A Study of the Interactions Between Milk Proteins and Soy Proteins

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A STUDY OF THE INTERACTIONS BETWEEN MILK PROTEINS AND SOY PROTEINS

by

Venkatachalam Narayanaswamy

A dissertation submitted in partial fulfillment of the requirements for the degree of DOCTOR OF PHILOSOPHY in Nutrition and Food Sciences

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ABSTRACT

A Study of the Interaction Between Milk Proteins and Soy Proteins

by

Venkatachalam Narayanaswamy, Doctor of Philosophy
Utah State University, 1997

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

This research investigates the protein interactions that occur when soy protein is added to milk and subjected to renneting or heating. Milk was fortified with 20% soy protein and enzymic coagulation studied at 35°C at various pH's and CaCl₂ levels. The first part deals with the interaction between milk and soy proteins during rennet-induced milk coagulation. The first goal was to determine how soy proteins affected milk coagulation. The effects of native versus heat-denatured soy proteins on rennet coagulation time and curd firmness were compared. Immunogold labeling along with transmission electron microscopy was used to identify and localize soy proteins in coagulated milk. Partitioning of β-conglycinin and glycinin, the two main soy protein fractions, between cheese and whey was determined by electrophoresis.

Soy proteins affected milk coagulation to the greatest extent at pH 6.6. Both heat-denatured and native soy proteins increased rennet coagulation time. Only heat-denatured soy proteins affected final curd firmness. Most of β-conglycinin was lost in whey, whereas glycinin was retained in curd.
Soy proteins existed in the curd as aggregates that were less electron dense than casein micelles. At pH 6.6, heat-denatured soy proteins were fibrous and adhered to the surfaces of casein micelle, preventing direct micelle-micelle contact. This would delay aggregation rate and decrease curd firmness by decreasing the number and strength of links between casein micelles. Native soy proteins did not bind to the casein micelles but rather were physically trapped within curd. Their effect of delaying aggregation is thought to be a function of their binding of calcium. Adding CaCl$_2$ or lowering the pH to 6.3 or 6.0 helped restore coagulation properties.

The second goal was to determine what heat-induced interaction occurs between milk and soy proteins, specifically between $\kappa$-casein and glycinin. Both $\kappa$-casein and glycinin are heat labile and form insoluble aggregates when heated. When glycinin and $\kappa$-casein were heated together, some acidic polypeptides of glycinin crosslinked with $\kappa$-casein via disulfide linkages. However, when disulfide linkage was prevented by adding $\beta$-mercaptoethanol, non-covalent interactions between $\kappa$-casein and both acidic and basic polypeptides of glycinin occurred that prevented the heat precipitation of glycinin. This non-covalent interaction between glycinin polypeptides and $\kappa$-casein may explain why the heat-treated soy proteins became attached to the surfaces of casein micelles during rennet coagulation of milk.
To my mother Rajam Narayanaswamy; father S. Narayanaswamy; wife Aparna Rao; and sister Usha Ramakrishnan.
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First and foremost, my humble prayers to the ALMIGHTY for giving me opportunities over the years to study, learn, and continue learning.

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Venkatachalam Narayanaswamy
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AP = acidic polypeptide of glycinin
\( \beta\text{-CG} = \beta\text{-Conglycinin} \)
BP = basic polypeptide of glycinin
G = Glycinin
K = \( \kappa\text{-casein} \)
K_F = curd firming rate
RCT = rennet coagulation time
RF = relative firmness
RU = rennet units where US single strength rennet \( \sim 90 \text{ RU/ml} \)
SEM = scanning electron microscopy
SPI = soy protein isolate (s)
SPI_H = heat-treated soy protein isolate
SPI_M = commercial (modified) soy protein isolate
SPI_N = native soy protein isolate prepared in the laboratory
SPI_{NCW} = SPI_N dispersed in cold water
SPI_{NHW} = SPI_N dispersed in hot water
SU = subunit of glycinin consisting of 1 AP and 1 BP linked by a disulfide bond
TEM = transmission electron microscopy
UHT = ultra high temperature
CHAPTER 1
GENERAL INTRODUCTION

Vegetable proteins such as soy proteins can substitute for milk, meat, poultry, sea food, or egg proteins in a variety of products. They can be modified to tailor their functionality for specific applications. In addition, they are readily available and cost less than animal proteins. As a result, their use as ingredients in food products is increasing. At present, the use of soy proteins to replace milk proteins is limited to commercial products such as nondairy creamers, whipped toppings, frozen desserts, and infant formulas. The use of soy proteins in other dairy products such as cheese or cheese analogs or yogurt is still in the research stage.

Researchers have used soy protein or soy-milk blends to make cheese or cheese analogs. Numerous studies report the effect of soy proteins on various rennet coagulum properties such as coagulation time, curd firmness, and syneresis. But it is still unclear as to how soy proteins affect rennet coagulation and coagulum properties. A good understanding of this mechanism could help to develop new approaches for mixing soy proteins with milk proteins in food manufacture. This may be in countries with inadequate milk supply to meet consumer needs. It may also provide information on how the functional properties of milk-protein-based food may be modified by addition of soy proteins.

Considerable fundamental research is still needed to understand the relationship between physicochemical properties of soy proteins and their functionality in milk or during rennet coagulation. The overall objective of this study is to determine how soy proteins interact with milk proteins to better
understand how they can be used in combined food systems. This requires a study of the effect of soy protein (and its fractions) on the coagulation properties of milk; its effect on curd microstructure; and how it partitions between the curd and whey. Specific objectives of the research were:

1. Determine the effect of unmodified soy protein isolate on the coagulum properties (rennet coagulation time, curd firmness, syneresis) of milk and the extent to which it is incorporated into cheese curd;
2. Determine which of the soy protein fractions (β-conglycinin or glycinin) has the greatest effect on the coagulation properties of milk;
3. Identify and localize soy protein isolate that is incorporated in the cheese curd;
4. Identify and localize glycinin and β-conglycinin incorporated in the cheese curd;
5. Determine whether heat denaturation of soy proteins impairs their ability to be used in conjunction with milk in making cheese;
6. Use information gathered above to explain how soy protein interacts with milk proteins during milk coagulation;
7. Determine if heat treatments can be used to increase the interaction between soy protein and milk protein fractions.
CHAPTER 2
USE OF SOY PROTEINS IN CHEESE MAKING: A REVIEW

INTRODUCTION

Vegetable proteins like soy proteins offer excellent functional properties, in addition to their easy availability and low cost. This has resulted in their increased use as ingredients to replace meat, egg, or milk proteins in various products. Such partial replacement can reduce product costs. There have been many attempts to partially replace milk proteins with soy proteins in cheese. Although some of the effects of adding soy proteins on coagulum properties are known, how soy proteins affect milk coagulation is still unclear. The overall objective of this study is to determine how soy proteins interact with milk proteins during milk coagulation. A basic understanding of this phenomenon would permit a more scientific approach to adding soy protein rather than by trial and error.

In this review, past research on rennet-induced coagulum properties of milk fortified with soy proteins is summarized. An overview of chemistry of milk proteins, soy proteins, and milk coagulation is presented followed by a description of work by various researchers on how soy proteins influence various milk coagulum properties such as rennet coagulation time, curd firmness, syneresis, yield, and curd microstructure. Then, various theoretical mechanisms of how soy proteins interfere with milk coagulation are presented followed by new approaches that could be taken to better understand a milk-soy protein system.
MILK PROTEINS

Bovine milk contains 30-35 g protein per liter. Caseins account for approximately 80% of total milk proteins and whey proteins constitute the rest. Caseins occur as large and highly hydrated sponge-like colloidal particles called micelles (63). These micelles have a very open structure and proteins occupy only about a quarter of the total volume (43). On a dry basis, these micelles contain 92% protein and 8% inorganic salts, mainly calcium phosphate and small amounts of magnesium, sodium, potassium, and citrate (93, 101). Casein micelles exhibit a broad statistical distribution of sizes (43, 63) with a probable weight average diameter of 160 nm as determined by inelastic light scattering (63).

Caseins

Caseins represent four gene products: $\alpha_{s1}$-casein, $\alpha_{s2}$-casein, $\beta$-casein, and $\kappa$-casein occurring in the ratio of 3.5:1:3:1, respectively. Each of these caseins has several genetic variants (101). Strongly hydrophobic and charged amino acid residues are not uniformly distributed along their polypeptide chains. They exist mainly as random coils with very little $\alpha$-helices or $\beta$-sheets in their secondary structure (101). They are phosphorylated to different extents at seryl residues: $\alpha_{s1}$-casein–8-9; $\alpha_{s2}$-casein–10-13; $\beta$-casein–4-5; and $\kappa$-casein–1. Due to high phosphorylation, $\alpha_{s1}$-, $\alpha_{s2}$-, and $\beta$-caseins can bind to Ca++, and aggregate to form precipitates. On the other hand, $\kappa$-casein, with least phosphorylation, does not precipitate in the presence of Ca++. In fact, $\kappa$-casein protects other caseins from calcium-induced precipitation in milk (101). Consequently, specific interactions between different casein components in the presence of calcium form the basis for casein micelle formation (93).
Composition of Casein Micelles

The composition of micelles varies with the micelle size. The fractional content of $\alpha_s$-casein (sum of $\alpha_{s1}$- and $\alpha_{s2}$-casein) increases slightly, $\beta$-casein increases appreciably, whereas $\kappa$-casein decreases strongly with increasing micellar size (93). Moreover, different caseins are not uniformly distributed within a casein micelle. The outer layer of the micelle is composed of nearly equimolar amounts of $\alpha_s$-casein (sum of $\alpha_{s1}$- and $\alpha_{s2}$-casein) and $\kappa$-casein, but only small amounts of $\beta$-casein (25). On the other hand, the interior of the micelle consists of equimolar amounts of $\alpha_s$- and $\beta$-caseins with a very small amount of $\kappa$-casein (25). Thus $\kappa$-casein is predominantly found on the surface of the micelle. It is linked to the remainder of the micelle via para-$\kappa$-casein, the N-terminal two-thirds of $\kappa$-casein (24).

