5-1994

Fate of β-Lactoglobulin, α-Lactalbumin, and Casein Proteins in Ultrafiltered Concentrated Milk After Ultra-high Temperature Processing

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FATE OF β-LACTOGLOBULIN, α-LACTALBUMIN, AND CASEIN PROTEINS IN ULTRAFILTERED CONCENTRATED MILK AFTER ULTRA-HIGH TEMPERATURE PROCESSING

by

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A dissertation submitted in partial fulfillment
of the requirements for the degree

of

DOCTOR OF PHILOSOPHY

in

Nutrition and Food Sciences

Approved:

UTAH STATE UNIVERSITY
Logan, Utah

1994

ACKNOWLEDGMENTS

I thank the Western Center for Dairy Protein Research and Technology, and the Utah Agricultural Experiment Station for funding this project. I also thank the Organization of American States (OAS) for a fellowship. I am especially grateful to my advisor, Dr. Donald J. McMahon, for sound advice, encouragement, and support. I extend appreciation to committee members, Drs. Rodney J. Brown, Danny J. Blubaugh, and Conly Hansen. Special thanks go to Dr. Paul A. Savello, who served as committee member and was also a source of inspiration throughout my stay at Utah State University. I also extend appreciation to Dr. Charlotte P. Brennand for serving as my first advisor; Dr. Nabil N. Youssef for invaluable instructions and advice in electron microscopy; my colleague and friend, William McManus, for technical assistance and much practical training in electron microscopy; Gary Pederson for developing and printing micrographs; and Lynn Burgess for technical assistance.

I express appreciation to my dad, Christopher, my sister, Rosamund, and my daughter, Markiesha. I am especially grateful to my wife, Debbie, for her understanding, love, and assistance throughout the years of graduate study and research.

Mark Christopher Alleyne

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ABSTRACT

Fate of β-Lactoglobulin, α-Lactalbumin, and Casein Proteins in Ultrafiltered Concentrated Milk after Ultra-high Temperature Processing

by

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The problem of age gelation in ultra-high temperature (UHT) sterilized milk retentate (ultrafiltered 3x concentrated) is investigated in this work. Transmission electron microscopy (TEM), utilizing the microcube encapsulation technique and protocols for immunolocalization of milk proteins, provides insight into the phenomenon of age gelation of UHT-sterilized, ultrafiltered (UF) milk retentate. Primary antibodies (specific for the native as well as the complexed forms of milk proteins) and secondary antibodies (conjugated to gold probes) are used to elucidate the positions of the milk proteins in various samples of milk from the stage of milking through UHT sterilization and storage for 12 months, by which time gelation had occurred. The movement of the milk proteins is charted and these data are used to determine the role of the proteins in age gelation of UHT-sterilized UF milk retentate.

Heat-denatured β-lactoglobulin and α-lactalbumin form complexes within the serum as well as with the casein components of the micelles. UHT sterilization not only denatures β-lactoglobulin and α-lactalbumin, but catalyzes the reaction of these whey proteins and κ-casein, leading to the successful formation of the complex. Complexing of β-lactoglobulin and κ-casein competitively weakens the complex of κ-casein to other casein

fractions of the micelle. This leads to migration of κ-casein from the micelle to the serum, compromising the role of κ-casein in stabilizing the casein proteins within the micellar moiety. The time-dependent loss of κ-casein from the micelle would expose the calcium-insoluble micellar α_{s1}-casein and β-casein to the serum calcium. Subsequent to this, some α_{s1}-casein and β-casein are also released from the micelles, and gelation of the milk occurs. No information was obtained on location of α_{s2}-casein. The release of κ-casein from the micelles thus apparently represents the critical factor in the phenomenon of age gelation in UHT-sterilized milk concentrates.

(229 pages)

CHAPTER I

GENERAL INTRODUCTION

UHT-Sterilized Milk Concentrates

Advances in the processing and packaging of dairy foods have led to the production of milk and other dairy foods that can be stored at room temperature for up to six months (Miller, 1985). Ultra-high temperature (UHT) sterilization of milk, in combination with aseptic filling techniques and hermetically sealed packaging, have been used for many years in some foreign countries to produce shelf-stable milk (McBean and Speckmann, 1988).

The success of dairy production in the United States requires that more attention be given to milk utilization and marketing. The thrust in recent years has been to develop new dairy products rather than concentrate on higher milk production.

More international markets for dairy products could be established if success in manufacturing stable products from surplus dairy production could be achieved. This would assist in the reduction of surpluses of milk and enhance the competitive position of the USA in its production of new and better quality products. The production of such products having a long shelf life should be an established priority. In 1981 the FDA approved the aseptic packaging procedure used in packaging UHT-sterilized milk for use in the USA (McBean and Speckmann, 1988).

The UHT-sterilized milk project at Utah State University includes the production of stable, rehydratable milk concentrates with long shelf life at ambient temperatures. The success of this project could assist in making USA dairy products more widely available on the world market. Another benefit from this project is that it will furnish information on extended shelf life of dairy products. Such information will be especially useful to dairy processors interested in entering the expanding food service business, where extension of shelf life is important.

The objectives of this study were:

1. To develop a more useful method of pre-fixation encapsulation of fluid milk for transmission electron microscopy.
2. To establish an alternative protocol to low-temperature embedding methods for immunolocalization studies of milk proteins.
3. To characterize the nature of immunogold-labeled α-lactalbumin and β-lactoglobulin in dairy products.
4. To determine the usefulness of developed immunolocalization techniques in relating labeling of β-lactoglobulin to the manufacture processes of dairy products.
5. To determine the location of α-lactalbumin, β-lactoglobulin, α_{s1}-casein, α_{s2}-casein, β-casein, and κ-casein in milk at various stages from fresh whole milk to UF concentrated (3x), UHT-sterilized milk.
6. To relate location of α-lactalbumin, β-lactoglobulin, α_{s1}-casein, α_{s2}-casein, β-casein, and κ-casein in UF concentrated (3x) UHT-sterilized milk with changes occurring in milk through UHT heating and storage, and thus propose a mechanism for age gelation.

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CHAPTER II

LITERATURE REVIEW

Gelation of UHT-Sterilized Milk Concentrates

Much research has been done to date on the production of UHT-sterilized milk concentrates. However, irreversible gelation of UHT-sterilized milk concentrates has hindered the commercial application of this process as a means of lowering transport costs. Gelation occurs when the product is stored at room temperature. The viscosity of the product usually remains constant for a period of time before suddenly rising as gelation sets in. This gel is the result of casein micelles forming a network, but why it happens is uncertain (Walstra and Jenness, 1984).

Work has been done on developing stable, UHT-sterilized milk concentrates, but to date no satisfactory solution has been found to prevent age gelation. Many factors influence the time taken for age gelation of UHT-sterilized milk concentrates to set in (Harwalker, 1982). These include composition of milk (van Boekel *et al.*, 1989), quality of milk (Adams *et al.*, 1976), severity of heat treatment (Darling, 1980; Fox, 1982), homogenization (Sweetsur and Muir, 1980a; Muir, 1984), temperature of storage (Andrews, 1975), concentration of milk (Whitney, 1977; Muir and Sweetsur, 1978; Sweetsur and Muir, 1980a; Fox, 1982; McMahon and Brown, 1984b), addition of calcium-sequestering agents (Kocak and Zadow, 1985), addition of carbohydrates (Lonergan, 1978; Kudo, 1980a; de Wit, 1981), addition of reducing agents (Singh and Fox, 1987a), and enzyme treatment (Snoeren and Both, 1981). The precise mechanism of how these processing and storage conditions influence gelation is not fully understood. Milk gelation is also affected by pH (Singh and Fox, 1985 and 1987b), ionic strength, and calcium and phosphate concentration (Tumerman and Webb, 1965).

Hypothesized Mechanism of Age Gelation

There are many hypotheses on the mechanism of age gelation of UHT-sterilized milk concentrates. Two different mechanisms have been suggested. One implicates proteolysis of casein, predisposing the micelles to aggregation (Creamer and Matheson, 1980; Snoeren and Both, 1981; Haque *et al.*, 1987). The other involves physico-chemical reactions leading to chemical cross linkages between micelles (Dziuba, 1979; Creamer and Matheson, 1980; Doi *et al.*, 1983).

Proteolysis Hypothesis

Proteolytic enzymes in milk, of microbial or native origin, survive UHT treatments or are reactivated during storage (Corradini, 1975). These cause flavor changes in the milk during storage (Renner, 1988). Some evidence supports the idea that age gelation occurs in a manner similar to rennet coagulation of milk in cheese manufacturing (Samel *et al.*, 1971; Hostettler, 1972). The coagulation kinetics for both processes are similarly characterized by a lag phase during which viscosity decreases, followed by a period of no change, and finally undergoing rapid thickening. The kinetics for nonenzymic gelation would yield a linear increase in viscosity without a lag phase or the rapid increase in viscosity shortly before gelation (Payens, 1978 and 1982). When proteolysis is inhibited in unconcentrated milk samples, no gelation is observed, but the controlled samples (without protease inhibitors) age gelled (Driessen, 1983). The proteolysis hypothesis alone, however, does not adequately explain age gelation of concentrated milks. Age gelation of commercially sterilized products has occurred, where neither proteases nor proteolysis was detected (Samel *et al.*, 1971). UHT-sterilized concentrated casein micelle dispersions, with and without proteolysis, gelled after identical storage periods (de Koning and Kaper, 1981 and 1985). The evidence suggests that unconcentrated sterilized milk age

gels through proteolysis while concentrated sterilized milk age gels without proteolysis (Hostettler, 1972; de Koning *et al.*, 1985).

Physico-chemical Hypothesis

Many physico-chemical processes have been implicated in age gelation of UHT-sterilized concentrated milk (Samel *et al.*, 1971; de Koning and Kaper, 1981; Manji and Kakuda, 1988).

Whey proteins denature during heat processing in the range 70-90°C, and as milk is heated to ~70-90°C a complex is formed between denatured whey proteins and casein. When the milk is heated to temperatures between 120-140°C, much of the denatured whey protein is present uncomplexed with casein (Burton, 1984). It has been suggested that the β-lactoglobulin-casein complex has a tendency to aggregate with time, leading to sedimentation and coagulation (Morr and Richter, 1988).

The breakdown of κ-casein during storage leading to its inability to stabilize the casein micelle can also cause age gelation (Singh *et al.*, 1989). Dissociation of κ-casein from the micelle surface during storage of UHT-sterilized milk may trigger the loss of stability, and this may be caused by changes in salt equilibrium, especially calcium, magnesium, and phosphate, during storage (Singh *et al.*, 1989). During heat treatment, some protein dissociates from the casein micelles. Heating skim milk and whey protein-free milk at sterilization temperatures causes a substantial increase in the level of soluble casein, consisting of 40% κ-casein (Singh *et al.*, 1989).

Changes in calcium and other mineral equilibria during storage have been suggested as factors affecting the stability of UHT products during storage (Corradini, 1975).

Polymerization of casein and whey proteins by Maillard-type reactions has also been implicated in age gelation of UHT concentrated milk (Andrews and Cheeseman, 1971; Andrews, 1975).

Sulfhydryl-disulfide interchange reactions involving various proteins have also been implicated in age gelation of UHT concentrated milk (Patrick and Swaisgood, 1976).

A decrease in surface energy of some micelles with time, creating an electrostatic difference which promotes aggregation of micelles, has been suggested as contributing to nonenzymic age gelation (Graf and Bauer, 1976).

An increase in nonsedimentable casein (at >100,000 x g) due to partial disaggregation of casein micelles may modify surface properties of casein micelles and expose regions on their surfaces that promote micellar interaction (Harwalker, 1982). This has also been suggested as contributing to age gelation.

Effect of Heating Milk

Heat sterilization of milk produces several changes. UHT sterilization generally produces less severe changes than retort sterilization (Zadow, 1986). Changes that affect proteins (de Wit, 1981; de Wit and Klarenbeek, 1981; Fox, 1981a), enzymes (Snoeren and Both, 1981), and mineral balance (Mattick and Hallett, 1929; Pyne and McHenry, 1955; Tessier and Rose, 1964) of milk are the ones most likely to influence age gelation.

Heat treatment results in an association between whey protein and casein (Sawyer, 1969). Whey proteins become denatured and either interact with micellar κ-casein to become sedimentable with casein or coprecipitate (with casein) at the isoelectric point of casein (Hostettler, 1972).

The extent of complex formation between denatured β-lactoglobulin and κ-casein significantly modifies the properties of casein micelles. These complex formations are predominantly through disulfide bridges, but hydrophobic and ionic interactions are also involved (Haque *et al.*, 1987; Haque and Kinsella, 1987 and 1988; Hill, 1989).

Some rearrangement of casein components within the micellar structure also occurs through UHT treatment of milk (Morr, 1969; Harwalker, 1982). The amount of casein not sedimentable on high speed centrifugation (>100,000 x g) increases as does the size of

sedimented micelles (Harwalker, 1982). These micelles increase two or three fold in size in UHT-sterilized concentrated milk (Schmidt, 1968; Harwalker, 1982).

Changes in the inorganic constituents of milk also occur as a result of heat treatment (Harwalker, 1982). Ionic calcium and magnesium concentrations decrease through their precipitation as phosphate and citrate salts (Aoki and Imamura, 1974). As a result of its association with casein micelles, heat-precipitated calcium phosphate does not sediment (Evenhuis and de Vries, 1956), and when the milk cools, colloidal calcium phosphate slowly dissolves to restore equilibrium (Pyne, 1958; Kannan and Jenness, 1961; Fox *et al.*, 1967). The role of calcium in gelation is not clear (Harwalker, 1982).

Effect of Additives

The addition of calcium-sequestering agents such as phosphates or citrates reduces the availability of calcium and promotes gelation (Sweetsur and Muir, 1980b; McMahon *et al.*, 1991). However, some protection against gelation has been achieved by adding polyphosphates to milk (Kocak and Zadow, 1985). This effect with the polyphosphates is thought to result from their ability to complex with casein micelles. This results in charge repulsion between micelles, thus preventing their interaction (Harwalker, 1982).

Addition of lactose stabilizes β-lactoglobulin (de Wit, 1981) and milk stability can be increased by hydrolyzing lactose or by adding other sugars. Replacement of lactose with glucose increases milk stability but replacement by sucrose does not (Lonergan, 1978; Kudo, 1980a). Lactose and other sugars inhibit denaturation of whey proteins (Hillier *et al.*, 1979).

Urea added to milk before heating stabilizes it (Pyne, 1958; Muir *et al.*, 1978). Lactose and urea added simultaneously have a great stabilizing effect on milk (Kudo, 1980a).

Effect of Storage

The stability of the colloidal calcium-caseinate-phosphate complex in sterilized milk is susceptible to coagulation by agents such as ethanol, rennet, and calcium, and decreases on storage. This loss in stability is gradual with time. Sensitivity to calcium increases, but no consistent relationship between sensitivity of calcium and gelation of UHT-sterilized concentrated milk exists (Harwalker, 1982).

No discernible pattern of mineral distribution satisfactorily explains the mechanism of gelation through storage. There is, however, a change in the distribution of mineral components in UHT-sterilized milk during storage (Parry, 1974). Initially, some of the calcium phosphate precipitated by UHT treatment dissociates. Prolonged storage causes precipitation of some forms of calcium phosphate (Fox, 1981a, 1981b and 1982). Colloidal calcium phosphate is necessary for the stability of casein micelles, and changes in the mineral balance affect the casein integrity (Lonergan, 1978; Fox and Hearn, 1978).

The casein micelles of UHT-sterilized milk undergo a number of changes during storage. Such changes are measured using ultracentrifuge sedimentation, gel permeation, chromatography, and electron microscopy. In addition to an increase in amount of nonsedimentable casein there is an increased formation of protein polymers (Aoki and Imamura, 1974). Covalent bonding or cross linking of casein polypeptide chains through carboxyl intermediates produces these polymers. The carboxyl intermediates are produced by Maillard-type reactions (Andrews, 1975). Disulfide bonds may also link polypeptide chains. Time and temperature of storage determine the extent of polymerization. The relationship of polymerization to gelation is not clear. UHT-sterilized milk stored at 4°C gels sooner than milk stored at 30°C even though less polymerization occurs at 4°C (Andrews, 1975).

Changes in casein micelle distribution have been observed to occur with gelation. Microstructures seen through electron microscopy show that during storage casein micelles

associate increasingly during the period in which viscosity rapidly increases, leading to gelation (Harwalker and Vreenan, 1978). Gelation results from gradual changes in casein micelles rather than a sudden coalescence of micelles (Schmidt, 1968). A great increase in nonmicellar particles occurs with storage, but the effect of this on gelation is not clear (Aoki and Imamura, 1974; Harwalker, 1982).

Effect of Concentration

Gelation of milk is favored by a high concentration of milk solids (Morr, 1967b). Heat coagulation of milk is a function of the milk solids concentration (Whitney, 1977). Concentration normally retards heat denaturation of protein solutions. Concentrating milk to total solid levels of 9, 28, and 44% reduces denaturation (at 80°C for 20 min) by 40, 60, and 80% (Whitney, 1977). Concentration affects individual proteins differently. As concentration is increased, denaturation of α-lactalbumin becomes easier while denaturation of β-lactoglobulin becomes more difficult. The complex protein system of milk and whey is destabilized by increased concentration (Muir and Sweetsur, 1978; Sweetsur and Muir, 1980a; Fox, 1982). The effects of concentration include the closer packing of casein micelles and the precipitation of calcium phosphate with a concomitant decrease in pH (Fox, 1982). The drop in pH causes the precipitation of proteins which are soluble at normal concentration. Flocculation of casein occurs within 3 weeks at -8°C in concentrated milk with 3x solids (Lonergan, 1978).

α-Lactalbumin

α-Lactalbumin constitutes ~4% of total milk protein and up to 25% of whey protein (Farrell, 1988). It exhibits a strong structural relationship to lysozyme (Farrell, 1988) and has the unique biochemical role as the specifier protein of the lactose synthetase system (Ebner and Schanbacher, 1974). In this system, it interacts with galactosyl transferase to promote the transfer of galactose from UDP galactose to glucose, producing lactose in the

process (Walstra and Jenness, 1984). The Michaelis constant *K*m (of the enzyme) for substrate glucose in this reaction is lowered from ~1400 mM to 4 mM by the presence of α-lactalbumin (Walstra and Jenness, 1984). Here the α-lactalbumin does not appear to participate directly in the catalytic reaction but acts as an enzyme modifier (Walstra and Jenness, 1984).

α-Lactalbumin is much more structurally stable than β-lactoglobulin because it has no free sulfhydryl groups although it does contain four disulfide bonds (Farrell, 1988). The secondary structure of α-lactalbumin confers compactness on the molecule, which has a monomer weight of 14.17 kDa (Farrell, 1988). α-Lactalbumin is a calcium-binding protein (Hiraoka *et al.*, 1980), and removal of calcium results in profound conformational changes equivalent to those occurring through acid denaturation (Kronman *et al.*, 1981; Permyakov *et al.*, 1981). These changes include the irreversible unfolding of the molecule and a decrease in denaturation temperature by 20°C (Bernal and Jelen, 1984). An increase in the calcium ion concentration up to .4 mg/ml slows the heat denaturation of α-lactalbumin (Hillier *et al.*, 1979). Calcium and other metal ions may strongly influence the stability of α-lactalbumin (Farrell, 1988).

There is a slow conformational change at pH 4 as calcium is released from carboxyl groups on the protein surface. α-Lactalbumin is denatured at pH < 4 and then undergoes an association reaction which requires an elevated protein concentration (Kronman *et al.*, 1964). In whey concentrates, both calcium binding and acid denaturation of α-lactalbumin may play a role in the retention or loss of the protein functional properties (Farrell, 1988). The free sulfhydryl of β-lactoglobulin can promote complex formation with α-lactalbumin through disulfide interactions at elevated temperatures (Hunziker and Tarassuk, 1965).

α-Lactalbumin has a denaturation temperature of 62°C and an enthalpy of denaturation of 253 KJ/mol(17.8 J/g), making it the whey protein least resistant to unfolding when milk is heated (de Wit and Klarenbeek, 1984). Immunodiffusion of heated

skim milk determined that denaturation of α-lactalbumin is first order (Lyster, 1970), but because its heat denaturation at pH 6 is reversible, α-lactalbumin is considered the most stable serum protein (Larson and Rolleri, 1955; de Wit, 1981). It is in fact stable against heat-induced aggregation because it renatures easily when cooled (Brown, 1988). Heating of α-lactalbumin results in a reversible conformational change through four pairs of disulfide bonds within the molecule (Lyster, 1979). Denaturation of α-lactalbumin is 80-90% reversible (Ruegg *et al.*, 1977). Addition of .28 mM *p*-chloromercuribenzoate to skim milk before heating reduces the rate of denaturation of α-lactalbumin from 25-fold at 85°C to ~3-fold at 155°C (Brown, 1988). Denaturation of α-lactalbumin is slower at pH 4 than at pH 6 or 9 (Hillier *et al.*, 1979), but α-lactalbumin is partially denatured at pH 4 without heating (Kronman *et al.*, 1964). There is no effect on the rate of denaturation of α-lactalbumin at 78°C or 100°C within the pH range 6.2-6.9 when NaOH or HCl is added to skim milk (Lyster, 1970). The rate of denaturation increases at these temperatures outside this pH range when these reagents are added to skim milk (Brown, 1988).

β-Lactoglobulin

Frank and Braunitzer (1967) published their partial amino acid sequence for bovine β-lactoglobulin A and B, which has since led to much of the understanding of the structure of β-lactoglobulin. β-Lactoglobulin is one of the most heat-stable serum proteins. It has a denaturation temperature of 78°C and exhibits another thermal reaction at ~140°C caused by the breaking of disulfide bonds and further unfolding of the molecule (Watanabe and Klostermeyer, 1976; de Wit, 1981). A pH of 6 favors reversible denaturation at 78°C while a pH of 7.5 favors irreversible denaturation at 140°C (de Wit and Klarenbeek, 1984). β-Lactoglobulin is sensitive to pH denaturation and is more stable at lower pH (e.g., 4) than at higher pH (e.g., 9) (Hillier *et al.*, 1979).

β-Lactoglobulin exists as an 18.3 kDa monomer below pH 3 but as a 36 kDa dimer above pH 3 (McKenzie, 1971). Each dimer contains four disulfide linkages and two thiol

groups. Increased thiol activity of β-lactoglobulin at high pH results in decreased stability of the molecule. These thiol groups are unreactive in native β-lactoglobulin, but when the dimer molecule reversibly dissociates through heating, a significant increase in activity occurs (de Wit and Klarenbeek, 1984). After dissociation, the monomers produced (Sawyer, 1969; McKenzie, 1971) unfold and then polymerize by sulfhydryl interchange followed by further aggregation (Harwalker, 1980b). These complexes produced can be found in milk heated at 100°C for 30 min at pH 6.5 (Creamer *et al.*, 1978). Less compact, thin strands of β-lactoglobulin are produced when milk is heated at pH 6.8 since net negative charges on individual protein molecules at this higher pH hinder interaction. Above pH 6.8, however, there is increased ability of the free thiol groups to interact because of favorable conformational changes and this leads to more compact structures (Dunnill and Green, 1966). Irreversible denaturation of β-lactoglobulin occurs above pH 7.5 (Kinsella, 1984).

Flavor changes occur in milk through heat treatment as free sulfhydryl groups appear on β-lactoglobulin before the protein is completely denatured. Of all milk proteins, β-lactoglobulin contains the highest sulfhydryl content (Hutton and Patton, 1952). Prolonged heating of β-lactoglobulin results in more extensive unfolding of individual protein chains, leading to bond breaking at disulfide linkages and exchange reactions with other proteins. Heating conditions that lead to β-lactoglobulin denaturation produce very active sulfhydryl groups which increase the rate of denaturation (Lyster, 1970).

The heat stability of β-lactoglobulin is affected by the ionic environment. As the ionic strength increases at specific pH, β-lactoglobulin shows less stability to heat, and, as a result higher rates of precipitation are observed (Harwalker, 1980a and b).

Addition of calcium up to .4 mg/ml causes a decrease in the solubility of β-lactoglobulin at any pH (Hillier *et al.*, 1979; de Wit 1981).

Lyster (1970) determined that the denaturation of β-lactoglobulin is second order. More recently, Dalgleish (1990) demonstrated that in the heating range 75-90°C, it is pseudo-first order. Harwalker (1980a) determined that the enthalpies of denaturation at pH 4.5 and 6.5 are 14.96 and 10.73 J/g.

Casein

It is difficult to define caseins in a way that includes all proteins belonging to the class and excludes all others (Walstra and Jenness, 1984). For bovine milk, the common property of low solubility at pH 4.6 serves as a basis for a convenient operational definition. At this pH all caseins, except some of the proteolytic derivatives, precipitate (Walstra and Jenness, 1984). Their solubility is so much less than that of any of the whey proteins at this pH that a clear-cut separation is possible (Walstra and Jenness, 1984).

Casein constitutes ~80% of milk protein and is primarily found as a colloidal dispersion of large protein-mineral complexes called casein micelles. This high concentration of casein relative to other milk proteins results in its dominance in determining protein-dependent characteristics of milk during processing (Schmidt, 1980). None of the four kinds of caseins has a highly organized secondary structure (Walstra and Jenness, 1984). Analyses suggest the presence of short lengths of α-helix and β-sheet structures (Walstra and Jenness, 1984) and recently molecular modeling of the caseins has been undertaken which presents their possible configurations (Farrell *et al.*, 1993). Their ionizable groups are accessible to titration and other side chains to reaction. Neither the reactivity of such groups nor the optical rotation is materially increased in the presence of denaturing agents or by heating. Their conformation appears to be much like that of denatured globular proteins (Walstra and Jenness, 1984). The presence of uniformly distributed proline residues helps to prevent a close-packed orderly secondary conformation (Walstra and Jenness, 1984). The proteins of casein micelles are thus arranged in a complex quaternary conformation, which provides stability on the structure (most globular

proteins are stabilized by their tertiary structures). Various casein proteins provide a protective environment for each other against solvents and other outside influences (McMahon and Brown, 1984a).

Casein micelles have been implicated in the age gelation of UHT-sterilized milk products through partial hydrolysis of milk proteins by residual proteolytic enzymes (Harwalker, 1982). Casein micelles in cooled milk show partial disaggregation, releasing β-casein and other components that may function as lipolytic enzymes (Morr and Richter, 1988). This release of casein subunits causes several changes in the physicochemical properties of the casein micelles (Morr and Richter, 1988). These include increased solvation of the micelles (Morr, 1973a), release of inorganic phosphorous and calcium from the micelles on standing (Morr and Richter, 1988), and a reduction in the ratio of micelle to total casein content from 85-95% at 35-40°C to 75-80% at 0-5°C (Morr, 1973b). As a result of these changes, casein micelles reversibly disaggregate from 2-3 Å at 35-40°C to .1-.25 Å at 0-5°C. They also become less electron dense and more translucent (Morr, 1973b). These chemical and physicochemical changes in casein micelles upon cooling of milk lead to a more viscous fluid with an increased tendency to foam (Morr and Richter, 1988).

Casein micelle proteins are represented primarily by α_{s1}-, α_{s2}-, β-, and κ-casein in approximate proportions of 3:.8:3:1 (Davies and Law, 1980). Compositionally, the hallmark of the caseins is ester-bound phosphate. All the casein polypeptide chains have at least one such group per molecule while none of the whey proteins have any (Walstra and Jenness, 1984). Depending on the genetic variant, α_{s1}-casein has 8 or 9 phosphate groups. α_{s2}-Casein has two disulfide bonds which can interact with the disulfide bonds of β-lactoglobulin on severe heat treatment. α_{s2}-Casein represents the most hydrophilic of the caseins and is very sensitive to calcium ion concentration. It has 10 to 13 phosphate groups (Swaisgood, 1982; Kinsella, 1984).

β-Casein has 4 or 5 phosphate groups depending on the genetic variant and is extremely dipolar and amphiphilic. It contains 16% proline and is mainly a random coil with two separate hydrophilic and hydrophobic domains (Swaisgood, 1982). Both heating and cooling have been reported to move β-casein from serum into the micelles (Dzurec and Zall, 1985). It has also been reported that milk stored at 4°C can have as much as 40% of β-casein dissociated from the micelles (Schmutz and Puhan, 1981). Calcium added to milk prevents β-casein from leaving the micelles regardless of the temperature treatment (Carpenter and Brown, 1985). Proteinases in milk cleave β-casein to yield γ-casein and proteose-peptone fractions (Pearce, 1980; Swaisgood, 1982).

κ-Casein like α_{S2}-casein has two disulfide bonds capable of interacting with those of β-lactoglobulin to form cross-links. These disulfide groups are confined within the hydrophobic N-terminal two-thirds of the molecule. The charged, polar, hydrophilic C-terminal makes up the rest of the molecule. A varying number of carbohydrate moieties and one phosphate group are found in the molecule. Most recent models of the casein micelle have κ-casein taking up most of its surface area (Heth and Swaisgood, 1982) from where it protects the other calcium-sensitive caseins from precipitation by calcium ions. κ-Casein is not susceptible to calcium ion binding (Swaisgood, 1982).

Milk casein also exists as serum casein (Rose, 1968; Downey and Murphy, 1970). Rose (1968) found that ~10% of the total casein in warm milk was serum casein. His conclusion that the serum caseins appeared not to be in equilibrium with the micellar casein has been refuted by later research (Creamer *et al.*, 1977; Ali *et al.*, 1980; Davies and Law, 1983).

Casein and Serum Protein Interactions

The properties of κ-casein are altered as a result of heat denaturation of β-lactoglobulin (Zittle *et al.*, 1962). Interaction through disulfide linkages occurs between κ-casein and β-lactoglobulin when these are heated together or when κ-casein is added to

heated β-lactoglobulin (Morr *et al.*, 1962; Morr, 1965; Sawyer, 1969; Snoeren and van der Spek, 1977). This interaction occurs optimally at pH 6.8 (de Wit, 1981) and at 85-90°C. Formation of these complexes decreases with ionic strength and as the pH is increased from 6.8 to 7.3 (Smits and Van Brouwershaven, 1980). The presence of calcium salt favors complex formation and more severe heat treatments increase the sensitivity of the serum protein to calcium ions. Ionic interactions, disulfide interchange, and hydrophobic bonding are all suspected to be involved in these complex formations (Hill, 1989).

α_{S2}-Casein also forms disulfide bridges when heated with β-lactoglobulin and this can interfere with the complex formation between κ-casein and β-lactoglobulin (Kudo, 1980b; Kinsella, 1984). The total amount of whey protein attached to casein increases as heat treatment is intensified, but the ratio of individual whey proteins attached remains constant (Farah, 1979). Very little direct interchange occurs between α-lactalbumin and κ-casein when these are heated together (Hartman and Swanson, 1965), but the complex formed between α-lactalbumin and β-lactoglobulin interacts with κ-casein (Hunziker and Tarassuk, 1965; Elfagm and Wheelock, 1977). The degree of denaturation of α-lactalbumin is greater when heated with β-lactoglobulin than when heated alone and increases with pH from 6.4 to 7.2. Denaturation is optimum between 70°C and 85°C. The denaturation of β-lactoglobulin is not affected by the presence of α-lactalbumin, but formation of the complexes between α-lactalbumin and β-lactoglobulin is facilitated by the presence of casein (Elfagm and Wheelock, 1978a and b). α-Lactalbumin reduces the direct interaction of κ-casein and β-lactoglobulin (Baer *et al.*, 1976; Elfagm and Wheelock, 1977 and 1978a). Complexes formed between κ-casein and β-casein, and between κ-casein and α_{S1}-casein (Doi *et al.*, 1979), may interfere with κ-casein and β-lactoglobulin complex formation.

Three classes of micelle models exist (Farrell, 1988). These include the "Coat-Core Models" (Parry and Carroll, 1969; Waugh *et al.*, 1970; Walstra, 1979), the "Internal

Structure Models" (Rose, 1969; Garnier and Ribadeau-Dumas, 1970; Pepper and Farrell, 1982), and the "Submicellar Models" (Shimmin and Hill, 1964; Morr, 1967a; Slattery and Evard, 1973). The most popular Coat-Core model with κ-casein on the outer casein micelle surface (Waugh *et al.*, 1970; Shahani, 1974; Walstra, 1979; Heth and Swaisgood, 1982; McMahon and Brown, 1984a) allows the possibility of heat-induced coagulation of milk being the result of serum proteins interacting with κ-casein on the micelle surface. The serum proteins further interact with each other to interconnect micelles (Brown, 1988). Ashoor *et al.* (1971) demonstrated that the other casein components also form part of the "coat" of the micelles. The other Coat-Core model (Parry and Carroll, 1969) has κ-casein as the core molecule surrounded by the other casein molecules. The Internal Structure models are based upon the properties of isolated casein components which are responsible for the formation of the internal structures of the micelles (Farrell, 1988). The Submicellar models propose that the micelles are made up of many discrete submicelles (diameter ~10 nm) joined together by calcium phosphate linkages (Shimmin and Hill, 1964; Morr, 1967a; Farrell, 1988).

Processes Involved in Making UHT-Sterilized Concentrated Milk

UHT Sterilization

Heat treatment of milk in dairy technology varies in intensity from pasteurization (72°C for 15 s) to sterilization (typically 120°C for 20 min or 142°C for several seconds) (Creamer and Matheson, 1980; Douglas *et al.*, 1981). Retort sterilization uses 110-120°C for 20-40 min while UHT sterilization is done at 120-140°C for several seconds (Creamer and Matheson, 1980). Sterilization is effective in inactivating enzymes and this effect can be used as a measure of the efficacy of the treatment. Pasteurization kills spoilage and pathogenic organisms in milk (Parnell-Clunies *et al.*, 1988) while sterilization is meant to destroy all microorganisms, including spores (Walstra and Jenness, 1984). The efficacy of

commercial sterilization naturally depends on the type and number of spores present (Walstra and Jenness, 1984).

UHT-sterilized milk is regarded as having superior organoleptic qualities compared to retort sterilized milk. UHT sterilization utilizes the higher thermal coefficient of biological reactions leading to faster destruction of microorganisms without compromising too much on flavor, color, and nutrient value of the product (Zadow, 1986). UHT treatment invariably results in some cooked flavor (Morr, 1985), Maillard browning and vitamin loss (Freeman and Mangino, 1981) occurring in the milk. Age gelation, however, represents the major problem resulting from UHT sterilization of milk (Corradini, 1975; Snoeren *et al.*, 1979). Gelation occurs more readily in UHT-sterilized concentrated milk than in UHT-sterilized milk (Harwalker, 1982).

UHT sterilization can be affected through different combinations of temperature and time. Raw milk is usually forewarmed to 80-85°C in an indirect heat exchanger before being heated to the final UHT temperature (Morr and Richter, 1988). The UHT temperature can be achieved through direct heating or by indirect heat exchange.

Direct heating is performed either by injecting steam into milk or vice versa (van den Berg, 1988). As a result of the direct contact of milk and steam, the steam gives up its latent heat to the cooler milk and very quickly (less than 1 s) brings the milk to the sterilization temperature (van den Berg, 1988). After holding for a period of 3-10 s, the milk passes to a vacuum chamber where excess water resulting from the condensed steam is removed by evaporation (van den Berg, 1988). Heat extracted from the milk is used to effect this evaporation, allowing instantaneous cooling to the immediate post-sterilization temperature (van den Berg, 1988).

Direct heating at 142°C for 4 s is ineffective in inactivating native milk proteinases or those produced by psychrotrophic bacteria during cold storage of raw milk (Snoeren *et al.*, 1979; Snoeren and Both, 1981). These enzymes exhibit some reactivation after high-

temperature, short-time heat treatment (Walstra and Jenness, 1984). Sterilized milk produced under these conditions is therefore susceptible to age gelation via proteolysis (Snoeren *et al.*, 1979; Snoeren and Both, 1981).

Indirect heating of milk requires a longer time to reach UHT temperature and this leads to more product defects than with direct heating (Freeman and Mangino, 1981). The longer heating time of indirect heating effectively inactivates proteinases found in milk. This may, however, lead to product "burn-on" on the heat exchanger, thus reducing its efficiency (Freeman and Mangino, 1981).

Membrane Processes for Concentrating Milk

Milk can be concentrated by evaporation of water from it (McKenzie and Murphy, 1970) or by membrane processes such as reverse osmosis (RO) and ultrafiltration (UF) (Walstra and Jenness, 1984).

Reverse osmosis or hyperfiltration differs from UF by the relative lower porosity of the membranes and in the application of a much higher pressure (Karel, 1975; Walstra and Jenness, 1984). When the "holes" within the membrane are about the same size as the diffusing molecules, it is usually accepted that transport occurs by "solution" or "activated" diffusion (Karel, 1975). The RO membrane does not act so much as a filter, but more as a material in which water "dissolves" and thus passes through, leaving the solutes behind (Walstra and Jenness, 1984). Its prime advantage over evaporative concentration is energy saving (Walstra and Jenness, 1984).

Ultrafiltration was introduced initially to the world dairy industry as a means of separating protein from sweet and acid whey (Kosikowski, 1982). The process has become firmly entrenched and large units are now in operation in many countries, including France, New Zealand, and the United States.

Skim milk is cycled at a specific temperature or combination of temperatures across a membrane, cellulose acetate with polyvinyl chloride backing or polysulfone, in a forced

turbulent flow. The temperature used depends upon the concentration of protein solids desired (Kosikowski, 1982). The membranes may be plates, straight tubes, or spiraling spaghetti-like tubes, and are mounted for support on stainless steel standards. The membranes have a maximum average pore diameter of 30 Å, and the milk moves under a pressure of 45-50 psi (Kosikowski, 1982). Cycling of the milk through the interior of the membrane is maintained until the concentrate on the inside attains the maximum protein solids desired or until the soluble components of the skim milk concentrate can no longer pass through. The material that passes through the membrane is the permeate or ultrafiltrate which has some of the properties of standard whey but is quite different otherwise. The material on the inner side of the membrane is the retentate or concentrate which moves across the membrane to the holding tank (Kosikowski, 1982).

UF employs the same principle as RO in concentrating milk. The membrane is effectively a filter through which small molecules like sugars, salts, and flavor compounds can pass while colloidal particles and macromolecules are retained (Karel, 1975; Walstra and Jenness, 1984). For milk this implies that globular proteins, casein micelles, fat globules, and cells do not pass. The permeate is thus sterile (Walstra and Jenness, 1984).

Water and small solute molecules are forced through the membrane by the higher pressure on the inside of the porous barrier. The water and small solute molecules always flow in this direction in contrast to dialysis where, owing to osmosis, there is usually a net flow of solvent in the reverse direction. As with pervaporation, plugging of the membrane pores presents a problem with UF. Denaturation of protein at the surface of the membrane is also a possibility with UF and pervaporation (McKenzie and Murphy, 1970).

UF does not affect serum casein nor the calcium and phosphate content of casein micelles (Yan *et al.*, 1979); neither does it affect the size distribution of casein micelles. Combined with diafiltration, it can remove up to 99% lactose, 42% phosphate, and 36% calcium from skim milk (Lonergan, 1982).

Less than 10% whey protein is lost through UF, depending on the pore size of the membrane (Chapman *et al.*, 1974). No loss of fat globules or fat-soluble vitamins occurs. Through their association with proteins, vitamin B12, and folic acid are also retained (Yan *et al.*, 1979).

Electron Microscopy

The resolution of the modern transmission electron microscope approaches the theoretical limit of ~5Å. This theoretical limit allows transmission electron microscopy (TEM) to be used in visualizing the smallest protein. However, the major problem with proteins is not resolution, but contrast. A protein may have a diameter of 50Å, but may fail to scatter enough electrons out of the beam to produce a satisfactory image that can be clearly delineated from the instrument "noise". Contrast for protein may be increased by positive (electron) staining (McKenzie, 1970).

