as described in Chapter 2, this phenomenon of fusing of supramolecules may be explained. On successful collision between two casein supramolecules that have undergone κ-casein hydrolysis, they become part of a single aggregate. The polymeric strands of the two supramolecules can make further contacts as stretching and
compaction may occur due to the momentum of collision. Increasing the number of contact points between two spherical aggregating particles may lead to the disappearance of the size and shape of individual entities.

4.2. High Heat Treatment

Whey proteins interact with caseins upon heating and this interaction is pH dependent (Creamer, Berry, & Matheson, 1978; Creamer & Matheson, 1980). In Figs. 5.3, 5.5, and 5.6 appendages or protuberances were seen on casein supramolecules. These have been identified as aggregated whey proteins (especially β-lactoglobulin) that have complexed with κ-caseins on the supramolecular surface (Harwalkar, 1989; McMahon, Yousif, & Kalab, 1993). The increase in size of casein supramolecules during heat treatment has been attributed to this attachment of whey proteins. This interaction between β-lactoglobulin and κ-casein has also been identified as the cause for decreased rennetability of high heat treated milk as it decreases the accessibility of chymosin to κ-casein on the surface of the supramolecule. Below the native pH of milk (pH 6.7), a decrease in pH before heat treatment results in a larger increase in casein supramolecular size. The size increase in casein supramolecules at pH 6.55, 6.6, and 6.7 was determined by Anema and Li, (2003) to be 30 to 35, 20 to 25, and 5 to10 nm, respectively. This corresponds to 75 to 80% of denatured whey proteins being associated with casein supramolecules at pH 6.5 and only ~30% at pH 6.7. This difference in association of whey proteins with small changes in pH has not yet been explained (Anema & Li, 2003).

Based on the node and strand polymeric structure for casein supramolecules, and its partial dissociation with decrease in pH (Chapter 4), it is likely that more sites for attachment of whey proteins are exposed with decrease in pH. Small amounts of κ-
casein can be present inside the supramolecular particle, and consequently, these may be exposed during any partial dissociation of casein supramolecules, allowing greater interaction with whey proteins.

At pH 6.4, electron dense compact aggregates were seen on the periphery of the supramolecule (Fig. 5.4b). This implies that the whey protein-casein interaction takes place on the surface of the supramolecule rather than in serum. At pH 7.0, large aggregates of electron dense material were seen surrounding casein supramolecules. These aggregates of κ-casein and whey proteins are formed in the serum outside the supramolecular surface as κ-caseins partially dissociate from the supramolecule at pH >6.7 (Kudo, 1980; Anema & Klostermeyer, 1997). The association of whey proteins with caseins is of practical significance as seen in commercial samples of evaporated and ultra-high temperature treated milk samples (Fig. 5.6).

4.3. Ethanol Treatment

In presence of ethanol, the milk protein system undergoes destabilization (Horne, 1992). Alcohols reduce the dielectric constant that in turn collapses the stabilizing outer C-terminal region of κ-casein on the casein supramolecule surface. This collapse reduces the casein supramolecular stability by reducing the net negative charge. Collapse and partial dissociation of the casein supramolecule is seen in Fig. 5.7b.

As seen in Fig. 5.8, casein supramolecules dissociate into smaller compact particles and their aggregates on heating in presence of ethanol. Unlike independent particles, there were inter-connected particles that could be considered as part of a larger aggregate. This dissociation of casein supramolecules when heated in presence of ethanol has been reported by Zadow (1984) and O’Connell et al. (2001). This
dissociation is similar to the effect of 2,2,2-trifluoroethanol on caseins (Horne & Davidson, 1987). Horne and Davidson (1987) reported that the dissociated particles in 100% trifluoroethanol/milk mixture were approximately the same hydrodynamic size as in native milk, but were dissimilar in molecular weight and sedimentation properties. As seen in Fig. 5.8, the dissociated casein supramolecules still remain interconnected and possess a large amount of void volume which can contribute to greater hydrodynamic radius while maintaining low molecular weight and consequently low sedimentation.