Stability of Casein Micelles

The isoelectric point of caseins is around pH 4.6 and therefore at normal pH of milk (6.7), the caseins carry an overall negative charge. However, this overall negative charge on the surface of casein micelles only partially contributes to micellar stabilization via electrostatic repulsion (63). $\kappa$-Casein present on the surface of casein micelles plays an important role in the stability of casein micelles (43, 93). The C-terminal part of $\kappa$-casein, called the macropeptide, is hydrophilic (101) and sticks into the surrounding solvent as hair-like protrusions (44, 45, 118). This does not allow casein micelles to approach very close to each other because of steric hindrance, thereby helping to stabilize casein micelles against colloidal instability (24, 43, 44, 45, 118).
SOY PROTEINS

Soybean protein is heterogeneous in nature and about 90-95% of nitrogenous material in defatted soybean meal is water extractable at alkaline pH (121). The extractable proteins are made up of 2S, 7S, 11S, and 15S protein components based on their ultracentrifugal sedimentation rates (121) and are called globulins. The relative percentages of 7S and 11S fractions reported in literature vary widely (30, 52, 124).

2S and 15S Proteins

The 2S fraction accounts for about 8-22% of extractable soybean proteins (52, 79). This fraction consists of Kunitz and Bowman-Birk protease inhibitors (trypsin inhibitors), cytochrome C, α-conglycinin, and several unidentified proteins (79, 121). The 15S fraction makes about 5-11% of the total extractable protein with a molecular weight of more than 5 x 10^5 Da (52, 121). It has not been studied extensively and may consist of aggregated 11S proteins (121).

7S Proteins

The 7S fraction accounts for about 35-37% of extractable proteins (52, 121). It consists of β- and γ-conglycinins (52, 77) and enzymes such as lipoxygenase and β-amylase (121). β-Conglycinin, which undergoes reversible dimerization, comprises about 85% of 7S proteins. The nondimerizing γ-conglycinin, also called hemagglutinin, with a molecular weight of 104,000 Da, represents 9% of 7S proteins (11, 30, 52, 124).

β-Conglycinin is an oligomeric glycoprotein consisting of 3 subunits: α, α' and β (103, 105, 106) held together by hydrophobic forces and hydrogen bonding. β-Conglycinin is heterogeneous with respect to subunit composition
(102). At least seven different types (B₀, B₁, B₂, B₃, B₄, B₅, and B₆) containing various amounts of three subunits have been identified (30). The molecular weights of α- and α' subunits are around 57,000 to 59,000 Da and that of the β-subunit is around 42,000 to 44,000 Da (11), although higher molecular weights for these subunits have been reported. The molecular weight of β-conglycinin is in the range of 150,000 Da and 190,000 Da (79), depending on its subunit composition.

11S Proteins

The 11S fraction is made up of a single protein, glycinin. It makes up about 31% of total extractable soybean protein (121) although higher amounts up to 50% have been reported (52). Its approximate molecular weight is 350,000 Da (79, 88) but its exact quaternary structure has not been determined. It consists of 12-14 heterogeneous polypeptides termed acidic and basic polypeptides. An acidic and a basic polypeptide (MW: 40,000 and 20,000 Da, respectively) are held together by a disulfide bond (88) to form a subunit of 60,000 Da. Three subunits combine by hydrophobic forces to form a glycinin monomer. Two monomers held together by electrostatic and/or hydrogen bonds form the glycinin dimer (88).

At least seven different acidic and five different basic polypeptides have been identified (11). Each acidic polypeptide is nonrandomly linked to a specific basic polypeptide (79) and each subunit, consisting of a specific acidic and basic polypeptide, is synthesized together as they are encoded by a single gene (79). Some of the different subunit pairings are A₁αB₂, A₁bB₁b, A₂B₁a, A₃B₄, and A₅A₄B₃ (79).
Physicochemical Properties of 
β-Conglycinin and Glycinin

Glycinin and β-conglycinin undergo various reactions depending on conditions of pH, ionic strength and temperature. These proteins have different isoelectric points (pl). The α-, α’- and β-subunits of β-conglycinin have isoelectric points of 4.9, 5.2, and 5.7-6.0, respectively, and this gives it an apparent pl of 4.8 (11, 104). On the other hand, the acidic polypeptides and basic polypeptides of glycinin have a pl of about 4.6-5.4 and 8.0-8.5 respectively, giving glycinin an apparent pl of 6.4 (11, 104).

Effect of ionic strength. Both β-conglycinin and glycinin undergo association-dissociation reaction depending on ionic strength. β-Conglycinin has a sedimentation coefficient of 7S at 0.5 M ionic strength. When the ionic strength is decreased to 0.1, it associates face to face to form an aggregate with sedimentation coefficient of 9.5S. This association is electrostatic and reversible (53). Glycinin (11S), on the other hand, dimerizes to 15S form on reducing the ionic strength from 0.5 to 0.1. But on reducing the ionic strength further to 0.01 M, part of 11S dissociates to glycinin monomer form which has a sedimentation coefficient of 7S (14, 53).

Metal binding capacity. Soy proteins can bind cations, especially Ca++ (5, 38, 56, 88, 95, 107). Alkali treatment of soy proteins increases its Ca++ binding property (56). Although phytic acid associated with soy proteins helps to form a soy protein-phytate-Ca complex (38), glycinin and β-conglycinin can bind calcium ions independent of phytic acid (5, 56). Glycinin binds more Ca++ than β-conglycinin (5). In addition to Ca++, soy proteins can bind other cations like Mg++ (6), Zn (49, 81), and Fe (98).
**Effect of heat.** Glycinin and β-conglycinin denature when heated and subsequently aggregate. β-Conglycinin undergoes denaturation at 75°C while glycinin denatures at 90°C (42). The denaturation temperature of these proteins increases with ionic strength (42). On heating these proteins, the polypeptides forming their subunits initially dissociate, unfold, and then subsequently aggregate (53). For example, when glycinin is heated, its subunits (made up of one acidic and one basic polypeptide) undergo sulfhydryl-disulfide exchange reactions (124), liberating constituent acidic and basic polypeptides. The acidic polypeptides associate via disulfide bonds (123) while the basic polypeptides associate via hydrophobic interaction, forming insoluble aggregates (32). The composition of the final species depends on protein concentration (124). However, when glycinin is heated in the presence of β-conglycinin, they interact (26, 32, 53, 108, 109, 110, 124). Specifically, the β-subunit of conglycinin interacts with the basic polypeptide of glycinin via electrostatic interaction (109) and prevents the precipitation of basic polypeptides of glycinin. In addition, the α and α’ subunits of β-conglycinin interact with acidic polypeptides of glycinin via disulfide interaction (124). Similarly, when soy proteins are heated in the presence of the meat protein myosin, they interact (39, 85, 86, 87). The basic polypeptide of glycinin interacts with the heavy chain of myosin via hydrophobic interaction while the acidic polypeptide of glycinin has little or no interaction with myosin (85, 86). Although β-conglycinin decreases the extent of heat induced-aggregation of myosin heavy chains, specific interactions with β-conglycinin subunits are unclear (87).
MILK COAGULATION

Milk proteins, especially caseins, can be coagulated by adding either enzyme or acid (10). During cheese making, an enzyme, chymosin, is added to clot casein micelles (43). Three separate but overlapping stages occur during enzymic coagulation of milk: enzymic proteolysis (primary phase), aggregation (secondary phase), and gelation (64). After enzyme addition, initially there occurs a lag phase wherein the average particle weight decreases due to \( \kappa \)-casein hydrolysis (24). This is followed by a period where the particle weight increases explosively due to aggregation (10, 24), culminating in the formation of a gel or a coagulum.

**Enzymic Phase**

The hydrolysis of \( \kappa \)-casein by chymosin initiates enzymic milk coagulation (24). During the primary phase, the enzyme attacks \( \kappa \)-casein at Phe\(_{105}\)-Met\(_{106}\) bond (47), the junction between the hydrophobic para-\( \kappa \)-casein (residues 1-105) and the hydrophilic macropetide (residues 106-169) (24).

The rate of proteolysis of \( \kappa \)-casein is affected by various factors such as concentration of enzyme, pH, ionic strength, and temperature (10, 24). Increasing the enzyme concentration increases the rate of proteolysis (114) and decreases rennet coagulation time (RCT) (62). Lowering the pH increases the proteolysis rate (111). Ionic strength has different effects depending on concentration and type of added ions. Increasing the ionic strength by adding Ca\(^{++}\) increases enzyme activity initially and reaches a maximum value at 8 mM of added Ca\(^{++}\). A further increase in Ca\(^{++}\) lowers the rate of proteolysis (10, 24). Different effects of temperature on rate of proteolysis have been reported. Some (24) report that there is no difference in the rate of proteolysis (\( k_M \) values)
between 25°C and 40°C, although others (80) report a Q-value of about 2 for the enzymic reaction.

**Aggregation**

After enzymic hydrolysis, the macropeptide diffuses into the serum leaving para-κ-casein residue on the micelle. Removing the hairy macropeptide portion from micellar κ-casein decreases the overall negative charge (24) and hydrodynamic radii (119) of the micelles, and creates reactive sites on their surface (24, 65). Electrostatic and steric repulsive forces between the micelles consequently decrease. The resulting unstable para-κ-casein micelles subsequently aggregate via reactive sites to form a clot (24). Electrostatic and hydrophobic interactions play an important role in the aggregation of renneted casein micelles (10, 24, 43, 64, 118).

The hydrolysis of few κ-casein molecules on the surface of casein micelles is insufficient to bring about its aggregation and coagulation (21, 24). A proportion of micellar κ-casein needs to be hydrolyzed before a micelle becomes capable of aggregating (24). Once sufficient hydrolysis of micellar surface κ-casein occurs, it can participate in aggregation (24). Consequently, the fraction of successful collisions between micelles resulting in aggregation increases and the average particle weight increases exponentially.