The most obvious application of electron microscopy in milk protein studies is in elucidation of the structure of the casein micelle. Difficulties exist in preparing suitable specimen for examination without drastically altering the micelle structure in milk. The first use of electron microscopy in micelle studies appears to be by Nitschmann (1949) who fixed the micelles with formaldehyde and showed that the particles were essentially spherical in shape (McKenzie, 1970). Since then many studies have been done with electron microscopy on elucidating structures of micelles. Some of the earlier studies are cited in the following literature: Hostettler and Imhof, 1952; Shimmin and Hill, 1965; Rose and Colvin, 1966a and b; Knoop and Wortmann, 1967; Carroll *et al.*, 1968; Buchheim, 1969; Eggmann, 1969.

Two methods of electron microscopy are widely used. These are scanning electron microscopy (SEM) and TEM. SEM is adequate for structure of surface morphology as well as internal structures of milk particles. TEM techniques such as freeze-fracturing and

thin sectioning are needed to identify individual constituents such as casein micelles and fat particles (Schmidt, 1982; Caric and Kalab, 1987).

Immunolocalization of Proteins Using Gold Labeled Antibodies

In 1971, Faulk and Taylor introduced their "immunocolloid" method for the study of cell surface antigens (Faulk and Taylor, 1971). Since then a large number of studies have documented the potential of colloidal gold as a marker for immunocytochemistry (Horisberger, 1979 and 1981; Goodman *et al.*, 1980; De Mey, 1983). Much of the basic methodology for preparing standardized gold sols and stable gold probes has been established (De Waele *et al.*, 1983). Colloidal gold particles have undergone an enormous evolution since their introduction by Faulk and Taylor in 1971 (Leunissen and De Mey, 1989). These are formed by chemical reduction of an aqueous solution of tetrachloroauric acid by condensation method. The gold particles are then converted to gold probes (Leunissen and De Mey, 1989) by reaction with a protein having the property of binding immunoglobulins (Bendayan, 1989). Two proteins, protein A isolated from staphylococci (Forsgren and Sjoquist, 1966) and protein G isolated from streptococci (Bjorck and Kronvall, 1984), are commonly used for this purpose to produce protein A-gold and protein G-gold complexes, respectively (Bendayan, 1989). Alternatively, immunoglobulin-gold complexes may be produced by reacting an immunoglobulin (secondary) with colloidal gold. The gold complexes are used as components in indirect two-step immunolabeling. The first step involves the interaction of a specific (primary) immunoglobulin with the antigen under investigation in the prepared tissue section. In the second step, the molecules of protein A or G surrounding the gold particle or the molecules of immunoglobulin-gold complex interact with the Fc fragment of the primary immunoglobulin. The presence of the gold particle thus allows the indirect localization of

the antigenic site. Electron microscopy can then be used to elucidate the position of the antigen under investigation (Bendayan, 1989).

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CHAPTER III

AN APPARATUS FOR A NEW MICROCUBE ENCAPSULATION OF FLUID MILK IN PREPARATION FOR TRANSMISSION ELECTRON MICROSCOPY[1]

ABSTRACT

A simple apparatus has been developed for a new "microcube" encapsulation of fluid milk samples in their prefixation preparation for electron microscopy. The new technique is based on making cubic wells in an agar gel layer, filling them with fluid milk samples, and sealing them with another agar gel layer. The individual wells are then separated by cutting from the initial block, providing .5 mm walls around the samples. The embedded material (milk, buttermilk, yogurt, etc.) is fixed, dehydrated, and embedded in a resin for transmission electron microscopy. The procedure is simpler, more versatile, reliable, and reproducible than other encapsulation methods used to prepare similar food samples. Agar gel tubes used in the other methods have several disadvantages such as the need for manual dexterity of the experimenter to make them, and difficulty in sealing the filled capsules properly. Results obtained by the microcube procedure were compared with results obtained by two methods using agar gel tubes and also by mixing a warm agar sol with fluid food samples. This latter method is simpler than agar encapsulation, but shows agar strands in the micrographs of the milk samples. This is particularly undesirable when investigating, for example, intermicellar strands of gelled UHT-sterilized milk concentrates. Microcube encapsulation produces superior quality images of the fluid food structure.

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INTRODUCTION

Preparation of fluid milk for electron microscopy requires the use of specific techniques in order to obtain artifact-free images of the samples (Carroll *et al.*, 1968; Stewart *et al.*, 1972; Andrews *et al.*, 1977; Davies *et al.*, 1978, Kalab, 1983; Heertje *et al.*, 1985; Farah and Ruegg, 1989). Fluid or gelled milk samples destined for electron microscopic examination must not disintegrate while they are fixed, dehydrated, and mounted or embedded. Various methods have been employed to achieve this objective both in scanning electron microscopy (SEM) and transmission electron microscopy (TEM).

Mixing of fluid milk with agar sol followed by gelling the mixture and treating the resulting gel as a solid sample has often been used in TEM because this procedure is simple. However, dilution of the sample with the agar sol alters the distribution of structural elements in the sample and agar strands present in the gel contaminate the sample and are visible under the electron microscope (Kalab, 1981; Harada *et al.*, 1991). Their images may interfere with other structural components such as fat globule membrane fragments (Kalab, 1980). Enclosing a small volume of the sample in a capsule permeable for fixatives and dehydrating agents promised to avoid some of the problems. Agar encapsulation was pioneered by Salyaev (1968) and his technique has been adapted and modified by several authors (Henstra and Schmidt, 1970 and 1974; Jewell, 1981; Allan-Wojtas and Kalab, 1984; Kalab, 1987 and 1988; Veliky and Kalab, 1990), particularly for use in SEM. In the original and modified procedures, the sample is aspirated into a narrow agar tube (Henstra and Schmidt, 1970 and 1974; Jewell, 1981; Allan-Wojtas and Kalab, 1984). The sample may alternatively be aspirated into a Pasteur pipette, which is subsequently coated with agar gel and the sample transferred from the Pasteur pipette into the gel tube formed by withdrawing the pipette from the agar gel coating (Kalab, 1987 and 1988). The sample-containing gel tube is sealed with warm agar sol.

Other hydrocolloids (sodium alginate) with low-temperature gelling properties have also been used to encapsulate viscous food samples (Veliky and Kalab, 1990). A somewhat different encapsulation procedure for SEM was used by Teggatz and Morris (1990). Holes (2 mm diameter x 2 mm depth) drilled into aluminum SEM stubs were filled with fluid food samples and the stubs were dipped into 3% agar sol at 45°C which subsequently solidified. After fixation and dehydration, the agar layer on top of each stub was lifted off and mounted upside-down on a clean SEM stub using double-coated tape. The samples were surrounded by a coat of carbon paint and coated with a layer of gold-palladium before being viewed in a scanning electron microscope at 6 kV (Teggatz and Morris, 1990).

All reported agar encapsulation procedures require a relatively high degree of manual dexterity of the experimenter both during preparation and sealing of the capsules, particularly because capsules used in TEM are considerably smaller than those used for SEM. Perfect sealing of fluid milk samples is difficult to achieve and the samples may slowly leak out.

The objective of this study was to develop a more practical method of encapsulating fluid milk samples in prefixation preparation for electron microscopy. The new method of encapsulation milk samples of both low and high viscosity is described in this chapter. The problematic aspiration step of other encapsulation methods is eliminated and the samples are sealed more efficiently. Results obtained using this new method are compared with those obtained by other methods.

MATERIALS AND METHODS

Reagents

Agar was purchased from Difco Laboratories (Detroit, Michigan); glutaraldehyde, osmium tetroxide, propylene oxide, and Epon-Araldite epoxy resin were obtained from

Electron Microscopy Sciences (Fort Washington, Pennsylvania). All other chemicals were analytical reagent grade.

Samples

Whole milk, stirred yogurt, and 8-month-old ultra-high temperature (UHT) sterilized ultrafiltered milk retentate (concentrated 3x by volume) were obtained from the Gary H. Richardson Dairy Products Laboratory, Utah State University. Cultured buttermilk was a commercial sample.

Microcube Encapsulation Compared with Encapsulation in Agar Gel Tubes

A kit for microcube encapsulation was designed and constructed. It consists of (a) a stainless steel mold with a row of 12 or 24 teeth of dimensions 1 x 1 x 2 mm (height x width x length) separated by 2-mm gaps (Figure 3.1) for simultaneous preparation of wells in agar gel, and (b) a fiberglass mask (bottomless rectangle) of inner dimensions 2 x 10 x 100 mm (height x width x length) for casting a block of agar gel of similar dimensions. The ends of the mold project 1 mm below the teeth so that when positioned over the block of agar gel, there is a 1-mm thick agar gel layer left beneath the bottoms of the wells.

Microcube Encapsulation

The fiberglass mask was placed on a square sheet of fiberglass (side: 200 mm) and the joints were sealed with a 4% agar sol kept at 50°C and stirred continuously with a magnetic bar. Two milliliters of the same agar sol was deposited in the opening of the mask using a plastic transfer pipette. After 5 s when gelation had just begun, the mold was pressed into the 2 x 10 x 100 mm (height x width x length) block and held in place for 5 s before being carefully removed to leave cubic wells impressed in the agar block (Figure 3.2a). All the wells in the block were filled with 2 mm^3 aliquots of one fluid food sample through a 25G 7/8 needle (Becton Dickinson, New Jersey) from a 1-ml syringe. In each

Figure 3.1. Stainless steel mold used to prepare microcube samples **(a)** stereographic view, **(b)** lateral view.

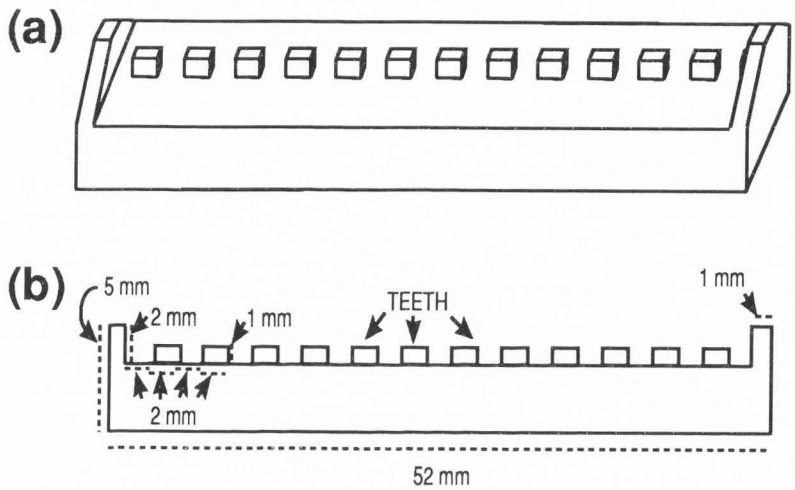
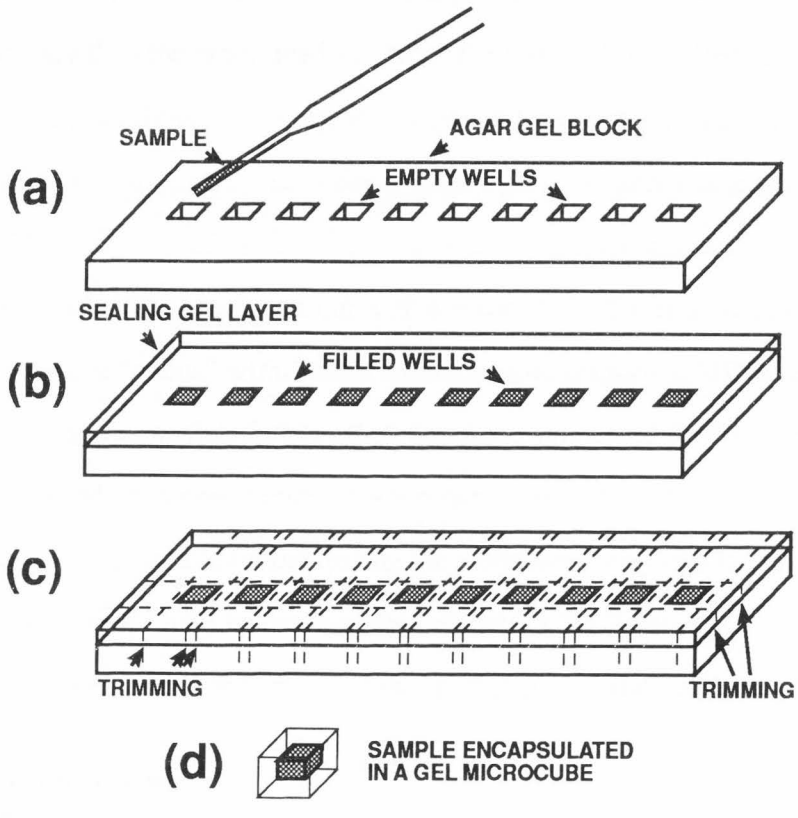

(a)
(b)
5 mm
2 mm
1 mm
TEETH
1 mm
2 mm
52 mm

Figure 3.2. Steps involved in the preparation of microcube samples: **(a)** filling empty cubic wells impressed in the agar gel block, **(b)** sealing filled wells in agar gel block, **(c)** trimming "cubes" from agar gel block, **(d)** gel "microcube" with encapsulated sample.


(a)
SAMPLE
AGAR GEL BLOCK
EMPTY WELLS
SEALING GEL LAYER
(b)
FILLED WELLS
(c)
TRIMMING
TRIMMING
(d)
SAMPLE ENCAPSULATED
IN A GEL MICROCUBE

case the sample was drawn into the syringe before attaching the needle to dispense materials into the wells. Preliminary studies compared results of filling wells with different gauge needles to determine if the shear forces on sample using the smaller bore needles affected the extended structures of samples. No detected differences in the extended structures of the samples were observed in samples dispensed with 16G 1, 21G 1/2, and 25G 7/8 needles. The 25G 7/8 needles were subsequently used because they filled the wells more efficiently than the other needles. After the last well was filled, another layer of agar sol at 50°C was placed over the block to a depth of 1 mm where it gelled, thus covering and sealing the samples in the wells (Figure 3.2b). A razor blade was used to trim the agar seal from the top of the mask and the entire solidified agar gel block was removed from the mask. The block was cut with a razor blade .5 mm around each well (Figure 3.2c) to produce "cubes" with 2 mm^3 of the sample (Figure 3.2d). The "cubes" were examined for leaks and other defects, then fixed and processed for TEM as described below in the Electron Microscopy section. Preliminary studies had shown that (a) agar sol concentrations < 4% were unsuitable for sealing the wells since they displaced the sample (especially the less viscous ones) from the wells and (b) 3 x 3 x 2 mm agar "cubes" with 2 mm^3 of milk sample were small enough to allow satisfactory fixation and dehydration.

Salyaev Encapsulation in Agar Gel Tubes

Capsules were made by dipping a stainless-steel rod (.5 mm in diameter) repeatedly into a 4% agar sol (stirred continuously with a magnetic bar at 45°C) and manipulating the rod to form a thin (~.5 mm) agar gel tube around it. The lower end of the solid tube was then cut off with a razor blade. Milk samples were aspirated into the tube by immersing its open end into the sample and pulling the steel rod upwards as a piston. After enough milk sample had been aspirated into the tube (a length of approximately 12 mm), its bottom end

was wiped with tissue paper and the tube was sealed with warm (45°C) agar sol producing a capsule.

A 4-mm length portion of the capsule was cut off from the bottom and a drop of agar sol was applied with a transfer pipette to seal the upper end, producing a microcapsule. This was repeated to produce three microcapsules from each capsule. All microcapsules were examined for leaks and other defects.

Kalab Encapsulation in Agar Gel Tubes

A glass Pasteur pipette with inner diameter of 1 mm was drawn out into a capillary tube with inner diameter of ~.5 mm. The milk sample was aspirated into the capillary tube to a length of ~2 mm. The lower end of the tube was wiped clean with tissue paper and sealed with a droplet of the agar sol. After the sealed end had solidified, the capillary tube was dipped into the agar sol and then manipulated to form a thin layer of agar gel on the glass surface around the sample. Dipping was repeated twice to form a uniform agar gel layer around the glass surface. This agar gel sleeve was trimmed at the upper end of the sample and removed. The capillary tube was then withdrawn from the agar gel sleeve resulting in the sample sliding from the glass tube into the gel tube. The freed upper end of the agar gel tube was then trimmed with a blade approximately .5 mm above the sample and sealed with a droplet of the agar sol producing a microcapsule. This was repeated for each milk sample to produce the desired number of microcapsules. All microcapsules were examined for leaks and other defects.

Electron Microscopy

Agar gel capsules obtained by three encapsulation methods (Salyaev, 1968; Kalab, 1987 and 1988, and our new microcube method) containing whole milk, buttermilk, and yogurt samples were fixed at 20°C for 24 h in 1.5% glutaraldehyde in .1 M phosphate buffer (pH 6.6 for whole milk and pH 4.5 for buttermilk and yogurt). After the fixation

had been completed, these samples as well as samples obtained by mixing milk with agar sol were washed with .1 M phosphate buffer, pH 6.6, and post-fixed with a buffered (.1 M phosphate buffer, pH 6.6) 1% osmium tetroxide, dehydrated in a graded ethanol series of 30, 50, 70, 95, and 100% ethanol, infiltrated with propylene oxide, and embedded in Epon-Araldite epoxy resin. Thin sections (90 nm thick, Sorvall MT-2 Porter-Blum ultramicrotome) from the epoxy resin blocks were stained with a uranyl acetate solution in methanol for 15 min, followed by lead acetate staining for 5 min (Youssef, 1985). TEM was carried out using a Zeiss CEM 902 electron microscope operated at 80 kV. Micrographs were taken on Kodak SO 163 sheet film and printed on Kodak polycontrast III RC paper.

Microcube Encapsulation Compared with Agar Sol Mixing

Microcube encapsulation of UHT-UF milk retentate and yogurt was performed as described in Experiment (a). Half of these samples were placed in vials containing 1.5% glutaraldehyde in .1 M phosphate buffer (pH 6.6 for UHT milk and pH 4.5 for yogurt) at 4°C for 1 h and subsequently refrigerated at 6°C for 24 h in a fresh 1.5% glutaraldehyde solution. The other half were placed in vials containing 1.5% glutaraldehyde in .1 M phosphate buffer (pH 6.6 for UHT milk and pH 4.5 for yogurt) at 45°C for 1 h and subsequently refrigerated at 6°C for 24 h in a fresh 1.5% glutaraldehyde solution.

Mixing with Agar Sol

UHT-sterilized concentrated milk and yogurt were each divided into two 5-ml aliquots and prepared as follows:

<u>Fixation with glutaraldehyde at 45°C</u>
<u>followed by solidification with agar</u>

The samples were fixed in a 4.5% glutaraldehyde solution by mixing 5 ml of sample with .5 ml of a 50% aqueous glutaraldehyde solution. Fixation proceeded for 10 min at

20°C. The samples were heated to 45°C and 5 ml of a warm (45°C) 3% agar solution were added. The samples were vortex mixed, poured into Petri dishes, and allowed to solidify. The sample-agar gel was cut into 1 mm^3 cubes and placed in labeled vials containing 1.5% glutaraldehyde in .1 M phosphate buffer (pH 6.6 for UHT milk and pH 4.5 for yogurt) at 6°C for 24 h.

Solidification of samples with agar
followed by glutaraldehyde fixation at 4°C

The UHT milk and yogurt samples were mixed with 3% agar sol at 45°C, solidified by cooling in Petri dishes and cut into 1 mm^3 cubes. These cubes were fixed with 1.5% glutaraldehyde at 4°C for 1 h and subsequently refrigerated at 6°C in a fresh 1.5% glutaraldehyde solution for 24 h.

RESULTS AND DISCUSSION

Microcube Encapsulation Compared with Encapsulation in Agar Gel Tubes

The first criterion used to assess the quality of encapsulation was to evaluate how well the samples were sealed inside the agar gel capsules. Results obtained by the microcube encapsulation method were compared with results obtained from the methods of Salyaev (1968) and Kalab (1987 and 1988). The numbers of leaky capsules detected after glutaraldehyde fixation for each method are shown in Table 3.1. The χ^2 analysis of these data shows the proportion of intact capsules to be significantly higher ($P = .05$) for the microcube method when compared to the other methods singly and collectively. There was no significant difference between the Salyaev and Kalab methods. After the production of milk-filled agar gel capsules using both methods, buttermilk and yogurt samples were found adhering to the inner walls of the microcapsules in the form of rings rather than uniformly filling the capsules (Figure 3.3). This phenomenon may be attributed to inadequately filled capsules. This problem was also observed with less viscous milk

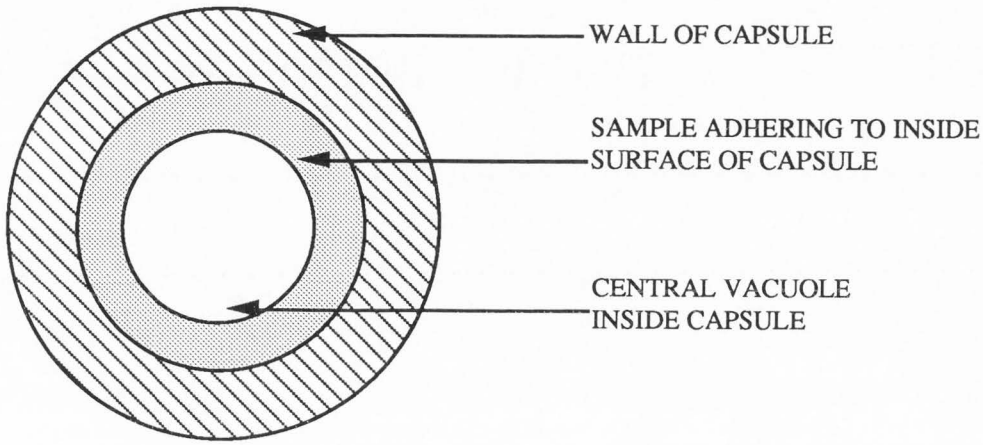
Table 3. 1. Percentage of intact capsules produced from three methods of encapsulating milk samples.

	Salyaev Method	Kalab Method	Microcube Method
Intact Capsules	28	33	50
Leaky Capsules	22	17	0
Percentage Intact	56%	66%	100%

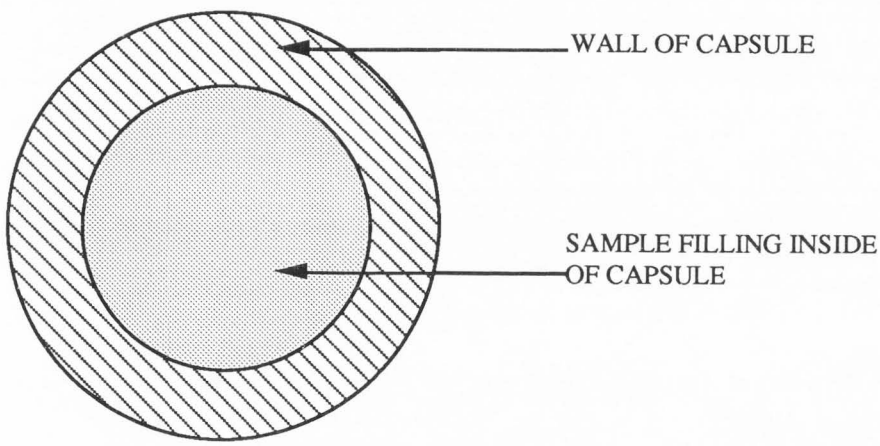
Table 3. 2. Percentage of good blocks produced from three methods of encapsulating milk samples.

	Salyaev Method	Kalab Method	Microcube Method
Good Blocks	37	38	50
Defective Blocks	13	12	0
Percentage Good	74%	76%	100%

Figure 3.3. Diagram comparing **(a)** defective microcapsule with **(b)** nondefective microcapsule. Defective microcapsule shows large vacuole within the sample matrix.



(a) DEFECTIVE CAPSULE



(b) NONDEFECTIVE CAPSULE

samples. This was due to the relatively low total solids content of milk. Replacement of the milk serum with aqueous glutaraldehyde during fixation and, in particular, dehydration in organic solvents destabilized the casein micelle suspension and resulted in sedimentation and adherence of the casein micelles to the well inner walls. This phenomenon was not evident with the microcube method since it apparently produced sedimentation only onto the "bottom" walls of the cubes resulting in intact samples. Throughout the fixation and dehydration processes, the "microcubes" were kept in a fixed position and the liquids removed and added using transfer pipettes. This method was also used with the capsules, but their shapes resulted in shifts of position which could cause sedimentation of sample around the insides of the capsules.

Of 50 blocks per method examined, the proportions that displayed this defect are shown in Table 3.2. From χ^2 analysis, the proportion of defective blocks was significantly lower ($P = .05$) for the microcube method compared to the other methods singly and collectively. There was no significant difference between the Salyaev and Kalab methods. Micrographs produced from the three techniques were quite similar (Figures 3.4 to 3.6). Distortion of fat globules shape observed in Figure 3.5 is due to melting of fat globules when the microcapsules and microcube were sealed using hot agar. The top layer of agar in this procedure cools rapidly after being poured, but some melting of fat does occur as is evident in Figure 3.5. The microcube technique can, however, be used with low-gelling-temperature agarose (Goff *et al.*, 1987; Liboff *et al.*, 1988) and (with slight modification) ultralow-gelling-temperature agarose. With these agaroses the procedure would be suitable for sample encapsulation of cold samples.

In addition to being simpler than the techniques of Salyaev and Kalab, the microcube method proved more reliable. It does not require a high degree of manual dexterity, produces reproducible results, and enables encapsulation of a wider viscosity range of fluid dairy foods.

Figure 3.4. Transmission electron micrographs of whole milk prepared using **(a)** Salyaev method, **(b)** Kalab method, **(c)** microcube method.

c: casein micelles.

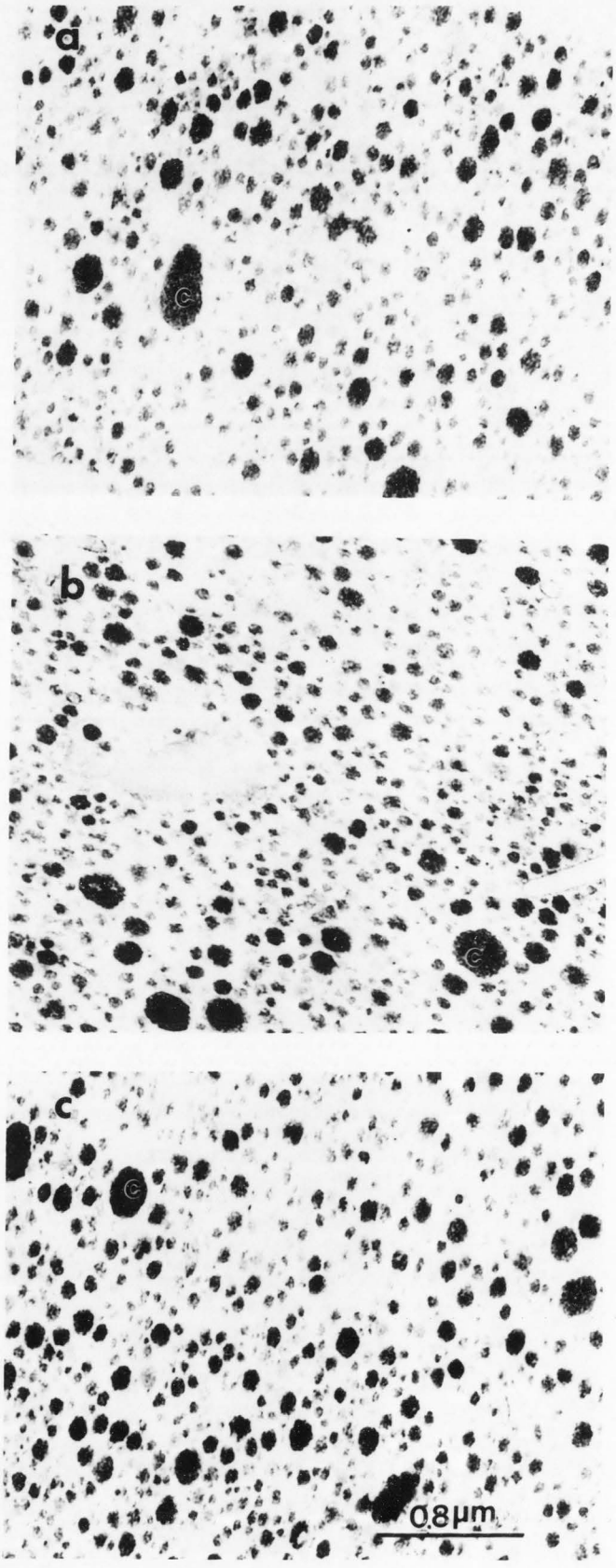

a
b
c
0.8µm

Figure 3.5. Transmission electron micrographs of cultured buttermilk prepared using **(a)** Salyaev method, **(b)** Kalab method, **(c)** microcube method.

c: casein micelles; small arrows: fat globule membrane fragments; large arrows: intact fat globules.

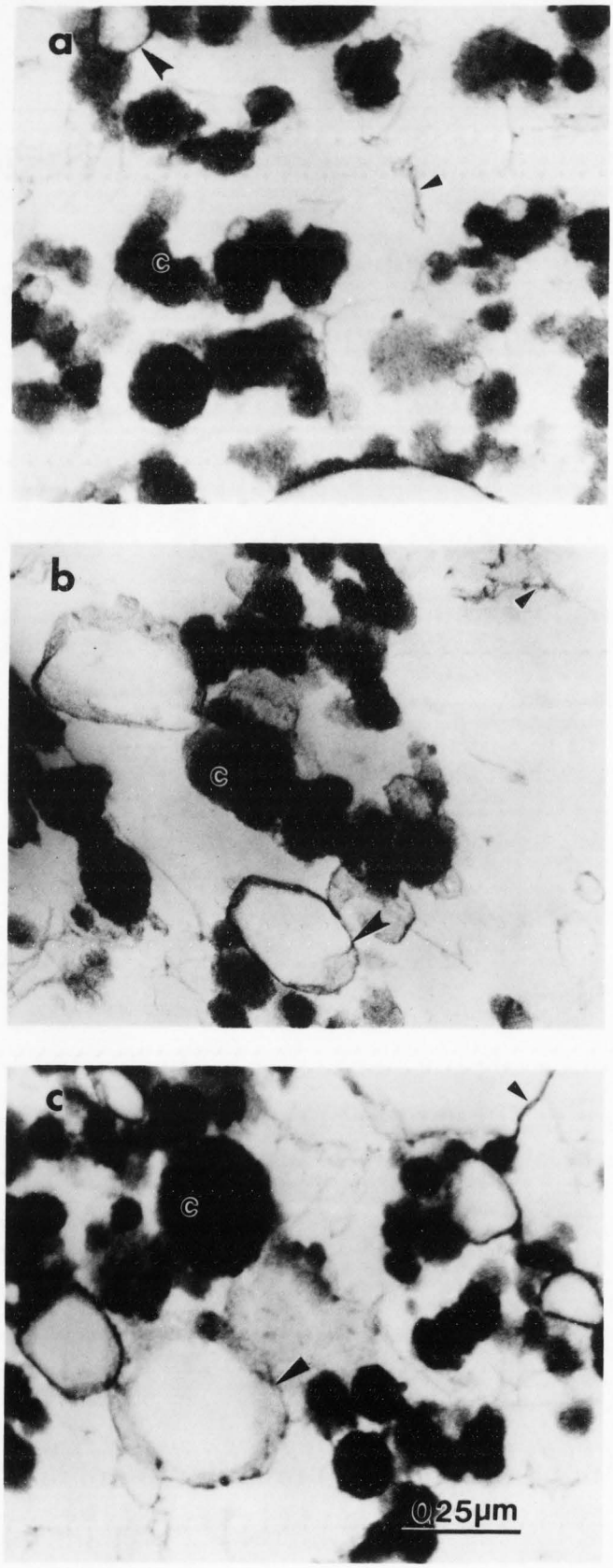
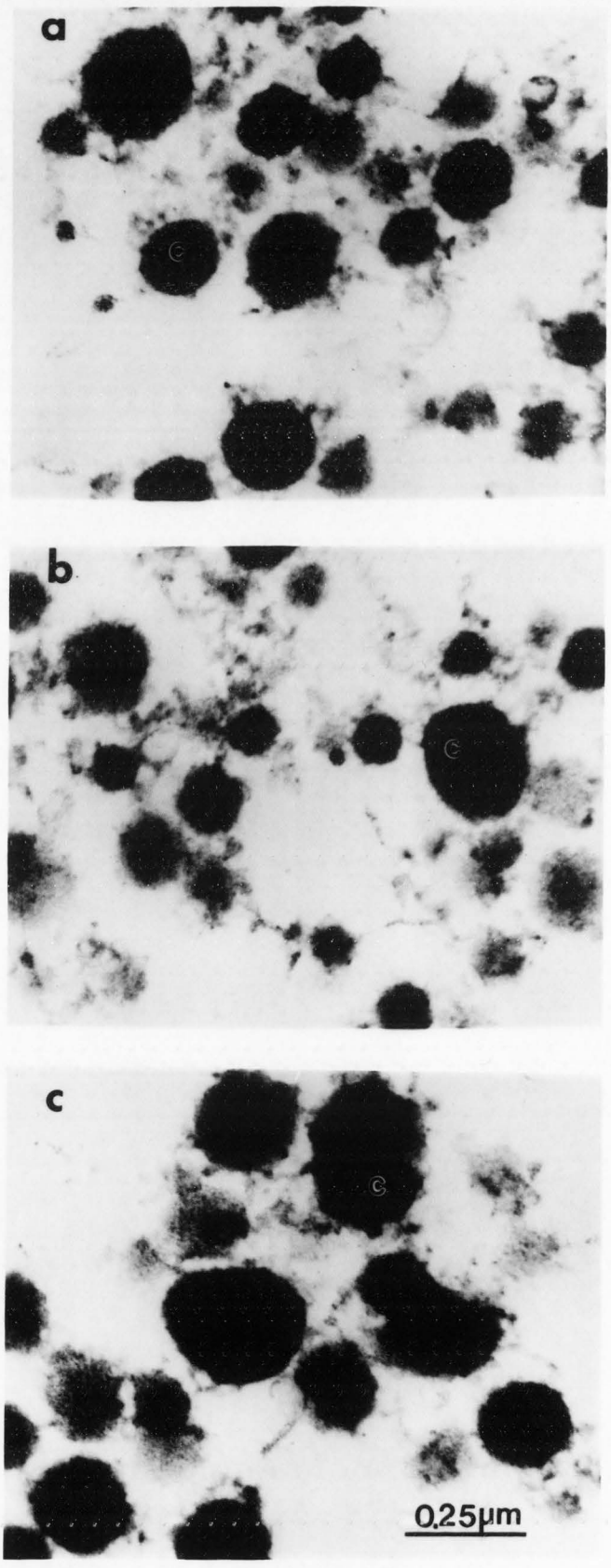

a
C
b
C
c
C
0.25μm

Figure 3.6. Transmission electron micrographs of stirred yogurt prepared using **(a)** Salyaev method, **(b)** Kalab method, **(c)** microcube method.

c: casein micelles.


a
b
c
0.25µm

Microcube Encapsulation Compared with Agar Sol Mixing

The agar sol method was simplest of all the techniques examined. Problems associated with Salyaev and Kalab encapsulation techniques include difficulties in aspiration (especially with viscous materials), sealing the capsules, and handling bulky capsules resulting from strengthening them with additional agar gel. These problems can adversely affect the quality of micrographs. The agar sol mix method avoids these problems, but it has other drawbacks such as dilution of the samples and presence of visible agar strands.

Both methods under study showed casein micelles in aged ultra-high temperature (UHT) treated milk samples (Figures 3.7a, 3.7b, 3.8a, and 3.8b) to be connected by strands (tendrillar appendages), giving them a characteristic star shape while casein micelles in yogurt were seen as discrete round structures (Figures 3.7c, 3.7d, 3.8c, and 3.8d). Agar strands were noticeable in the micrographs of agar sol mix samples (Figure 3.8) and in the case of the UHT milk samples (Figures 3.8a and 3.8b) it was difficult to distinguish the tendrillar appendages (connecting micelles) from these agar strands. These strands were present even when purified agarose (electrophoretic grade) was used to solidify milk. No artifacts caused by polymerization of glutaraldehyde through heating to 45°C were observed.

No artifacts of electron-dense granules as observed by Harwalkar and Kalab (1986 and 1988) and McMahon *et al.* (1991) were found in any of the micrographs produced in this investigation. The granules have been shown to be a complex of osmium tetroxide and glutaraldehyde (Parnell-Clunies *et al.*, 1986). The use of low concentrations of the fixatives (1.5% glutaraldehyde and 1% osmium tetroxide) are believed to be partially responsible for this result.

Figure 3.7. Transmission electron micrographs of **(a)** UHT milk concentrate fixed at 4°C, **(b)** UHT milk concentrate fixed at 45°C, **(c)** yogurt fixed at 4°C, **(d)** yogurt fixed at 45°C, all prepared by microcube encapsulation.

b: bacteria; c: casein micelles; arrows: tendrillar appendages connecting casein micelles.