Contrary to this explanation, O'Connell et al. (2001a), using confocal laser scattering microscopy, showed that the dissociated particles formed were smaller than the original casein supramolecules. The interconnected particles or the larger aggregates may be few in numbers and in the process of dissociation which might not have been seen in confocal microscopy.

The nuclear magnetic spectra of both urea (6 mol/L) treated milk and milk heated to 70 °C in presence of ethanol were similar (O'Connell, Kelly, Fox & de Kruif, 2001b). Urea dissociates casein supramolecules by enhancing protein solubility and by inhibiting hydrophobic bonding. The increase in repulsive forces between caseins and solvent quality may result in dissociation of casein supramolecules when heated in presence of ethanol (O'Connell et al., 2001b). Horne and Davidson (1987) attributed this dissociation of casein supramolecules to high helix development in caseins in presence of alcohols. For all these to happen, the individual molecules of caseins that constitute the supramolecule have to be surrounded by ethanol which is possible if the supramolecule had a sponge-like polymeric structure as described in Chapter 2. The loss of internal
structure and compactness of the small aggregates imply rearrangement of individual monomeric caseins.

5. Conclusions

The changes in structure of casein supramolecule during change in temperature can be monitored using transmission electron microscopy. Renneting of milk at 5 °C caused no aggregation of casein supramolecules until 30 min. At 35 °C, aggregated particles were seen at 15 min. By 30 min of renneting, the individual supramolecules that formed the aggregate started to fuse together. When milk was heated at lower pH, there was more whey protein association on the casein supramolecule surface. At pH 7.3, larger aggregates of whey proteins were formed in the serum outside the casein supramolecule surface. Heat treatment of a mixture of milk and ethanol dissociated the casein supramolecules into smaller particles of which some were interconnected. All these changes in structure can be explained using the interlocked node and strand structure of casein introduced in Chapter 2.

References


 CHAPTER 6

GENERAL SUMMARY

Physical methods of fixation, uranyl staining, flash freezing, and freeze drying in sample preparation can be successfully used to image the quaternary structure of caseins using TEM. Such a method of sample preparation yields images without altering the native conformation of the proteins.

Dispersion and solubilization of dried milk proteins require longer hydration times and/or high shear. Microstructure of sodium and calcium caseinate differ significantly from native casein found in milk. Sodium caseinate lacks the spherical colloidal structure while calcium caseinate forms compact and highly electron dense spherical aggregates. Fortification of milk with dried milk proteins destabilizes the coagulation properties and microstructure of rennet gels depending on the nature of the protein. These coagulation properties can be brought back to that of original milk by addition of relevant salts. In case of sodium caseinate and calcium caseinate, addition of calcium chloride and potassium phosphate respectively reduced their adverse effect on coagulation properties.

During acidification at 40 °C there was progressive partial breakup of the casein supramolecule from outside to inside and was visualized as tendrils extending from the periphery of casein supramolecule. These polymers formed small (<50 nm) loosely entangled aggregates outside the supramolecule until pH 5.9. Further reduction in pH to the isoelectric points of caseins might have contributed to the changes in charge associated with the various aggregates resulting in their aggregation and reassociation with casein supramolecules. Calcium chelation using EDTA at 5 °C resulted in
disruption of the casein supramolecules resulting in linear polymers and their cross-links. The disrupted system also formed particles with long tendrils and loosely entangled proteins. At 40 °C dissociation of the supramolecule was not as extensive as at 5 °C. The resultant proteins aggregates had similar appearance of native supramolecule with multiple ring-like structures.

The changes in structure of casein supramolecule during change in temperature was monitored using transmission electron microscopy. Renneting of milk at 5 °C caused no aggregation of casein supramolecules until 30 min. At 35 °C, aggregated particles were seen at 15 min. By 30 min of renneting, the individual supramolecules that formed the aggregate started to fuse together. When milk was heated at lower pH, there was more whey protein association on the casein supramolecule surface. At pH 7.3, larger aggregates of whey proteins were formed in the serum outside the casein supramolecule surface. Heat treatment of a mixture of milk and ethanol dissociated the casein supramolecules into smaller particles of which some were interconnected.