Enzymic hydrolysis and subsequent aggregation are not two separate steps. In fact, hydrolysis of κ-casein and aggregation of para-casein micelles proceed simultaneously (65, 84). Initially the concentration of such aggregating species is low and therefore a lag time is observed before coagulation is detected (84). Although enzymic hydrolysis is almost complete at RCT (15, 36), aggregation of para-casein micelles begins much before visual coagulation occurs (66). In fact, significant aggregation is detectable even at 20% of true gelation time (65).
Various factors like temperature, pH, and ionic strength or added ions affect rate of aggregation of renneted casein micelles (10, 22, 24, 64, 70, 111, 112).

**Ionic strength.** Ionic calcium concentration affects aggregation rate (24), which increases with Ca$$^{++}$$ concentration at temperatures below 45°C (22). Adding Ca$$^{++}$$ up to 0.04 M increases both rate of aggregation (curd firming rate) and curd firmness (67). It decreases the surface charge of casein as well as para-κ-casein micelles and reduces the intermicellar repulsion (24). Moreover, Ca$$^{++}$$ increases the effective hydrophobicity of the micelles (61) by exposing buried tryptophan and tyrosine residues to the solvent (82). The decreased inter-micellar repulsion and the increased micellar hydrophobicity (10) result in a much stronger interaction between the micelles. This allows casein micelles with a lower degree of κ-casein hydrolysis to aggregate (41) and coagulate faster (lower RCT). Thus, added Ca$$^{++}$$ helps to reduce the overall energy barrier for coalescence between two casein particles by either decreasing the size of the reactive site on the surface of casein micelles or by decreasing the amount of macropeptide that must be removed to create an active site (65).

However, higher levels of Ca$$^{++}$$ (> 0.05 M) decrease both the aggregation rate (curd firming rate) and final curd firmness by possibly interfering with electrostatic interactions due to high ionic strength (67). Similarly, adding NaCl decreases the rate of coagulation sharply at 25 and 30°C, but has little effect at 50°C (22). Sodium ions can compete with calcium ion binding sites in caseins, leading to a decrease in the calcium-mediated interactions between the caseins, resulting in decreased coagulation rate. Moreover, they can shield electrostatic interactions.

**Temperature.** Aggregation rate is temperature dependent ($$Q_{10}$$ value of around 11-12) (17, 18) and increases sharply with temperature (22). At
temperatures below 15°C, renneted milk does not clot (24) but it coagulates on subsequent warming (22). Aggregation rate approaches its theoretical maximum value at temperatures above 45°C (22). An increase in aggregation rate with temperatures suggests that hydrophobic interactions are involved in the aggregation process (10, 24, 64).

**pH.** As pH decreases, rennet clotting time decreases (70, 99, 100, 111), aggregation rate increases, and the extent of hydrolysis required for aggregation decreases (24, 70, 111). Although lowering the pH increases enzymic activity (24, 111), it has a greater influence on the rate of aggregation (24, 70). Several things change as pH is lowered. The composition of the micelles changes with pH (24). Moreover, the electrostatic repulsion between the micelles decreases because of decreased surface charge and electrical double layer on the micelles (10). In addition, the state of micellar calcium phosphate, free Ca++, and steric repulsion of κ-casein changes (10, 24). These factors have a greater effect on aggregation rate. The lower pH neutralizes some of the negative charge within the micelles, decreases the overall charge and steric repulsion of the micelles, increases hydrophobic interactions between the casein micelles, and increases the rate of coagulation, once some of the stabilizing κ-casein is hydrolyzed by rennet (24).

**Chemical additives.** Different chemical additives have different effects on rennet coagulation (33, 34, 37, 46, 61). Positively charged additives like salmine, cetyl- trimethyl ammonium bromide, cetyl pyridinium chloride, and poly-L-lysine bind strongly to casein micelles while some amines like spermidine and spermine bind only weakly to casein micelles. Some of these additives decrease RCT of milk. These additives shield charged groups, thereby neutralizing the negative charge and enhancing the hydrophobicity of
the micelles. This increases the rate of aggregation of casein micelles after rennet treatment and decreases RCT of milk. On the other hand, negatively charged additives like SDS, poly-L-glutamate, which adsorb strongly to casein micelles, increase RCT of milk by binding Ca++. Different proteins bind to different extents with caseins and affect milk coagulation differently. There seems to be no correlation between charge or hydrophobicity of proteins and their ability to bind to casein micelles or affect rennet coagulation. Many negatively charged proteins adsorb strongly to casein micelles and decrease RCT of milk, but poly-L-glutamate (highly negatively charged) increases RCT of milk. On the other hand, positively charged proteins, which bind weakly to casein micelles, either increase or decrease RCT of milk. Poly-L-lysine (highly positively charged), which binds strongly to casein micelles, decreases RCT of milk. Perhaps, the effects are determined by the local-structure of these proteins rather than properties averaged over the whole molecule.

Additives like fatty acids (0.4%), trisodium citrate (15 mM), phytic acid (5 mM), and disodium EDTA (10 mM) increase RCT and decrease firmness by binding Ca++ (46). Lowering the pH partially restores RCT and firmness. Adding 20 mM Ca++ completely restores RCT and firmness in case of trisodium citrate, but only partially in case of phytic acid. Adding disodium phosphate (0.1%) hastens coagulation but retards firming rate although the final curd firmness is not affected (68). Additives that bind and sequester Ca++ can have a significant effect on RCT and curd firming rate, as well as final curd firmness.

Additives like 1,8-anilinonaphthalenesulfonate and Nile Red bind to hydrophobic regions of casein micelles and subsequently decrease the rate of aggregation of rennet treated casein micelles (31). Additives that interfere with
hydrophobic interactions between the para-casein micelles can thus have a significant effect on milk coagulation.

**Heat treatment.** Heat treatment of milk affects milk coagulation (24, 100, 113). As milk is heated above 70°C, β-lactoglobulin forms a complex with micellar κ-casein via hydrophobic and disulfide interaction (23, 40, 96) and this complex remains attached to the micelles. This decreases the rate of chymosin-induced proteolysis (92). Further, the complex prevents direct micelle-micelle contact and fusion during aggregation, resulting in a weak coagulum (69).

**Gelation**

Transmission electron microscopy has been used to monitor changes occurring during the formation of rennet-induced gel and manufacture of cheddar cheese (35, 36, 51). The micelles are far apart in milk and remain as such for up to 60% of RCT. At 80% of RCT, the micelles form aggregates by direct contact or via bridges, involving large areas of their surfaces. At RCT, most of the micelles are aggregated into chains, and various chains interlink to form a loose irregular network. At 200% of RCT, a loose network is clearly seen. Chains of micelles group together to form loose, convoluted strands at 300% of RCT. These chains form strands roughly 5 micelles thick and the distance between adjacent strands is some 10 micelles long. The resulting three-dimensional gel network has large openings up to 10 µm in diameter and entraps much serum (35, 36). The aggregation process continues for hours after rennet addition (12, 120). The number or the strength of links formed between the casein micelles or both, continuously increases with time (36) and the gel becomes firmer (12, 35, 120). This eventually leads to fusion between adjacent micelles and the strands become more compact. The gel then
contracts, resulting in syneresis and a consequent increase in gel firmness (35, 36).

**USE OF SOY PROTEINS IN FOOD PRODUCTS**

Soy proteins offer many functional properties (9, 52, 115) and can be used as ingredients in different food products. They have been used commercially to partially replace meat, poultry, sea food (19, 20, 55, 115, 116, 117), and cereal proteins (9, 28, 55, 115). At present, soy proteins are used to replace milk proteins in nondairy creamers, whipped toppings, frozen desserts, and infant formulas (54, 55) but their use in other dairy products or its analogs is still in the research stage (50, 97). Researchers have used soy protein or soy-milk blends to make cheese or cheese analogs (1, 3, 27, 48, 50, 57, 58, 71, 72, 73, 74, 76, 78, 83, 89, 90, 97, 125) and yogurt-type products (16, 29, 54, 60, 97, 122).

**USE OF SOY PROTEINS IN SOFT CHEESE MAKING**

Soy protein has been added as soy milk (1, 2, 3, 71, 83, 89, 90) or soy extracts (57, 59) or soy flour (76) or soy proteins isolates (73, 74, 75, 76, 91) to milk in order to make cheese. However, soy protein isolate is preferred to other forms because it provides a concentrated means of adding soy proteins and contains fewer undesirable compounds.

**EFFECT OF SOY PROTEINS ON MILK COAGULUM PROPERTIES**

**Rennet Coagulation Time**

The effect of soy proteins on the rennet coagulation time (RCT) of milk is unclear. Some researchers (2, 71, 89, 91) report that adding soy proteins increases RCT of milk with higher levels of soy proteins having a greater effect.
on RCT (2, 71, 91). Coagulating milk at a lower pH or adding CaCl₂ or increasing the quantity of rennet used for coagulation helps to decrease the effect of soy proteins on the RCT of milk (2, 71). Some researchers (74), however, report no effect of soy proteins on RCT. The type of soy proteins used in the study or different methods used to estimate RCT may give different results.

**Curd Firmness**

Adding soy proteins lowers the curd firmness of milk (57, 59, 71, 74, 76, 91). Increasing the proportion of soy proteins further decreases curd firmness (2, 71, 74, 91). On the other hand, adding CaCl₂ either increases (73) or has no effect (57, 71) on curd firmness of the milk-soy blends. The type of soy proteins used or the mode of measuring curd firmness or both may have something to do with different results being reported. However, coagulating the milk-soy blend at a lower pH helps to increase the firmness (71).

There is no agreement between various studies on the extent of reduction of milk curd firmness caused by adding soy proteins. This is because, in most cases, soy-milk blend contained fewer caseins than the control. This is in itself would reduce the firmness. Moreover, different researchers used different types of soy proteins viz. soy protein extracts (57), soy milk (71), heated soy milk (2, 89), chemically modified soy proteins (59), commercial soy protein isolates (76), and alkali-treated commercial soy protein isolates (73, 74). A difference in the physicochemical behavior of these proteins in milk could yield different results.