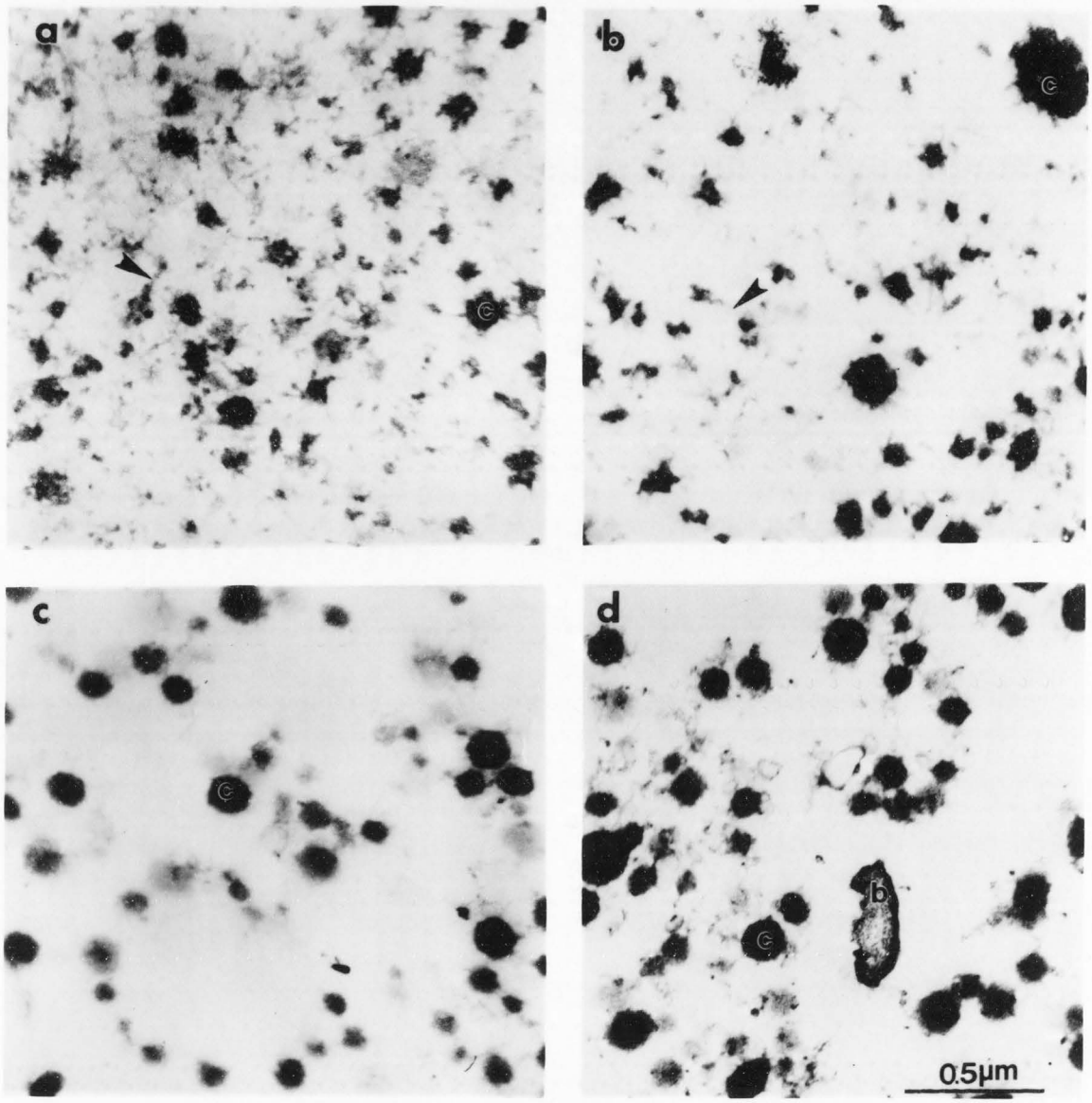
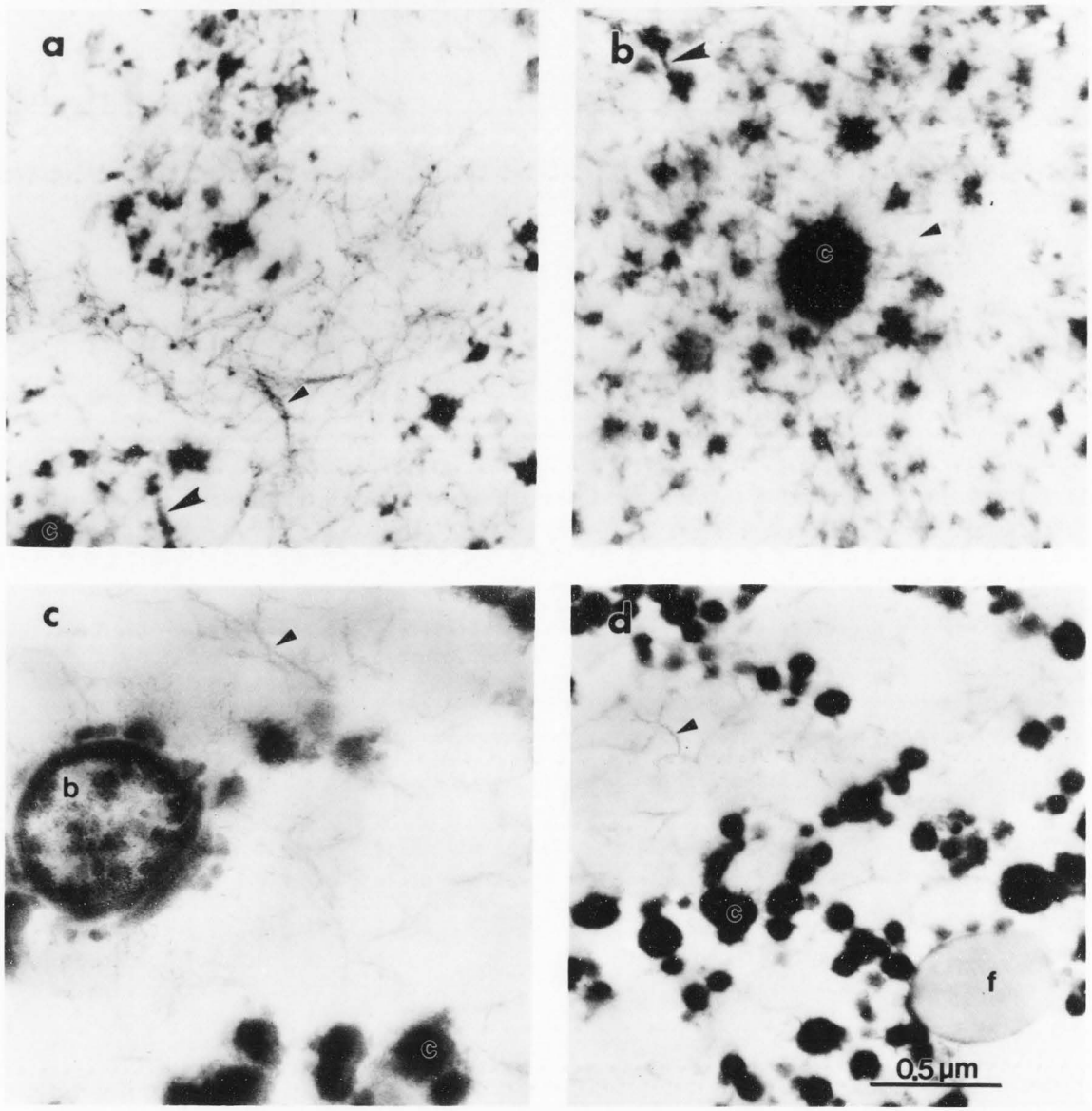

a
b
c
d
0.5µm

Figure 3.8. Transmission electron micrographs of **(a)** UHT milk concentrate fixed at 4°C, **(b)** UHT milk concentrate fixed at 45°C, **(c)** yogurt fixed at 4°C, **(d)** yogurt fixed at 45°C, all prepared by mixing with agar sol.

b: bacteria; c: casein micelles; f: fat globules; large arrows: tendrillar appendages; small arrows: agar strands.


a
b
c
d
b
f
0.5 µm

CONCLUSION

The microcube encapsulation method is a simple, versatile, reliable, and reproducible method for prefixation preparation of fluid dairy products. It is more reliable than the agar gel tube methods of Salyaev and Kalab in sealing the encapsulated fluid and does not require the production of individual agar gel tubes into which the sample is aspirated. The microcube method will prove beneficial to researchers in structural studies of fluid foods because it allows a greater number of samples to be prepared in a simpler way and ensures a considerably lower incidence of sample leakage.

There was no apparent effect of fixing with glutaraldehyde at 45°C rather than at 4°C. Fixation at 20°C to 25°C is therefore adequate unless the state of the material at another temperature is under investigation.

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CHAPTER IV

COMPARING ANTIGENICITY OF β-LACTOGLOBULIN IN ULTRA-HIGH TEMPERATURE-STERILIZED ULTRAFILTERED (3x) MILK CONCENTRATE AND WHEY AFTER DIFFERENT MODES OF ALDEHYDE FIXATION[1]

ABSTRACT

A protocol has been established for aldehyde fixation and immunogold labeling of β-lactoglobulin in milk samples, using trichloroacetic acid precipitated milk whey protein from direct ultra-high-temperature sterilized milk retentate (ultrafiltration concentrated 3x by volume reduction). Microcube prefixation encapsulation was used for all samples. Aldehyde degradation of antigen was investigated using enzyme-linked immunosorbent assay (ELISA). Antigenicity of the samples is reduced by both aldehyde fixatives, but paraformaldehyde is less deleterious than glutaraldehyde. Paraformaldehyde (4%) with fixation times of 2 h, 3 h, 4 h, and 5 h and glutaraldehyde (1.5%) with fixation times of .5 h, 1 h, 2 h, and 3 h were investigated to determine the optimal fixation time for maximum protein antigenicity. The 4 h fixation with paraformaldehyde (4%) and 1 h fixation with glutaraldehyde (1.5%) best preserves antigenicity while providing adequate fixation of the protein. The paraformaldehyde fixation results in better sections at labeling. LR White resin polymerized at 50°C was satisfactory for the embedding of samples. Teleosteam fish gelatin (.1%) with normal goat serum (.1%) in 20 mM Tris buffer without bovine serum albumin proved to be an adequate blocking agent. The protocol localizes β-lactoglobulin in gelled and fresh UHT-sterilized UF milk retentate.

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[1] Coauthored by M. C. Alleyne, D. J. McMahon and W. McManus.

INTRODUCTION

Fixation is the process which preserves the fine structures of biological materials with minimal alterations to the morphology and is used for electron microscope immunochemistry to avoid the artifactual diffusion of soluble tissue components (Beesley and Dougan, 1991), and to allow prolonged storage of the biological specimen (Leenan *et al.*, 1982). It must be recognized, however, that fixation itself is a major artifact (Brandtzaeg, 1982) since the fluid or semifluid nature of the living cell is lost.

Except for formaldehyde, the use of aldehydes as fixatives is a comparatively recent development (Hayat, 1989) pioneered mainly by Sabatini *et al.* (1963), who demonstrated the usefulness of aldehydes (particularly glutaraldehyde) to electron microscopy (Hayat, 1989). For electron microscopic immunochemistry, the fixative should stabilize the antigen without destroying its antibody-binding capacity (antigenicity); aldehyde fixation is usually used for this process (Beesley and Dougan, 1991). Osmium tetroxide reduces or even destroys antigenicity (Sitte *et al.*, 1989) and is not used for this purpose.

Aldehyde fixation is used to preserve as much antigenicity as possible, but the resulting ultrastructure can be very poor especially with formaldehyde alone (Beesley and Dougan, 1991). The fixation method used depends on the antibody. Formaldehyde (4%) is used to stabilize antigens prior to immunolabeling with monoclonal antibodies while the stronger fixative glutaraldehyde (2.5%) is used when employing polyclonal antibodies, which have much wider specificity (Beesley, 1988).

The optimal duration of fixation for most tissues is not known, and an arbitrary standardized duration of 1-4 h at room temperature or 4°C is often used (Hayat, 1989). The optimal duration of fixation for a specific tissue is controlled by the type of fixative, specimen size, type of specimen, temperature, buffer type, staining method, and the objective of the study. Very little is known of the effects of over-fixation except that the extraction of tissue constituents increases. Fixation times now used are safe, but are longer

than necessary and should be modified to retain as much cellular materials as possible (Hayat, 1989).

Immunological labeling seeks to adjust the concentration of reagents to obtain a high signal with minimal noise (Beesley and Dougan, 1991). A high concentration of reagents results in considerable background or nonspecific labeling, which can be prevented by pretreating the antigens with blocking agents that attach to any sticky nonimmunological sites. Preincubating the sample with gelatin in buffer containing bovine serum albumin (BSA) reduces background labeling (Beesley and Dougan, 1991). Phosphate buffered saline (PBS) is usually used for all incubations (Slot and Geuze, 1984).

Low-temperature embedding techniques have been used to study protein interactions in biological systems (Armbruster and Kellenberger, 1986; Carlemalm and Villiger, 1989; Rudick *et al.*, 1991) and to study food ultrastructure (Armbruster and Desai, 1992). The low-temperature resin, Lowicryl K4M, appears to be the resin of choice for these procedures (Carlemalm *et al.*, 1982), which include the progressive lowering of temperature (PLT) technique (Carlemalm *et al.*, 1982 and 1986; Chiovetti *et al.*, 1986; Hobot, 1991) and the freeze-substitution method (Rebhun, 1972; Sitte *et al.*, 1986; Steinbrecht and Muller, 1987).

The PLT technique requires aldehyde fixation. After fixation, dehydration, and embedding, the temperature of the medium is progressively lowered to inhibit leaching of components and to preserve the antigens *in situ* (Sitte *et al.*, 1989). Finally after the specimen has been transferred into pure monomeric mixture at between -35°C and -80°C, UV light polymerization is carried out (Sitte *et al.*, 1989).

Freeze-substitution may be conducted in closed capsules in dry ice at -79°C (Sitte *et al.*, 1989). It does not require aldehyde fixation, but stabilizing compounds may be added to the substitution medium. Ice in the frozen tissue is replaced with an organic solvent

(usually acetone) at a temperature higher than that at which the specimen was frozen (Hayat, 1989). The solvent is replaced by a resin. Fixation occurs at a low temperature during the substitution process, and the procedure apparently involves less extraction and translocation of solutes than with fixation and dehydration at room temperature.

It is also possible to use ultrathin frozen sections that do not require the use of resins (Slot and Geuze, 1984). The tissue is frozen and sectioned at -100°C, and the sections are thawed before immunolabeling (Beesley and Dougan, 1991). This may be the most sensitive method for antigen preparation.

It is preferable to test the responsiveness of the antibody using Western blot analysis, ELISA, or immunoelectrophoresis before the sample is immunolabeled (Beesley and Dougan, 1991). This test is best done under immunocytochemical conditions using known positive samples.

It is not known whether the advantages of low-temperature embedding in polyacryl justify the additional labor (Sitte *et al.*, 1989).

The objective of this study was to establish an alternative protocol to low-temperature embedding for immunocytochemical studies of β-lactoglobulin in UHT-sterilized concentrated milk, using LR White resin as the embedding medium with a polymerization temperature of 50°C.

MATERIALS AND METHODS

Reagents

Agar was obtained from Difco Laboratories (Detroit, Michigan); paraformaldehyde, glutaraldehyde, normal goat serum (NGS), secondary antibody (goat anti-mouse) IgG conjugated to 10 nm gold probes (batch # 9458), and LR White resin (medium grade) were obtained from Electron Microscopy Sciences (Fort Washington, Pennsylvania). Teleosteam fish gelatin was obtained from Sigma Chem. Co. (St. Louis, Missouri).

Primary antibodies (mouse anti-β-lactoglobulin and mouse anti-α_{s1}-casein) were obtained from J. J. Statsny, University of Illinois, Chicago. These monoclonal antibodies were raised against purified proteins (Kuzmanoff *et al.*, 1991; Kuzmanoff and Beattie, 1991). All other chemicals were analytical reagent grade.

Samples

A 10-month-old gelled ultra-high temperature (UHT) sterilized milk sample (ultrafiltration concentrated 3x by volume reduction) was obtained from the Gary H. Richardson Dairy Products Laboratory, Utah State University, and divided into two parts. One part was used without further treatment. The other part was ultracentrifuged at 100,000 x g (38,500 rpm) for 1 h at 20°C in a Sorvall Ultracentrifuge to sediment all the casein micelles. The whey proteins in the supernatant were precipitated by addition of 24% trichloroacetic acid (TCA) in the volume ratio of 1:1 and filtered to separate the precipitate.

A 6-hour-old ungelled UHT-sterilized milk sample (ultrafiltration concentrated 3x by volume reduction) and fresh skim milk were also obtained from the Gary H. Richardson Dairy Products Laboratory, Utah State University.

ELISA

This procedure was used to screen fixatives in order to optimize antigenicity of β-lactoglobulin. Skim milk, as a source of β-lactoglobulin, was adsorbed to ELISA plates (Corning Inc., Corning, New York). The ELISA procedure from "Monoclonal Antibody Screening Kit, Mouse Immunoglobulins" (Hyclone Laboratories, Inc., Logan, Utah) was followed with modifications: A fixation step was added after the milk proteins had adsorbed to the plates. Glutaraldehyde in concentrations of .25%, .5%, and 1% and paraformaldehyde in concentrations of 1%, 2%, and 4% were used as fixatives in PBS buffer (pH 6.6) for 6 min. Blocking was done with .1% Teleosteam fish gelatin with .1% NGS in PBS buffer. The primary antibody (mouse anti-β-lactoglobulin) was diluted

1:5000 (antibody:PBS buffer). ELISA plates were read to determine the optical density at 490 nm (OD_{490}) on a Biotek EL 309 Microplate Autoreader (Bio Tek Instruments, Winooski, Vermont). There were six replicates of each aldehyde concentration. A positive control (with no fixative) and a negative control (with a primary antibody to a non-milk protein) were utilized to establish the optical density range for the system. Data were analyzed using ANOVA. Treatments were structured as one factor with eight levels. Various differences between controls, aldehydes, and concentrations were evaluated.

Preparation of Blocks

Glutaraldehyde (1.5%) and paraformaldehyde (4%) fixatives were prepared in .1 M phosphate buffer (pH 6.6) to fix samples. Each of the three samples (TCA-precipitated whey, gelled UHT-sterilized UF milk retentate, and fresh UHT-sterilized UF milk retentate) were encapsulated in agar (4%) using the microcube technique (Alleyne *et al.*, 1993). After encapsulation each sample was divided into groups and subjected to eight methods of aldehyde fixation as follows: (1) glutaraldehyde (1.5%) for .5 h, (2) glutaraldehyde (1.5%) for 1 h, (3) glutaraldehyde (1.5%) for 2 h, (4) glutaraldehyde (1.5%) for 3 h, (5) paraformaldehyde (4%) for 2h, (6) paraformaldehyde (4%) for 3 h, (7) paraformaldehyde (4%) for 4 h, and (8) paraformaldehyde (4%) for 5 h. They were then dehydrated in an ascending ethanol series (50%, 70%, 95%, 100%), infiltrated in LR White resin and embedded at 50°C for 21 h.

Immunogold Labeling

We conducted preliminary studies to determine (1) the most efficient blocking agent, (2) the optimal ratio of primary and secondary antibodies to buffer dilution, (3) the optimal duration of each step in the labeling procedure, and (4) the optimal duration of staining.

Thin sections, 90 nm thick (Sorvall MT-2 Porter-Blum ultramicrotome), from the LR White blocks were floated for 15 min on the blocking agent (.1% fish gelatin with .1% NGS in 20 mM Tris buffer without BSA) at pH 6.6 and then rinsed for 5 min in 50 mM Tris saline buffer (pH 7.4) (Zymed, 1991). They were floated on the primary antibody (mouse anti-β-lactoglobulin) in buffer, ratio 1:2000 (antibody:50 mM Tris saline buffer), and refrigerated for 24 h in a humidity chamber. The control was floated on PBS (in place of the primary antibody) in the humidity chamber . All grids were then rinsed for 5 min in 50 mM Tris saline buffer (pH 7.4) (Zymed, 1991) and floated on the secondary antibody (goat anti-mouse) IgG conjugated to 10 nm gold probes in buffer, ratio 1:38 (antibody:50 mM Tris saline buffer) in a humidity chamber at room temperature for 3 h. Preliminary investigations to select the optimum probe size for these investigations showed that 10 nm probes gave reduced labeling density compared to 5 nm probes, in agreement with other researchers (van Bergen en Henegouwen and Leunissen, 1986; Yokota, 1988; Gu and D'Andrea, 1989; Ghitescu and Bendayan, 1990; Hansen *et al.*, 1992; Giberson and Demaree, 1994). However, similar labeling patterns were observed with both sizes of probes, and the 10 nm probes were much more distinguishable in the micrographs. Grids were rinsed (6 x 5 min) with double-distilled water and stained with uranyl acetate (20 min) followed by lead acetate (5 min) (Youssef, 1985). Transmission electron microscopy (TEM) was carried out using a Zeiss CEM 902 electron microscope operated at 80 kV. Micrographs were taken on Kodak SO 163 sheet film and printed on Kodak polycontrast III RC paper.

RESULTS AND DISCUSSION

ELISA

Tests of the specificity of anti-β-lactoglobulin for β-lactoglobulin determined that a dilution of 1:5000 (primary antibody:PBS buffer) yielded conclusive results with an

antigen concentration of ~5 mg/ml. Under these conditions the ELISA of β-lactoglobulin vs. anti-β-lactoglobulin had an OD_{490} of .280 ±.0076, the ELISA of skim milk vs. anti-β-lactoglobulin had an OD_{490} of .260 ± .0076, and the ELISA of β-lactoglobulin vs. anti-α_{S1}-casein (the negative control) had an OD_{490} of .017 ±.0039.

Results of the ELISA procedure allowed us to optimize the antigenicity of β-lactoglobulin. The positive control sample (with no fixative used) yielded an average optical density of .070 ±.0065. The negative control sample yielded an average optical density of .003 ±.0039. There was no significant difference ($P = .90$) between β-lactoglobulin treated with either 1%, 2%, or 4% concentrations of paraformaldehyde (Figure 4.1) although all had lower OD_{490} than the positive control. There were significant differences ($P = .016$) between the β-lactoglobulin treated with .25%, .5%, and 1% concentrations of glutaraldehyde (Figure 4.2). Paraformaldehyde fixation reduced the antigenicity of β-lactoglobulin compared to the native unfixed protein and antigenicity was ~67% that of the positive control. The antigenicity of all paraformaldehyde treatments was similar and acceptable for immuno-electron microscopy (Hayat, 1981). The greater the concentration of glutaraldehyde, the greater was the reduction of antigenicity of β-lactoglobulin. Only at the lowest concentration of glutaraldehyde (.25%) was antigenicity similar to that of paraformaldehyde. Antigenicity at glutaraldehyde concentrations of .5% and 1% was 52% and 28% of the positive control.

Immunogold Labeling

In preliminary studies, various combinations and concentrations of Tween 20 and Tris buffered saline (TBS) with NGS failed to satisfactorily reduce nonspecific labeling. We tested Teleosteam fish gelatin (Armbruster and Desai, 1992) .1% in 20 mM Tris buffer without BSA (pH 6.6), with and without .1% NGS as a blocking agent. We used samples of TCA-precipitated whey gel fixed for 4 h with 4% paraformaldehyde. The blocking efficiencies of the fish gelatin with and without NGS are shown in Figure 4.3. There was

Figure 4.1. Least square means for average optical density of ELISA with paraformaldehyde-treated β-lactoglobulin. Negative control was a non-species primary antibody (anti-*Nosema locustae* in mouse).

No treatment is equivalent to a positive control. a,b,c: within main effects, means with no common letters differ significantly ($P < .05$). Error bar = 1 standard deviation.

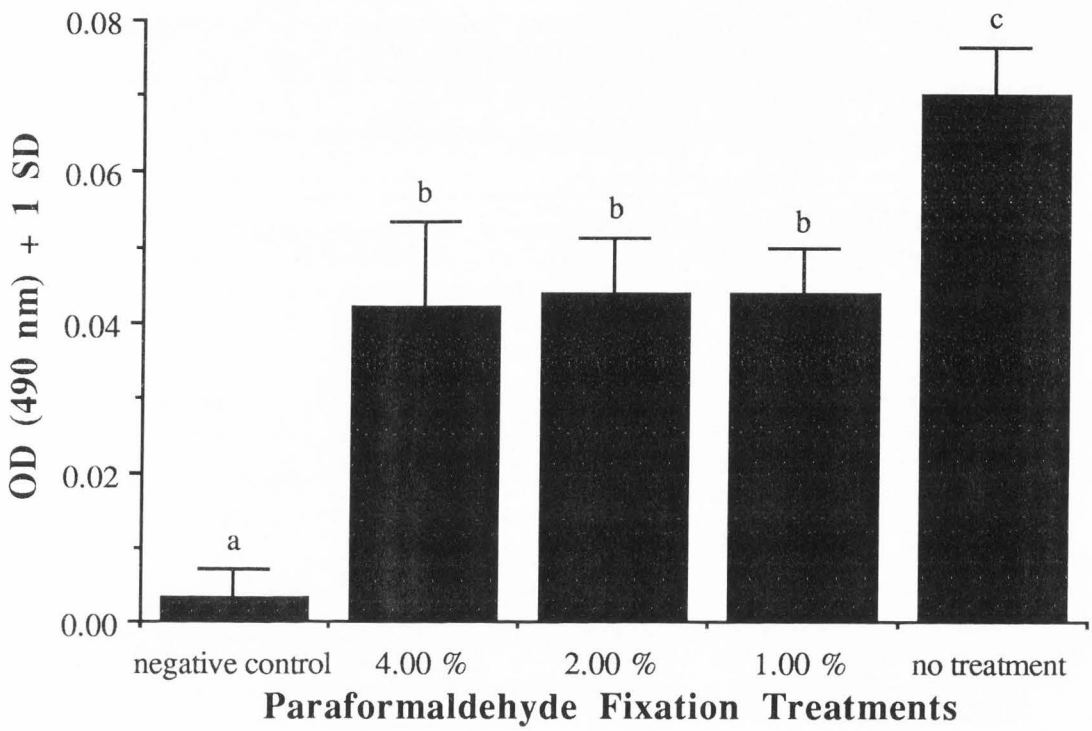

0.08
0.06
0.04
0.02
0.00
OD (490 nm) + 1 SD
a
b
b
b
c
negative control
4.00 %
2.00 %
1.00 %
no treatment
Paraformaldehyde Fixation Treatments

Figure 4.2. Least square means for average optical density of ELISA with glutaraldehyde-treated β-lactoglobulin. Negative control was a non-species primary antibody (anti-*Nosema locustae* in mouse).

No treatment is equivalent to a positive control. a,b,c,d,e: within main effects, means with no common letters differ significantly ($P < .05$). Error bar = 1 standard deviation.

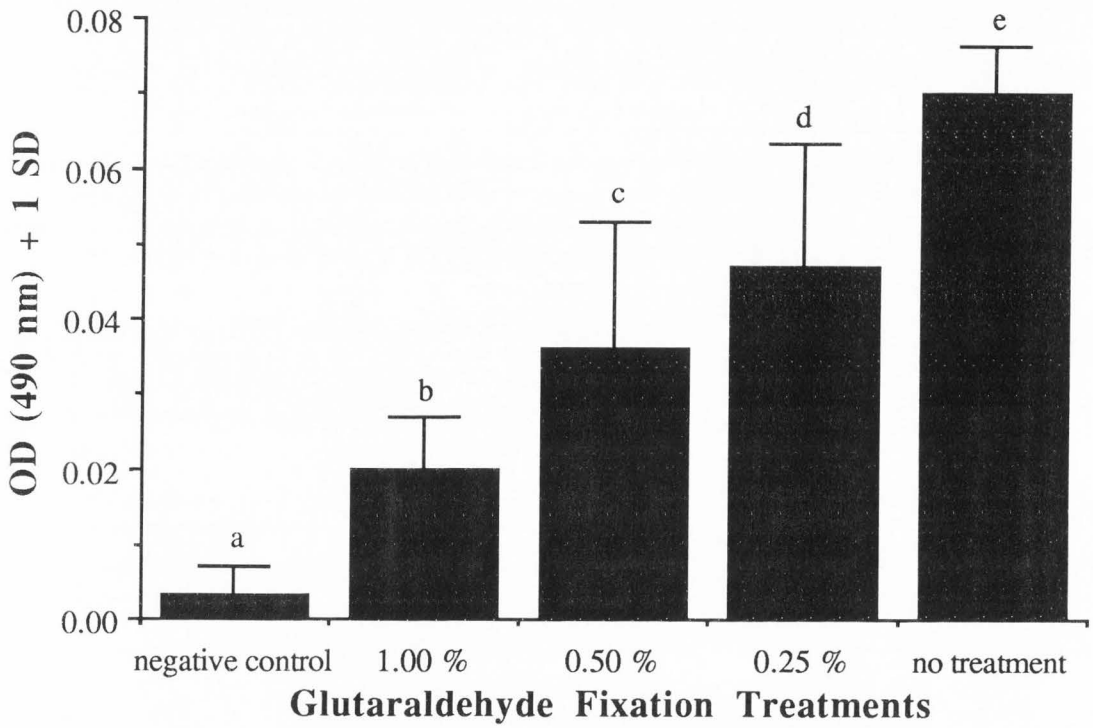
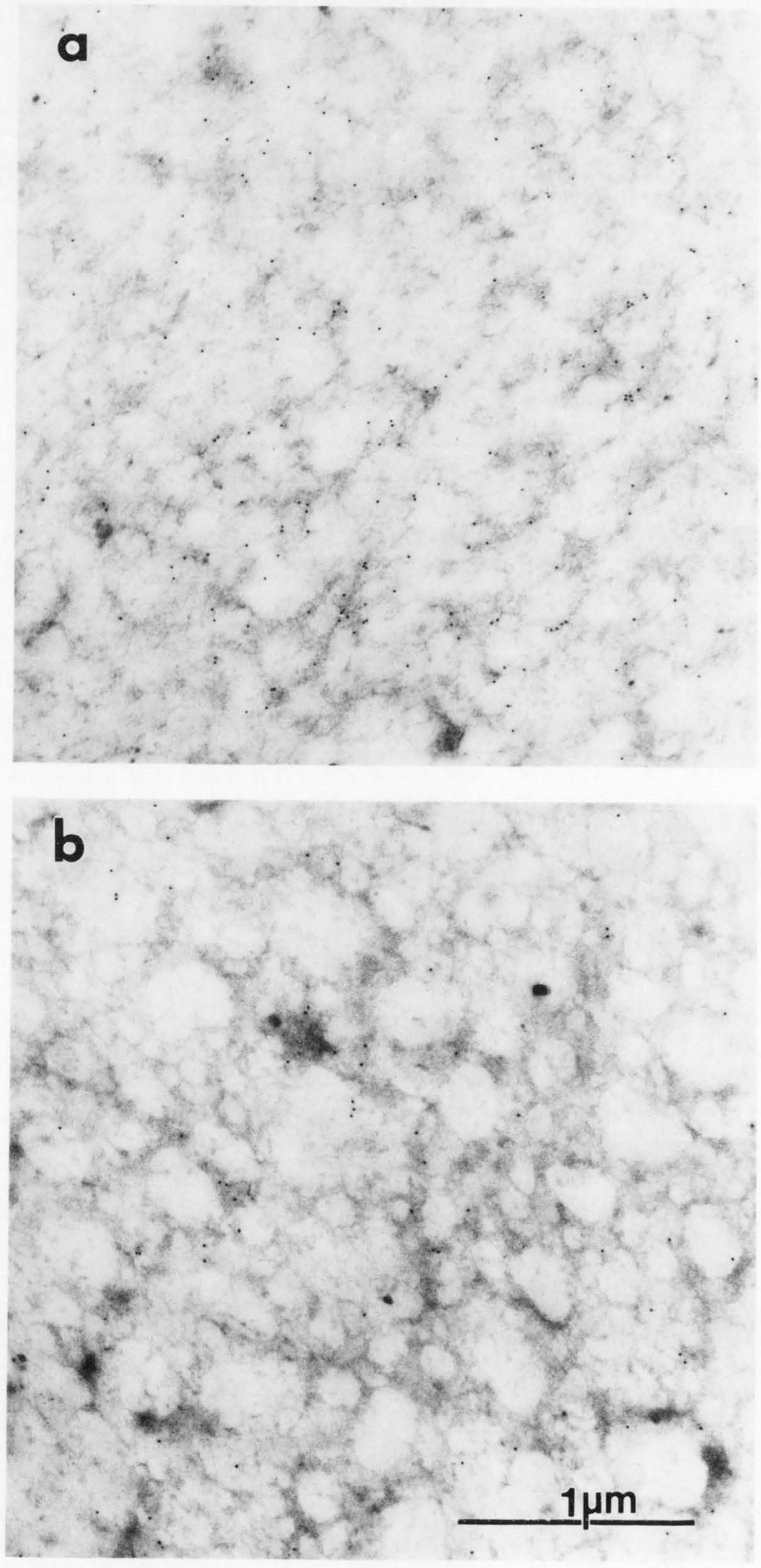

0.08
0.06
0.04
0.02
0.00
OD (490 nm) + 1 SD
a
b
c
d
e
negative control
1.00 %
0.50 %
0.25 %
no treatment
Glutaraldehyde Fixation Treatments

Figure 4.3. Transmission electron micrographs of controls: immunogold-labeled TCA-precipitated whey gel fixed for 4 h with 4% paraformaldehyde comparing the blocking efficiency of the fish gelatin **(a)** without NGS, **(b)** with NGS. With NGS **(b)** there was less labeling (noise) than without NGS **(a)**.


a
b
1µm

much more labeling (noise) without the NGS so we used fish gelatin with NGS as the blocking agent. Preliminary studies using fish muscle sample as a control showed no labeling for β-lactoglobulin when β-lactoglobulin antigen was used at concentration levels of 1:3000 to 1:500 (antibody:buffer).

TCA-precipitated whey gel fixed for 4 h with 4% paraformaldehyde required higher concentrations of primary antibody than that used in the ELISA test because the embedded material contained a lower concentration of β-lactoglobulin antigen. The probe density with an antibody:buffer dilution of 1:2000 was similar to that with 1:1000 dilution. The 1:3000 dilution markedly reduced probe density (Figure 4.4). Consequently, a dilution of 1:2000 was used for the primary labeling of β-lactoglobulin.

Among the glutaraldehyde (1.5%) fixed samples (Figure 4.5), 1 h of fixation resulted in more dense labeling of the protein matrix of TCA-precipitated whey gel (Figure 4.5b). With paraformaldehyde (4%) fixed samples (Figure 4.6), 4 h of fixation resulted in more dense labeling of the protein matrix (Figure 4.6c). The shorter periods of fixation (.5 h for glutaraldehyde, 2 and 3 h for paraformaldehyde) did not adequately stabilize the protein and therefore allowed leaching during dehydration and embedding, resulting in reduced gold labeling. The longer periods of fixation (2 and 3 h for glutaraldehyde, 5 h for paraformaldehyde) reduced the antigenicity of the protein through diffusion of enzymes and extraction of cellular materials (Hayat, 1981). This also reduced labeling of the samples. As shown in Figures 4.5b and 4.6c, the probe density and micrograph quality are similar, but fixation with paraformaldehyde generally resulted in a cleaner labeled section than those fixed with glutaraldehyde. Samples fixed with glutaraldehyde had positive fixed charges because all surface cations are not masked (Hayat, 1981). These charges may attract extraneous materials during immunolabeling and produce dirtier sections. Consequently, we used paraformaldehyde (4%) with a fixation period of 4 h for our studies of β-lactoglobulin.

Figure 4.4. Transmission electron micrographs of immunogold-labeled, TCA-precipitated whey gel fixed for 4 h with 4% paraformaldehyde comparing the dilutions of primary antibody with buffer **(a)** 1:1000, **(b)** 1:2000, **(c)** 1:3000. Dilution of 1:2000 produced similar probe density as the 1:1000 while the 1:3000 dilution resulted in a marked reduction in probe density.

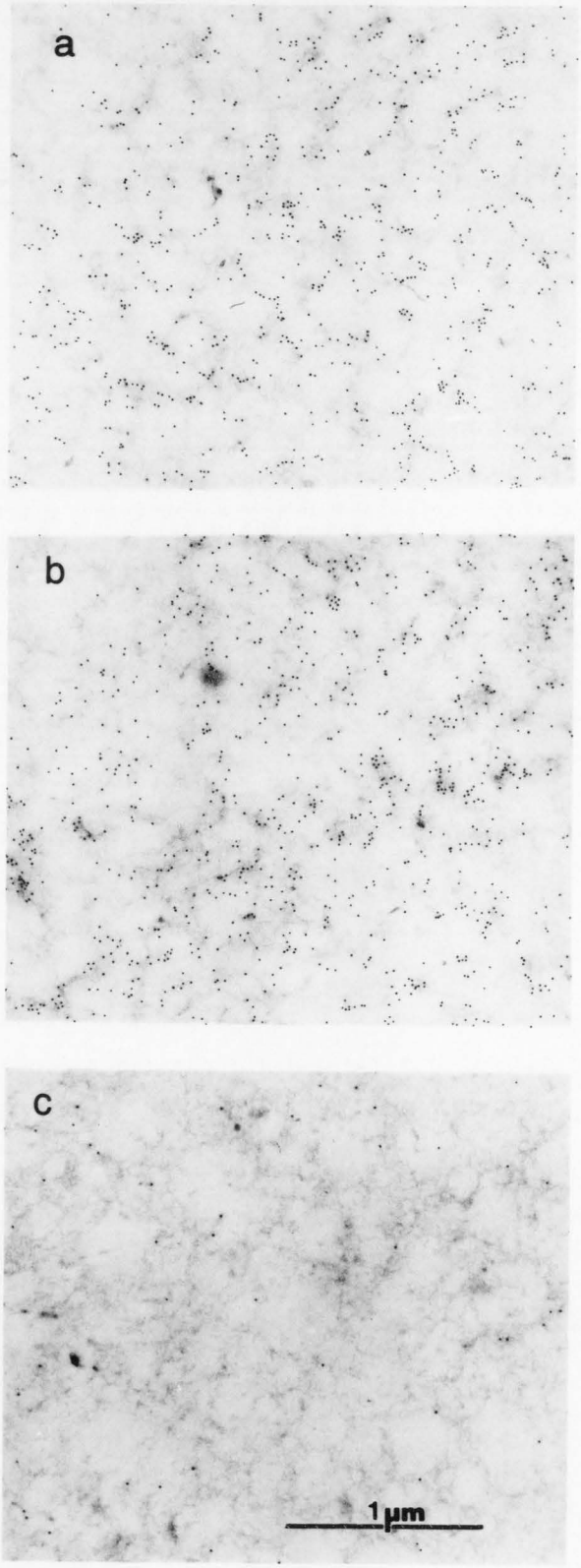

a
b
c
1µm

Figure 4.5. Transmission electron micrographs of immunogold-labeled, TCA-precipitated whey gel fixed with 1.5% glutaraldehyde for periods of **(a)** .5 h, **(b)** 1 h, **(c)** 2 h, **(d)** 3 h. There was a higher density of labeling with the 1-h fixation **(b)**.