These studies provide evidence to support an irregular supramolecular structure for the colloidal casein particles in milk. This irregular structure, supports an open structure in which different caseins can attach to calcium phosphate nanoclusters preventing calcium phosphate crystallization in the mammary gland. Chains of proteins can then grow until they encounter a chain terminating protein or bond with another chain. Also, different dissociation and aggregation behavior of casein supramolecules may be explained using this model. Overall, this study has put forth a molecular model for the casein supramolecule that satisfies the principles of self aggregation, interdependence, and diversity that are often observed in nature. Synthesis of casein
supramolecules in the mammary gland rely on a controlled synergy between two concomitant aggregation processes. Calcium phosphate is formed into clusters because of its low solubility, and caseins are simultaneously undergoing polymerization because of their calcium sensitivity and hydrophobic nature. Precipitation of calcium phosphate is limited to formation of nanoclusters by binding of caseins via their phosphoserine side chains, and the polymerization of the caseins is limited to colloidal size by the chain-terminating influence of κ-casein.
APPENDIX
Fig. A.1. Micrographs of micelle formation by (a) β- (250,000×) and (b) κ-casein in the absence of calcium (140,000×)
Fig. A.2. Micrographs of casein supramolecules in (a) human (b) pigmy goat and (c) mare milks
Fig. A.3. Micrographs of pasteurized skim milk diluted using skim milk ultrafiltrate (a) 7,000× (b) 140,000×

Fig. A.4. Electron micrograph (7000×) showing artifact formation (linear aggregates) formed when excess proteins were not washed after adsorption on to the parlodion film.
Fig. A.5. Micrographs of pasteurized skim milk stained at pH 6.7 (a) 20,000× (b) 85,000×
CURRICULUM VITAE

Bonney S. Oommen

(February 2004)

Candidate for the Degree of Doctor of Philosophy

Dissertation: Casein Supramolecules: Structure and Coagulation Properties

Major Field: Nutrition and Food Sciences

Education:
Ph.D., Nutrition and Food Sciences, Utah State University, Logan, Utah, 2004
M. S., Dairy Science, South Dakota State University, Brookings, South Dakota, 1999
B. Tech., Dairy Technology, Gujarat Agricultural University, Anand, India, 1996

Awards
Gandhi Fellowship, Dept. of Nutrition and Food Sciences, Utah State University, 2000.
Gamma Sigma Delta, South Dakota State University.
Merit Scholarship, Gujarat Agricultural University.
C.K. Desai Gold Medal, Gujarat Agricultural University.

Experience

Research Scientist, Research and Development.
Process and technology development for production of nutritional dairy ingredients
Member of mergers and acquisition team for corporate development.

Intern., Research and Development.
Developed methods for separation, isolation, and characterization of proteins using chromatography, PAGE, HPLC, and mass spectrometry.

Graduate Research Assistant.
Studied: Functionality of Mozarella cheese made using bacteria of different proteolytic activity; Structure and functional relationship of Feta cheese using electron microscopy; Casein microstructure and its influence on coagulation properties.
Proposed and modeled a new quaternary structure for casein supramolecules (micelles).
Graduate Research Assistant.
Manufactured cheese and Whey Protein Concentrates.
Studied: Chemical, sensory, and rheological properties of cheese; functionality of whey proteins; and analyzed data statistically using SAS®.
Tested and implemented new manufacturing protocol to increase Cheddar cheese yield by increasing fat and protein retention in cheese.

Trainee.
Responsible for production of ice cream, cheese (Cheddar, Emmental, Mozzarella, Paneer), butter, ghee, fluid milk and its quality assurance.

Publications


Presentations


Oommen, B. S., V. V. Mistry, and M. G. Nair. 1999. Effect of homogenization of cream on the yield and functionality of Cheddar cheese made from milk supplemented with ultrafiltered milk. J. Dairy Sci. 82 (Suppl. 1): (abstr.).

Nair, M. G., B. S. Oommen, and V. V. Mistry. 1999. Yield and functionality of Cheddar cheese as influenced by homogenization of cream J. Dairy Sci. 82 (Suppl. 1): (abstr.).