Chemically modified soy proteins have been added to milk and coagulation has been studied in order to understand how soy proteins affect milk coagulation (59). Soy proteins with their amino acid side chain residues modified by different agents have different effects on milk curd firmness.
Increasing negative charge on soy proteins by extensive acetylation or succinylation or decreasing the negative charge by modifying with ethylenediamine in presence of ethyldimethylaminopropyl carbodiimide (EDC) decreases the curd firmness. On the other hand, increasing the negative charge by alkylation with iodoacetate or lower levels of acetylation or succinylation did not affect curd firmness. Adding bulky neutral hydrophobic groups via modification with EDC tended to lower curd firmness (approached significance) but adding neutral groups via modification with N-ethylmaleimide did not affect curd firmness. Partially dissociating the soy proteins by treating with alkali either increases (59) or decreases (76) or has no effect on the curd firmness (59). Chemically modified soy proteins do not follow a definite pattern in the way they affect curd firmness. Lee and Marshall (59) believe that this difference in the behavior of chemically modified soy proteins could be due to the modification or due to changes in the conformation of the protein that occurs during modification or both.

Enzyme-hydrolyzed soy proteins have been added to milk to see whether such proteins would improve curd firmness compared to unmodified soy proteins (75). Soy proteins hydrolyzed using different enzymes have different effects on coagulum firmness. Soy proteins treated with pepsin or trypsin produce coagulums that have a higher firmness than coagulum containing unhydrolyzed soy protein. On the other hand, coagulum containing chymotrypsin-hydrolyzed soy protein has a lower firmness than unhydrolyzed soy protein (75).

**Syneresis**

Milk-soy blend coagulum has a higher moisture (less syneresis) as compared to the control without soy protein (57, 71, 73, 74, 76, 89). The looser
network of casein formed in the presence of soy proteins, plus the higher water-holding capacity of soy proteins, could contribute towards higher moisture content of curd.

Yield

Cheese curd containing soy protein (57, 89) or 11S protein rich fraction (57) has lower solids and fat content than the control curd. This is because soy milk or extract has different composition than bovine milk. It has less fat, carbohydrate, and ash content than milk (71) although the total protein content is about the same (71, 89).

Curd Microstructure

Electron microscopy has been used to study the effect of soy proteins on milk curd microstructure. In studies using scanning electron microscopy (SEM), only boiled soy proteins, which existed as large aggregates, could be identified in the curd matrix (58). Others (74) were unable to identify soy proteins in the microstructure although the effect of soy protein on the development of curd structure could be seen. Soy proteins seem to delay the formation of casein micelle clusters, chains and strands (74). Some researchers (58, 74) believe that soy proteins in their native subunit structure state were intermingled among casein micelles throughout the network structure, either bound to the micelles or entrapped within or between the micelles. However, how soy proteins exist in the curd is still unknown.

HOW SOY PROTEINS AFFECT MILK COAGULATION:

PROPOSED MECHANISMS

Several mechanisms have been proposed to explain how soy proteins affect
milk coagulation. Some authors believe that soy proteins form a gel network independent of the casein micelle network. This soy protein network could interfere with the micelle network and affect gel formation (57). Based on the effect of chemically modified soy proteins on milk curd firmness (59), the authors believed that soy proteins (pl ~ 4.6) having negative charge at pH 6.0 could bind to casein micelles and increase the repulsive potential between the rennet modified casein micelles. This would decrease the probability of micellar contacts and reduce the number of linkages formed between the micelles. In addition, they could also be entrapped within the micelles. Soy proteins getting either adsorbed on the surface of casein micelles or entrapped within the micelles could block hydrophobic and other binding sites of casein micelles. This could affect both the electrostatic and hydrophobic interactions between para-casein micelles during milk coagulation (59), reduce the strength of linkages formed between casein micelles, and form a weak coagulum.

Mohamed and Morris (74) believe that soy proteins affect milk coagulation physically without binding to the micelles. Soy proteins, by being physically present between the micelles, may progressively restrict their movement and prevent them from coming into close contact. This would decrease the rate of aggregation of para-casein micelles and affect coagulum structure and firmness (74).

Soy proteins can bind Ca++ (5, 56), affect calcium equilibria in milk, and therefore affect milk coagulation (73). Soy proteins lowered ionic Ca++ concentration of milk very minimally (<5%) but decreased curd firmness significantly (73). Further, adding CaCl₂ did not restore curd firmness (73). The authors (73) therefore concluded that the large molecular size of soy proteins (180,000-350,000 Da as compared to ~20,000 Da of caseins) and its high
water-holding capacity may play an important role in the way it interferes with milk coagulation (73, 74). Some authors (71, 89) believe that milk proteins and soy proteins interact via disulfide bonds and interfere with milk coagulation in a similar manner to whey proteins.

Although different mechanisms have been proposed to explain how soy proteins affect milk coagulation, there is still no evidence supporting either of these views. It is still unclear how soy proteins interfere with milk proteins during rennet-induced milk coagulation.

**CONCLUSIONS**

Most of the studies used either heat-treated soy extracts and soy milk or chemically and enzymically modified soy protein isolates. Proteins may become partially denatured or unfolded or both, and may aggregate during heating or chemical modification treatments. Modified proteins have different physico-chemical properties (solubility, protein conformation, state of denaturation or aggregation or dissociation, charge profile) than unmodified proteins and can behave differently when added to a food system. Consequently, the effect of chemically or heat-modified soy protein on the coagulum properties may be due to any of these induced changes. Studies using unmodified soy proteins will help in understanding the behavior of soy proteins in milk and during rennet coagulation. It is not known how unmodified proteins behave during rennet coagulation in comparison to heat-denatured proteins.

The extent of curd firmness reduction caused by soy proteins is unclear because the amount of casein (w/w) in soy-fortified milk was less than the control in most cases. This, in itself, would reduce coagulum firmness. Most of
these studies were designed to simply measure the impact of adding soy proteins to milk (as occurs industrially) rather than to eliminate the confounding effects caused by changing the components of the coagulating system. Consequently, few strong conclusions could be drawn from such experiments.

Soy proteins are heterogeneous in nature and the isolate is a mixture of different fractions (2S, 7S, 11S, and 15S) (121). The major fractions glycinin (11S) and β-conglycinin (main component of 7S) have different physico-chemical and functional properties (94). The behavior of the soy protein isolate depends upon how these two major fractions behave. But, there is no information available on how the different fractions, β-conglycinin and glycinin, behave in milk or during rennet milk coagulation.

The location of soy proteins in the curd or the manner in which they exist in the curd could not be determined by scanning electron microscopy. Either soy proteins exist in their native structure so that they are too small to be seen using SEM, or they exist in another form, which makes it difficult to be distinguished from casein micelles. Higher resolution can be obtained using transmission electron microscopy; moreover, it may be easier to discern soy proteins from milk casein micelles because a cross section of the curd is usually seen. If soy proteins could be localized in the curd, useful information on the microstructure of the curd matrix, distribution of soy proteins in the curd, and the type of interaction of soy proteins with curd structure could be obtained. One approach could be to use antibodies to localize soy proteins in the curd. Immunogold-labeling in conjunction with TEM has been used to identify carbohydrate- and protein-based additives in food products (7) or study the storage-related changes in the distribution of various milk proteins in ultra-high temperature
(UHT) processed concentrated milk (4) or study the distribution of different caseins in casein micelles (13).

It can be concluded that although numerous studies have been conducted to study the effects of soy proteins on milk coagulation and coagulum properties, it is still unclear as to how soy proteins affect milk coagulation. Considerable research is still needed to understand the relationship between physicochemical properties of soy proteins and their functionality in milk or during rennet coagulation in order to successfully utilize milk-soy blends (8).

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CHAPTER 3
IMMUNOLOCALIZATION AND ELECTROPHORETIC
CHARACTERIZATION OF SOY PROTEINS
IN RENNET COAGULUM OF
MILK-SOY BLENDS

ABSTRACT

Milk-soy blends containing native soy protein isolate (SPI\textsubscript{N}) or modified isolate (SPI\textsubscript{M}) were prepared by adding soy protein isolate dispersions (8.2%) to skim milk in a 1:11 ratio. After adding 0.05% CaCl\textsubscript{2}, the pH was adjusted to 6.42. The blends were rennet or rennet-acid coagulated at 35\textdegree{}C, and examined using immunogold labeling in conjunction with transmission electron microscopy (TEM) to localize soy proteins in the coagulum. The extent of syneresis as well as loss of soy proteins was also analyzed. Soy proteins from both the native and modified SPI existed as aggregates in the coagulum. The size of SPI\textsubscript{N} aggregates was approximately 0.5-1.0 µm. The SPI\textsubscript{M} aggregates were bigger and had a more open structure. These aggregates were physically trapped between casein micelle chains and did not bind to casein micelles. There were also small soy protein particles from the SPI\textsubscript{M} that were attached to the surface of para-casein micelles, which prevented their fusion with other para-casein micelles. Adding soy proteins decreased coagulum syneresis from 81.1% in the control to 69.5% with SPI\textsubscript{N} added and 58.3% with SPI\textsubscript{M}. Most of the soy glycinin was retained in curd, whereas most of the $\beta$-conglycinin was lost in whey as shown by SDS-PAGE.
INTRODUCTION

The effect of soy proteins on various properties of rennet-induced milk coagulum has been studied (1, 13, 14, 15, 16, 17, 18, 19, 20, 21, 22). Although such coagulums tend to have a softer (less firmer) texture and retain more moisture (less syneresis) than the control, very little is known about how soy proteins affect milk coagulation. A knowledge of how soy proteins exist in the coagulum or how its main fractions, β-conglycinin (major protein in 7S fraction) and glycinin (11S fraction), partition between coagulum and whey would help to understand their behavior during milk coagulation.