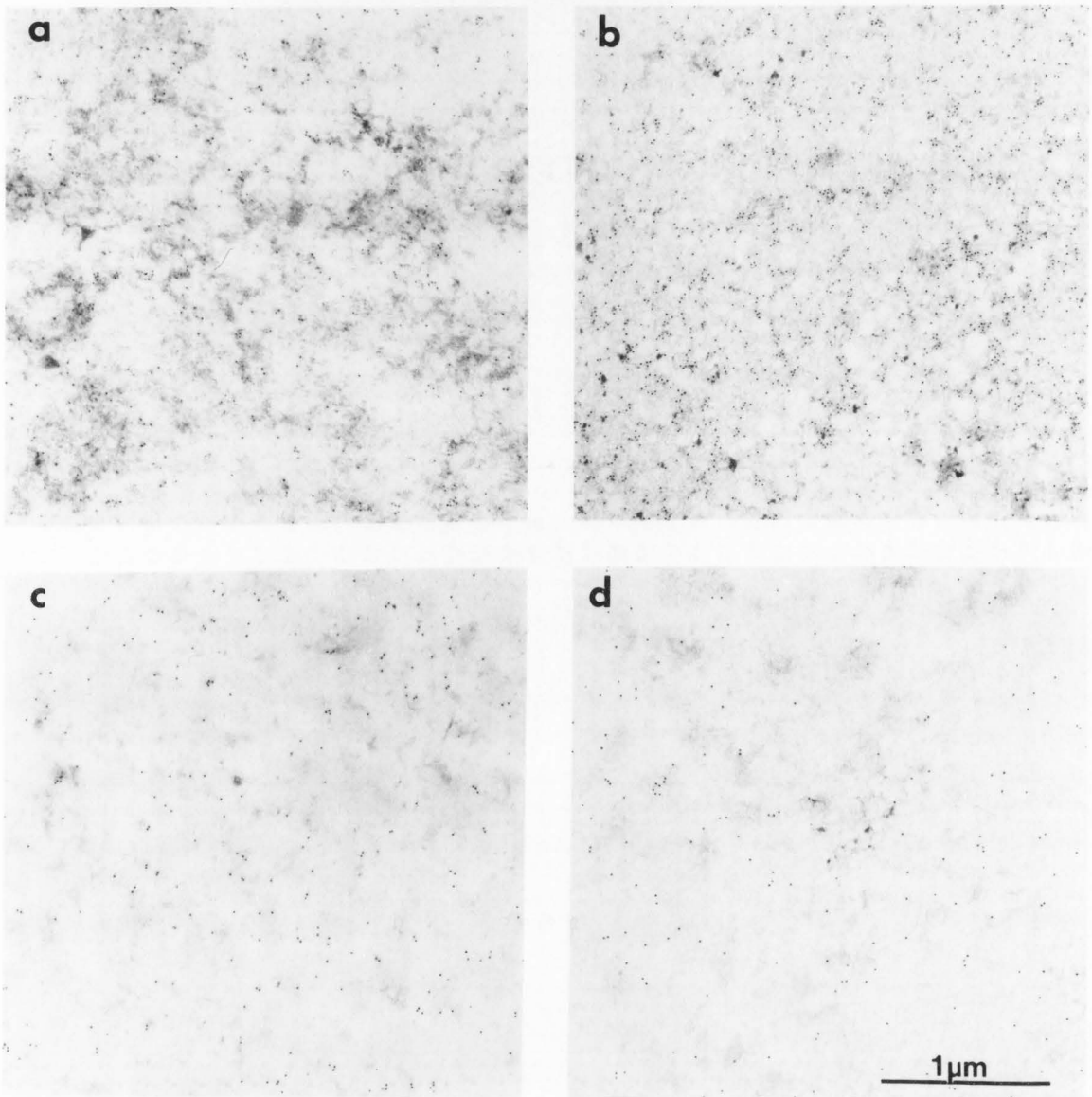
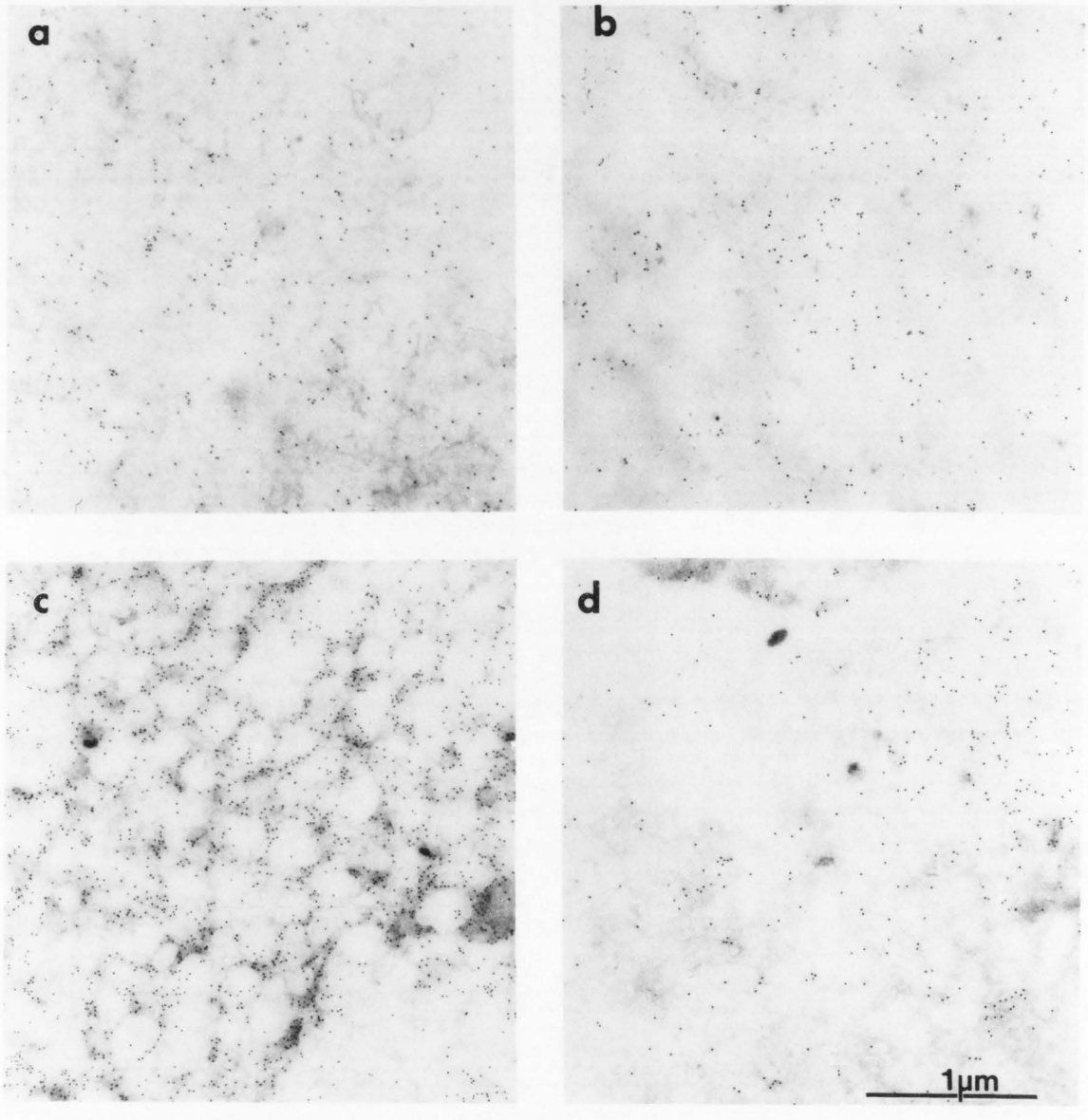

a
b
c
d
1µm

Figure 4.6. Transmission electron micrographs of immunogold-labeled, TCA-precipitated whey gel fixed with 4% paraformaldehyde for periods of **(a)** 2 h, **(b)** 3 h, **(c)** 4 h, **(d)** 5 h. There was a higher density of labeling with the 4-h fixation **(c)**.


a
b
c
d
1µm

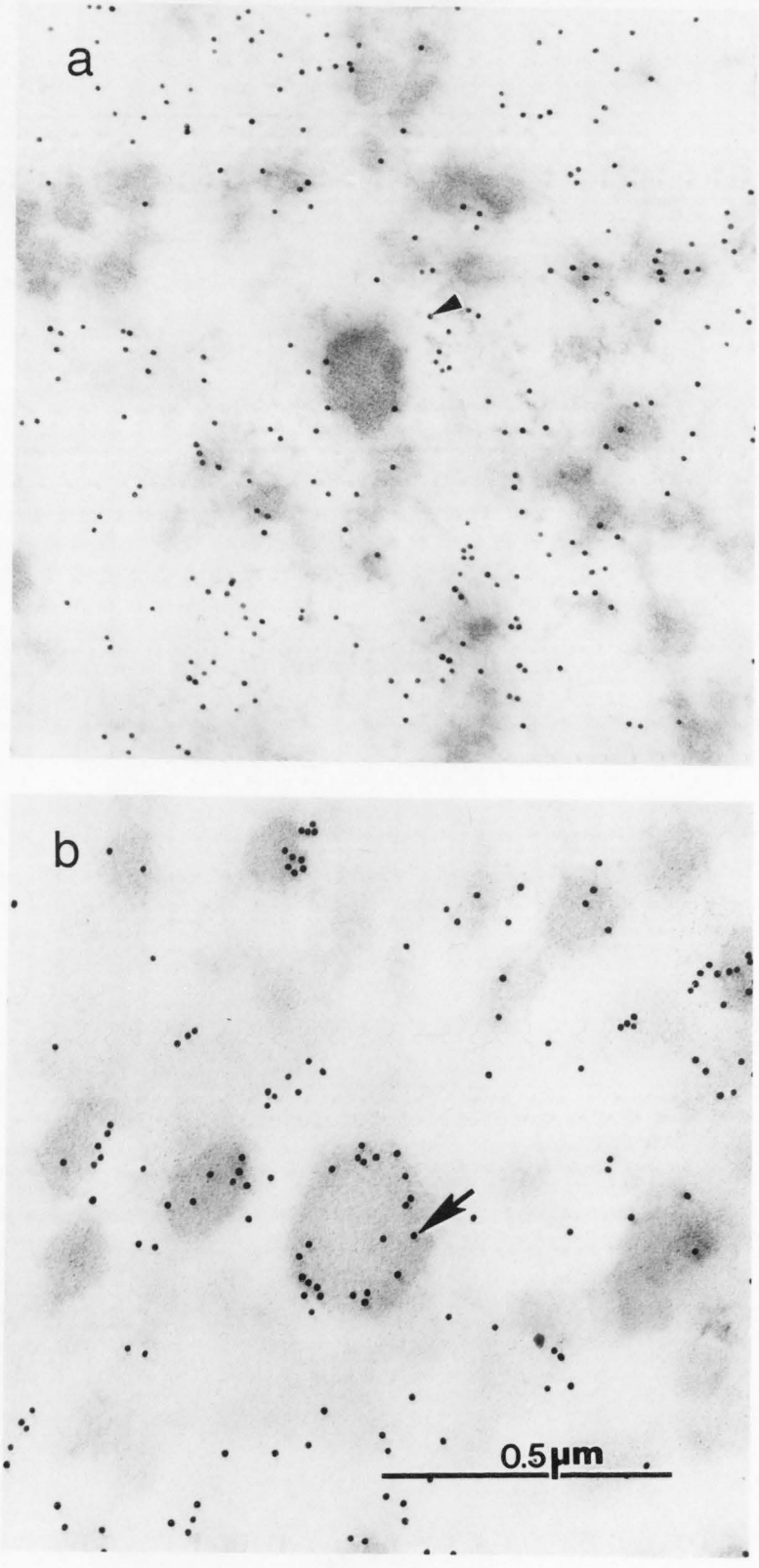
We investigated the optimal poststaining procedure for uranyl acetate and lead citrate by varying the staining times of combinations of these two reagents. Twenty minutes (5 min at 45°C and 15 min at room temperature) with uranyl acetate and 5 min with lead acetate produced the best stains.

The labeling of β-lactoglobulin in fresh UHT and gelled UHT milk samples (Figure 4.7) showed that the paraformaldehyde fixation alone did not appear to adversely affect the ultrastructure. The gelled sample had more label on the intermicellar matrix than on the micelles, and the label on the micelles appeared mainly on the surface (Figure 4.7a). The micelles of the fresh sample appeared smaller than those of the gelled sample, and there was more dense labeling on the micelles than on the matrix between micelles (Figure 4.7b). The labeling of the micelles was mainly on the surface.

Label on the micelles was presumed to be from the formation of the complex between β-lactoglobulin and κ-casein (Morr *et al.*, 1962; Morr, 1965; Sawyer, 1969; Snoeren and van der Spek, 1977) and/or α_{S2}-casein and β-lactoglobulin (Kudo, 1980; Kinsella, 1984). The labeled matrix between micelles in the fresh UHT sample suggests that a complex, probably between β-lactoglobulin and α-lactalbumin (Elfagm and Wheelock, 1978a and b) and/or β-lactoglobulin and serum κ-casein (Morr *et al.*, 1962; Morr, 1965; Sawyer, 1969; Snoeren and van der Spek, 1977), was present. As the UHT samples aged, the β-lactoglobulin-κ-casein complex on the micellar surface migrated to the intermicellar spaces and some coalescence of smaller micelles was observed. This migration and the possible interaction with the existing complex of the intermicellar spaces (Hunziker and Tarassuk, 1965; Elfagm and Wheelock, 1977) partially explain why and how age gelation occurs in UHT-sterilized milk.

Figure 4.7. Transmission electron micrographs of immunogold-labeled, gelled, UHT milk and fresh UHT milk. **(a)** The micrograph of the gelled sample showed labeling generally between micelles (small arrows) with reduced density on the micelles (large arrows). **(b)** The micrograph of the fresh sample showed labeling concentrated mainly on the surface and within the micelles (large arrows).

mc: micelles; mx: matrix.


a
b
0.5µm

CONCLUSION

The results of both the ELISA and immunolocalization assay indicate that paraformaldehyde is superior to glutaraldehyde as a fixative for immunogold labeling of β-lactoglobulin. Paraformaldehyde, at 4% concentration and 4 h incubation, provides adequate fixation and preserves antigenicity. The effectiveness of .25% glutaraldehyde as a fixative warrants additional study. Teleosteam fish gelatin and NGS in buffer adequately blocked samples. Uranyl acetate (20 min) followed by lead citrate (5 min) adequately stained immunogold-labeled milk samples. Samples embedded in LR White resin at 50°C were as good as samples embedded in polyacryl at low temperature. Our procedures, however, are considerably less laborious and tedious than the low-temperature embedding methods.

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CHAPTER V

CHARACTERIZATION OF IMMUNOLOCALIZED β-LACTOGLOBULIN AND α-LACTALBUMIN IN WHEY SAMPLES[1]

ABSTRACT

Samples of anti-β-lactoglobulin and anti-α-lactalbumin were used in Western blot analyses and immunolocalization studies of whey at various stages of preparation from fresh samples to ultra-high-temperature sterilized samples. The antibodies were specific for the native as well as the complexed forms of β-lactoglobulin and α-lactalbumin. The native form of the whey proteins was more susceptible to leaching through the fixation, dehydration, and embedding processes prior to electron microscopy, than was the complexed form. Most of the labeling for β-lactoglobulin and α-lactalbumin was on these proteins complexed with each other or with other milk proteins.

INTRODUCTION

β-Lactoglobulin is denatured at 78°C and disulfide bonds break and when the molecule unfolds further at 140°C (Watanabe and Klostermeyer, 1976; de Wit, 1981). β-Lactoglobulin is sensitive to pH denaturation and is more stable at lower pH than at higher pH (Hillier *et al.*, 1979). Irreversible denaturation of β-lactoglobulin occurs above pH 7.5 (Kinsella, 1984). Increased thiol activity of β-lactoglobulin at high pH reduces the stability of the molecule. These thiol groups are unreactive in native β-lactoglobulin, but the dimer molecule reversibly dissociates when heated (de Wit and Klarenbeek, 1984), and the monomers produced unfold (Sawyer, 1969; McKenzie, 1971), polymerize by sulfhydryl interchange, and undergo further aggregation (Harwalker, 1980). Prolonged heating of β-lactoglobulin results in more extensive unfolding of individual protein chains,

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[1] Coauthored by M. C. Alleyne, D. J. McMahon, W. McManus and L. Burgess.

leading to bond breaking at disulfide linkages and exchange reactions with other proteins. Heating conditions conducive to β-lactoglobulin denaturation produce very active sulfhydryl groups which increase the rate of denaturation (Lyster, 1970). In-container sterilization and vat heating almost completely denature β-lactoglobulin, whereas UHT processes denature to an intermediate level (Burton, 1988; Parnell-Clunies *et al.*, 1988). Pasteurization processes cause very little denaturation (Burton, 1988). Heat-denatured β-lactoglobulin forms complexes with α-lactalbumin (Hunziker and Tarassuk, 1965), α_{S2}-casein (Kudo, 1980; Kinsella, 1984), β-casein (Elfagm and Wheelock, 1978a and b) and κ-casein (Doi *et al.*, 1979). The presence of α-lactalbumin reduces the direct interaction of β-lactoglobulin and κ-casein (Baer *et al.*, 1976; Elfagm and Wheelock, 1977, 1978a and b), but does not affect the denaturation process of β-lactoglobulin (Elfagm and Wheelock, 1978a and b).

α-Lactalbumin is more stable than is β-lactoglobulin and contains four disulfide bonds but no free sulfhydryl groups (Farrell, 1988). Denaturation of α-lactalbumin occurs at 62°C but is reversible upon cooling (de Wit , 1981). In-container sterilization and vat heating, which utilize long residence times, extensively denature α-lactalbumin (Burton, 1988; Parnell-Clunies *et al.*, 1988). α-Lactalbumin heated with β-lactoglobulin experiences more denaturation than when heated alone (Elfagm and Wheelock, 1978a and b). The complex formed between β-lactoglobulin and α-lactalbumin results from a temperature-induced disulfide reaction, promoted by the free sulfhydryl group of β-lactoglobulin (Hunziker and Tarassuk, 1965). The presence of casein facilitates these reactions (Elfagm and Wheelock, 1978a and b).

β-Lactoglobulin and other whey proteins have been localized using gold-labeled antibodies and various immunolocalization techniques. These include embedding in Araldite resin polymerized at 60°C to localize denatured α-lactalbumin and denatured

β-lactoglobulin on the micellar surfaces in yogurt (Mottar *et al.*, 1989), embedding in Lowicryl K4M resin polymerized at -35°C to localize β-lactoglobulin in the ultrastructure of low fat frozen desserts, salad dressing, and process cheese (Armbruster and Desai, 1992), and embedding in LR White resin (London Resin Co., Basingstoke, UK) polymerized at 50°C, to localize β-lactoglobulin in whey and UHT-sterilized UF milk retentate (Alleyne *et al.*, 1994). Immunolocalization probably locates β-lactoglobulin complexed with other milk proteins. The complexes are more stable than are the free proteins during extraction, fixation, and dehydration processes required for electron microscopy.

The objectives of this study were (1) to compare the specificity of primary antibodies: anti-β-lactoglobulin and anti-α-lactalbumin in the native and the complexed forms of the complementary protein and (2) to compare the susceptibility of native and complexed forms of β-lactoglobulin and α-lactalbumin to leaching from the samples during the fixation, dehydration, and embedding processes of electron microscopy preparation. We made these comparisons using Western blot analyses and immunolocalization procedures (Alleyne *et al.*, 1994) with embedding in LR White and polymerization at 50°C.

MATERIALS AND METHODS

Reagents

Agar was obtained from Difco Laboratories (Detroit, Michigan); paraformaldehyde, secondary antibody (goat anti-mouse) IgG conjugated to 10 nm gold probes (batch # 9458), and LR White medium grade were obtained from Electron Microscopy Sciences (Fort Washington, Pennsylvania). Teleosteam fish gelatin was obtained from Sigma Chem. Co. (St. Louis, Missouri). Primary antibodies (mouse anti-β-lactoglobulin and mouse anti-α-lactalbumin) were obtained from J. J. Statsny, University of Illinois, Chicago. These monoclonal antibodies were raised against purified proteins (Kuzmanoff *et al.*, 1990; Kuzmanoff and Beattie, 1991). β-Lactoglobulin and α-lactalbumin were

prepared using FPLC (Hollar *et al.*, 1991). All other chemicals were analytical reagent grade.

Samples

Fresh whole milk and skim milk at room temperature were obtained from the Gary H. Richardson Dairy Products Laboratory, Utah State University. The fresh whole milk at ~25°C was treated with .1 M HCl to reduce the pH to 4.6, the point at which casein precipitated. The clear serum was decanted, filtered through cheese cloth, and filtered under reduced pressure through Whatman #4 filter paper (Whatman International Ltd., Maidstone, England). The serum was adjusted to pH 6.6 with .1 M NaOH and collected for analysis. The skim milk was treated with .1 M HCl until the pH reached 4.6. The acid-treated milk was left for 2 h. It was decanted and the clear serum was collected. The clear serum was filtered through cheese cloth and, under reduced pressure, through Whatman #4 filter paper (Whatman International Ltd., Maidstone, England), and adjusted to pH 6.6 with .1 M NaOH. A sample of this serum was collected for analysis. The rest of the skim milk serum was pasteurized (63°C for 30 min), cooled to 50°C, and collected for analysis. A three-module in-series UF system with spiral wound polysulfone membranes (Osmonics Inc., Minnetonka, Minnesota; 20 kDa nominal molecular weight cutoff, 15 m^2 membrane area) was used to concentrate the skim milk serum, and a sample of this was collected for analysis. The rest of the concentrated milk was homogenized (Model 3DDL Homogenizer, Crepaco Inc., Chicago, Illinois) at 13.8 MPa with 3.4 MPa second stage pressure and sterilized in an Alfa-Laval SteriLab® UHT Pilot System for UHT processing. The sample was heated to 140°C and held for 4 s by indirect and direct heat exchange to aseptic conditions. The UHT-sterilized samples (direct and indirect) were packaged in sterile plastic containers in an Alfa-Laval SteriCab® filling station and collected for analysis.

Western Blot

Samples of whole milk whey, skim milk whey, pasteurized skim milk whey, UF (3x concentrated) whey, direct UHT-sterilized whey, indirect UHT-sterilized whey, β-lactoglobulin, and α-lactalbumin were used as the protein samples. The nondenaturing gel (15% acrylamide) was run at 15 mA for stacking and at 20 mA for separation until blue dye precipitated to the bottom. The gel protein (.1 mg/lane) samples were transferred to nitrocellulose membrane at 14-15 V for 5 h in transfer buffer (pH 6.6) consisting of 800 ml methanol, 9.66 g Tris base, 44.6 g glycine in 4 L distilled water. All preceding steps were done on a shaker at room temperature. Blocking was done in PBS and 1% fish gelatin for 3 h and washed three times in PBS and .5% Tween 20 for 10 min per wash. The primary antibody was diluted at 1:750 in PBS and was added and left on a shaker for 1 h. The membrane was washed three times in PBS and .5% Tween 20 for 10 min per wash. The secondary antibody was diluted at 1:2000 in PBS, and was added and left on a shaker for 1 h. The gel was again washed three times in PBS and .5% Tween 20 for 10 min per wash. Diaminobenzidine (DAB) substrate, consisting of 50 mg 3, 3' diaminobenzidine, 10 ml .3% $CoCl_2$, 90 ml PBS, and 100 µl 30% H_2O_2, was added to blot until bands developed. The reaction was stopped by rinsing in distilled water. The blot was preserved by drying it on filter paper at 50°C. Samples of whole milk whey, skim milk whey, pasteurized skim milk whey, UF (3x concentrated) whey, direct UHT-sterilized whey, indirect UHT-sterilized whey, β-lactoglobulin, and α-lactalbumin were stored in a freezer at -70°C for 2 months and the Western blot analyses were repeated after this storage period.

Electron Microscopy

Prepared samples of whole milk whey, skim milk whey, pasteurized milk whey, UF (3x concentrated) whey, direct UHT-sterilized whey, and indirect UHT-sterilized whey were encapsulated in 4% agar using the microcube technique (Alleyne *et al.*, 1993). The samples were fixed in paraformaldehyde (4%) for 4 h (Alleyne *et al.*, 1994). They were

dehydrated in an ascending ethanol series (50%, 70%, 95%, 100%), infiltrated in LR White resin, and embedded at 50°C for 21 h.

Immunogold Labeling

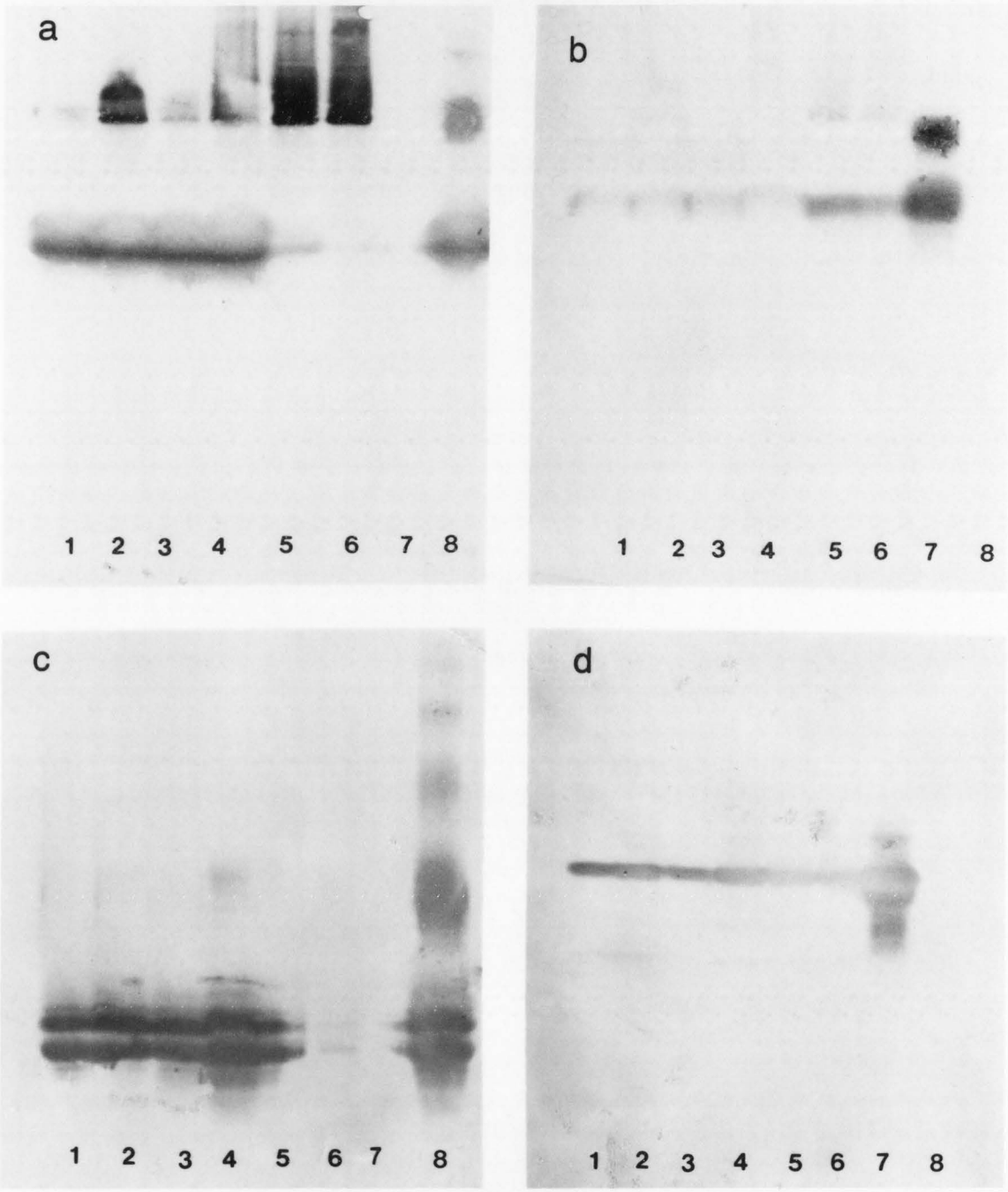
Thin sections (90 nm thick, Sorvall MT-2 Porter-Blum ultramicrotome) from the LR White blocks were immunogold labeled according to Alleyne *et al.* (1994). Sections were floated for 15 min on the blocking agent (.1% fish gelatin with .1% NGS in 20 mM Tris buffer without BSA) at pH 6.6 and rinsed for 5 min in 50 mM Tris saline buffer (pH 7.4) (Zymed, 1991). They were floated on the primary antibody (mouse anti-β-lactoglobulin or anti-α-lactalbumin) in buffer, ratio 1:2000 for β-lactoglobulin, 1:1000 for α-lactalbumin (antibody:50 mM Tris saline buffer), and refrigerated for 24 h in a humidity chamber. The control was floated on PBS in the humidity chamber in place of the primary antibody. All grids were rinsed for 5 min in 50 mM Tris saline buffer (pH 7.4) (Zymed, 1991) and then floated on the secondary antibody (goat anti-mouse) IgG conjugated to 10 nm gold probes in buffer, ratio 1:38 (antibody:50 mM Tris saline buffer) in a humidity chamber at room temperature for 3 h. Grids were rinsed (6 x 5 min) with double-distilled water and stained with uranyl acetate (20 min) followed by lead acetate (5 min). Transmission electron microscopy (TEM) was carried out using a Zeiss CEM 902 electron microscope operated at 80 kV. Micrographs were taken on Kodak SO 163 sheet film and were printed on Kodak polycontrast III RC paper.

RESULTS AND DISCUSSION

Western Blot

The results of the Western blots (Figure 5.1) for anti-β-lactoglobulin and anti-α-lactalbumin were similar, but the staining with anti-β-lactoglobulin was more intense than that with anti-α-lactalbumin. This indicates that anti-β-lactoglobulin bound more strongly to protein than did anti-α-lactalbumin. The negative controls (α-lactalbumin for

Figure 5.1. Western blot for antibodies vs. whey samples: **(a)** anti-β-lactoglobulin vs. fresh samples, **(b)** anti-α-lactalbumin vs. fresh samples, **(c)** anti-β-lactoglobulin vs. freeze-stored samples, **(d)** anti-α-lactalbumin vs. freeze-stored samples.
LANES: 1. whole milk whey, 2. skim milk whey, 3. pasteurized whey, 4. ultrafiltered whey, 5. direct UHT whey, 6. indirect UHT whey, 7. α-lactalbumin (control), 8. β-lactoglobulin (control). There was interaction between the antibodies and the narrow bands (uncomplexed protein) and diffused bands (complexed proteins) of the fresh samples, and the narrow bands (uncomplexed protein) of the freeze-stored samples. There was interaction between antibodies and the corresponding positive controls, but no reaction with the corresponding negative controls.


a
1 2 3 4 5 6 7 8
b
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c
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d
1 2 3 4 5 6 7 8

anti-β-lactoglobulin and β-lactoglobulin for anti-α-lactalbumin) for all blots did not react with the antibodies.

In the fresh whey samples anti-β-lactoglobulin reacted with the two native bands of β-lactoglobulin in the samples of whole milk whey, skim milk whey, pasteurized whey, and UF whey. The antibody also reacted with the wider diffused band of the UF concentrated milk (suggesting some polymerization of β-lactoglobulin during UF) and the UHT-sterilized samples (Figure 5.1a). In the UHT-sterilized samples the lower band of β-lactoglobulin was almost completely replaced by the wider diffused band (especially in the indirect UHT-processed samples using plate heat exchangers), indicating that most of the native protein was converted to the complexed or denatured state. Anti-α-lactalbumin reacted with the single native band of α-lactalbumin in all samples and with the wider diffused band of the UF and UHT-sterilized samples (Figure 5.1b). The lower band of α-lactalbumin was still present in the UHT-sterilized samples, indicating that considerable native α-lactalbumin remained after these treatments.

These results suggested whey proteins start to complex during the UF stage of sample preparation and that complexing intensifies through UHT sterilization. The labeling of the narrow and diffused bands showed that both antibodies are specific for the native as well as the complexed forms of the whey proteins.

In the blots for the freeze-stored samples, the antibody reacted with the native double band of β-lactoglobulin (Figure 5.1c) and the native single band of α-lactalbumin (Figure 5.1d). These were the only reactions between antibody and protein and were the only distinct bands in the samples. The reason for the absence of the diffused band (which had been presumed to represent high molecular-weight polymers) is not understood at this time.

The multiple bands observed for the controls reflect the presence of impurities in these samples.

Immunogold Labeling

There was no labeling in any of the controls. The intensity of labeling for α-lactalbumin (Figure 5.2) generally reflected the following order: whole < skim < pasteurized < UF < direct UHT < indirect UHT. The increased labeling between pasteurized and UF (3x) samples was partially due to the differences in concentration of α-lactalbumin and by the occurrence of some aggregation of α-lactalbumin as shown by Western blots (Figure 5.1).

Figure 5.3 shows labeling of whey samples for β-lactoglobulin. Labeling intensity was generally higher than with α-lactalbumin at every stage of the experiment, but the order of labeling intensity was similar. This suggests that native or uncomplexed whey proteins (predominant in whole milk, skim milk, and pasteurized milk samples) were more easily lost than were complexed whey proteins (predominant in UHT-sterilized milk samples), during fixation, dehydration, and embedding. More complexing apparently occurred with indirect UHT sterilization than with direct UHT sterilization, perhaps due to the longer exposure to high temperature with the indirect method of heating (97 s residence time compared with > 1 s with direct steam injection). The labeling in the whole milk whey and skim milk whey samples suggested that a small percentage of uncomplexed whey proteins is, however, retained during sample preparation.

CONCLUSION

The antibodies that we used were specific for the native as well as the denatured and aggregated forms of β-lactoglobulin and α-lactalbumin. The native form of these proteins were, however, more susceptible than were the complexed form to leaching through the fixation, dehydration, and embedding processes of electron microscopy preparation.

Figure 5.2. Transmission electron micrographs of immunogold-labeled α-lactalbumin in whey samples **(a)** whole milk, **(b)** skim milk, **(c)** pasteurized, **(d)** UF, **(e)** direct UHT, **(f)** indirect UHT. Labeling for α-lactalbumin showed a general increase in labeling intensity in the order: whole < skim < pasteurized < UF < direct UHT < indirect UHT.

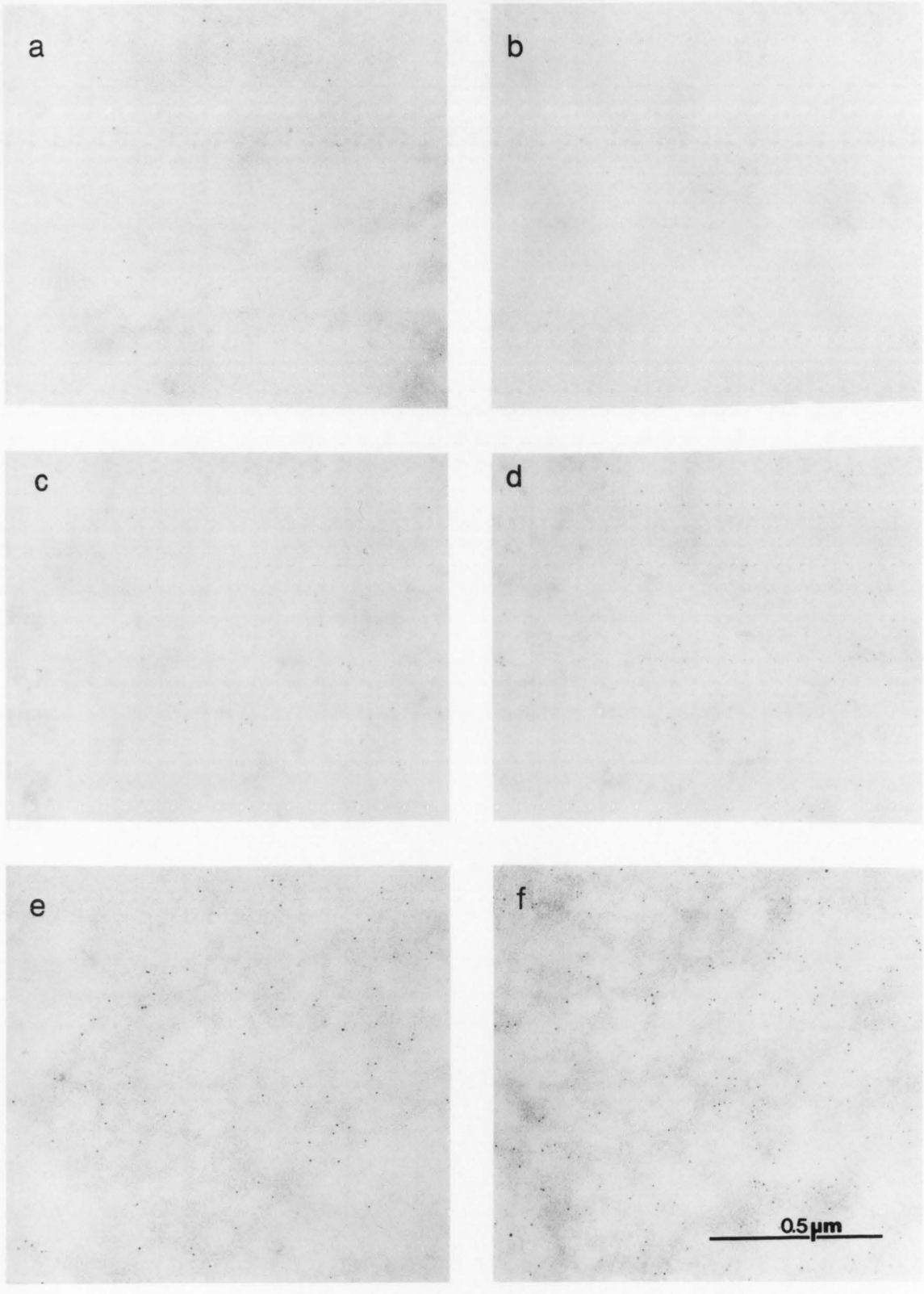
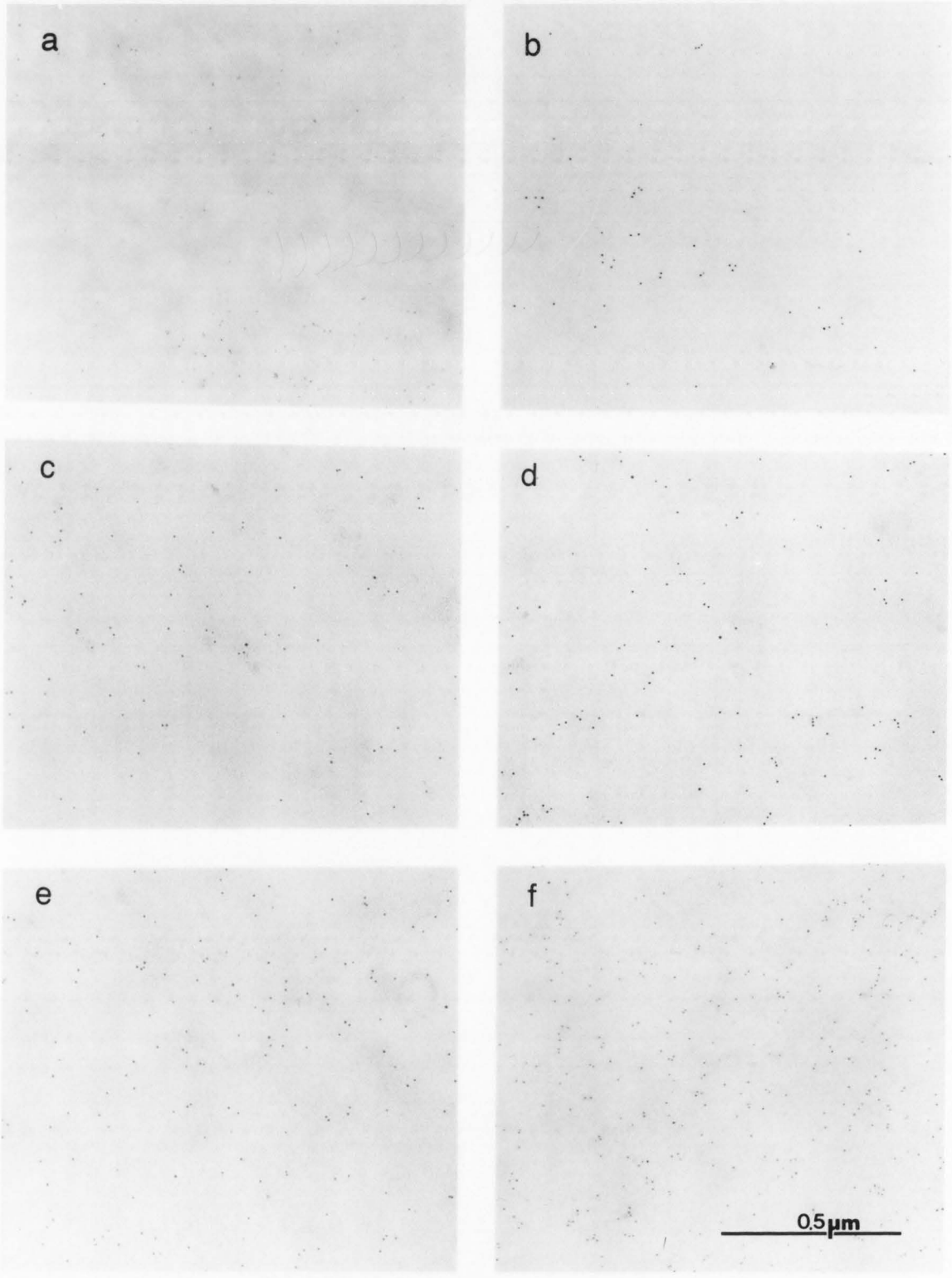

a
b
c
d
e
f
0.5 µm

Figure 5.3. Transmission electron micrographs of immunogold-labeled β-lactoglobulin in whey samples **(a)** whole milk, **(b)** skim milk, **(c)** pasteurized, **(d)** UF, **(e)** direct UHT, **(f)** indirect UHT. Labeling for β-lactoglobulin showed a general increase in labeling intensity in the order: whole < skim < pasteurized < UF < direct UHT < indirect UHT.


a
b
c
d
e
f
0.5µm

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CHAPTER VI

IMMUNOLOCALIZATION OF β-LACTOGLOBULIN IN PROCESSED MILK, YOGURT, AND CHEESE SAMPLES[1]

ABSTRACT

We identified complexed β-lactoglobulin in Ricotta cheese, evaporated milk, condensed milk, yogurt, cottage cheese, Feta cheese, Queso Blanco cheese, Mozzarella cheese, Cheddar cheese, process cheese, and process cheese food using immunolocalization techniques and embedding in LR White resin polymerized at 50°C. The location and relative abundance of β-lactoglobulin corresponded to the effect of the manufacturing process on causing denaturation and aggregation of the whey proteins in the processed dairy foods. More stable β-lactoglobulin complexes were produced around an optimum temperature and low pH. Identifying β-lactoglobulin complexes in other food systems should be useful in determining how manufacturing affects this protein in foods.