Attempts to locate soy proteins in curd using scanning electron microscopy (SEM) have been unsuccessful. Lee and Marshall (14) could locate only boiled soy proteins while Mohamed and Morris (18) observed a delay in curd structure development in the presence of soy proteins, but were unable to identify soy proteins in the coagulum using SEM. The authors (14, 18), therefore, believed that soy proteins in the coagulum exist in their native subunit structure (glycinin ~ 12 polypeptides and β-conglycinin ~ 3 polypeptides) commingled with caseins.

While individual proteins are too small to be seen using SEM, using immunogold labeling in conjunction with transmission electron microscopy offers an approach to identify and localize soy proteins in the curd. Such a technique has been used to study protein distribution in casein micelles (6), identify carbohydrate and protein-based additives in food products (3), and study the changes in the distribution of various milk proteins during storage of UHT-processed concentrated milk (2). So our objective was to mix milk with soy protein isolate (SPI), rennet coagulate the milk-soy mixtures, and then
localize either native or denatured soy proteins using immunogold labeling. In addition, we wanted to study the partitioning of soy proteins between curd and whey using SDS-PAGE.

MATERIALS AND METHODS

Skim Milk

Whole milk was obtained from the Utah State University Dairy Products laboratory, skimmed (3000 x g for 1 h at 4°C), preserved by adding sodium azide (0.05%), and filtered (GF/A filter paper, Whatman International Ltd., Maidstone, England) to produce skim milk with 3.1% protein. It was then refrigerated overnight.

Soy Protein Isolates

Two SPI--an unmodified SPI (SPI$_N$) containing native soy proteins and a modified SPI (SPI$_M$)--were used. The SPI$_N$ was prepared from defatted soy flour (Protein Technologies International, St. Louis, MO) according to Kolar et al. (12). Soy flour was stirred for 1 h with 15 volumes of deionized distilled water while the pH was continuously adjusted to 7.5. It was then centrifuged at 9000 x g for 30 min and the decanted supernatant was filtered through Whatman 4 filter paper. The supernatant was acidified to pH 4.5 to precipitate the proteins and the resulting slurry was again centrifuged at 9000 x g for 30 min and the pellet collected. It was washed twice with deionized distilled water, redispersed in deionized distilled water by continuously adjusting the pH to 7.5, and then freeze dried. The SPI$_M$ (Protein Technologies International, ST. Louis, MO) had been heat denatured, partially hydrolyzed, and partially deamidated during its manufacture. The protein content of both of these preparations was ~83% on a wet basis.
Preparation of Soy Dispersion

An 8.2% SPI dispersion was prepared by mixing SPI\textsubscript{N} or SPI\textsubscript{M} with hot water (72 ± 2 °C) using a bench top homogenizer (Omni 4000, Omni International Inc., Gainesville, VA) at 8000 rpm for about 3 min (SPI\textsubscript{NHW} and SPI\textsubscript{M}, respectively). These were stored overnight at 4 °C. Another dispersion of SPI\textsubscript{N} (SPI\textsubscript{NW}) was prepared in water at 22 ± 2 °C (without using the homogenizer) immediately prior to testing.

Preparation of Milk-Soy Blends

The SPI dispersions and skim milk were warmed to room temperature and mixed in 1:11 ratio to give 20% fortification of milk protein with soy protein. Adding SPI\textsubscript{N} increased the blend pH and a predetermined amount of 1N HCl was immediately added to decrease the pH to 6.6. No acid was added to SPI\textsubscript{M} blend because it increased the pH by only 0.02 to 0.23 units. A control sample was made by adding deionized water to skim milk (1:11) to maintain comparable casein levels. The above milk samples were warmed to 35°C and pH of the control and samples containing SPI were again adjusted to milk pH. Calcium chloride was then added at 0.05% level to all samples and the pH was adjusted to 6.42 ± 0.02 (which was the pH of milk with 0.05% CaCl\textsubscript{2} added).

Rennet Coagulum Formation

Diluted rennet, 1.8 rennet units (RU)/ml (7), was prepared by diluting single strength calf rennet (Rhone Poulenc, WI) with distilled water. American single strength rennet was assumed equivalent to 90 RU/ml. The control, milk, and milk-soy blends were either rennet or rennet-acid coagulated at 35°C. To 200 ml of each sample at 35°C, either 4 ml of diluted rennet or 0.4 ml of diluted rennet plus 4 g glucono-δ-lactone (GDL) was added for coagulation. After 30
min, the curd was cut, and allowed to heal for 10 min, very gently stirred, heated for another 10 min, and then prepared for electron microscopy.

**Electron Microscopy**

Curd was cut to approximately 1-mm$^3$ pieces and treated further according to Alleyne (2) with slight modifications. The curd was fixed in 4% paraformaldehyde (in 0.1 M phosphate buffer, pH 6.7) for 4 h. It was then dehydrated using increasing concentrations of graded ethanol solutions (1 x 10 min in 50% ethanol, 2 x 10 min and then overnight in 70% ethanol, 2 x 15 min in 95% ethanol, and 3 x 15 min in 100% ethanol). After dehydration, it was infiltrated (2 x 1 h and then overnight) in 100% LR White resin (Electron Microscopy Sciences, Fort Washington, PA), transferred to gelatin capsules (Electron Microscopy Sciences), and embedded at 50°C for 21 h. The hardened resin was removed from gelatin capsule by immersing the capsule in water and excess resin was trimmed to expose the sample. Ultrathin sections were cut using a MT2B ultramicrotome (Research and Manufacturing Co. Inc., Tucson, AZ) and collected on 300-mesh nickel grids. The grids were then coated with carbon to give rigidity to the sections to prevent tearing in the presence of electron beam.

Appropriate blocking conditions and primary antibody dilutions were determined by ELISA. The sections (in grids) were first blocked in 20 mM Tris buffer, pH 7.4 containing 0.225% fish gelatin (Sigma Chemical Co., St. Louis, MO) and 0.5% normal goat serum (Sigma Chemical Co.) for 15 min, and rinsed once for 5 min in 50 mM Tris saline buffer, pH 7.4. They were then incubated overnight with rabbit anti-soy protein polyclonal antibody (Sigma Chemical Co.), diluted 1:1000 in 50 mM Tris saline buffer, pH 7.4, and thereafter rinsed 6 x 5 min each in 50 mM Tris saline buffer, pH 7.4. This was followed by
incubation for 3 h with 5-nm gold labeled, goat anti-rabbit antibody (Ted Pella Inc., Redding, CA), diluted 1:25 in 50 mM Tris saline buffer, pH 7.4, and rinsed 6 x 5 min with distilled water. They were then post stained with uranyl acetate (5 min at 45°C and 5 min at room temperature) and lead citrate (5 min at room temperature) and subsequently air dried.

Stained sections were then examined using a Zeiss CEM 902 transmission electron microscope (Zeiss Inc., Thornwood, NY) at 80 kV. Images were recorded on Kodak (Eastman Kodak Co., Rochester, NY) electron image film 50-163.

**Curd Syneresis**

Curd syneresis was determined by coagulating the milk blends with rennet or rennet combined with GDL at 35°C and then centrifuging them at 250 x g at 25°C for 20 min. Ten grams of pH-adjusted milk sample was weighed into centrifuge tubes and heated to 35°C. Then either 0.2 ml of diluted rennet or 0.02 ml of diluted rennet plus 0.2 g GDL was added and incubated at 35°C for 30 min. After coagulation, two perpendicular cuts were made in the curd. The samples were then centrifuged at 250 x g at 25°C for 20 min. After centrifugation, the volume of released whey was measured and expressed as a percentage of total moisture (g) initially present in the sample.

**Electrophoresis**

Partitioning of soy protein fractions (β-conglycinin and glycinin) between whey and curd was determined using SDS-PAGE under reducing conditions. Electrophoresis was done in a Biorad mini-protean® II system (Biorad Laboratories, Hercules, CA) according to the Biorad Mini-Protean® II electrophoresis cell instruction manual (4) using 5% stacking gel and 20%
resolving gel. To 0.5 ml of filtered cheese whey (obtained from curd prepared for electron microscopy), 0.5 ml of Tris (20 mM) EDTA (2 mM) SDS (5%) buffer, pH 8.0, was added and the pH of the solution was readjusted to 8.0. After adding β-mercaptoethanol (50 µl), the solutions were heated in boiling water bath for 5 min. Then 3 µl of 4.5% bromophenol blue and 110 µl of glycerol were added, and 3 µl of sample was loaded on to the gel. The SPI dispersions (SPI\textsubscript{N} and SPI\textsubscript{M}) were diluted 1:11 with distilled water and used as standards, representing the amount of soy proteins added to milk. They were prepared for electrophoresis as described above. The gels were run at 100 V for 2.5 h. The gels were stained overnight in a solution containing 0.025% Coomassie blue, 40% methanol, and 7% acetic acid. They were then destained first in 50% methanol, 10% acetic acid solution for 2 h followed by overnight destaining in 5% methanol, 7% acetic acid solution.

**RESULTS AND DISCUSSION**

**Whey**

When milk is rennet-coagulated, the proteins in milk are partitioned. The caseins remain in the curd while the soluble whey proteins (e.g., β-lactoglobulin and α-lactalbumin) get exuded from the curd into the whey (Lanes 9 and 5, Figure 3.1). A similar partitioning of soy proteins also occurred. Lanes 1 and 10 in Figure 3.1 show the various proteins that comprise 7S (β-conglycinin) and 11S (glycinin) fractions of soy proteins. β-Conglycinin (MW = 180,000 Da) is made up of α, α' and β subunits (5). On the other hand, glycinin (MW = 360,000 Da) consists of six subunits with each subunit consisting of one acidic (AP) and one basic polypeptide (BP) (5).