INTRODUCTION

Localization of Milk Proteins

Various microscopy methods have been employed to identify milk proteins in foods, including the use of periodic acid-methenamine to localize κ-casein in fresh milk (Kudo *et al.*, 1979), gold-labeled lectin to localize κ-casein in micelles of milk (Horisberger and Vonlanthen, 1980; Horisberger and Rouvet-Vauthey, 1984), gold particles to label α_{S1}-, β-, and κ-casein components before allowing these to reassemble into micelles (Schmidt and Both, 1982), ferritin-labeled antibodies to localize κ-casein on micelles of milk (Carroll and Farrell, 1983), gold-labeled antibodies to locate κ-casein in micelles of milk (Horisberger and Vauthey, 1984), fluorescent dye Eosin Y to locate milk proteins in

[1] Coauthored by M. C. Alleyne and D. J. McMahon.

milk chocolate samples (Heathcock, 1985), fluorescent dye Acridine Orange to detect structural differences between casein matrices of various cheeses (Yiu, 1985), fluorescent dyes Acridine Orange and Fast Green FCF to reveal the distribution of compact protein, which caused grittiness in cheese preparations (Modler *et al.*, 1989), and gold-labeled antibodies to localize κ-casein on the surface of micelles in milk (Schmidt and Buchheim, 1992).

β-Lactoglobulin and other whey proteins have been localized using gold-labeled antibodies and various immunolocalization techniques. These include embedding in Araldite resin polymerized at 60°C to localize denatured α-lactalbumin and denatured β-lactoglobulin on the micellar surfaces in yogurt (Mottar *et al.*, 1989), embedding in Lowicryl K4M resin polymerized at -35°C, to identify β-lactoglobulin in the ultrastructure of low fat frozen desserts, salad dressing, and process cheese (Armbruster and Desai, 1992) and embedding in LR White resin (London Resin Co., Basingstoke, UK) polymerized at 50°C, to localize β-lactoglobulin in whey and UHT-sterilized UF milk retentate (Alleyne *et al.*, 1994a and b).

Resin Embedding

LR White and Lowicryl K4M embedding with colloidal gold techniques are two very sensitive methods of processing tissue into resin while retaining tissue structure and reactivity (Newman and Hobot, 1989). Low-temperature embedding techniques have been successfully used to study protein interactions in food ultrastructure (Horisberger and Vauthey, 1984; Horisberger and Rouvet-Vauthey, 1984; Armbruster and Desai, 1992). The low-temperature resin, Lowicryl K4M, appears to be the resin of choice for these procedures (Carlemalm *et al.*, 1982), which involve the progressive lowering of temperature (Carlemalm *et al.*, 1982 and 1986; Chiovetti *et al.*, 1986; Hobot, 1991). Following aldehyde fixation of the sample at ~0°C, dehydration commences at 0°C and continues while increasing the concentration of ethanol until the temperature is lowered to

-35°C to -50°C (Hayat, 1989). This inhibits leaching of components and preserves the antigens *in situ* (Sitte *et al.*, 1989). Lowering of temperature is achieved through several methods, including the use of Blazers low-temperature embedding apparatus, ice mixed with sodium chloride, household chest-type freezer, and mixtures of *o*- and *m*-xylene with crushed ice (Chiovetti, 1987). After the specimen has been transferred into pure resin, which has been degassed with a vacuum pump (Fryer *et al.*, 1983), UV light polymerization at between -35°C and -80°C is carried out for at least 24 h. Samples may be polymerized in either Beem or gelatin capsules. A capsule holder must allow the capsules to receive UV radiation from all sides. If volume of the capsule exceeds 1 ml, temperatures may increase during polymerization (Chiovetti, 1987). Polymerization is carried out in specially constructed chambers that fit into a deep chest-type freezer. After polymerization at low temperature, the capsules are cured under UV radiation for another 2-3 days at room temperature. Sectioning must be performed with a diamond knife. An excellent review on Lowicryls and low-temperature embedding for colloidal-gold methods is given by Hobot (1989).

It is easier, quicker, cheaper, and safer to fix and embed samples in LR White resin (Newman, 1987 and 1989; Newman and Hobot, 1989; Newman *et al.*, 1983) than to utilize low-temperature techniques with Lowicryl K4M resin.

Denaturation of β-Lactoglobulin

β-Lactoglobulin has a denaturation temperature of 78°C. Heating conditions conducive to β-lactoglobulin denaturation produce very active sulfhydryl groups, which increase the rate of denaturation (Lyster, 1970). Another thermal reaction occurs at ~140°C when disulfide bonds break and the molecule unfolds (Watanabe and Klostermeyer, 1976; de Wit, 1981). In-container sterilization and vat heating cause almost complete denaturation of β-lactoglobulin; UHT processes produce an intermediate level of

denaturation while pasteurization processes cause very little denaturation (Burton, 1988; Parnell-Clunies *et al.*, 1988).

α-Lactalbumin is more structurally stable than β-lactoglobulin and contains four disulfide bonds but no free sulfhydryl groups (Farrell, 1988). The free sulfhydryl of β-lactoglobulin can promote complex formation with α-lactalbumin through disulfide interactions at elevated temperatures (Hunziker and Tarassuk, 1965).

β-Lactoglobulin also forms complexes with α_{S2}-casein (Kudo, 1980b; Kinsella, 1984), β-casein (Elfagm and Wheelock, 1978a and b) and κ-casein (Doi *et al.*, 1979). β-Lactoglobulin and κ-casein interact through disulfide linkages when heated together or when κ-casein is added to heated β-lactoglobulin (Morr *et al.*, 1962; Morr, 1965; Sawyer, 1969; Snoeren and van der Spek, 1977). As well as disulfide interchange, ionic interactions and hydrophobic bonding may be involved in these complex formations (Hill, 1989). β-Lactoglobulin is identified in foods using immunolocalization techniques mainly as part of these complexes (Alleyne *et al.*, 1994b).

Whey Proteins in Dairy Foods

Ultrafiltration (UF) in dairy processing allows increased incorporation of whey proteins into traditional dairy products (Mortensen, 1984). UF is also employed to isolate whey proteins and to produce whey protein concentrates (WPC), which are then used in non-dairy and dairy products like ice cream and baby foods. Chapman *et al.* (1974) used UF concentrated whole milk to make hard cheese, soft cheese, and yogurt. They found the yield of soft cheese to be 41% greater than that from normal whole milk and the make time to be half that of the normal process.

Yogurt is heated before fermentation to denature whey proteins. β-Lactoglobulin and whey proteins may be necessary components in the formation of protein matrices and core-and-shell structures in acid-heat-induced milk gels (Harwalker and Kalab, 1988; Kalab *et al.*, 1991).

We employed immunolocalization procedures involving LR White with polymerization at 50°C using the protocol of Alleyne *et al.* (1994a) for immunolocalization of β-lactoglobulin. The objective of this study was to localize complexed β-lactoglobulin in different dairy products, and to correlate the manufacturing process with the location and relative abundance of this whey protein.

MATERIALS AND METHODS

Reagents

Agar was obtained from Difco Laboratories (Detroit, Michigan); paraformaldehyde, secondary antibody (goat anti-mouse) IgG conjugated to 10 nm gold probes (batch # 9458), and LR White medium grade were obtained from Electron Microscopy Sciences (Fort Washington, Pennsylvania). Teleosteam gelatin was obtained from Sigma Chem. Co. (St. Louis, Missouri). Monoclonal primary antibody (mouse anti-β-lactoglobulin), raised against purified protein (Kuzmanoff and Beattie, 1991), was obtained from J. J. Statsny, University of Illinois, Chicago. All other chemicals were analytical reagent grade.

Samples

Commercial samples of stirred yogurt, cottage cheese, process cheese food, and Cheddar cheese were obtained from the Gary H. Richardson Dairy Products Laboratory, Utah State University. Commercial samples of sweetened condensed milk, evaporated milk, Mozzarella cheese, Queso Blanco cheese, process cheese, and Ricotta cheese were obtained from a supermarket. Feta cheese was made at Utah State University.

Samples of yogurt, cottage cheese, sweetened condensed milk, and evaporated milk were encapsulated in 4% agar using the microcube technique (Alleyne *et al.*, 1993). The other cheese samples were cut into cubes of sides ~1 mm. All samples were then fixed in paraformaldehyde (4%) for 4 h (Alleyne *et al.*, 1994a). They were then dehydrated in an

ascending ethanol series (50%, 70%, 95%, 100%), infiltrated in LR White resin, and embedded at 50°C for 21 h.

Immunogold Labeling

Thin sections (90 nm thick, Sorvall MT-2 Porter-Blum ultramicrotome) from the LR White blocks were immunogold labeled according to Alleyne *et al.* (1994a). Sections were floated for 15 min on the blocking agent (.1% fish gelatin with .1% NGS in 20 mM Tris buffer without BSA) at pH 6.6 and then rinsed for 5 min in 50 mM Tris saline buffer (pH 7.4) (Zymed, 1991). They were floated on the primary antibody (mouse anti-β-lactoglobulin) in buffer, ratio 1:2000 (antibody:50 mM Tris saline buffer), and refrigerated for 24 h in a humidity chamber. The control was floated on PBS in the humidity chamber in place of the primary antibody. All grids were rinsed for 5 min in 50 mM Tris saline buffer (pH 7.4) (Zymed, 1991) and floated on the secondary antibody (goat anti-mouse) IgG conjugated to 10 nm gold probes in buffer, ratio 1:38 (antibody:50 mM Tris saline buffer) in a humidity chamber at room temperature for 3 h. Grids were then rinsed (6 x 5 min) with double-distilled water and were stained with uranyl acetate (20 min) followed by lead acetate (5 min). Transmission electron microscopy (TEM) was carried out using a Zeiss CEM 902 electron microscope operated at 80 kV. Micrographs were taken on Kodak SO 163 sheet film and were printed on Kodak polycontrast III RC paper.

RESULTS AND DISCUSSION

Resin Embedding

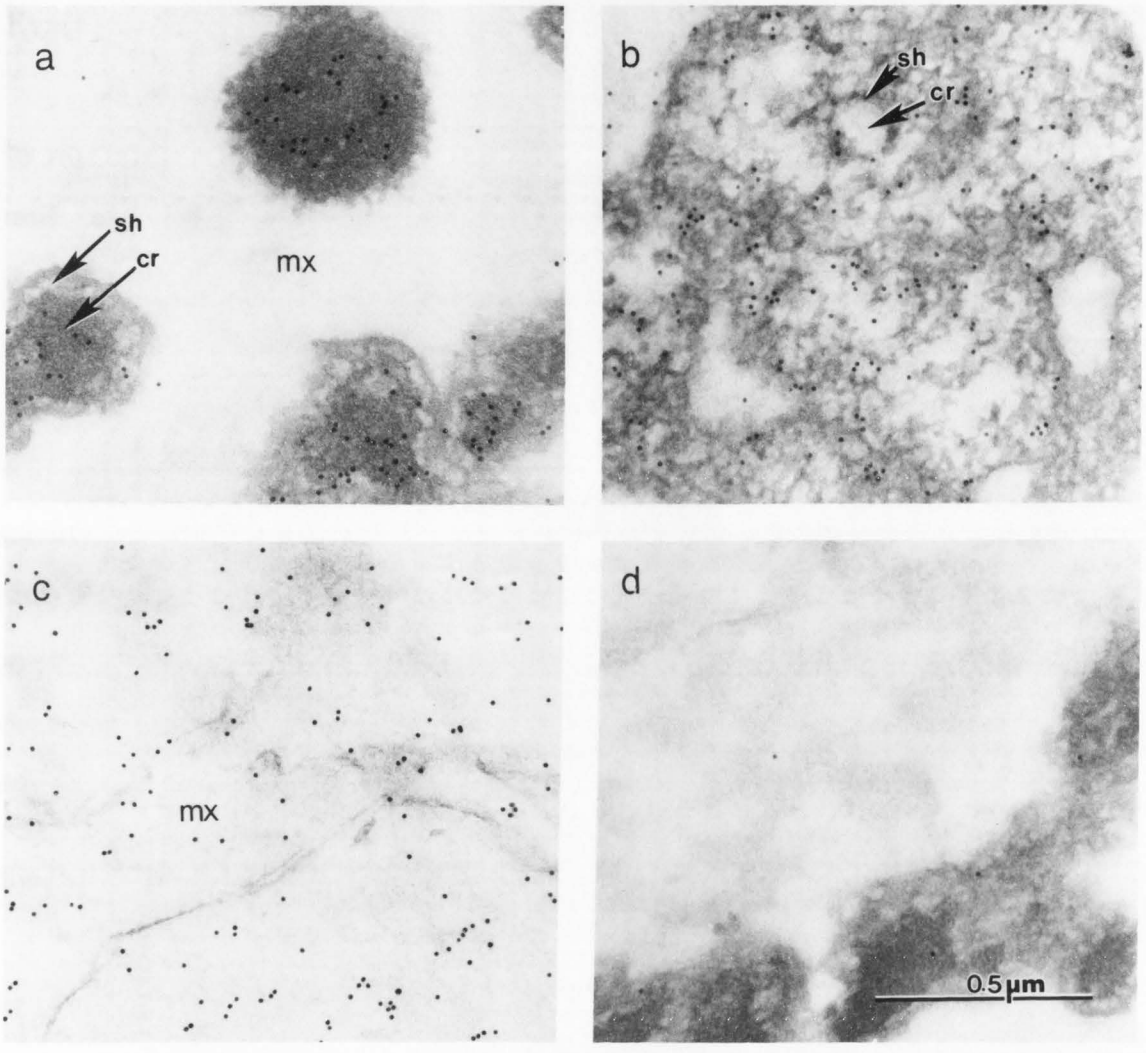
The quality of labeled micrographs produced using LR White resin was comparable to that obtained by Armbruster and Desai (1992) who used low-temperature embedding techniques with Lowicryl K4M. Newman and Hobot (1989) also compared immunocolloidal gold labeling on LR White and Lowicryl K4M using sections of pancreas. They found little difference in microstructure or gold particle counts and concluded that LR

White resin was excellent for routine cytochemical and immunocytochemical problems. We would recommend the use of LR White resin because fixation, dehydration, and infiltration are performed at room temperature, polymerization in gelatin capsules is done at 50°C, and sectioning can be performed with a glass knife. Use of Lowicryl K4M and the progressive lowering of temperature would be recommended if fixation reduces sensitivity and where a radical reduction in glutaraldehyde concentration to .2% or less is required.

Ricotta Cheese

Ricotta cheese is a soft, bland, semi-sweet whey cheese made from the coagulable whey proteins from cheese such as Cheddar, Swiss, and Provolone. The Ricotta cheese used in this experiment was made with 10% pasteurized whole milk added to pasteurized whey (according to the label). The milk-whey mixture was then acidified and the temperature raised to 80-100°C (Bassette and Acosta, 1988). We observed two types of diffused core-and-shell micellar matrix (Figures 6.1a and 6.1b) and a diffused intermicellar whey protein matrix (Figure 6.1c). In Ricotta cheese the core-and-shell matrix of Figure 6.1a was more dense and more compact than that of Figure 6.1b. A core-and-shell matrix has been observed for such acid-heat coagulated cheeses (Harwalker and Kalab, 1988; Kalab *et al.*, 1991; Kalab, 1993). The matrices all showed relatively heavy labeling for β-lactoglobulin. The use of whey as the main ingredient in this cheese and the formation of larger β-lactoglobulin aggregates accounted for the overall heavy labeling of this product. Figure 6.1d shows the ultrastructure of Ricotta cheese used as a control for labeling. The general lack of labeling for this control (compared to Figures 6.1a and 6.1b) typified the results of all controls in this experiment.

Figure 6.1. Transmission electron micrographs of immunogold-labeled Ricotta cheese: **(a)** compact core-and-shell structure with heavy labeling for β-lactoglobulin, **(b)** extended core-and-shell structure with heavy labeling for β-lactoglobulin, **(c)** diffused intermicellar whey protein matrix with heavy labeling for β-lactoglobulin, **(d)** immunogold-labeled control for Ricotta cheese showing slight background labeling for β-lactoglobulin.
cr: core, sh: shell, mx: intermicellar matrix.


a
sh
cr
mx
b
sh
cr
c
mx
d
0.5 µm

Evaporated Milk

Evaporated milk is sometimes called unsweetened condensed milk and is produced by vacuum evaporating ~60% of water from whole milk (Lampert, 1965). Standardized milk is warmed to a temperature of 88-99°C and is then drawn into a vacuum pan where it is condensed to the required concentration. After evaporation the milk is homogenized, cooled, canned, and pressurize sterilized at 117°C for 15 min, 126°C for 2 min, or up to 150°C for ~5 s (Bassette and Acosta, 1988). The ultrastructure of evaporated milk (Figure 6.2) showed round micelles, an irregular intermicellar matrix, and fat globules with membranes mostly surrounded by attached micelles. This characteristic ultrastructure is similar to that observed by Heintzberger *et al.* (1972). Gold-labeled β-lactoglobulin appears on the micellar surfaces and within the intermicellar spaces. Labeling appears more dense within the intermicellar spaces than on the micellar surfaces. This is probably due to a higher concentration of β-lactoglobulin complex within the intermicellar matrix.

Sweetened Condensed Milk

Sweetened condensed milk is essentially sweetened evaporated milk. The addition of 18% sucrose to evaporated milk confers adequate preservation without pressurized sterilization (Bassette and Acosta, 1988). After whole milk is warmed for 15-30 min at a temperature of 82-93°C (Lampert, 1965), syrup of 65% sucrose or a mixture of sucrose and dextrose is added at ~88°C. The sugar-milk mixture is evaporated in a vacuum pan at ~57°C to the required concentration and is then cooled rapidly to ~27°C and canned. The ultrastructure of condensed milk (Figure 6.3) resembled that of evaporated milk (Figure 6.2), but had a less dense intermicellar matrix and less labeling. A similar structure was observed in gels of UF homogenized milk retentate (Gavaric *et al.*, 1989), gels made from UHT-UF homogenized milk retentate (McMahon *et al.*, 1991), and Labneh made from homogenized milk (Tamine *et al.*, 1989 and 1991). Labeling of β-lactoglobulin was mainly on the micelles and appeared to be on the surface as well as throughout the micelle

Figure 6.2. Transmission electron micrograph of immunogold-labeled evaporated milk showing even distribution of labeling for β-lactoglobulin.

mc: micelles; f: fat globule; mb: membrane, mx: intermicellar matrix.

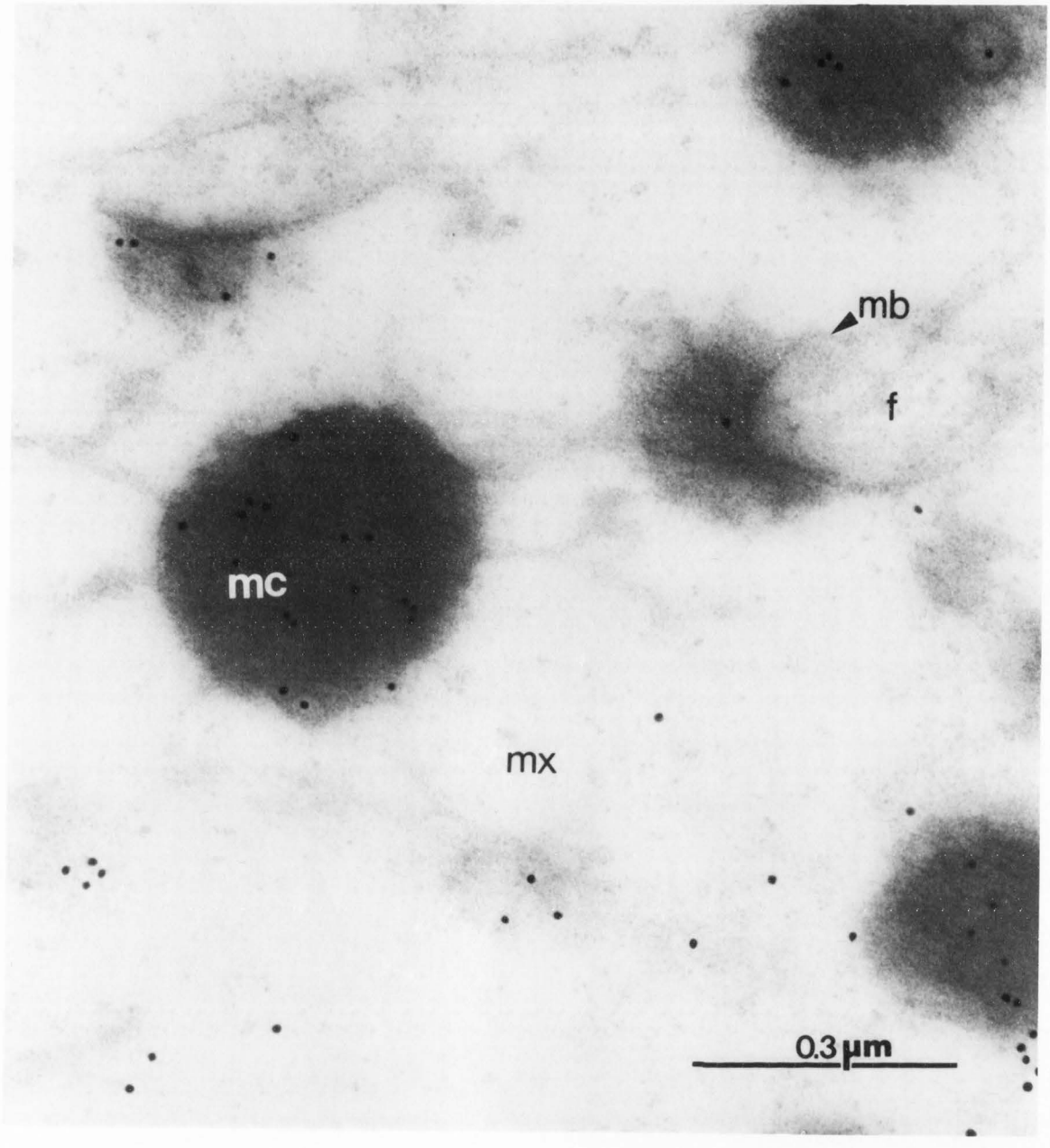
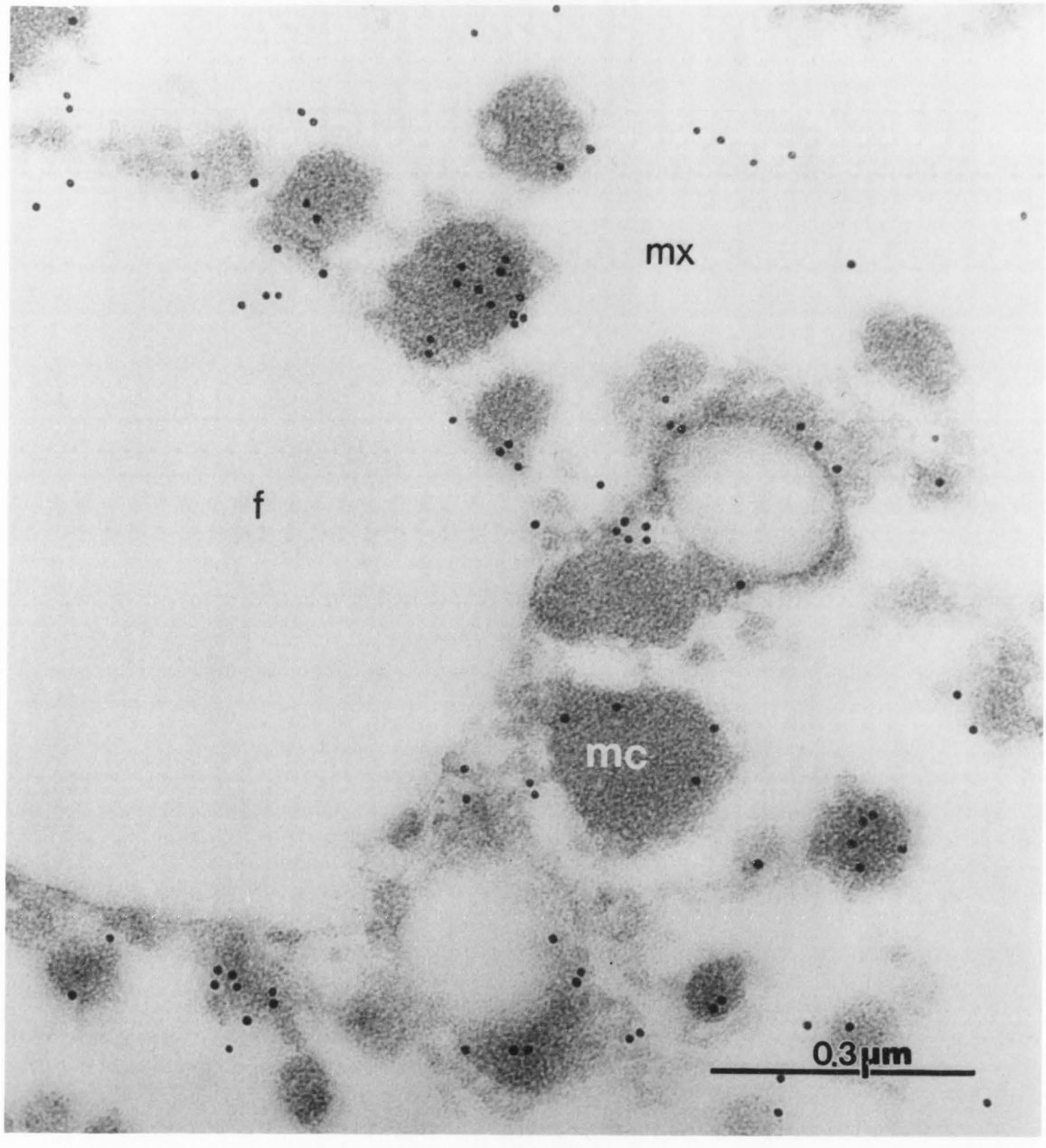

mb
f
mc
mx
0.3 µm

Figure 6.3. Transmission electron micrograph of immunogold-labeled sweetened condensed milk showing slight labeling for β-lactoglobulin on the micelles and in the intermicellar spaces.

mc: micelles; f: fat globule; mx: intermicellar matrix.


mx
f
mc
0.3 µm

interior. Less β-lactoglobulin complexing would occur because of lower processing temperature and slower denaturation through the stabilizing effect of added sugar (Lonergan, 1978; Hillier *et al.*, 1979; Kudo, 1980a).

Yogurt

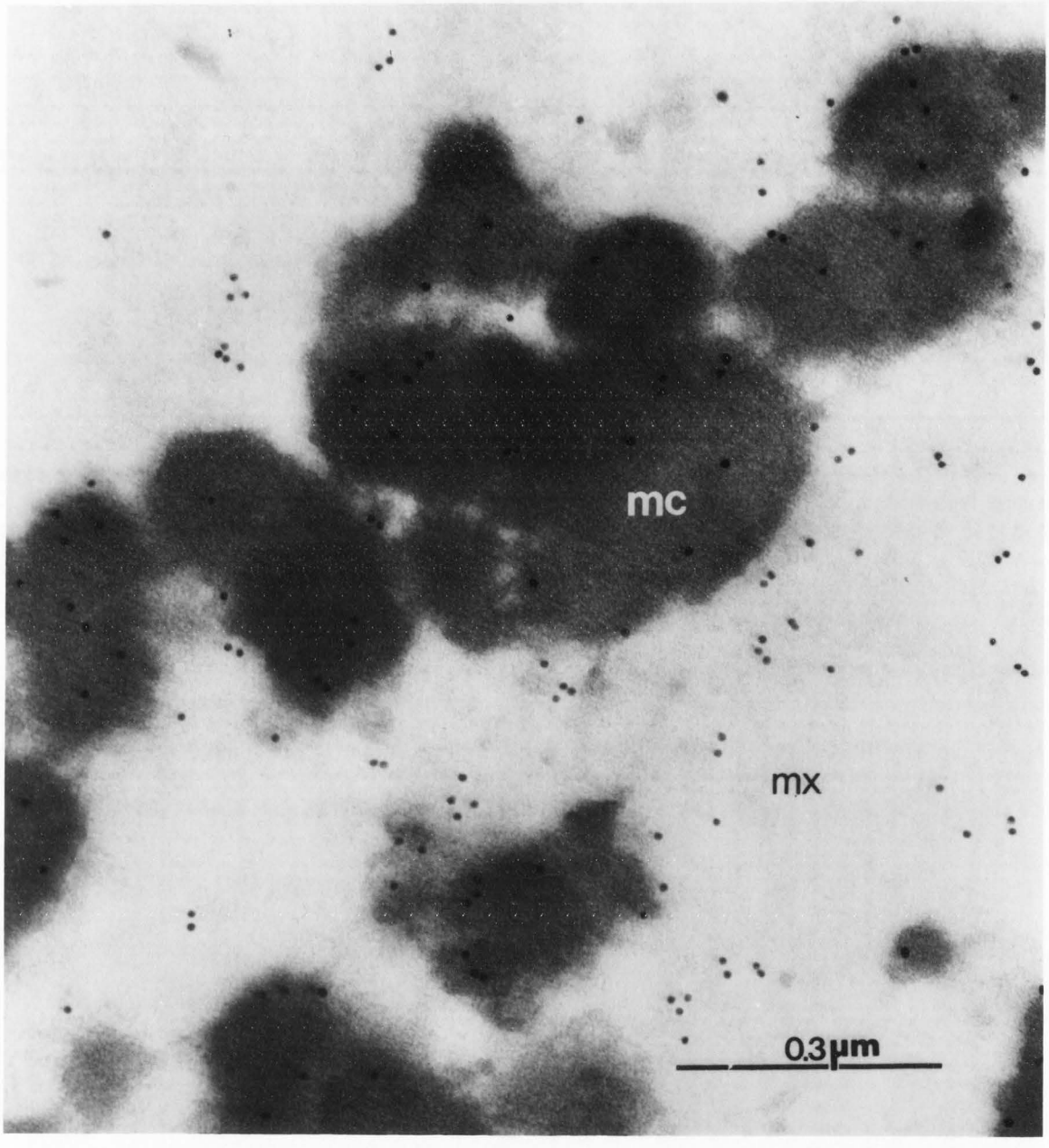
Yogurt is produced by controlled fermentation of milk (whole, part skim, or skimmed) by a mixed culture of lactic acid bacteria (Schmidt, 1992). Manufacture typically involves heating of the milk to 85°C and holding for 20 min. Denaturation of whey proteins, and complexing with caseins, improves behavior of the gel particles and produces a smoother gel (Modler *et al.*, 1983). At Utah State University, the milk was fortified with skim milk powder, homogenized, mixed with sugar, inoculated with active cultures, poured into cans, and incubated until pH decreased to 5.2. It was cooled to 4°C overnight and then stirred, at which time pH was 4.2. The ultrastructure of the low-fat, stirred yogurt (Figure 6.4) showed round micelles of various sizes linked in an irregular protein matrix. This structure is characteristic of yogurt as reported by others (Kalab *et al.*, 1983; Tamine *et al.*, 1984).

There was moderately heavy labeling of β-lactoglobulin, distributed evenly on the micelles and within the intermicellar spaces. Labeling that appeared within the micelle may actually be surface β-lactoglobulin that is present on the sectioned surface of the specimen, rather than β-lactoglobulin present in the interior of the micelles. The low pH of the product stabilizes β-lactoglobulin (Hillier *et al.*, 1979), and the holding temperature of 85°C is optimum for complexing between β-lactoglobulin and κ-casein (de Wit, 1981).

Cottage Cheese

Cottage cheese is a soft, unripened, acid cheese made with coagulated curd from various combinations of skim milk, partially condensed skim milk, and/or reconstituted low-heat nonfat, dry milk. The curd can be formed by either lactic acid produced by starter

Figure 6.4. Transmission electron micrograph of immunogold-labeled stirred skim milk yogurt showing labeling for β-lactoglobulin on the micelles and in the intermicellar spaces. mc: micellar matrix, mx: intermicellar matrix.


mc
mx
0.3µm

cultures or edible food grade acids (Bassette and Acosta, 1988). At Utah State University, direct acid cottage cheese is manufactured from skim milk pasteurized at 80°C for 28 s and cooled to 4°C. After addition of phosphoric acid the milk is warmed to 32°C and glucono-δ-lactone is added. After 1 h the curd is cut at a pH of 4.8 and allowed to stand for 10 min before cooking for 90 min at 57°C to a final pH of 4.5. After cutting and draining, the curd is washed twice, first with cold water at 32.2°C for 5 min, and then with ice water/ice for 5 min. The curd is then drained and dressing added.

The ultrastructure of the cottage cheese (Figure 6.5) had a micellar matrix somewhat similar to that of yogurt (Figure 6.4), but with a more dense packing of micellar residue. This difference is a consequence of curd cutting and drainage. The micellar matrix was interspersed with fat globules and membranes. There was some labeling for β-lactoglobulin probably as a consequence of pasteurizing the milk at a temperature higher than legally required. This would cause some denaturation of β-lactoglobulin and its complexing with κ-casein.

Feta Cheese

Feta cheese is a white, soft, brine-ripened cheese (Bassette and Acosta, 1988). Calcium chloride and yogurt starter cultures are added to pasteurized whole milk, which is heated to 37°C at pH 6. Rennet is added and the milk allowed to set at 37°C for 30 min. After coagulation the curd was cut and allowed to heal for 20 min before the whey was drained. The final pH of the cheese was ~5.0. The Feta cheese (Figure 6.6) had an extended protein matrix which was very sparsely labeled for β-lactoglobulin. This is due to the absence of high-temperature processing and the drainage of whey after healing.

Queso Blanco Cheese

Queso Blanco cheese is a white, soft, creamy cheese that can be made in a variety of ways. It may be ripened or unripened and made from whole or part skim milk. The

Figure 6.5. Transmission electron micrograph of immunogold-labeled cottage cheese showing sparse labeling for β-lactoglobulin.

mc: micellar matrix, f: fat globule, mx: intermicellar matrix.

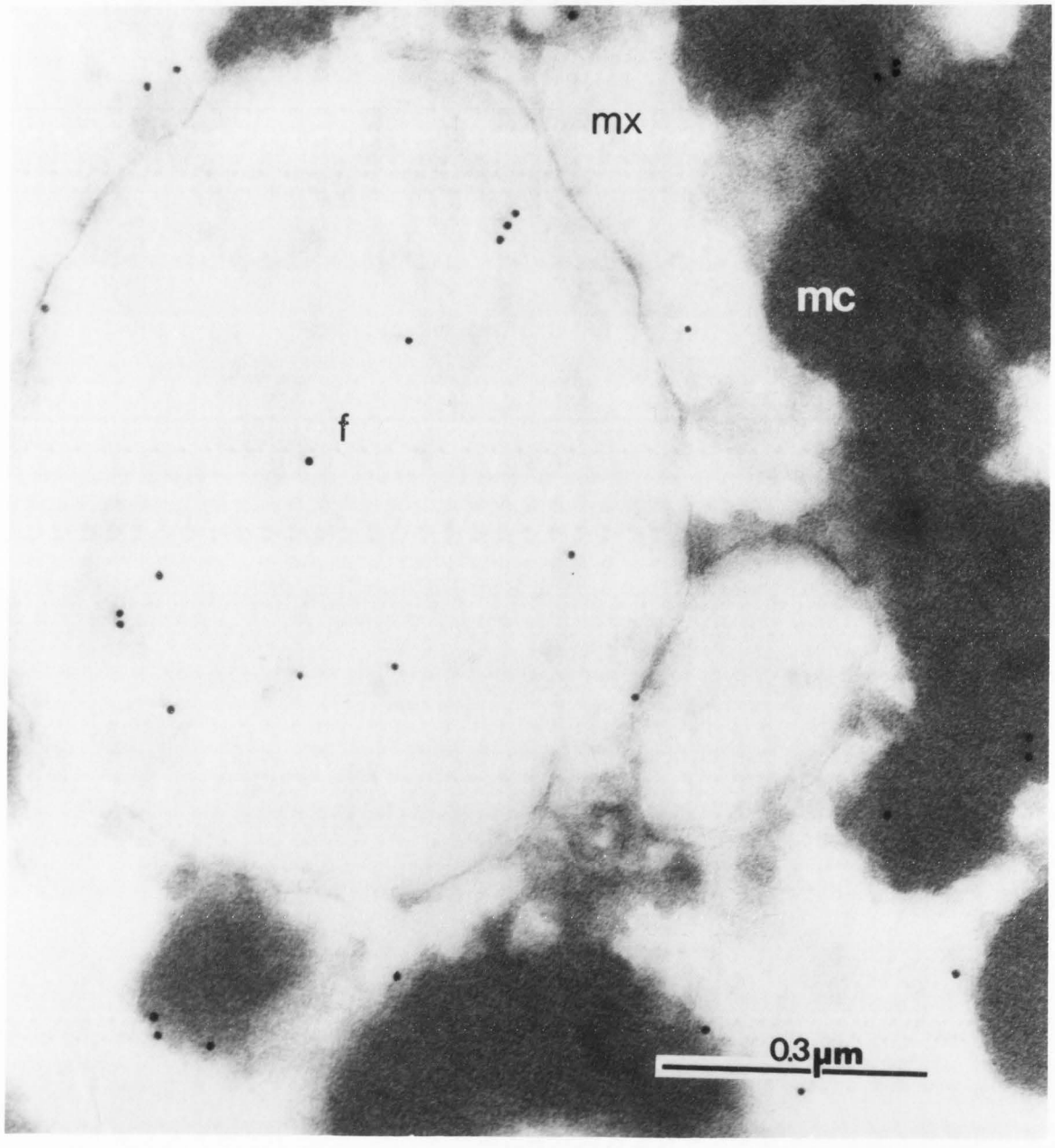
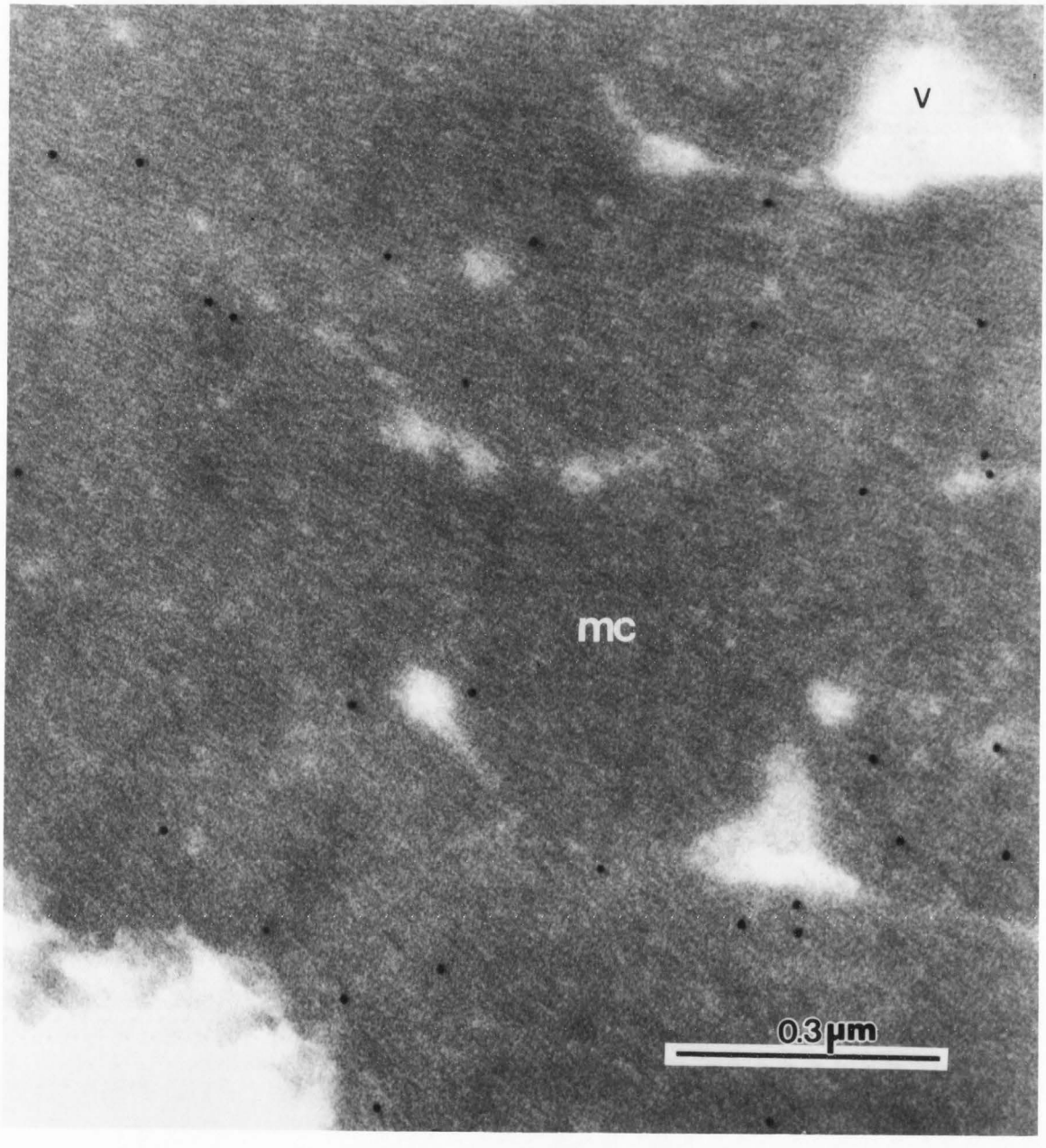

mx
mc
f
0.3µm

Figure 6.6. Transmission electron micrograph of immunogold-labeled unripened Feta cheese showing sparse labeling for β-lactoglobulin.

mc: micellar matrix, v: void area.


V
mc
0.3 µm

milk may be heated to 82°C and dilute food grade acid added to give instantaneous precipitation, or it can be rennet coagulated. The whey is quickly drained and the curd salted and pressed (Kosikowski, 1982). No information on the make procedure of the Queso Blanco used in this study was available. The ultrastructure of the Queso Blanco cheese (Figure 6.7) had an extended protein matrix without any core-and-shell structures. Labeling for β-lactoglobulin was similar to that of Feta cheese (Figure 6.6). From this it can be concluded that this Queso Blanco cheese had been made using rennet coagulation rather than heat coagulation.

Mozzarella Cheese

Mozzarella cheese is a soft, plastic-curd cheese that may be eaten fresh with little or no ripening or used in cooking. In the manufacture of Mozzarella cheese, pasteurized milk is heated to 34°C, inoculated with active cultures and set with rennet. The curd is cut and gently agitated and heated to 38°C. After drainage the curd is cheddared or dry stirred, then stretched in water at 82°C (with the cheese being heated to ~57°C), molded, cooled, and brined. The ultrastructure of fresh Mozzarella cheese (Figure 6.8) had an extended protein matrix surrounded by void spaces showing evidence of the stretching process involved in its manufacture. As shown by Oberg *et al.* (1993), the void spaces originate from columns of emulsified fat droplets. The matrix and void spaces showed sparse labeling for β-lactoglobulin. It was expected that there would be little if any heat-denatured whey protein in Mozzarella cheese because of the absence of high-temperature steps in its manufacture. The emulsification of fat globules during stretching may, however, cause capture of β-lactoglobulin onto the fat/water interface, and this would explain the observed labeling in the void spaces.

Figure 6.7. Transmission electron micrograph of immunogold-labeled ripened Queso Blanco cheese showing sparse labeling for β-lactoglobulin on the protein matrix.

mc: micellar matrix, v: void area.

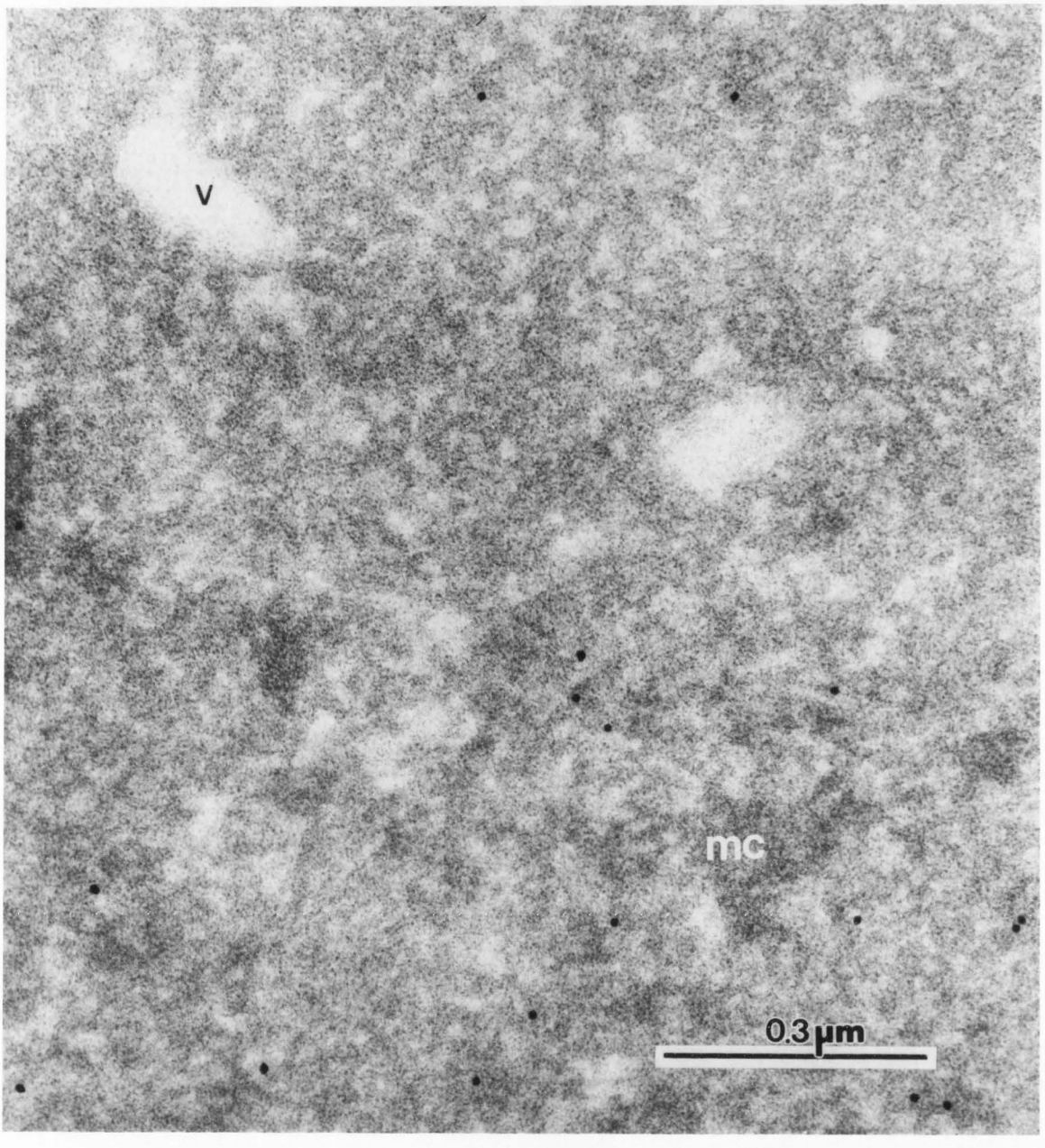
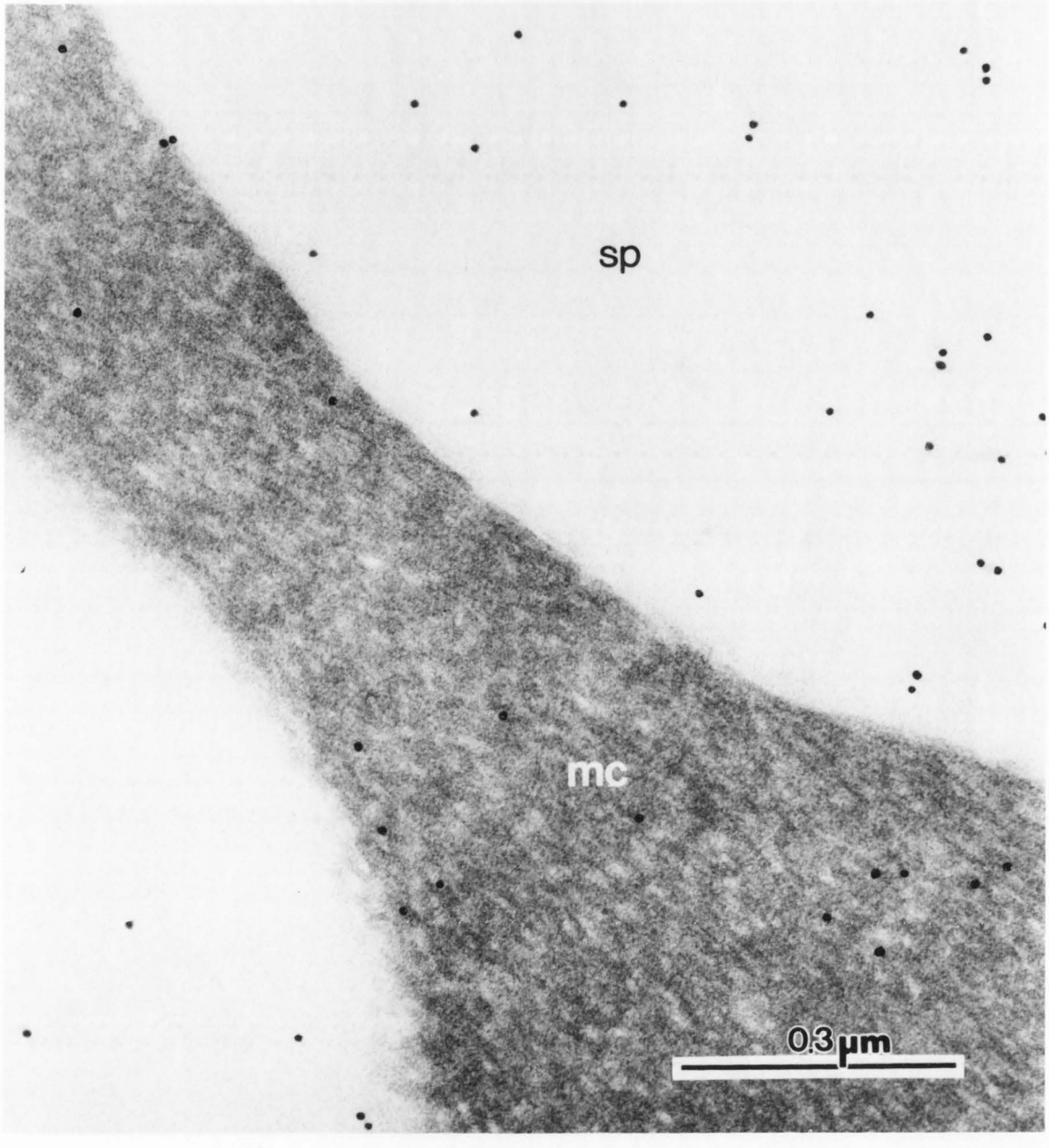

V
mc
0.3 µm

Figure 6.8. Transmission electron micrograph of immunogold-labeled, fresh Mozzarella cheese showing sparse labeling for β-lactoglobulin on the protein matrix.

mc: micellar matrix, sp: intermicellar space.


sp
mc
0.3 µm

Cheddar Cheese

We manufactured this hard, close-textured, bacteria-ripened cheese (Bassette and Acosta, 1988) by adding starter, color, and rennet to ultra-pasteurized (80°C for 28 s) milk at a temperature of 31°C. Curd was cut and cooked at 39°C for 30 min before the whey was drained. The curd was kept at this temperature for 30 min before cheddaring, then milled, salted, and pressed. The ultrastructure of Cheddar cheese (Figure 6.9) showed an extended protein matrix with void spaces. The matrix and void spaces showed sparse labeling for β-lactoglobulin. The higher-than-standard pasteurization conditions would cause a small amount of denaturation of whey proteins. These would then be incorporated into the curd, providing an increased yield. The presence of such β-lactoglobulin aggregates was evident in the appearance of doublet and triplet labeling in the void areas, as well as complexing with κ-casein in the casein matrix.

Pasteurized Process Cheese

Pasteurized process cheese results from mixing and heating several lots of natural cheese with suitable emulsifying salts, color, and salt into a homogenous plastic mass. The sample of process cheese used contained Cheddar cheese, milkfat, sodium citrate, sodium phosphate, and sorbic acid. The cooking temperature was 71-80°C. The ultrastructure of the process cheese (Figure 6.10) was a diffused compact protein matrix showing very sparse labeling for β-lactoglobulin. No labeling of β-lactoglobulin would be expected in U.S.A. pasteurized process cheese because whey proteins cannot legally be added. An exception would be if the cheese had been made using ultrafiltration, which would capture the whey proteins as well as the caseins. The extended microstructure showed cheese material interspersed with void spaces, which would presumably have contained the emulsified fat droplets.

Figure 6.9. Transmission electron micrograph of immunogold-labeled Cheddar cheese showing sparse labeling for β-lactoglobulin.

mc: micellar matrix, sp: intermicellar space.

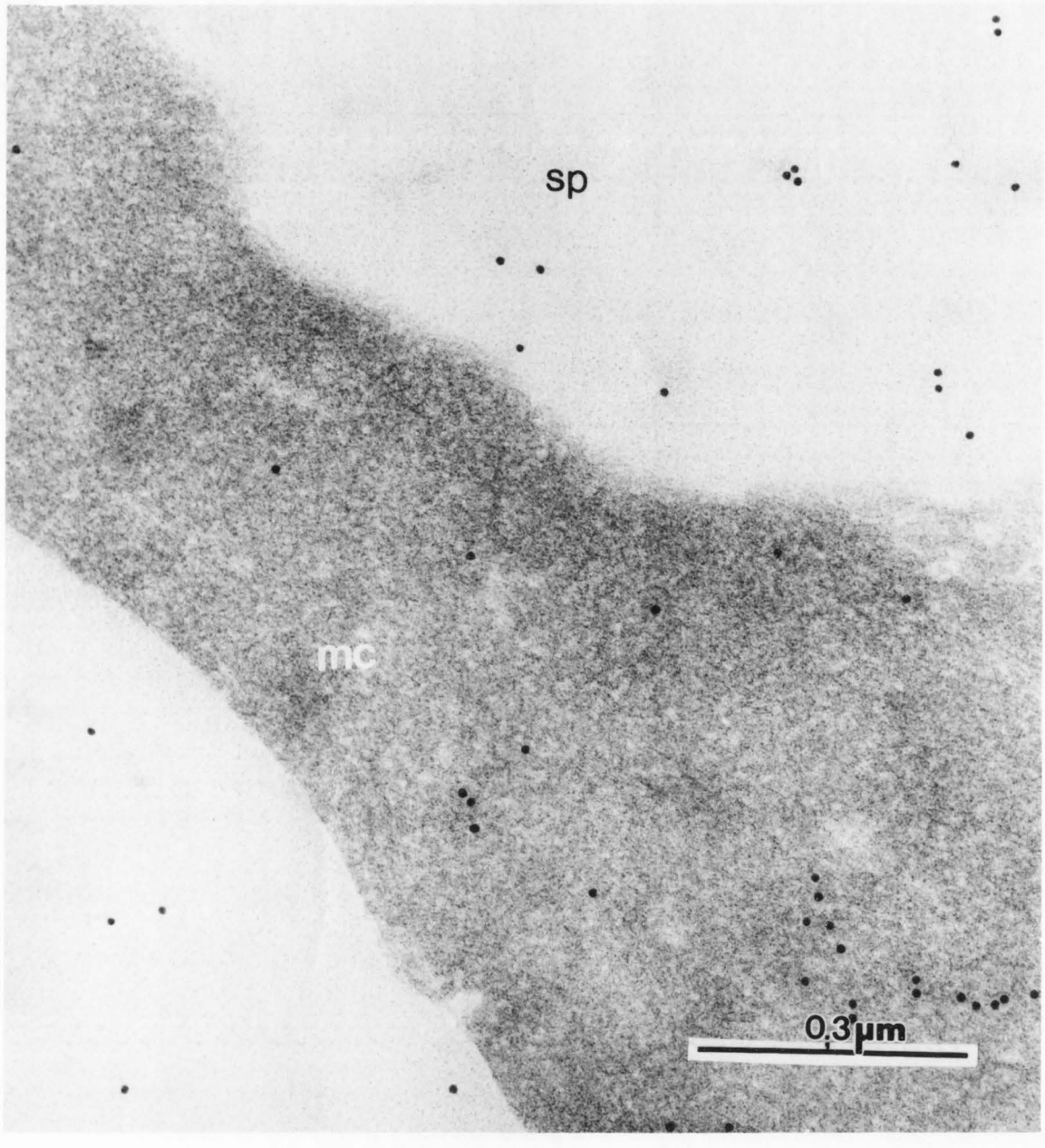
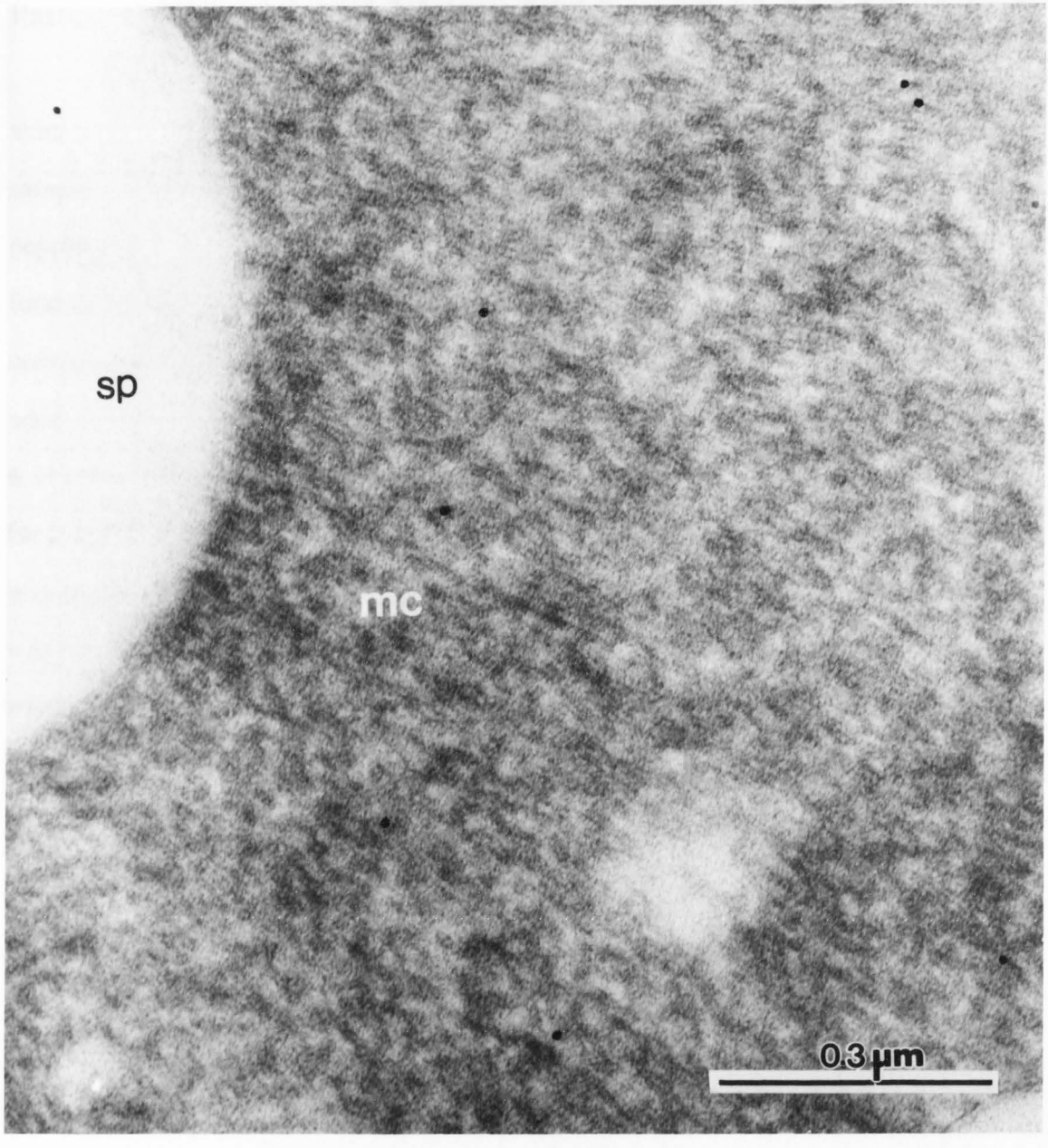

sp
mc
0,3 µm

Figure 6.10. Transmission electron micrograph of immunogold-labeled, pasteurized, process cheese showing sparse labeling for β-lactoglobulin on the protein matrix. mc: micellar matrix, sp: intermicellar space.


sp
mc
0.3 µm

Pasteurized Process Cheese Food

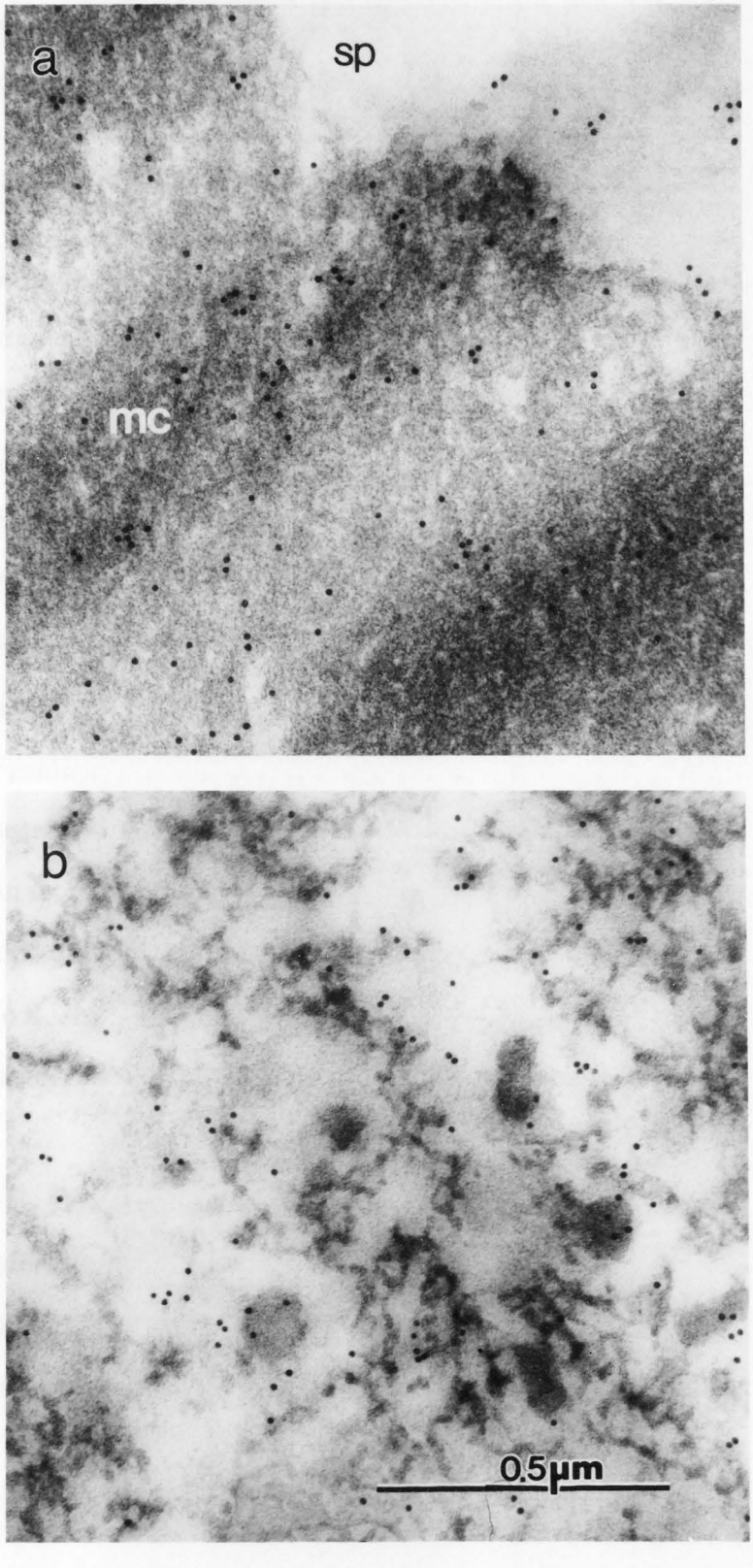
Pasteurized process cheese food is similar to process cheese, but additives such as skim milk, whey, cream, albumin, skim milk cheese, and organic acids are allowed. Our sample contained ~70% Cheddar cheese, 4% whey powder, taco sauce, and jalapeña peppers. The cooking temperature was 80°C. The ultrastructure of the process cheese food (Figure 6.11) was similar to that of process cheese (Figure 6.10), but with a less compact protein matrix. This was expected because of the increased moisture content and addition of whey powder. The extended microstructure showed cheese material (Figure 6.11a) interspersed with non-cheese materials (Figure 6.11b) and void spaces. Labeling for β-Lactoglobulin was distributed throughout the cheese and non-cheese material, and was more dense than that of process cheese (Figure 6.10). This higher degree of labeling was because of the higher cook-temperature of the process cheese food and the addition of whey powder to the product. The higher cook-temperature is optimum for complexing between β-lactoglobulin and κ-casein (de Wit, 1981). When whey proteins are added to process cheese, the meltability of process cheese is reduced (Savello *et al.*, 1989), which can be an advantage when the cheese is used as the protein portion of hot meals and snacks (Lankveld, 1984).

CONCLUSION

Immunolocalization, with embedding at 50°C in LR White resin, identified β-lactoglobulin complexes in dairy products. These data allowed conclusions to be drawn about the relationship between manufacturing processes and the location and relative abundance of this protein in the product. Labeling for β-lactoglobulin was observed when the products had received a high heat treatment during processing, or when whey proteins were added. Embedding at 50°C in LR White allowed for the immunolocalization of

Figure 6.11. Transmission electron micrographs of immunogold-labeled, pasteurized, process cheese food showing labeling for β-lactoglobulin mainly on the protein matrix **(a)** typical cheese matrix, **(b)** atypical matrix.

mc: micellar matrix, sp: intermicellar space.


a
sp
mc
b
0.5µm

β-lactoglobulin without the need to use low-temperature embedding. It avoids the laborious and tedious steps necessary for low-temperature embedding in polyacryl.

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CHAPTER VII

IMMUNOLOCALIZATION OF CASEINS AND WHEY PROTEINS IN MILK AT VARIOUS STAGES OF TREATMENT FROM FRESH WHOLE MILK TO ULTRA-HIGH TEMPERATURE-STERILIZED ULTRAFILTERED (3x) MILK CONCENTRATE[1]

ABSTRACT

Immunolocalization techniques were employed to elucidate the positions of β-lactoglobulin, α-lactalbumin, α_{s1}-casein, α_{s2}-casein, β-casein, and κ-casein in fresh whole milk, skim milk, pasteurized milk, ultrafiltered milk, and direct and indirect sterilized (110, 120, 130, and 140°C) milk.

Heating of milk during pasteurization and UHT sterilization affected the distribution and altered the conformational state of some milk proteins, especially of β-lactoglobulin whose interactions with whey and micellar casein protein depended on processing temperature. α-Lactalbumin and κ-casein were less affected by temperature. α_{s1}-Casein and β-casein were localized on the micelles, but heating did not affect the distribution of these proteins. α_{s2}-Casein did not respond to these immunolocalization procedures.

INTRODUCTION

Milk contains ~3.5% protein, of which 78% is casein and 22% is whey proteins (Johnson, 1974). Casein primarily occurs as a colloidal dispersion of large protein-mineral complexes called casein micelles. In bovine milk, caseins are insoluble at pH 4.6 and all except some of the proteolytic derivatives will precipitate. None of the four kinds of caseins has a highly organized secondary structure (Walstra and Jenness, 1984). They appear to contain short α-helices and β-sheets. Their conformation appears to be similar to

[1] Coauthored by M. C. Alleyne and D. J. McMahon.

that of denatured globular proteins. The complexed quaternary conformation provides structural stability. Casein micelle proteins are represented primarily by α_{s1}-, α_{s2}-, β-, and κ-casein in approximate proportions of 3:.8:3:1 (Walstra and Jenness, 1984). κ-Casein is not susceptible to calcium-ion binding, and it apparently occupies most of the micelle surface area (Heth and Swaisgood, 1982), protecting the other calcium-sensitive caseins from precipitation by calcium ions (Swaisgood, 1982; McMahon and Brown, 1984).

The composition of serum proteins is approximately 55% β-lactoglobulin, 12% α-lactalbumin, 10% proteose-peptone, 10-15% casein, and 8-13% immunoglobulins and enzymes (Parry, 1974). β-Lactoglobulin has a denaturation temperature of 78°C and exhibits another thermal reaction at ~140°C caused by the breaking of disulfide bonds break and the molecule unfolds (Watanabe and Klostermeyer, 1976; de Wit, 1981). β-Lactoglobulin can exist as an 18.3 kDa monomer, a 36 kDa dimer (McKenzie, 1971) or various polymers (Sawyer, 1969; McKenzie, 1971; Harwalker, 1980; de Wit and Klarenbeek, 1984).

α-Lactalbumin is a molecule with a monomer weight of 14.17 kDa (Farrell, 1988) and has a denaturation temperature of 62°C (de Wit and Klarenbeek, 1984). This conformational change is 80-90% reversible (Ruegg *et al.*, 1977), because α-lactalbumin has four pairs of disulfide bonds and no free sulfhydryl group through which it could aggregate (Lyster, 1979).

β-Lactoglobulin forms complexes with α-lactalbumin (Hunziker and Tarassuk, 1965), α_{s2}-casein (Kudo, 1980; Kinsella, 1984), β-casein (Elfagm and Wheelock, 1978a and b) and κ-casein (Doi *et al.*, 1979). α-Lactalbumin interferes with interaction of β-lactoglobulin and κ-casein (Baer *et al.*, 1976; Elfagm and Wheelock, 1977, 1978a and b), but does not affect the denaturation of β-lactoglobulin (Elfagm and Wheelock, 1978a

and b). Complexes formed between κ-casein and β-casein, and between κ-casein and α_{S1}-casein (Doi *et al.*, 1979) may interfere with the formation of complexes between κ-casein and β-lactoglobulin.

Various methods involving microscopy have been employed to identify milk proteins in foods, the most recent of which employed gold-labeled antibodies to identify β-lactoglobulin and casein in the ultrastructure of low fat frozen desserts, salad dressing, and process cheese (Armbruster and Desai, 1992), and to localize β-lactoglobulin in whey, UHT-sterilized UF milk retentate (Alleyne *et al.*, 1994a and b) yogurt, processed milk, and cheese (Alleyne and McMahon, 1994).

The objective of this study was to localize the β-lactoglobulin, α-lactalbumin, α_{S1}-casein, α_{S2}-casein, β-casein, and κ-casein in samples of milk at various stages of treatment, from fresh whole milk to UHT-sterilized UF-concentrated milk retentate, using the technique of Alleyne and McMahon (1994) and Alleyne *et al.* (1994a and b) of embedding in LR White resin (London Resin Co., Basingstoke, UK) with polymerization at 50°C.

MATERIALS AND METHODS

Reagents

Agar was obtained from Difco Laboratories (Detroit, Michigan); paraformaldehyde, secondary antibody (goat anti-mouse) IgG conjugated to 10 nm gold probes (batch # 9458), and LR White medium grade were obtained from Electron Microscopy Sciences (Fort Washington, Pennsylvania). Teleosteam fish gelatin was obtained from Sigma Chem. Co. (St. Louis, Missouri). Primary antibodies (mouse anti-protein) for proteins, α-lactalbumin, β-lactoglobulin, α_{S1}-casein, α_{S2}-casein, κ-casein, and β-casein, were obtained from J. J. Statsny, University of Illinois, Chicago. These monoclonal antibodies were raised against purified proteins (Kuzmanoff *et al.*, 1990a and b; Kuzmanoff *et al.*,

1991; Kuzmanoff and Beattie, 1991; Leung *et al.*, 1991). All other chemicals were analytical reagent grade.

Samples

Samples of fresh (4°C) whole milk and skim milk were obtained from the Gary H. Richardson Dairy Products Laboratory, Utah State University. Skim milk was pasteurized (63°C for 30 minutes), cooled to 50°C, and concentrated by ultrafiltration to 3x (volume reduction) to contain approximately 10% protein. Pasteurized and UF samples were collected for immunolocalization studies. Skim milk was concentrated with a three-module in-series UF system with spiral wound polysulfone membranes (Osmonics Inc., Minnetonka, Minnesota; 20 kDa nominal molecular weight cutoff, 15m^2 membrane area). The concentrated milk was homogenized (Model 3DDL Homogenizer, Crepaco Inc., Chicago, Illinois) at 13.8 MPa with 3.4 MPa second stage pressure. Indirect and direct heating of UF milk was accomplished with an Alfa-Laval SteriLab® UHT Pilot System for UHT processing (McMahon *et al.*, 1993). The concentrated milk was heated to high temperatures (110°C, 120°C, 130°C, and 140°C held for 4 s) by indirect (plate heat exchanger, residence time 97 s) and direct (steam injection, residence time < 1 s) heat exchange to aseptic conditions. The milk samples were packaged in sterile plastic containers in an Alfa-Laval SteriCab® filling station and samples were collected for immunolocalization studies.

Sample Preparation for Electron Microscopy Analysis

The 12 milk samples were (1) fresh whole milk, (2) skim milk, (3) pasteurized skim milk, (4) ultrafiltered pasteurized skim milk, (5) indirect 110°C concentrated milk, (6) indirect 120°C concentrated milk, (7) indirect 130°C concentrated milk, (8) indirect 140°C concentrated milk, (9) direct 110°C concentrated milk, (10) direct 120°C concentrated milk, (11) direct 130°C concentrated milk, and (12) direct 140°C concentrated milk. The

microcube technique (Alleyne *et al.*, 1993), a modification of the microencapsulation technique (Salyaev, 1968; Kalab, 1988), was used to contain the 12 milk samples for transmission electron microscopy (TEM).

Electron Microscopy and Immunogold Labeling

Our previously developed method for immunolocalization of milk proteins (Alleyne *et al.*, 1994a and b) was followed. Agar gel capsules obtained by the microcube method (Alleyne *et al.*, 1993) were fixed at 20°C for 4 h in 4% formaldehyde in .1 M phosphate buffer (pH 6.6). After completion of fixation, samples were washed with .1 M phosphate buffer, pH 6.6, and dehydrated in a graded ethanol series of 50, 70, 95, and 100% ethanol, infiltrated with LR White resin, and polymerized at 50°C for 21 h. Thin sections (90 nm thick, Sorvall MT-2 Porter-Blum ultramicrotome), from the LR White blocks, were collected on nickel grids. These were floated for 15 min on the blocking agent (.1% fish gelatin with .1% NGS in 20 mM Tris buffer without BSA) at pH 6.6 and rinsed for 5 min in 50 mM Tris saline buffer (pH 7.4) (Zymed, 1991). They were floated on the primary antibody, mouse anti-protein (for proteins: α-lactalbumin, β-lactoglobulin, α_{S1}-casein, α_{S2}-casein, κ-casein, and β-casein) and refrigerated for 24 h in a humidity chamber. (ELISA was used to determine the appropriate dilution of antibodies in 50 mM Tris saline buffer for each protein. An antibody:buffer ratio of 1:2000 was optimum for β-lactoglobulin, α_{S1}-casein, and β-casein, and 1:1000 was optimum for α-lactalbumin, α_{S2}-casein, and κ-casein.) The controls were floated on PBS in the humidity chamber in place of the primary antibody. All grids were rinsed for 5 min in 50 mM Tris saline buffer (pH 7.4) (Zymed, 1991) and were floated on the secondary antibody (goat anti-mouse) IgG conjugated to 5 nm gold probes in buffer, ratio 1:38 (antibody:50 mM Tris saline buffer) in a humidity chamber at room temperature for 3 h. Grids were rinsed (6 x 5 min) with double-distilled water and stained with uranyl acetate (20 min) followed by lead

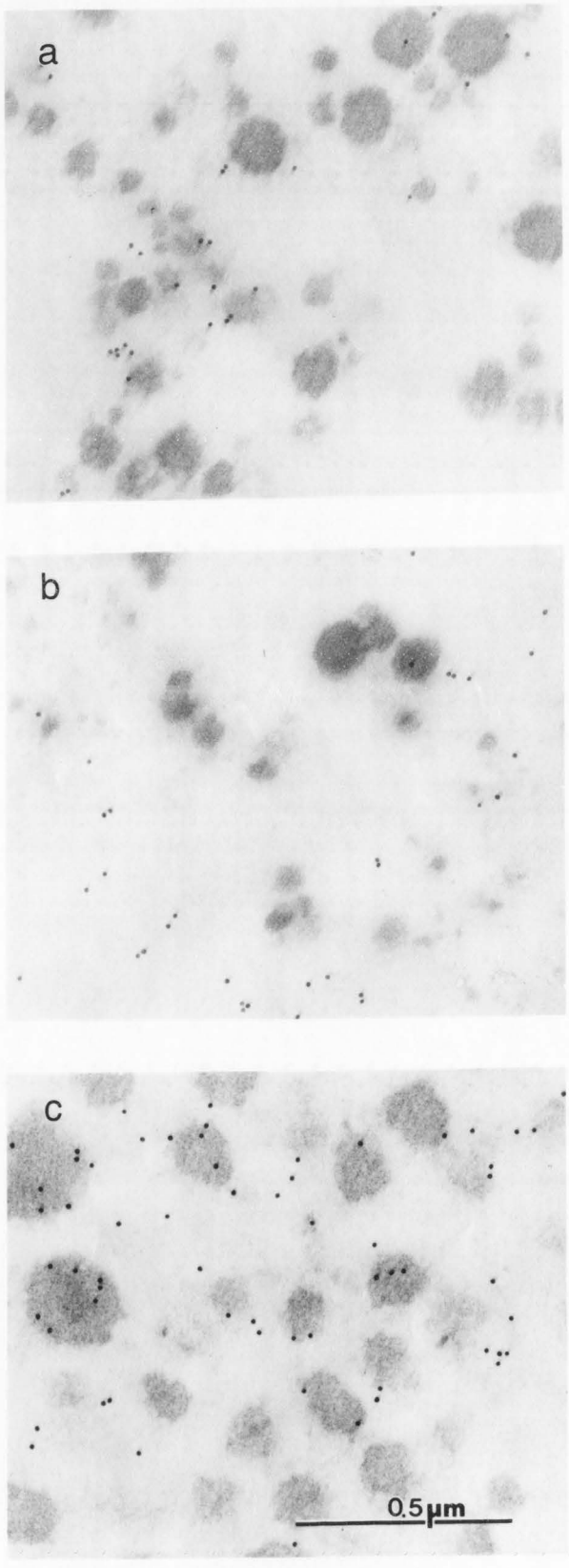
acetate (5 min) (Youssef, 1985). Transmission electron microscopy (TEM) was carried out using a Zeiss CEM 902 electron microscope operated at 80 kV. Micrographs were taken on Kodak SO 163 sheet film and were printed on Kodak polycontrast III RC paper.