The whey obtained from milk-soy blends, that were renneted at pH 6.4
Figure 3.1. SDS-PAGE analysis of rennet and rennet-acid cheese wheys. SPI_N-native soy protein isolate. SPI_M-modified soy protein isolate. SPI_NCW-cold water dispersed native soy protein isolate. SPI_NHW-hot water dispersed native soy protein isolate. Lanes 6, 7, 8, and 9--rennet wheys. Lanes 2, 3, 4, and 5--rennet-acid wheys. Lane 1--standard SPI_M (Std SPI_M); Lane 10--standard SPI_N (Std SPI_N); Lanes 5 and 9--control (C); Lanes 2 and 6--SPI_NCW; Lanes 3 and 7--SPI_NHW; Lanes 4 and 8--SPI_M. β-CG--β-conglycinin (made of α, α' and β subunits); G--glycinin (made of AP--acidic polypeptide and BP--basic polypeptide); BSA--bovine serum albumin; β-LG--β-lactoglobulin; α-LA--α-lactalbumin.
(Lanes 6-9), had reduced amounts of some of the soy protein fractions. For the native SPI, whether prepared in hot water (SPI\textsubscript{NHW}) or cold water (SPI\textsubscript{NCW}), there were only small amounts of acidic and basic polypeptides of glycinin in the whey. On the other hand, the level of $\alpha, \alpha'$, and $\beta$ subunits of $\beta$-conglycinin in whey were similar to those in the Lane 10 standard (representing the amount of soy protein added to milk). The different partitioning behavior of native $\beta$-conglycinin and glycinin could be due to their different physicochemical properties. The isoelectric point of glycinin is 6.4, whereas that of $\beta$-conglycinin is 4.8 (23). Therefore, under the conditions of this study (pH 6.42, 0.05% CaCl\textsubscript{2}), glycinin exists in a more insoluble form than $\beta$-conglycinin. Consequently, most of glycinin is retained in curd, whereas $\beta$-conglycinin is lost in the whey. When milk was coagulated at a lower pH (pH was 5.2 when rennet-acid coagulum was cut), the amount of soy proteins in the whey decreased for all soy-milk samples. There was virtually no trace of AP or BP subunits of glycinin in the whey (Lanes 2, 3, and 4, Figure 3.1).

**Milk Coagulum**

In the renneted-milk coagulum, the para-casein micelles were observed as electron-dense spheres that had aggregated into clumps and chains (Figure 3.2). Such a structure is typical of microstructure of milk curd before extensive curd syneresis (such as during cheesemaking) has occurred (8, 9, 10). Only slight nonspecific labeling by the anti-soy antibody was observed in the control samples confirming that no specific binding of antisoy-primary antibody to caseins occurred.

When native soy protein (SPI\textsubscript{NCW}) was added to milk and rennet coagulated, the soy protein that was retained in the coagulum was present as large aggregates (Figure 3.3). The soy proteins were less electron dense than
Figure 3.2. Transmission electron micrograph of the renneted control (skim milk diluted with water in the ratio 11:1). Short arrows = casein micelles. Note the smooth surface of casein micelles.
Figure 3.3. Transmission electron micrograph of renneted milk coagulum containing native soy protein isolate dispersed in cold water (SPI_{NCW}) immunogold labeled for soy protein. Black dots represent the location of soy protein. Short arrows = casein micelles, long arrows = soy proteins, arrow heads = physical separation between casein micelles and soy proteins.
caseins, suggesting they had a more open structure and were heavily labeled with the anti-soy immunogold system. The level of labeling on the caseins was the same as the nonspecific labeling observed with the control milk (Figure 3.2). The absence of any labeling on either casein or soy protein when the primary antibody was not added confirmed the specificity of the secondary antibody with gold particles (Figure 3.4).

These SPINcw aggregates were an order of magnitude larger than individual casein micelles. They were most likely composed of aggregates of glycinin because most of the β-conglycinin was lost in the whey. Moreover, glycinin exists in an aggregated form because the coagulum pH is very close to its the isoelectric point. These aggregates appeared to be physically trapped in the para-casein matrix without any chemical bonding between them. Even when the soy protein aggregate was adjacent to the para-casein micelles, there was a thin area of 5 to 15 nm separating them.

The microstructure of the coagulum did not change when soy proteins dispersed in hot water (SPI\textsubscript{NHW}) were added. The soy protein was entrapped within the para-casein network as large aggregates (~1 µm) very similar to those of SPI\textsubscript{NCW}. These aggregates often formed clusters of 5 to 10 µm size (Figure 3.5). At higher magnification (Figure 3.5b), the same heavy labeling of soy proteins and its physical separation from the casein micelles was seen, similar to that observed for SPI\textsubscript{NHW}. However, the distribution of these proteins was not uniform throughout the network as many para-casein groups had no soy protein aggregates in their proximity. This probably represents the relative distribution of these protein particles in the milk-soy blends. Whereas the casein micelles in such blends exist as a true colloidal dispersion, the soy proteins are present as a suspension of large protein aggregates of the
Figure 3.4. Transmission electron micrographs of renneted milk coagulum containing (a) commercial soy protein isolate, SPI_M and (b) cold water dispersed native soy protein isolate, SPI_NCW. Primary antibody was not used during immunostaining. Short arrows = casein micelles and long arrows = soy proteins.
Figure 3.5. Transmission electron micrograph of rennet milk coagulum containing native soy protein isolate dispersed in hot water, SPI_{NHW}. Higher magnification of the area outlined in (a) is shown in (b). Short arrows = casein micelles, long arrows = soy proteins, arrow heads = physical separation between casein micelles and soy proteins. Small black dots visible in (b) are the immunogold labels.
dimension shown in the micrograph.

The microstructure of the coagulum containing SPI\(_M\) was different from those with unmodified soy proteins. Some of the soy protein aggregates were about 5 to 10 \(\mu\)m in diameter (Figure 3.6) and had a more open structure than unmodified soy protein aggregates. Although most of the soy protein aggregates were present between para-casein micelle chains like those of SPI\(_N\), some soy proteins were attached to the surface of para-casein micelle (Figure 3.7b). The SPI\(_M\) was partially hydrolyzed during its manufacture. Its proteins were in a denatured state when analyzed by differential scanning calorimetry (data not shown). When heat denatured, the subunit structure of soy proteins is disrupted and the constituent acidic and basic polypeptides undergo sulfhydryl-disulfide exchange reactions (11, 24). Subsequently, highly reactive species are formed (24). The concentration of such species may increase on partial hydrolysis. These reactive species could bind to the surface of para-casein micelles as observed in the case of coagulum containing SPI\(_M\) (Figure 3.7b). When soy proteins were pretreated with different chemicals, they had different effects on milk coagulum strength (15). Thus the type of interaction of soy proteins with para-casein micelles in the coagulum may vary with the type of pretreatment given to soy proteins.

When sample pH was lowered during rennet coagulation, there was increased fusion of micelles (Figure 3.8). After just 50 min of adding rennet (at 1/10 the level for coagulation at pH 6.4), the casein micelles had fused into large strands (> 1 \(\mu\)m thick). No individual casein micelles could be identified. In contrast, the chains of micelles in pH 6.4 coagulum were typically < 0.2 \(\mu\)m thick. This acceleration of casein micelle fusion is well known in cheesemaking, and acid development by starter cultures is used to increase curd shrinkage.
Figure 3.6. Transmission electron micrograph of renneted milk coagulum containing commercial soy protein isolate, SPI_M, immunogold labeled for soy protein, showing large soy protein aggregate. Black dots represent the location of soy protein. Short arrows = casein micelles, long arrows = soy proteins.
Figure 3.7. Transmission electron micrograph of renneted milk coagulum containing commercial soy protein isolate, SPI$_M$, immunogold labeled for soy protein showing interaction between soy proteins and casein micelles. Figures (a) and (b) are at different magnifications. Black dots represent the location of soy protein. Short arrows = casein micelles, long arrows = soy proteins, short bold arrows = soy proteins attached to the surface of casein micelles.
Figure 3.8. Transmission electron micrograph of rennet-acid coagulum of the control (skim milk diluted with water in the ratio 1:11). Short arrows = casein micelles. Note the large fused casein micelles. Individual micelles are no longer discernible.
and whey expulsion. However, both native and modified soy proteins decreased casein micelle fusion. The individual casein micelles could be identified and soy proteins were fused with para-casein micelles (Figure 3.9).

**Syneresis**

Once the coagulum is formed, the paracasein micelle chains form thicker strands. Adjacent strands come closer expelling whey (syneresis). The coagulum shrinks and becomes firmer. However, in presence of soy proteins, the ability of the coagulum to expel whey decreased significantly. A similar effect has been observed by others (13, 15, 16, 17, 18, 19, 20).

Modified soy proteins inhibited syneresis to a greater extent than native soy proteins (Table 3.1). Presence of soy proteins on the surface of para-casein micelles, as with SPI_M (see Figure 3.7), would limit direct micelle-micelle contact by chemical interaction during aggregation and curd shrinkage. The large soy protein aggregates, especially those of SPI_M, could also decrease syneresis by physical intervention between the casein strands as they coalesce. Native soy proteins interacted only physically with the coagulum. On the other

<table>
<thead>
<tr>
<th>Treatment</th>
<th>Syneresis¹</th>
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<tbody>
<tr>
<td>Control</td>
<td>81.1 ± 0.3a</td>
</tr>
<tr>
<td>SPI_{NCW}</td>
<td>69.2 ± 0.3b</td>
</tr>
<tr>
<td>SPI_{NHW}</td>
<td>69.7 ± 0.3b</td>
</tr>
<tr>
<td>SPI_M</td>
<td>58.3 ± 1.6c</td>
</tr>
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¹Means followed by common letters were not significantly different at p=0.05
Figure 3.9. Transmission electron micrograph of rennet-acid coagulum containing (a) commercial soy protein isolate, SPI\textsubscript{M} and (b) native SPI, immunogold labeled for soy proteins. Black dots represent the location of soy protein. Short arrows = casein micelles, Long arrows = soy proteins, and f = soy proteins fused with casein micelles.
hand, the modified soy proteins interacted both chemically and physically with the coagulum para-casein micelles, which explains its greater effect on syneresis. Thus the state of proteins in SPI will have a large effect on syneresis depending on whether or not they block chemical fusion of the micelles. At a lower pH, however, presence of soy proteins did not affect syneresis. Both the control and coagulum containing soy proteins expelled about 90% of initial moisture.