RESULTS AND DISCUSSION

The micelles of all milk samples were generally spheroid but varied considerably in size. Some had a more open micellar structure (Figure 7.1a) (compare with Figures 3.4 to 3.8) due to the lack of osmium tetroxide staining in these preparations. Fixation with osmium tetroxide significantly reduces antigenicity of protein but imparts heavy metal staining to the samples. This staining confers a compact appearance on the micelles, which may be artifactitious. The open structure of the micelle is believed to be more representative of the actual structure. The outline of micelles in many samples appeared rough as though with short tendrillar appendages as reported by other investigators (Mohammad and Fox, 1987; Mottar *et al.*, 1987).

There was little labeling for α-lactalbumin, the order of which was whole = skim < pasteurized = UF < UHT 110°C < UHT 120°C < UHT 130°C < UHT 140°C. The labeling occurred mainly within the intermicellar matrix. Figure 7.1 shows the labeling of whole milk, pasteurized milk, and indirect UHT (140°C) samples. The heat treatment during pasteurization and UHT sterilization created complexes of α-lactalbumin, thus retaining more of this protein during sample preparation. Complexing of α-lactalbumin appeared to increase with temperature. At pH values below the isoionic point, the dimers, trimers, and aggregated polymers formed by α-lactalbumin are reversible (Kronman and Andreotti, 1964; Kronman *et al.*, 1964). A similar association may be responsible for the complexing of α-lactalbumin in this experiment (the labels were frequently observed as doublets and triplets), with aggregation increasing at higher temperatures.

Figure 7.1. Transmission electron micrographs of milk samples immunogold labeled for α-lactalbumin **(a)** whole milk, **(b)** pasteurized milk, **(c)** indirect UHT (140°C) milk. Labeling was concentrated on the intermicellar spaces.


a
b
c
0.5µm

β-Lactoglobulin was labeled more intensely than was α-lactalbumin, but both exhibited similar trends in labeling density (Figure 7.2). The labeling density of the UHT samples from 110-140°C or from direct to indirect did not differ much, but there was a definite trend in the pattern of labeling. From whole milk through UHT (110°C) samples (Figures 7.2a to 7.2c), the labeling was concentrated within the intermicellar matrix and often appeared as doublets, triplets, and higher order linear multiples. From UHT (120°C) to UHT (140°C) (Figures 7.2d to 7.2f), labeling occurred mainly on the surface of the micelles as well as on some intermicellar material. Complexing of β-lactoglobulin apparently increased at higher temperatures and complexing with the micellar casein occurred at higher UHT treatment. β-Lactoglobulin may initially complex with itself, α-lactalbumin, and/or serum caseins and complex with micellar κ-casein and/or α_{s2}-casein as temperatures increase. β-Lactoglobulin exists primarily as a dimer in the pH range 5.2-7.5 but dissociates into monomers below pH 3.5. The dimer dissociates to monomers and eventually aggregates above pH 8.0 (Whitney, 1988).

In all samples, there was considerable labeling for α_{s1}-casein, especially on the micelles (Figure 7.3). There appeared to be no change in labeling through heat treatment for this protein, and there was labeling throughout the micelles showing an even distribution of α_{s1}-casein. The UHT (140°C) sample had less labeling density near the surface of large micelles which, after UHT-induced complexing, would be predominantly β-lactoglobulin complexed with κ-casein.

From ELISA testing, the antibodies were observed to be active against α_{s2}-casein (with some cross reactivity with α_{s1}-casein) (Leung *et al.*, 1991), but none of our samples labeled for α_{s2}-casein perhaps because serum α_{s2}-casein may have been completely leached from the samples during prelabeling TEM preparation, or because the epitote of both micellar and serum α_{s2}-casein was not accessible to the primary antibody, due to

Figure 7.2. Transmission electron micrographs of milk samples immunogold labeled for β-lactoglobulin **(a)** whole milk, **(b)** pasteurized milk, **(c)** indirect UHT (110°C) milk, **(d)** indirect UHT (120°C) milk, **(e)** indirect UHT (130°C) milk, **(f)** indirect UHT (140°C) milk. The whole milk, pasteurized milk, and UHT (110°C) samples showed labeling concentrated within the intermicellar matrix, but from UHT (120°C) to UHT (140°C) the labeling density was mainly on the surface of the micelles.

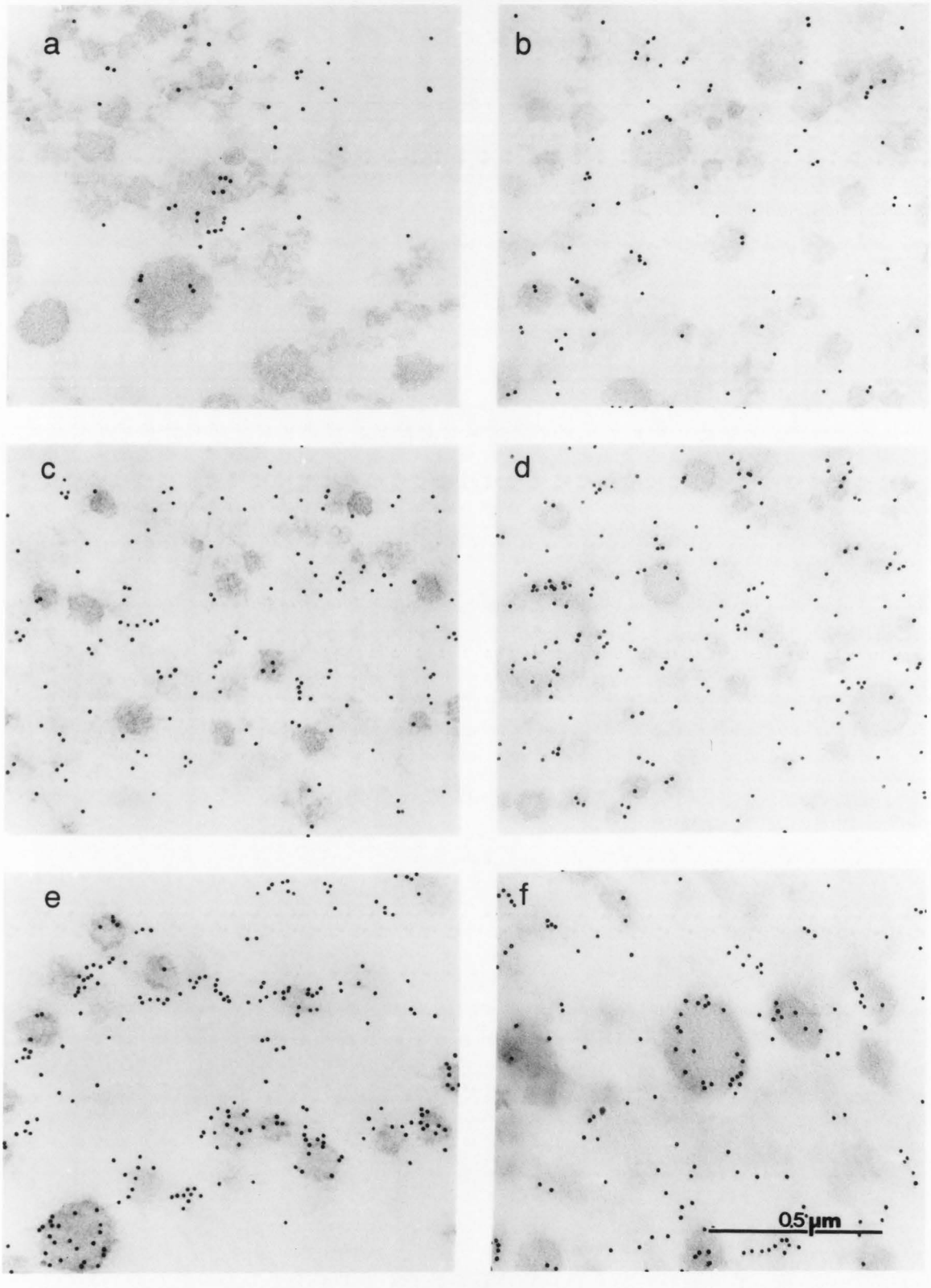
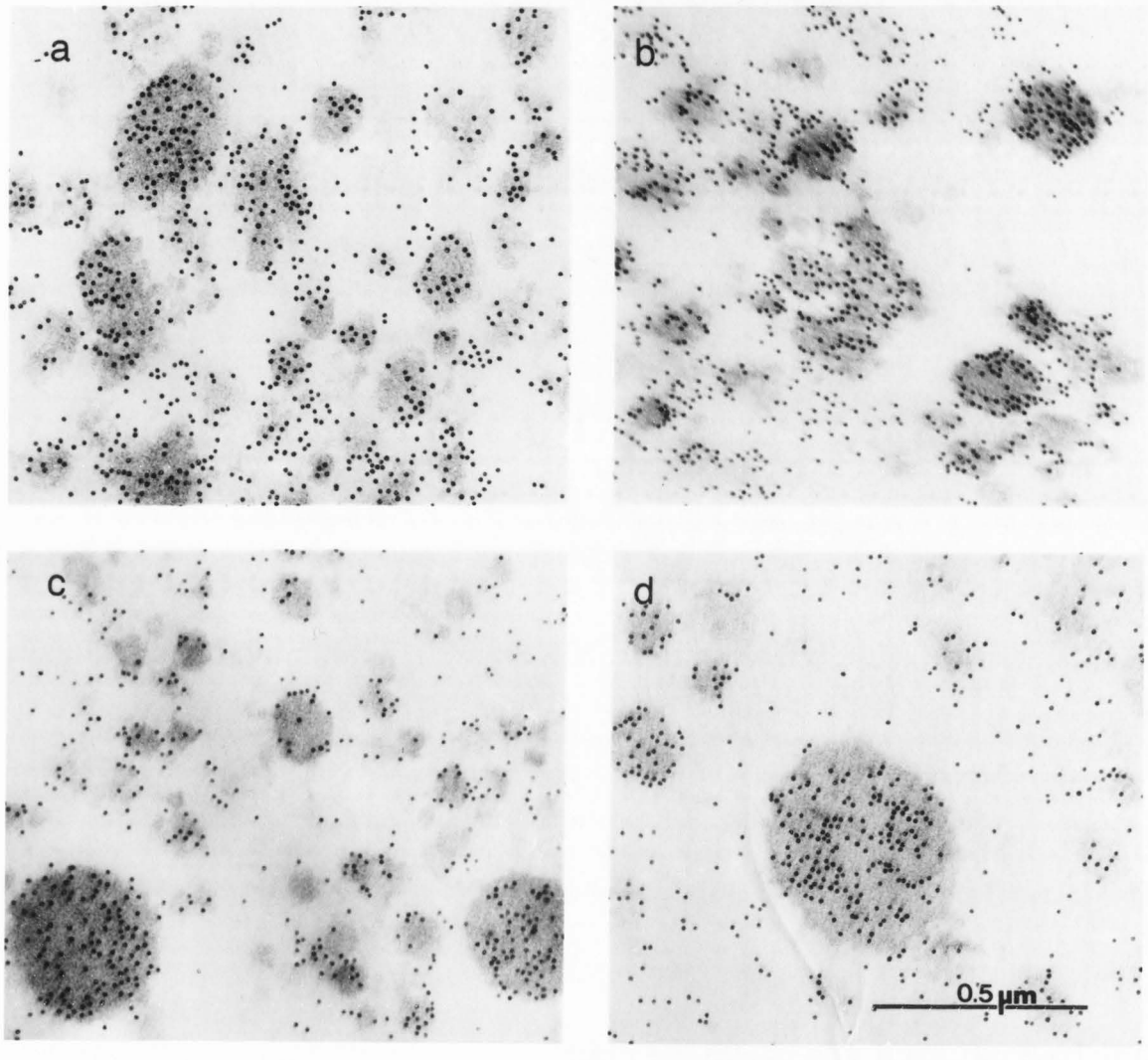

a
b
c
d
e
f
0.5 µm

Figure 7.3. Transmission electron micrographs of milk samples immunogold labeled for α_{S1}-casein **(a)** whole milk, **(b)** pasteurized milk, **(c)** indirect UHT (110°C) milk, **(d)** indirect UHT (140°C) milk. All samples showed heavy labeling for α_{S1}-casein, especially on the micelles.


a
b
c
d
0.5 µm

conformational or steric restrictions. This occurs when the antibody is raised to a different form of the protein than that found in the intact material. Figure 7.4 shows the labeling density on the direct UHT (130°C) sample (a), and the direct UHT (130°C) control sample (b). The degree of labeling in this control was typical of all controls in this experiment.

Labeling for β-casein was similar to that for α_{s1}-casein. In all samples, labeling was concentrated on the micelles (Figure 7.5) as well as some intermicellar labeling of the non-UHT samples. The absence of label from certain areas of the micelles indicated that β-casein was not uniformly distributed. The micelles in the indirect UHT (140°C) sample (Figure 7.5d) appeared to be more heavily labeled than the micelles in the other samples, perhaps because the higher temperature caused serum β-casein to migrate to the micellar surface and participate in complex formation with β-lactoglobulin and κ-casein.

All samples showed sparse labeling concentrated mainly in the intermicellar matrix for κ-casein (Figure 7.6). This suggests that only the serum κ-casein was being labeled. The micellar κ-casein through conformational or steric hindrances was not accessible to the primary antibody. No κ-casein was labeled within the micelle interior, supporting other work (Waugh *et al.*, 1970; Shahani, 1974; Walstra, 1979; Heth and Swaisgood, 1982) which suggested that κ-casein is located predominantly on the micelle surface. Being located on the micelle surface, the κ-casein molecules have a small probability of being oriented correctly to bind with the antibody, since labels attach only to proteins on the sectioned surface. The antibody for κ-casein may have been raised against parts of the κ-casein molecule that were hidden due to aggregation within the micelle surface. The antibody may have been raised against para-κ-casein. Heated samples were more heavily labeled than were the whole milk and skim milk samples due to heat-induced formation of more stable κ-casein/whey protein complexes.

Figure 7.4. **(a)** Transmission electron micrograph of direct UHT (130°C) milk sample immunogold labeled for α_{S2}-casein. None of the samples labeled for α_{S2}-casein. **(b)** Transmission electron micrograph of direct UHT (130°C) control milk sample immunogold labeled. This level of labeling was typical of all the controls in this experiment.

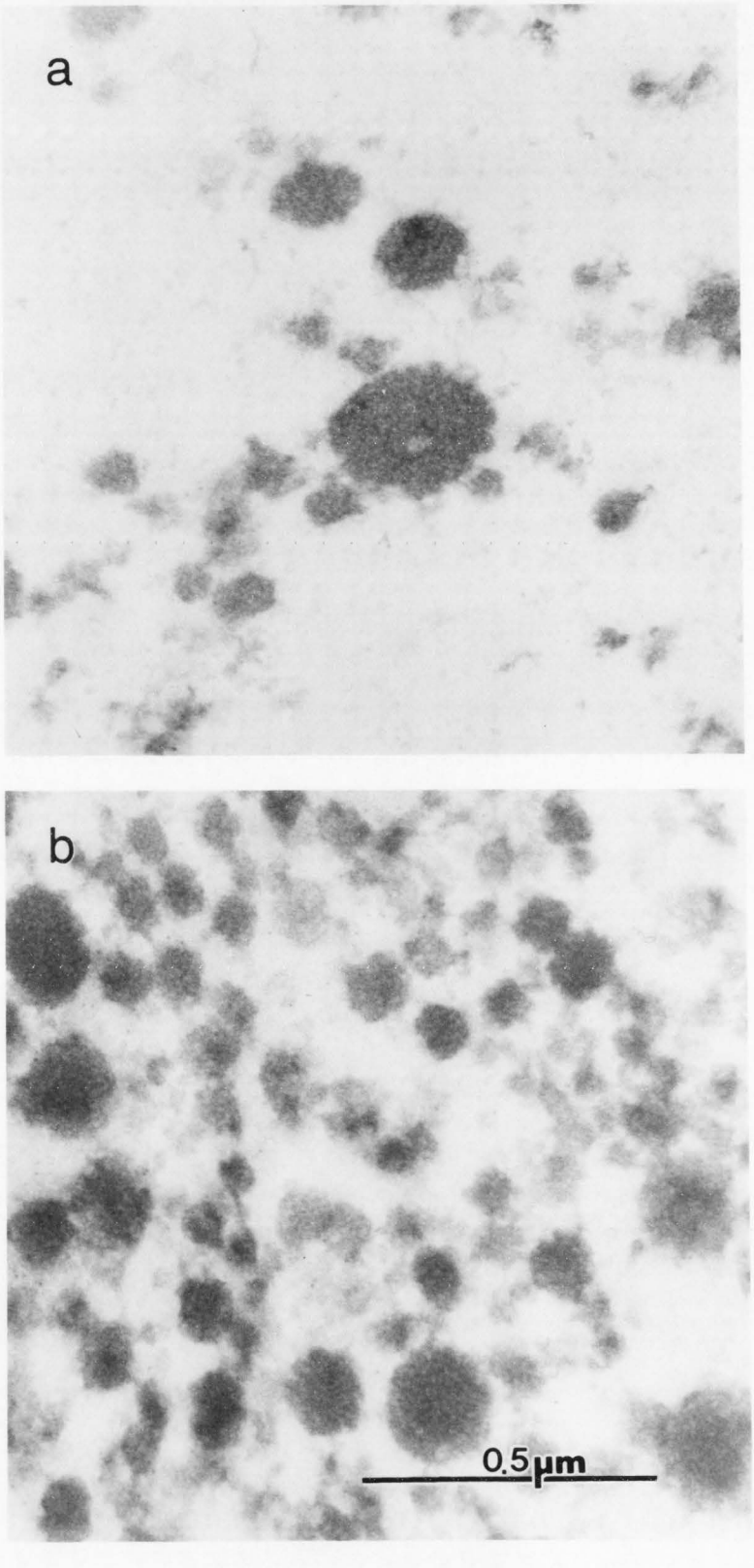

a
b
0.5µm

Figure 7.5. Transmission electron micrographs of milk samples immunogold labeled for β-casein: **(a)** whole milk, **(b)** pasteurized milk, **(c)** indirect UHT (110°C) milk, **(d)** indirect UHT (140°C) milk. All samples showed heavy labeling concentrated on the micelles.

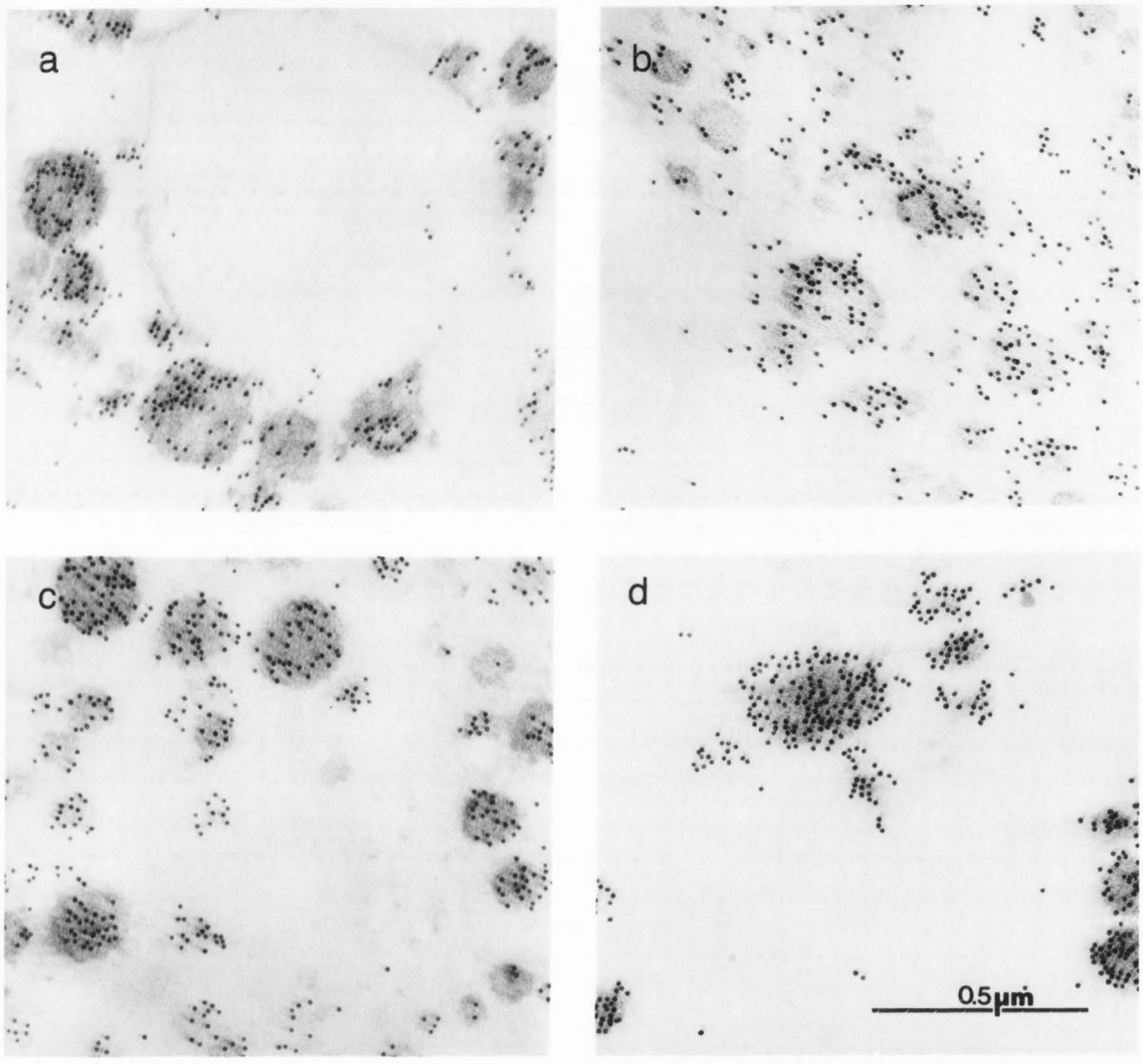
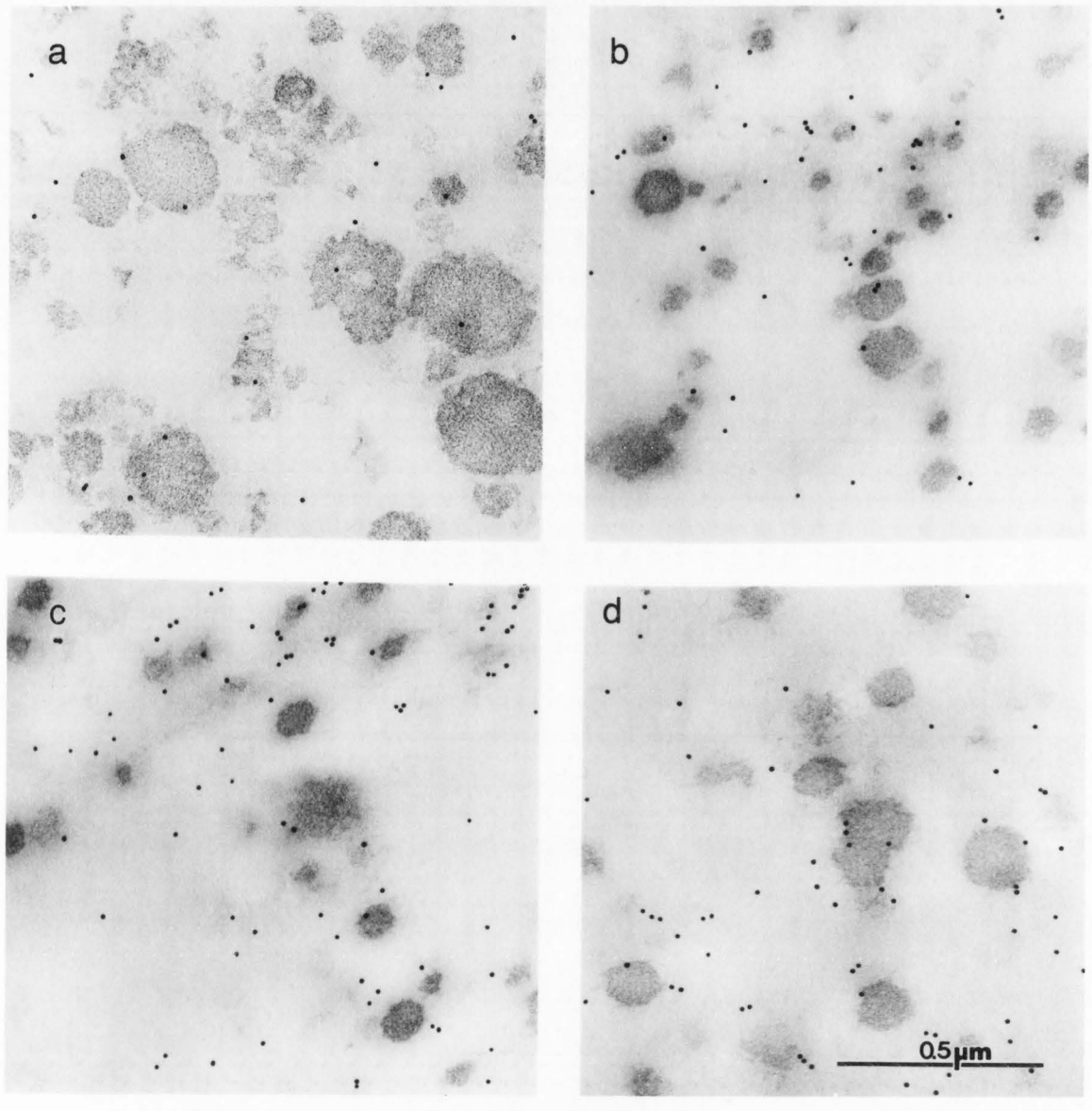

a
b
c
d
0.5µm

Figure 7.6. Transmission electron micrographs of milk samples immunogold labeled for κ-casein: **(a)** whole milk, **(b)** pasteurized milk, **(c)** indirect UHT (110°C) milk, **(d)** indirect UHT (140°C) milk. All samples showed sparse labeling concentrated mainly in the intermicellar matrix for κ-casein.


a
b
c
d
0.5µm

CONCLUSION

We labeled milk proteins by employing immunolocalization techniques. Heating of milk during pasteurization and UHT sterilization affected the distribution and conformational state of some milk proteins, particularly in β-lactoglobulin where heating induced interactions with whey and casein protein. Heating had less effect on α-lactalbumin and κ-casein. Labeling of α_{s1}-casein and β-casein was concentrated on the micelles, and heating did not affect the distribution of these proteins. α_{s2}-Casein did not respond to these immunolocalization procedures.

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CHAPTER VIII

IMMUNOLOCALIZATION OF CASEINS AND WHEY PROTEINS IN ULTRA-HIGH TEMPERATURE-STERILIZED ULTRAFILTERED (3x) MILK CONCENTRATE STORED AT ROOM TEMPERATURE UNTIL GELATION[1]

ABSTRACT

Immunolocalization techniques were employed to elucidate the positions of β-lactoglobulin, α_{S1}-casein, β-casein, and κ-casein in stored UHT-sterilized UF milk retentate from the day of preparation through to age gelation. The milk retentate had been stored at room temperature and sampling done bimonthly. Denatured β-lactoglobulin complexed on the micellar surface during UHT preparation and moved back to the intermicellar spaces during prolonged storage. The labeling of κ-casein was negligible just after UHT preparation, but began to increase with storage time forming linear patterns within the intermicellar matrix. α_{S1}-Casein and β-casein labeled heavily throughout the experiment. Labeling for these two casein moieties appeared very specific for the micelles just after UHT preparation until ~10 months of storage. At this time the labeling appeared on the intermicellar matrix, but still showed high specificity for portions of the micelles. Much of the structural integrity of the micelle was retained in the coagulum. Labeling for α-lactalbumin and α_{S2}-casein was slight and indeterminate.

A mechanism of age gelation of UHT-sterilized UF-concentrated milk was proposed. The loss of the β-lactoglobulin-κ-casein complex from the micelles to the serum exposed the calcium-insoluble micellar α_{S1}-casein and β-casein to the serum calcium. This reduced micelle stability and promoted coalescence of the micellar proteins, leading to coagulation. The tendrillar appendages appeared to be the β-lactoglobulin-κ-casein

[1] Coauthored by M. C. Alleyne and D. J. McMahon.

complex, which entrapped the micellar residues at gelation.

INTRODUCTION

Irreversible age gelation of UHT-sterilized milk concentrates has hindered the commercial application of this process. Gelation occurs when the product is stored at room temperature. The reason for this gelation is uncertain, but it is thought to be the result of casein micelles forming a network (Walstra and Jenness, 1984).

Factors which influence the time taken for age gelation of UHT-sterilized milk concentrates include composition of milk (van Boekel *et al.*, 1989), quality of milk (Adams *et al.*, 1976), severity of heat treatment (Darling, 1980; Fox, 1982), homogenization (Sweetsur and Muir, 1980; Muir, 1984), temperature of storage (Andrews, 1975), concentration of milk (Muir and Sweetsur, 1978; Sweetsur and Muir, 1980; Fox, 1982; McMahon and Brown, 1984), addition of calcium-sequestering agents (Kocak and Zadow, 1985), addition of carbohydrates (Lonergan, 1978; Kudo, 1980; de Wit, 1981), addition of reducing agents (Singh and Fox, 1987a), and enzyme treatment (Snoeren and Both, 1981). Milk gelation is also affected by pH (Singh and Fox, 1985 and 1987b), ionic strength, and calcium and phosphate concentration (Tumerman and Webb, 1965).

Two different mechanisms of age gelation of UHT-sterilized milk have been suggested. One implicates proteolysis of casein, predisposing the micelles to aggregation (Creamer and Matheson, 1980; Snoeren and Both, 1981; Haque *et al.*, 1987). The other involves physico-chemical reactions leading to chemical cross linkages between micelles (Dziuba, 1979; Creamer and Matheson, 1980; Doi *et al.*, 1983).

The proteolysis hypothesis implicates the survival or reactivation of proteolytic enzymes in unconcentrated sterilized milk during storage (Corradini, 1975), leading to age gelation in a manner similar to rennet coagulation of milk in cheese manufacturing (Samel *et*

al., 1971; Hostettler, 1972). The proteolysis hypothesis, however, does not explain age gelation in concentrated sterilized milk (Hostettler, 1972; de Koning *et al.*, 1985).

Physico-chemical processes implicated in age gelation of UHT-sterilized concentrated milk include complex formation between denatured whey proteins and casein (Burton, 1984), the breakdown of κ-casein during storage leading to its inability to stabilize the casein micelle (Singh *et al.*, 1989), changes in calcium and other mineral equilibria during storage affecting the stability of UHT products (Corradini, 1975), polymerization of casein and whey proteins by Maillard-type reactions (Andrews and Cheeseman, 1971; Andrews, 1975), sulfhydryl-disulfide interchange reactions involving various proteins (Patrick and Swaisgood, 1976), a decrease in surface energy of some micelles with time creating an electrostatic difference which promotes aggregation of micelles (Graf and Bauer, 1976), and an increase in nonsedimentable casein due to partial disaggregation of casein micelles leading to micellar interaction (Harwalker, 1982).

Heat sterilization of milk produces several changes in proteins (de Wit, 1981; de Wit and Klarenbeek, 1981; Fox, 1981), enzymes (Snoeren and Both, 1981), and mineral balance (Mattick and Hallett, 1929; Pyne and McHenry, 1955; Tessier and Rose, 1964) that are likely to influence age gelation. Heat treatment results in an association between whey protein and casein (Sawyer, 1969). Whey proteins become denatured and either interact with micellar κ-casein to become sedimentable with casein or coprecipitate (with casein) at the isoelectric point of casein (Hostettler, 1972). The extent of complex formation between denatured β-lactoglobulin and κ-casein significantly modifies the properties of casein micelles. These complex formations are predominantly through disulfide bridges, but hydrophobic and ionic interactions are also involved (Haque *et al.*, 1987; Haque and Kinsella, 1987 and 1988; Hill, 1989). Microstructures seen through electron microscopy show that during storage casein micelles associate increasingly during the period in which viscosity rapidly increases, leading to gelation (Harwalker and

Vreenan, 1978). A great increase in nonmicellar particles occurs with storage, but the effect of this on gelation is not clear (Aoki and Imamura, 1974; Harwalker, 1982).

In this study the localization of the β-lactoglobulin, α-lactalbumin, α_{s1}-casein, α_{s2}-casein, β-casein, and κ-casein in stored samples of direct and indirect UHT-sterilized milk concentrates was achieved using techniques of Alleyne and McMahon (1994a) and Alleyne *et al.* (1994a and b). The objective was to determine the relative positions of these milk proteins in the samples as the milk aged, and thus propose a mechanism for age gelation.

MATERIALS AND METHODS

Reagents

Agar was obtained from Difco Laboratories (Detroit, Michigan); paraformaldehyde, secondary antibody (goat anti-mouse) IgG conjugated to 10 nm gold probes (batch # 9458), and LR White medium grade were obtained from Electron Microscopy Sciences (Fort Washington, Pennsylvania). Teleosteam fish gelatin was obtained from Sigma Chem. Co. (St. Louis, Missouri). Primary antibodies (mouse anti-protein) for proteins, β-lactoglobulin, α-lactalbumin, α_{s1}-casein, α_{s2}-casein, β-casein, and κ-casein, were obtained from J. J. Statsny, University of Illinois, Chicago. These monoclonal antibodies were raised against purified proteins (Kuzmanoff *et al.*, 1990a and b; Kuzmanoff *et al.*, 1991; Kuzmanoff and Beattie, 1991; Leung *et al.*, 1991). All other chemicals were analytical reagent grade.

Samples

Skim milk were obtained from the Gary H. Richardson Dairy Products Laboratory, Utah State University. The skim milk was pasteurized (63°C for 30 minutes), cooled to 50°C, and concentrated by ultrafiltration to 3x (volume reduction) to contain approximately 10% protein. A three-module in-series UF system with spiral wound polysulfone

membranes (Osmonics Inc., Minnetonka, Minnesota; 20 kDa nominal molecular weight cutoff, 15m^2 membrane area) was used to concentrate the skim milk. The concentrated milk was then homogenized (Model 3DDL Homogenizer, Crepaco Inc., Chicago, Illinois) at 13.8 MPa with 3.4 MPa second stage pressure. For indirect and direct heating of UF milk an Alfa-Laval SteriLab® UHT Pilot System was used (McMahon *et al.*, 1993). The concentrated milk was heated to 140°C (held for 4 s) by indirect and direct heat exchange to aseptic conditions. The milk samples were packaged into sterile plastic containers in an Alfa-Laval SteriCab® filling station. Samples were taken immediately and immunolocalization studies conducted on them. The remainder of the samples were stored at room temperature and analyzed bimonthly through immunolocalization studies. The last analysis was conducted after 12 months of storage, at which time gelation had occurred.

Electron Microscopy and Immunogold Labeling

The microcube technique (Alleyne *et al.*, 1993) was used to contain the milk samples in agar gel capsules for electron microscopy. The agar gel capsules were fixed at 20°C for 4 h in 4% formaldehyde in .1 M phosphate buffer (pH 6.6) (Alleyne *et al.*, 1994a and b). After the fixation had been completed, these samples were washed with .1 M phosphate buffer, pH 6.6, and dehydrated in a graded ethanol series of 50, 70, 95, and 100% ethanol, infiltrated with LR White resin, and polymerized at 50°C for 21 h. Thin sections (90 nm thick, Sorvall MT-2 Porter-Blum ultramicrotome), from the LR White blocks, were collected on nickel grids. These were floated for 15 min on the blocking agent (.1% fish gelatin with .1% normal goat serum in 20 mM Tris buffer without bovine serum albumin) at pH 6.6 and rinsed for 5 min in 50 mM Tris saline buffer (pH 7.4) (Zymed, 1991). They were floated on the primary antibody, mouse anti-protein (for proteins: α-lactalbumin, β-lactoglobulin, α_{s1}-casein, α_{s2}-casein, κ-casein, and β-casein), and refrigerated for 24 h in a humidity chamber. (ELISA was used to determine the

appropriate dilutions of antibodies in 50 mM Tris saline buffer for each protein. An antibody:buffer ratio of 1:2000 was optimum for β-lactoglobulin, α_{S1}-casein, and β-casein, and 1:1000 was optimum for α-lactalbumin, α_{S2}-casein, and κ-casein.) The control was floated on phosphate buffered saline in place of the primary antibody in the humidity chamber. All grids were rinsed for 5 min in 50 mM Tris saline buffer (pH 7.4) (Zymed, 1991) and floated on the secondary antibody (goat anti-mouse) IgG conjugated to 10 nm gold probes in buffer, ratio 1:38 (antibody:50 mM Tris saline buffer) in a humidity chamber at room temperature for 3 h. Grids were rinsed (6 x 5 min) with double-distilled water and stained with uranyl acetate (20 min) followed by lead acetate (5 min) (Youssef, 1985). Transmission electron microscopy (TEM) was carried out using a Zeiss CEM 902 electron microscope operated at 80 kV. Micrographs were taken on Kodak SO 163 sheet film and printed on Kodak polycontrast III RC paper.

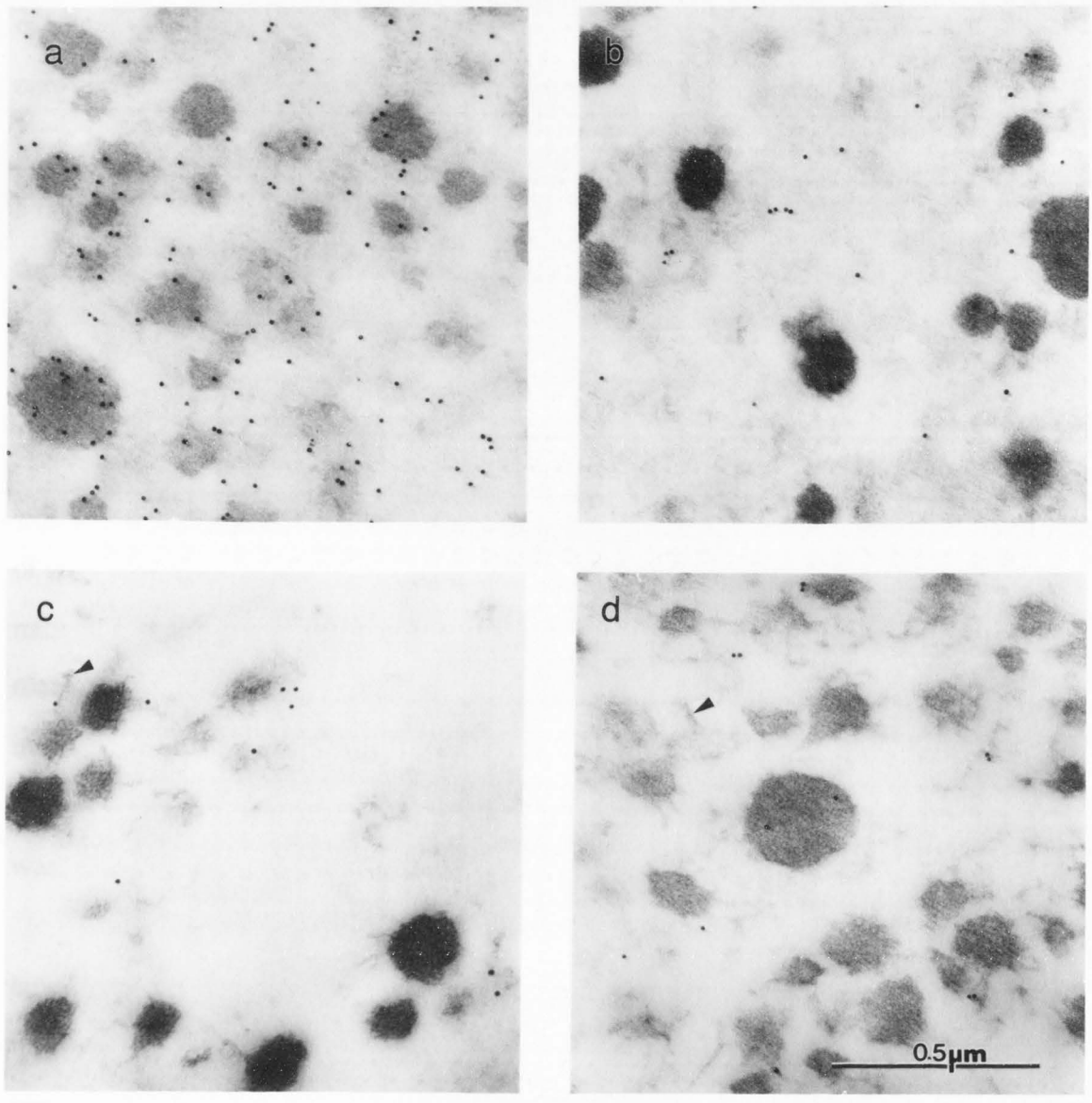
RESULTS AND DISCUSSION

Direct UHT samples began to gel an average 2 months earlier than indirect UHT samples, but indirect UHT samples showed a higher degree of labeling than the corresponding direct UHT samples. This reflected the greater degree of heat received by the indirect UHT samples through a longer exposure to heating which leads to more denaturation of whey proteins. Consequently, there was a higher degree of complexing (hence labeling) through denatured whey proteins. Both UHT treatments produced the same trends for all proteins.

Labeling for α-lactalbumin (Figure 8.1) showed the highest intensity of labeling immediately after UHT-sterilization (0 month of storage). Labeling at month 4, 8, and 12 was very sparse. This suggested that the complexed α-lactalbumin formed at UHT (140°C) sterilization breaks down with time, leading to leaching of the uncomplexed

Figure 8.1. Transmission electron micrographs of immunogold-labeled, stored, UHT-sterilized (140°C) milk concentrate, showing labeling for α-lactalbumin **(a)** month 0, **(b)** month 4, **(c)** month 8, **(d)** month 12. The highest intensity of labeling was at 0 month of storage. Labeling at month 4, 8, and 12 is very sparse.

arrows: tendrillar appendages connecting casein micelles.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α-lactalbumin during prelabeling TEM preparation. The known reversibility of conformational changes in α-lactalbumin brought about by heat processing supports this hypothesis. These aggregates would not be disulfide linked and, therefore, could revert to a nonaggregated state upon cooling. Alternatively, the complex formed may undergo further time-dependent aggregation, leading to the unavailability of the epitote for labeling.

Labeling for β-lactoglobulin was relatively heavy in all samples (Figure 8.2). At the beginning of storage (Figures 8.2a) the labeling was mainly associated with the surface of the micelles . At 4 months of storage the labeling was still associated with the micellar surfaces, but the intermicellar matrix was also well labeled. At 8, 10, and 12 months of storage the bulk of the labeling was on the intermicellar matrix with very little on the micellar surfaces. This gradual transfer of labeling from the micelles to the intermicellar matrix suggested a movement of the initial β-lactoglobulin-κ-casein complex into the intermicellar matrix on aging. The labeling, however, did not appear specifically on the tendrillar appendages between micelles in the gelled samples. This suggested that there was more intermicellar material than had been previously observed in electron micrographs.

All samples labeled heavily for α_{s1}-casein (Figure 8.3). At months 0 through 8 labeling was almost exclusively on the micelles, but at months 10 and 12 (at which time the milk concentrate had gelled) the micelles appeared less heavily labeled, and there was increased labeling in the intermicellar matrix. This suggested that on gelation some of the α_{s1}-casein migrated from the casein micelles to the intermicellar spaces.

Although immunolocalization for α_{s2}-casein was conducted, none of the samples showed labeling for α_{s2}-casein (Figure 8.4). The level of labeling observed in the control (Figure 8.4b) was typical for all the controls in this experiment.

All samples labeled heavily for β-casein (Figure 8.5). The absence of label from certain areas of the micelles indicated that β-casein was not uniformly distributed within the

Figure 8.2. Transmission electron micrographs of immunogold-labeled, stored, UHT-sterilized (140°C) milk concentrate, showing labeling for β-lactoglobulin **(a)** month 0, **(b)** month 2, **(c)** month 4, **(d)** month 8, **(e)** month 10, **(f)** month 12. At 0 and 2 months of storage the labeling was mainly associated with the surface of the micelles. At 4 months storage the labeling was still associated with the micellar surfaces, but the intermicellar matrix was also well labeled. At 8, 10, and 12 months storage the bulk of the labeling was on the intermicellar matrix with very little on the micellar surfaces.
arrows: tendrillar appendages connecting casein micelles.

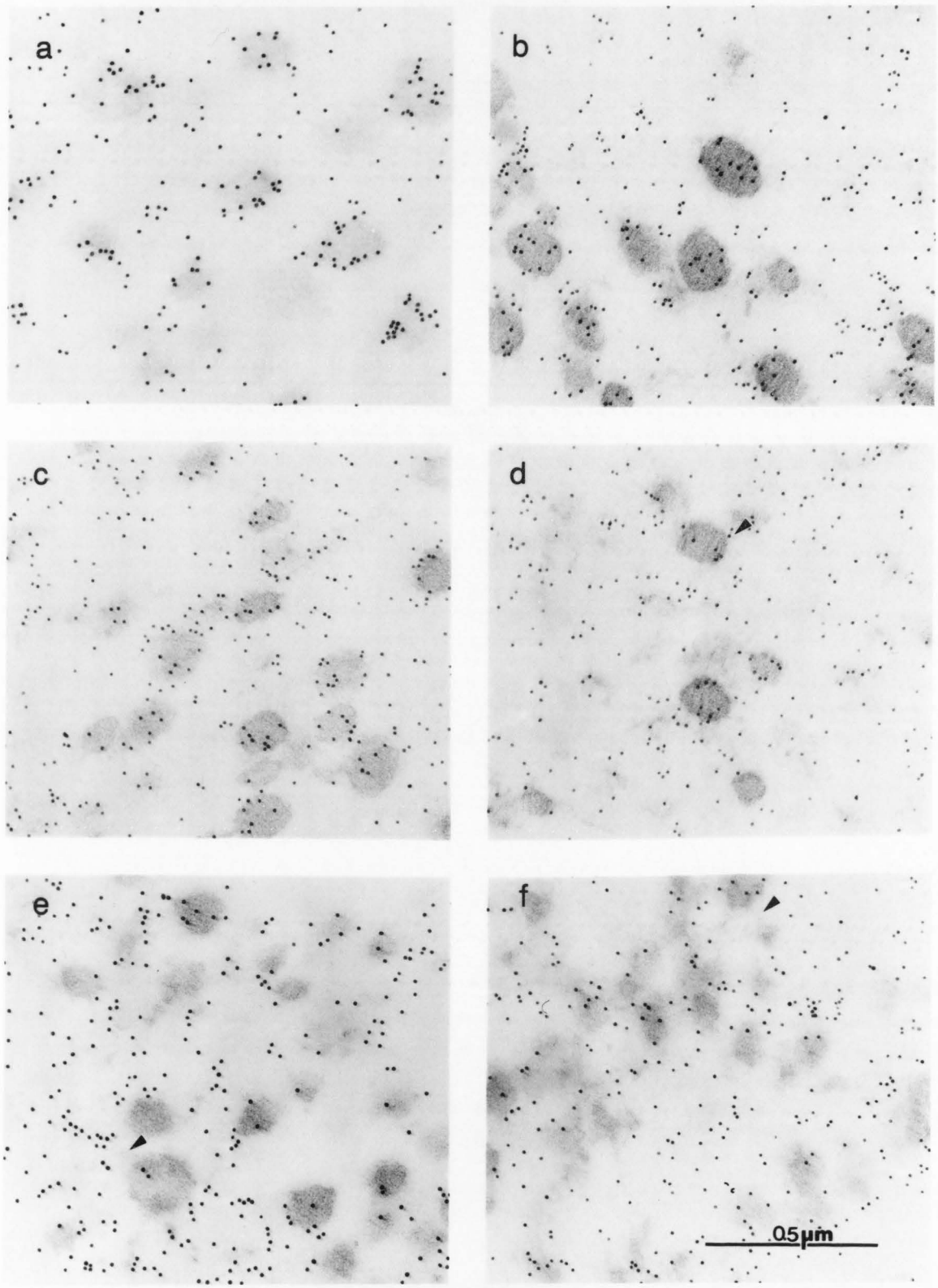
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Figure 8.3. Transmission electron micrographs of immunogold-labeled, stored, UHT-sterilized (140°C) milk concentrate, showing labeling for α_{S1}-casein **(a)** month 0, **(b)** month 8, **(c)** month 10, **(d)** month 12. At months 0 through 8 labeling was almost exclusively on the micelles, but at months 10 and 12 the micelles appeared less heavily labeled and there was increased labeling in the intermicellar matrix.
arrows: tendrillar appendages connecting casein micelles.

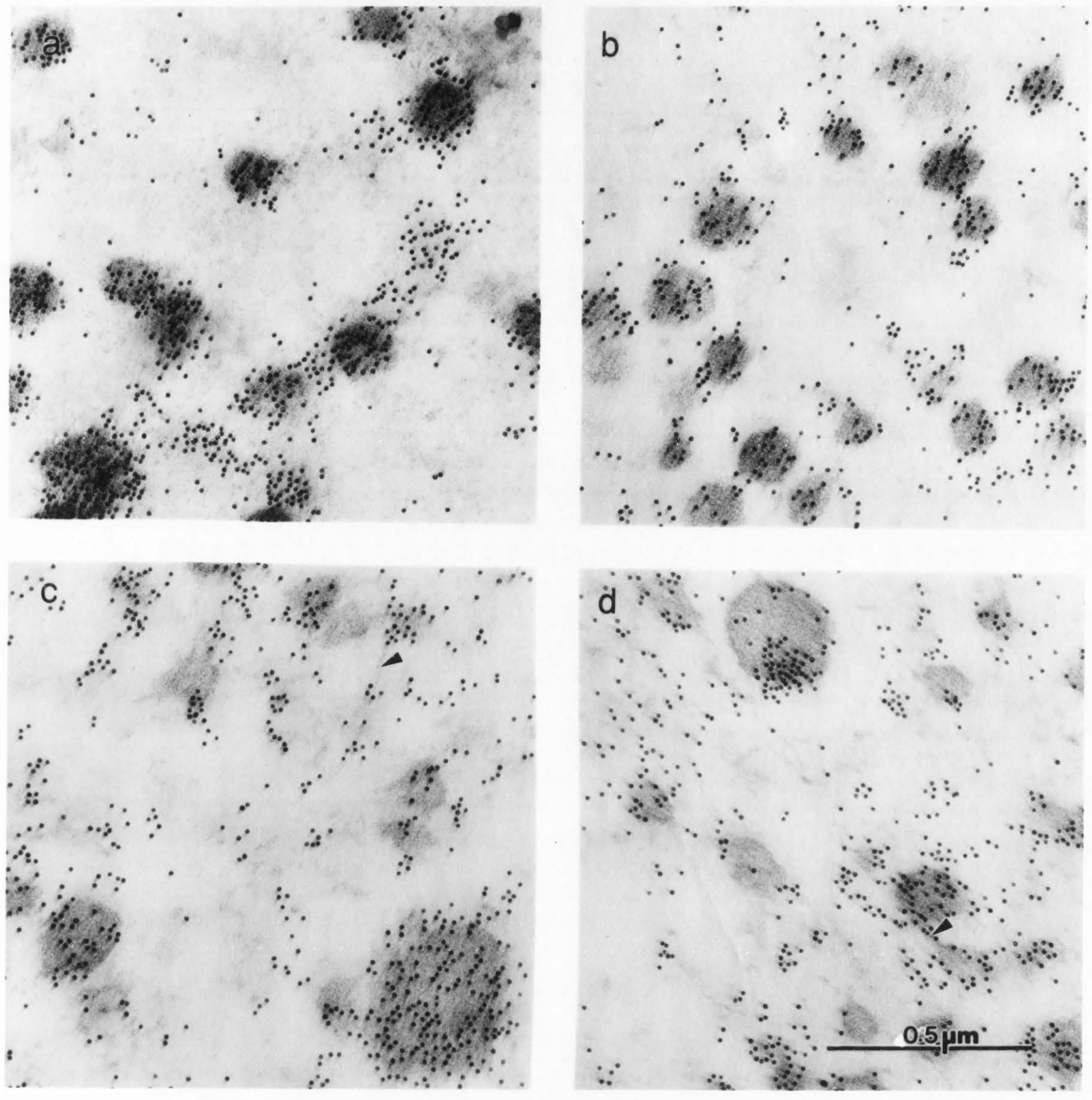
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Figure 8.4. **(a)** Transmission electron micrograph of 8 month milk sample immunogold labeled for α_{S2}-casein. None of the samples labeled for α_{S2}-casein. **(b)** Transmission electron micrograph of 8 month milk sample control immunogold labeled. This level of labeling was typical of all the controls in this experiment.
arrows: tendrillar appendages connecting casein micelles.

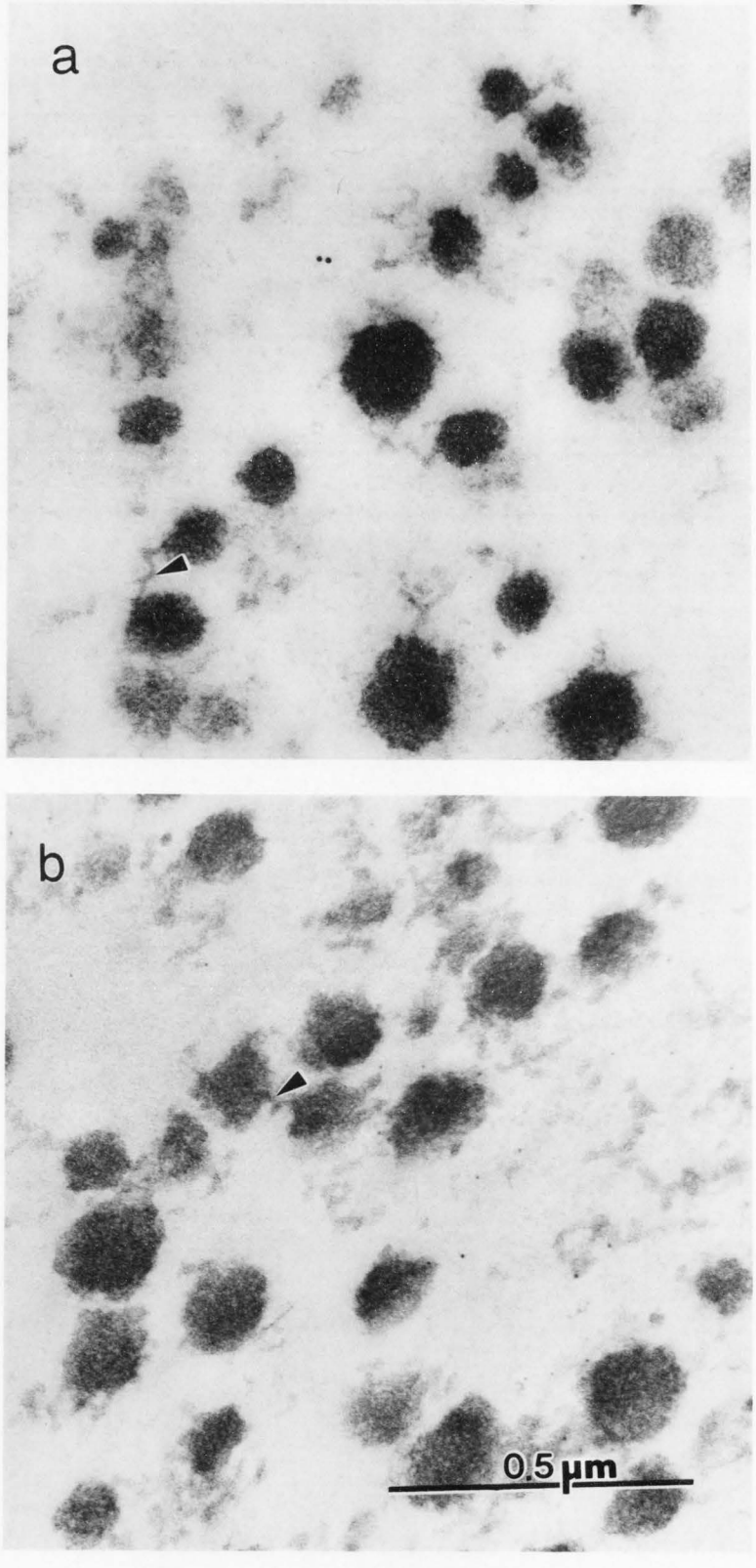
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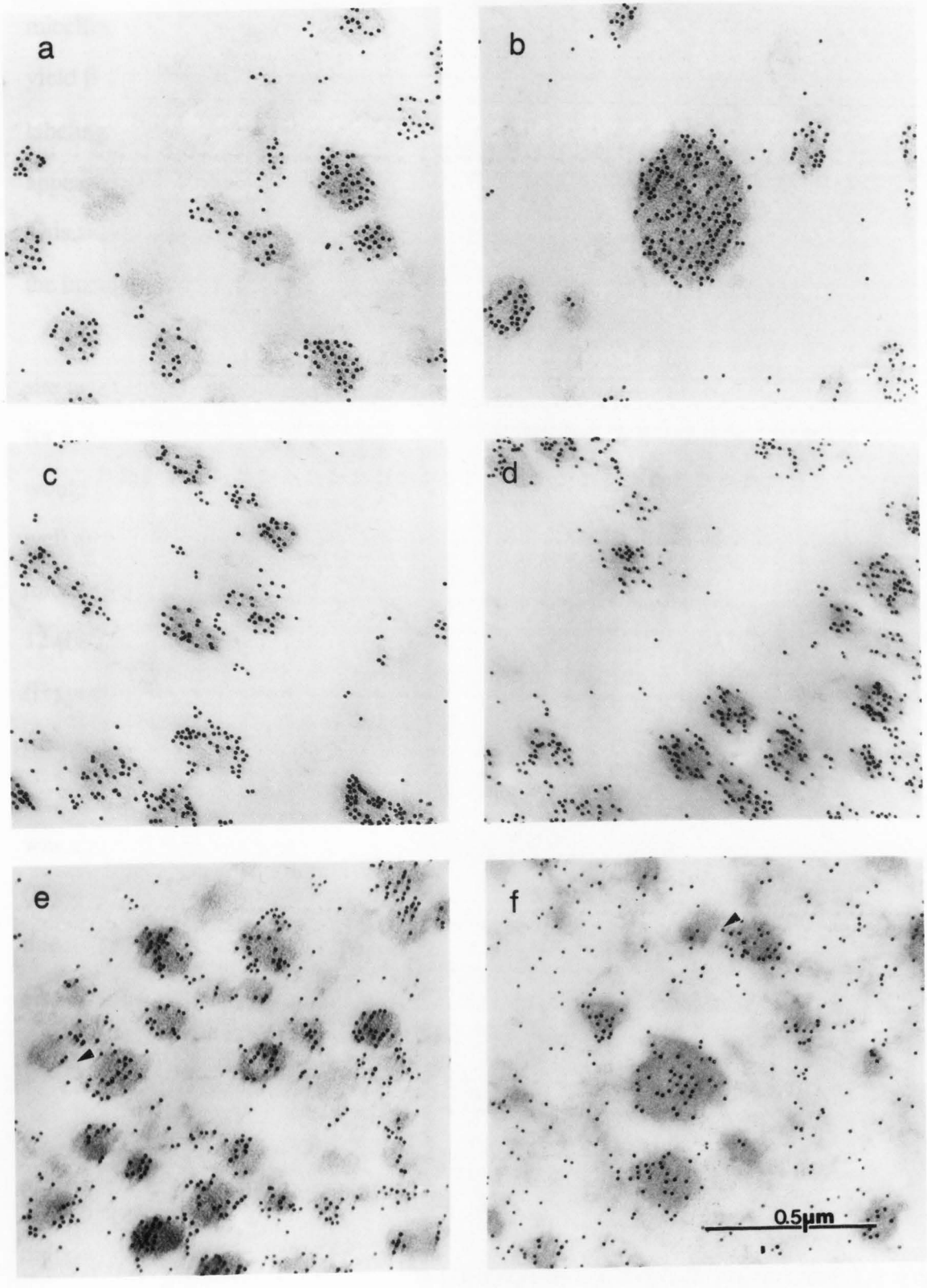
Figure 8.5. Transmission electron micrographs of immunogold-labeled, stored, UHT-sterilized (140°C) milk concentrate, showing labeling for β-casein **(a)** month 0, **(b)** month 4, **(c)** month 6, **(d)** month 8, **(e)** month 10, **(f)** month 12. At months 0 through 8 labeling was almost exclusively on the micelles, but at months 10 and 12 the micelles appeared less heavily labeled and there was increased labeling in the intermicellar matrix. arrows: tendrillar appendages connecting casein micelles.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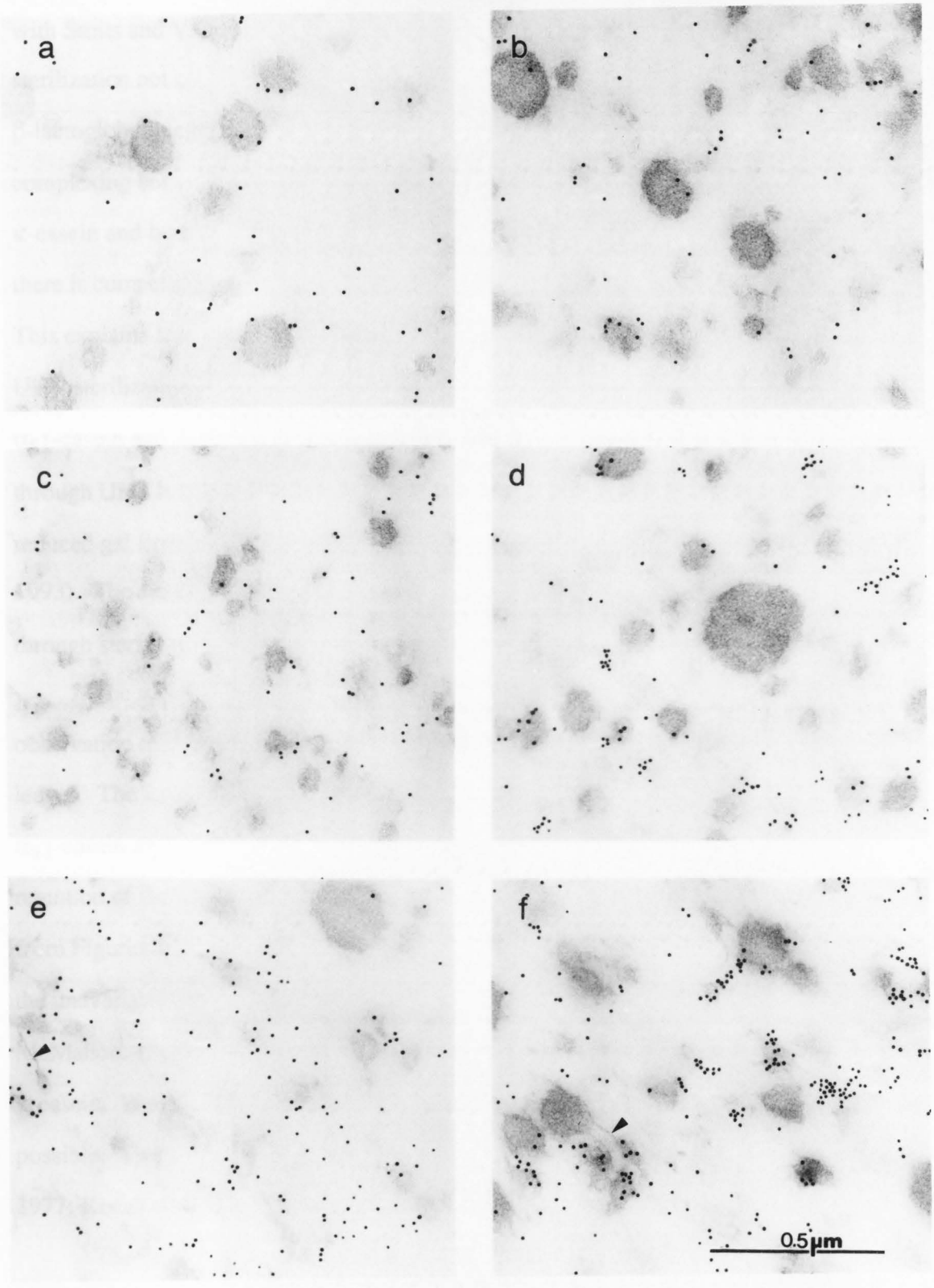
micelles. This may also be evidence of proteolysis of β-casein by residual proteinases to yield β-casein fragments (Pearce, 1980; Swaisgood, 1982). At months 0 through 8 labeling was almost exclusively on the micelles, but at months 10 and 12 the micelles appeared less heavily labeled, and there was increased labeling in the intermicellar matrix. This suggested that on gelation some of the β-casein migrated from the casein micelles to the intermicellar spaces.

Labeling for κ-casein increased as the samples aged (Figure 8.6) indicating that the site recognized by the antibodies became more available. This suggested that the antibody for κ-casein may have been raised against para-κ-casein which, in native casein micelles, would be embedded within the micelles. The labeling often traced out linear patterns as well as clumps within the intermicellar matrix. A few tendrillar appendages, between micelles, were seen at months 0-8 with a proliferation of these structures at months 10 and 12 (Figure 8.6). Months 0-4 showed sparse labeling within the intermicellar matrix (Figures 8.6a, 8.6b, and 8.6c). Months 6 and 8 showed heavier labeling within the matrix mainly between adjacent micelles (Figures 8.6d and 8.6e). The labeling at month 10 was very dispersed, but showed a pattern of linkage between micelles (Figure 8.6f). This trend was accentuated at month 12, showing linear clusters of labeling between adjacent micelles (Figure 8.6g). There was no definite labeling on the tendrillar appendages between linked micelles. These would be expected to consist of κ-casein and β-lactoglobulin, but are embedded within the section, and labeling only occurs against proteins that extend from the surface of the sections.

The defined movement of κ-casein and β-lactoglobulin on storage seemed to be connected to the process of age gelation. The results suggested that there was interaction between these proteins producing a complex. The pattern of β-lactoglobulin moving onto the micelles through heating and then gradually moving away into the serum during storage may indicate a destabilizing influence of the β-lactoglobulin on the κ-casein in agreement

Figure 8.6. Transmission electron micrographs of immunogold-labeled, stored, UHT-sterilized (140°C) milk concentrate, showing labeling for κ-casein **(a)** month 0, **(b)** month 2, **(c)** month 4, **(d)** month 8, **(e)** month 10, **(f)** month 12. The degree of labeling increased as the samples age, tracing out linear patterns within the intermicellar matrix. Tendrillar appendages between micelles were seen as early as month 0 through 8 with a proliferation of these structures at months 10 and 12.

arrows: tendrillar appendages connecting casein micelles.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with Smits and Van Brouwershaven (1980), and Farrell and Douglas (1983). UHT sterilization not only denatures β-lactoglobulin, but would catalyze the reaction of β-lactoglobulin and κ-casein, leading to the successful formation of the complex. Since complexing between κ-casein and β-lactoglobulin is impeded by complexing between κ-casein and both α_{S1}-casein and β-casein (Doi *et al.*, 1979), the converse is also true, and there is competition occurring amongst these three proteins for binding sites on κ-casein. This explains how the successful binding of β-lactoglobulin to κ-casein (promoted by the UHT-sterilization treatment) destabilizes the casein micelle relative to κ-casein binding to α_{S1}-casein and β-casein. The complexing of β-lactoglobulin with micellar κ-casein through UHT heating explains why there is an increase in rennet-coagulation time and reduced gel firmness of gels made from UHT-sterilized concentrated milk (McMahon *et al.*, 1993). The attachment of β-lactoglobulin impedes the rennet proteolysis of κ-casein through steric interference or conformational alteration of the casein protein.

The movement of all micellar casein into the intermicellar spaces on gelation is an observation of the gelling process where the contents of the micelle coagulate as κ-casein leaves. The loss of κ-casein from the micelle to the serum resulted in calcium-insoluble α_{S1}-casein and β-casein being exposed to the serum calcium ions. There was, however, retention of the integrity of α_{S1}-casein and β-casein in the micellar residue as evidenced from Figures 8.3 and 8.5. Micellar κ-casein did not label in these experiments, suggesting the unavailability of its epitote to the antibody as discussed previously (Alleyne and McMahon, 1994b). This indicated that the antibodies may have been raised against para-κ-casein. When the κ-casein leaves the micelle the epitote is exposed, and labeling is then possible. The lowering of pH of the UHT-sterilized milk on storage (Andrews *et al.*, 1977; Kocak and Zadow, 1985) may also play a role in age gelation (Bringe, 1988).

The linear labeling observed for κ-casein suggested that the tendrillar appendages are the β-lactoglobulin-κ-casein complex that dissociated from the micelles. There was no

definite labeling of these appendages for κ-casein or β-lactoglobulin because of their size and distribution within the milk sample. The tendrillar appendages are cylindrical structures ~10 nm in diameter and between 20-100 nm in length. The thickness of section used in these TEM preparation is ~90 nm and the visible intact tendrils appear to be beneath the resin and thus inaccessible to the primary antibodies. In cases where the tendrils are sectioned and available at the surface for labeling, labeling does appear, but the very thin remnant of the tendril is almost invisible in the matrix.

CONCLUSION

UHT sterilization of UF-concentrated milk denatured β-lactoglobulin, which formed complexes with micellar κ-casein. This interaction destabilized the κ-casein, predisposing it to dissociation from the micellar moiety. Consequently, the calcium-insoluble α_{S1}-casein and β-casein were exposed to the calcium ions in the serum leading to the coalescence of these molecules and some dissociation from the micelles. There was retention of the physical integrity of α_{S1}-casein and β-casein within the micellar residue.

The tendrillar appendages are the β-lactoglobulin-κ-casein complex remnants that are still physically associated with the surface of the coagulated micellar residues. Specific labeling of the appendages was difficult to observe because of the minute size and distribution of these structures within the sectioned milk sample. κ-Casein left the micelles through aging as a result of weakening of bonds with the rest of the casein micelle, brought about by the competitive binding of denatured β-lactoglobulin to κ-casein.

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CHAPTER IX

GENERAL SUMMARY

1. Transmission electron microscopy, utilizing the microcube encapsulation technique and protocols for immunolocalization of milk proteins, provided insight into the phenomenon of age gelation of UHT-sterilized UF concentrated milk.

2. Antibodies specific for the native as well as the complexed forms of milk proteins elucidated the positions of the milk proteins in various samples. These samples included fresh milk, skim milk, UF concentrated milk, heated, concentrated milk, UHT-sterilized, concentrated milk, and stored, UHT-sterilized, concentrated milk.

3. Heating of milk through pasteurization and UHT-sterilization affected the distribution and altered the native state of some milk proteins. Denatured β-lactoglobulin and α-lactalbumin formed complexes within the serum as well as with the casein components of the micelles.

4. Complexing of β-lactoglobulin and κ-casein destabilized the κ-casein molecule. This compromised the role of κ-casein in stabilizing the other casein proteins within the micellar moiety, leading to a time-dependent dissociation of κ-casein from the micelle. The remnants of the β-lactoglobulin-κ-casein complex constitute the tendrillar appendages observed in gelled milk.

5. The loss of κ-casein from the micelles to the serum exposed the calcium-insoluble micellar α_{s1}-casein and β-casein to the serum calcium. This resulted in their partial dissociation from the micelles and participation in gelation. The binding of denatured β-lactoglobulin to the κ-casein molecule apparently weakened the attachment of κ-casein to other casein fractions of the micelle.

APPENDICES

Permission Letter #1

November 10, 1993
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Permission Letter #2

November 10, 1993
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Dr. DJ McMahon
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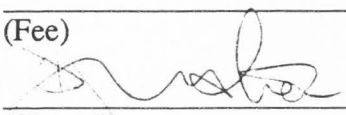

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Permission Letter #3

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Tel. no. (801) 750-3664

Dr. NN Youssef
Dept. of Biology
Utah State University
Logan, UT 84322-5500

Dear Dr. Youssef,

I am in the process of preparing my dissertation in the Nutrition and Food Sciences Department at Utah State University. I hope to complete in the Fall of 1993.

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(Alleyne MC, McMahon DJ, Youssef NN, Hekmat S. (1993). An apparatus for a new microcube encapsulation of fluid milk in preparation for transmission electron microscopy. Food Structure 12, 21-30).

_____
(Fee)

Nabil M. Youssef
(Signed)

Permission Letter #4

November 10, 1993
Mark Christopher Alleyne
Dept. Nutri. & Food Sciences
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Logan, UT 84322-8700
Tel. no. (801) 750-3664

S Hekmat
Dept. Nutri. & Food Sciences
Utah State University
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Dear Mrs. Hekmat,

I am in the process of preparing my dissertation in the Nutrition and Food Sciences Department at Utah State University. I hope to complete in the Fall of 1993.

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N A
(Fee)

Shirah Hekmat
(Signed)

Permission Letter #5

November 10, 1993
Mark Christopher Alleyne
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W. McManus
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Utah State University
Logan, UT 84322-5500

Dear Mr. McManus,

I am in the process of preparing my dissertation in the Nutrition and Food Sciences Department at Utah State University. I hope to complete in the Fall of 1993.

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(Alleyne MC, McMahon DJ, McManus W. (1994). Comparing antigenicity of β-lactoglobulin in ultra-high temperature-sterilized ultrafiltered (3x) milk concentrate and whey after different modes of aldehyde fixation. Food Struc. -Approved for submission.)

(Alleyne MC, McMahon DJ, McManus W. (1994). Characterization of immunolocalized β-lactoglobulin and α-lactalbumin in whey samples. Food Struc. -Approved for submission.)

(Fee)

(Signed)

Permission Letter #6

November 10, 1993
Mark Christopher Alleyne
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Tel. no. (801) 750-3664

L. Burgess
Dept. of Biology
Utah State University
Logan, UT 84322-5500

Dear Mr. Burgess,

I am in the process of preparing my dissertation in the Nutrition and Food Sciences Department at Utah State University. I hope to complete in the Fall of 1993.

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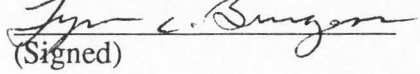
(Fee)



(Signed)

VITA

Mark Christopher Alleyne

Candidate for the Degree of Doctor of Philosophy

Utah State University

Dissertation: Fate of β-lactoglobulin, α-lactalbumin, and casein proteins in ultrafiltered concentrated milk after ultra-high temperature processing

Major Field: Nutrition and Food Sciences

Education:

1976-1979	B.S. Faculty of Natural Sciences University of the West Indies, Cave Hill Campus, Barbados Majors: Chemistry and Biology; 2nd Class Honors
1986-1988	MPhil. Chemistry Department University of the West Indies, Cave Hill Campus, Barbados Major: Chemistry European Development Fund Scholar
1990-1993	Ph.D. Department of Nutrition and Food Sciences Utah State University (USU), Logan, Utah Organization of American States Fellow; College of Family Life (USU) Widtsoe Scholar

Membership:

-Institute of Food Technologists
-American Dairy Science Association
-Microscopy Society of America
-American Association for the Advancement of Science
-National Wildlife Federation
-Phi Kappa Phi National Honor Society
-Phi Upsilon Omicron Honor Society
-Smithsonian Associates

EXPERIENCE:

1979-1984 CXC Chemistry and Biology Secondary School Teacher: Ministry of Education, Barbados.
1984-1985 Product Engineer: Corcom Electronics Industry, Barbados.
1985-1986 Private Tutor: Chemistry and Biology to Cambridge O' and A' level.
1986-1988 Research/Teaching Assistant: University of the West Indies, Cave Hill Campus, Barbados. Conducted research in Solar Crop Drying. Teaching assistant for Chemistry.
1987-1989 Assistant Examiner: Caribbean Examination Council (CXC), Barbados.
1988-1990 Chemistry Teacher: Queen's College, Barbados. Cambridge A' level .
1990-1993 Research /Teaching Assistant: Dept. of NFS, Utah State University.

Publications:

-The drying of the fruit of the dunk plant, Ziziphus Mauritiana. Report of the Meeting of Lab-Based Institutions, Wildey Conference Center, Caribbean Development Bank, Dec. 1-4, 1987.

-(1993). An apparatus for a new microcube encapsulation of fluid milk in preparation for transmission electron microscopy. Food Struc. 12, 21-30.

-(1994). Comparing antigenicity of β-lactoglobulin in ultra-high temperature-sterilized ultrafiltered (3x) milk concentrate and whey after different modes of aldehyde fixation. Food Struc. (Approved for submission).

-(1994). Characterization of immunolocalized β-lactoglobulin and α-lactalbumin in whey samples. Food Struc. (Approved for submission).

-(1994). Immunolocalization of β-lactoglobulin in processed milk, yogurt and cheese samples. Food Struc. (Approved for submission).

-(1994). Immunolocalization of caseins and whey proteins in milk at various stages of treatment from fresh whole milk to ultra-high temperature-sterilized ultrafiltered (3x) milk concentrate. J. Dairy Sci. (Approved for submission).

-(1994). Immunolocalization of caseins and whey proteins in ultra-high temperature-sterilized ultrafiltered (3x) milk concentrate stored at room temperature until gelation. J. Dairy Sci. (Approved for submission).

Seminars and Workshops:

-Short School and Workshop, "Materials, Science, Energy and Development" U.W.I., Mona, Jamaica - March 30-April 10, 1987.

-Meeting of Lab-Based Institutions, Caribbean Development Bank, Wildey, Barbados - December 1-4, 1987.

-Teaching Assistant Workshop, Utah State University - Sept. 13-19, 1990.

-American Dairy Science Association 86th Annual Meeting, Utah State University, Utah, August 12-15, 1991.

-Latin America and Caribbean Countries Leadership Seminar, Florida International University, Miami, May 18-29, 1993.

Personal:

Born in Barbados, West Indies
Married
One daughter.