Failure to locate soy proteins in milk coagulum using scanning electron microscopy (SEM) had led other researchers to conclude that soy proteins exist in an unaggregated state in the coagulum and occur commingled with caseins (14, 18). This, however, highlights the limitations of SEM in examining coagulum structures. Using TEM, higher magnifications can be used and positive identification of soy proteins can be made using immunogold labeling. In this study, the soy proteins were seen as large aggregates and, depending on the type of SPI used (native or modified) and sample pH (native milk pH or acidified milk), they may or may not have chemically bonded to the casein micelle network. Hence, if they were only physically entrapped, they could easily be lost from the fracture surface during SEM preparation. In addition, the need to metal coat the sample for SEM may also have masked the presence of soy protein. Thus, TEM may be a better technique in characterizing how different types of soy proteins exist in a rennet-induced milk coagulum and how they affect milk coagulation.

**CONCLUSIONS**

Immunogold labeling was successfully used to locate native and modified soy proteins in a milk coagulum. Soy proteins may exist as large aggregates
and as soy protein particles that bind to the para-casein micelle surfaces. Coagulum syneresis is retarded by adding soy proteins, particularly if the soy proteins bind to the micelle surfaces and prevent fusion. The soy proteins are partitioned between coagulum and whey with most of the glycinin being retained in the coagulum and most of the β-conglycinin being lost in the whey. The amount of soy proteins captured in renneted milk coagulum depends on the state of soy proteins used (native or modified) and the pH at which coagulation is conducted. Lowering milk pH results in almost 100% retention of glycinin in the coagulum, but β-conglycinin is still lost in the whey.

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Numerous studies report how soy proteins affect rennet coagulum properties of milk, but the mechanism by which soy proteins affect milk coagulation is not known. Milk-soy blends containing either native soy protein isolate (SPI\textsubscript{N}) or heat-denatured isolate (SPI\textsubscript{H}) were prepared by adding soy protein isolate dispersion (8.2%) to skim milk in the ratio 1:11 w/w. The Control and milk-soy blends at different pH's and CaCl\textsubscript{2} levels were prepared and rennet coagulated at 35°C to see the effect of soy proteins on curd firmness. We localized soy protein in the coagulum using immunogold labeling in conjunction with transmission electron microscopy (TEM) and studied the partitioning of different soy protein fractions between curd and whey using electrophoresis. We used this information to determine how soy proteins affect milk coagulation.

Soy proteins had the greatest effect on milk coagulum at pH 6.6. The SPI\textsubscript{H} increased rennet coagulation time (RCT) and decreased curd firmness (CF), whereas SPI\textsubscript{N} only increased RCT without affecting the final CF. Native soy protein was retained in the coagulum to a greater extent than heat-denatured soy proteins. In case of SPI\textsubscript{N}, β-conglycinin was completely lost in the whey, whereas α, α' subunits of β-conglycinin and acidic polypeptide of glycinin were lost in SPI\textsubscript{H} whey.

Soy proteins that were retained in curd existed as aggregates. The size of
these aggregates depended on the type of soy proteins, added CaCl$_2$, and pH. At pH 6.6, the SPI$_h$ aggregates were attached to casein micelle surface and prevented direct micelle-micelle contact. This delayed aggregation rate and decreased curd firmness by decreasing the number and strength of links between casein micelles. On the other hand, the SPI$_n$ aggregates (mainly glycinin) were physically trapped in curd without binding to micelles and delayed aggregation by binding calcium. Adding CaCl$_2$ or lowering the pH to 6.3 or 6.0 helped to restore coagulum properties. In these curds, both SPI$_n$ and SPI$_h$ soy proteins existed as aggregates that were physically trapped without binding to caseins.

**INTRODUCTION**

Soy proteins can offer economic advantage when used to partially replace proteins of animal origin, viz. milk, meat, poultry, seafood, or egg, in various food products. Their use as a food ingredient is therefore increasing (19, 20, 21, 46, 47), however, their use in specific dairy products like cheese is still being researched (18).

Numerous researchers have used soy protein or milk-soy blends to make cheese or cheese analogs (1, 23, 24, 25, 31, 32, 33, 34, 40, 41). These studies mainly discuss the effect of soy proteins on the various properties of rennet coagulum such as the rennet coagulation time, curd firmness, and syneresis. Although numerous theories have been put forward to explain how soy proteins affect milk coagulation, the mechanism is still unclear. A wide range of soy proteins was used by these researchers, including soy protein extracts (23), soy milk (31), heated soy milk (1, 40), chemically modified soy proteins (25), commercial soy protein isolate (34), and alkali-treated commercial soy protein.
isolate (32, 33). The inherent differences in these soy proteins could have been responsible for some of the differences in the results reported.

Soy proteins are heterogeneous in nature (8, 20) and their major fractions, glycinin and β-conglycinin, have different physicochemical properties (8, 35, 44). These properties may change during heating or alkali modification or the manufacture of commercial isolates. The effect of soy proteins on milk coagulation would depend to a large extent on the behavior of these two major soy protein fractions. However, very little information correlating their behavior in milk and their effect on milk coagulation is available. In addition, it is not known how heat-denatured soy proteins (as in heated soy milk) would compare with native undenatured soy proteins in their effect on milk coagulation.

Correlating the physicochemical properties of soy proteins to their behavior in milk or during milk coagulation may help to understand how they affect milk coagulation (5). In this study, we compared how undenatured soy proteins, heat-denatured soy proteins, and β-conglycinin affected various milk coagulum properties such as rennet clotting time, curd firming rate, and final curd firmness. In addition, we localized these different types of proteins in the curd using immunogold labeling in conjunction with transmission electron microscopy. Using SDS-PAGE, we determined how various soy protein fractions and their subunits partitioned between curd and whey under different conditions of the study. We used the information gathered from above to determine how soy proteins interact with milk during rennet-induced milk coagulation.
MATERIALS AND METHODS

Skim Milk

Whole milk was obtained from the Utah State University Dairy Products laboratory, skimmed (3000 x g for 1h at 4°C), preserved by adding sodium azide (0.05%), and filtered (GF/A filter paper, Whatman International Ltd., Maidstone, England) to produce skim milk with 3.1% protein. It was then refrigerated. Skim milk was heated at 60°C for 30 min before use, in order to restore its coagulating properties (2, 39).

Soy Protein Isolates

An unmodified soy protein isolate, SPI\textsubscript{N}, was prepared from defatted soy flour (Protein Technologies International, St. Louis, MO) according to Kolar et al. (21). Soy flour was stirred for 1 h with 15 volumes of deionized distilled water while the pH was continuously adjusted to 7.5. It was then centrifuged at 9000 x g for 30 min and the decanted supernatant was filtered through Whatman 4 filter paper. The supernatant was acidified to pH 4.5 to precipitate the proteins and the resulting slurry was again centrifuged at 9000 x g for 30 min and the pellet collected. It was washed twice with deionized distilled water, redispersed in deionized distilled water by continuously adjusting the pH to 7.5, and then freeze dried.

A heat-denatured soy protein isolate, SPI\textsubscript{H}, was prepared from SPI\textsubscript{N} as follows. A 3.5 ± 0.5 % solution of SPI\textsubscript{N} was heated in a 90°C water bath for 30 min, cooled in tap water, and then freeze dried. Denaturation of both β-conglycinin and glycinin was complete when this SPI\textsubscript{H} was tested using differential scanning calorimetry. The protein content of both SPI\textsubscript{N} and SPI\textsubscript{H} preparations was ~83% (N x 6.25) on a wet basis.
**β-Conglycinin**

Crude β-cONGLYcInin was prepared according to Nagano et al. (36). It was >90% pure as assessed by SDS-PAGE followed by densitometry. The protein content of the preparation was 85% (N x 6.25) on a wet basis. Two levels of β-cONGLyCININ (0.23% and 0.6%) were used in the milk coagulation study.

**Preparation of Soy Dispersion**

An 8.2% solution of SPI₈ or SPI₉ in water or 8% solution of β-cONGLyCININ in water was prepared, stirred for 1 h to allow hydration, and used immediately.

**Preparation of Milk-Soy Blends**

The SPI dispersions and skim milk were warmed to room temperature and mixed in the ratio 1:11 w/w to give 20% fortification of milk protein with soy protein. Adding soy proteins increased the pH of milk-soy blend and therefore a predetermined amount of 2N HCl was added immediately to decrease the pH approximately to the desired level (6.6 or 6.3 or 6.0). Calcium chloride (1 M) was added to some of the samples as required. A control blend was made by adding deionized water to skim milk (1:11 w/w) to maintain comparable casein levels. An undiluted skim milk sample was also tested. Three sets of study were done: 1) pH 6.6 with 4 levels of CaCl₂ (no calcium, 0.005%, 0.01%, 0.02%); 2) three pH levels (6.6, 6.3, 6.0); and 3) two pH levels (6.6 and 6.3) and two CaCl₂ levels (no calcium and 0.05%). All studies were replicated twice with two determinations of each sample per replicate. The above samples (skim milk, control, milk + SPI₈, milk + SPI₉, and milk + β-cONGLYcInIN) were warmed to 35°C and pH was continuously adjusted to the desired level until there was no change (~2.5 h) and was then used for coagulation studies. Statistica™ (Statsoft Inc., Tulsa, OK) was used for statistical analysis according to the
Coagulation Studies

Rennet coagulation time (RCT) and curd firmness (CF) were measured at 35°C using a Formagraph (Foss Inc., NY) according to McMahon and Brown (27). Single strength calf rennet (Rhone Poulenc, WI) was diluted 1:50 to 1.8 rennet units (RU)/ml. Ten grams of samples were weighed into sample cuvette wells and kept at 35°C for 30 min. Then 200 µl of diluted rennet was added and the development of curd firmness was studied in the recorder module, a 10-channel recording system, of the Formagraph. Each channel consists of pendulum (a wire loop) with a counterbalanced damper and an optical system. The sample oscillating system, light flashing unit, and a strip chart recorder are common to all 10 channels. The recorder module was started the instant enzyme was added. After mixing the enzyme-milk mixture, the sample (in each cuvette) is brought in contact with the immersed wire-loop pendulum and is subjected to linear oscillation. When milk remains uncoagulated, milk passes freely through the loop. The force transmitted from the coagulum to the pendulum is insufficient to tilt the pendulum. When coagulation occurs, the coagulum (in linear oscillation) pulls the pendulum along in synchronous motion. This motion of pendulum is recorded through the optical system at different lateral positions on a photosensitive paper. The observed rennet coagulation time (RCT) was determined by measuring the distance from the origin to the point where the baseline diverges. Curd firmness was determined by measuring the distance between the diverged lines (Formagraph units, mm) at specified intervals on the photosensitive paper. Then a graph of relative curd firmness (in mm) was plotted versus time after rennet addition.

Curd firmness at 2 x RCT was also measured and used as an indication of
relative curd firming rate, $K_F$. Measuring curd firmness at 2 x RCT removed the influence of RCT on curd firmness when curd firmness is measured at a fixed time after adding rennet. This facilitated easy comparison of curd firming rates for samples with different RCT's.

**Electron Microscopy**

Immunogold labeling of the cheese curd was done to localize soy proteins in the curd matrix. Milk blends at 35°C were rennet coagulated, and curd samples removed at twice the RCT were used for electron microscopy. Curd was cut to approximately 1-mm$^3$ pieces and treated further according to Alleyne (3) with slight modifications. The curd was fixed in 4% paraformaldehyde (in 0.1 $M$ phosphate buffer, pH 6.7) for 4 h. It was then dehydrated using increasing concentrations of graded ethanol solutions (1 x 10 min in 50% ethanol, 2 x 10 min and then overnight in 70% ethanol, 2 x 15 min in 95% ethanol, and 3 x 15 min in 100% ethanol). After dehydration, it was infiltrated (2 x 1 h and then for 48 h) in 100% LR White resin (Electron Microscopy Sciences, Fort Washington, PA), transferred to gelatin capsules (Electron Microscopy Sciences), and embedded at 50°C for 21 h. The hardened resin was removed from the gelatin capsule by immersing the capsule in water to dissolve the gelatin, and excess resin was trimmed to expose the sample. Ultrathin sections were cut using a MT2B ultramicrotome (Research and Manufacturing Co. Inc., Tucson, AZ) and collected on 300-mesh nickel grids. The grids were then coated with carbon to give rigidity to the sections to prevent tearing in the presence of electron beam.

The sections (in grids) were first blocked in 20 mM Tris buffer, pH 7.4, containing 0.5% fish gelatin solution (solution was 45% protein) and 0.5% normal goat serum for 15 min, and rinsed once for 5 min in 50 mM Tris saline buffer, pH 7.4. They were then incubated overnight with rabbit anti-soy antibody
(Sigma Chemical Co., St. Louis, MO), diluted 1:1000 in 50 mM Tris saline buffer, pH 7.4, and thereafter rinsed 6 x 5 min each in 50 mM Tris saline buffer, pH 7.4. This was followed by incubation for 3 h with 5 nm gold labeled--goat anti-rabbit antibody (Ted Pella Inc., Redding, CA), diluted 1:25 in 50 mM Tris saline buffer, pH 7.4, and rinsed 6 x 5 min with distilled water. They were then post stained with uranyl acetate (5 min at 45°C and 5 min at room temperature) and lead citrate (5 min at room temperature) and subsequently air dried. Stained sections were then examined using a Zeiss CEM 902 transmission electron microscope (Zeiss Inc., Thornwood, NY) at 80 kV. Images were recorded on Kodak (Eastman Kodak Co., Rochester, NY) electron image film 50-163.

**Electrophoresis**

Partitioning of soy protein fractions, β-conglycinin and glycinin, between whey and curd was determined using SDS-PAGE under reducing conditions. Electrophoresis was done in a Biorad Mini-Protean® II system (Biorad Laboratories, Hercules, CA) according to the Biorad Mini-Protean® II electrophoresis cell instruction manual (6) using 5% stacking gel and 20% resolving gel. To 0.5 ml of filtered cheese whey, 0.5 ml of Tris (20 mM) EDTA (2 mM) SDS (5%) buffer, pH 8.0, was added and the pH was readjusted to 8.0. After adding β-mercaptoethanol (50 µl), the samples were heated in boiling water bath for 5 min. Then 3 µl of 4.5% bromophenol blue and 110 µl of glycerol were added, and 3 µl was loaded on to the gel. The soy protein dispersions (SPI_N, SPI_H and β-conglycinin) were diluted 1:11 w/w with water and used as standards representing the amount of soy proteins added to milk. They were prepared for electrophoresis as described above. The gels were run at 100 V for 2.5 h. The gels were stained overnight in a solution containing
0.025% Coomassie blue, 40% methanol, and 7% acetic acid. They were then destained first in 50% methanol, 10% acetic acid solution for 2 h followed by overnight destaining in 5% methanol, 7% acetic acid solution.

RESULTS

Rennet Coagulation Time

Adding either native, SPI_N or heated, SPI_H soy proteins to milk significantly increased its RCT at normal milk pH of 6.6 (Tables 4.1 and 4.2). At normal conditions, with no added calcium, adding native or heat-denatured soy proteins nearly doubled the RCT of milk from 9 min to 16.4 to 18 min (Table 4.2). Adding 0.02% CaCl_2 overcame the effect of soy proteins on RCT of milk. Adding CaCl_2 up to 0.01% reduced the RCT of milk-soy blends but did not restore RCT to that of the control with no calcium at pH 6.6. However, adding 0.02% CaCl_2 restored the RCT of milk-soy blend to that of the control with no added calcium. The control milk with added calcium showed a corresponding decrease in RCT. Adding CaCl_2 to skim milk had less effect on RCT compared to the control sample, which had been diluted with water, which would account for the significant M x C interaction effect.

Similarly, lowering the pH from 6.6 to either 6.3 or 6.0 also helped to overcome the effect of SPI_N and SPI_H on the RCT of normal milk at pH 6.6 (Tables 4.3 and 4.4). When the pH of milk-soy blends was decreased to either 6.3 or 6.0, their RCT values were significantly lower than that of the control at pH 6.6 (Table 4.4). Combining pH and calcium study in the range 6.6 to 6.3 and 0 to 0.05% added CaCl_2, every main effect and all interactions significantly affected RCT (Tables 4.5 and 4.6). Adding 0.05% CaCl_2 and reducing the pH to 6.3 resulted in all samples (including skim milk) having the same RCT,
Table 4.1. Analysis of variance for rennet coagulation time (RCT) of coagulated milk as a function of adding soy protein isolate at pH 6.6 with different levels of added calcium and coagulated by rennet.

<table>
<thead>
<tr>
<th>Source</th>
<th>df</th>
<th>MS</th>
<th>P</th>
</tr>
</thead>
<tbody>
<tr>
<td>Replicate</td>
<td>1</td>
<td>4.10</td>
<td>0.168</td>
</tr>
<tr>
<td>Milk-blend (M)</td>
<td>3</td>
<td>193.6</td>
<td>0.000</td>
</tr>
<tr>
<td>Calcium (C)</td>
<td>3</td>
<td>64.64</td>
<td>0.000</td>
</tr>
<tr>
<td>M x C</td>
<td>9</td>
<td>5.135</td>
<td>0.0476</td>
</tr>
<tr>
<td>Error</td>
<td>15</td>
<td>1.957</td>
<td></td>
</tr>
<tr>
<td>Subsampling</td>
<td>32</td>
<td>0.186</td>
<td></td>
</tr>
</tbody>
</table>

Table 4.2. Mean rennet coagulation time, RCT\(^1\) in minutes, of coagulated milk as a function of adding soy protein isolate at pH 6.6 with different levels of added calcium and coagulated by rennet.

<table>
<thead>
<tr>
<th>Samples</th>
<th>% Calcium</th>
<th>added</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>0.0</td>
<td>0.005</td>
</tr>
<tr>
<td>Skim milk</td>
<td>9.0</td>
<td>9.1</td>
</tr>
<tr>
<td>Control(^2)</td>
<td>9.2</td>
<td>8.8</td>
</tr>
<tr>
<td>SPI(_N^3)</td>
<td>16.4</td>
<td>15.3</td>
</tr>
<tr>
<td>SPI(_H^4)</td>
<td>17.6</td>
<td>16.6</td>
</tr>
</tbody>
</table>

\(^1\) LSD\(_{0.05}\) = 2.1 min.
\(^2\) skim milk (11 parts) diluted with 1 part of water.
\(^3\) skim milk containing native soy protein isolate.
\(^4\) skim milk containing heat treated soy protein isolate.
Table 4.3. Analysis of variance for rennet coagulation time (RCT) of coagulated milk as a function of adding soy protein isolate at pH 6.6, 6.3, and 6.0 and coagulated by rennet.

<table>
<thead>
<tr>
<th>Source</th>
<th>df</th>
<th>MS</th>
<th>P</th>
</tr>
</thead>
<tbody>
<tr>
<td>Replicate</td>
<td>1</td>
<td>3.08</td>
<td>0.092</td>
</tr>
<tr>
<td>Milk-blend (M)</td>
<td>3</td>
<td>49.8</td>
<td>0.0000</td>
</tr>
<tr>
<td>pH (P)</td>
<td>2</td>
<td>526.4</td>
<td>0.0000</td>
</tr>
<tr>
<td>M x P</td>
<td>6</td>
<td>20.43</td>
<td>0.0000</td>
</tr>
<tr>
<td>Error</td>
<td>11</td>
<td>0.9027</td>
<td></td>
</tr>
<tr>
<td>Subsampling</td>
<td>24</td>
<td>0.1277</td>
<td></td>
</tr>
</tbody>
</table>

Table 4.4. Mean rennet coagulation time, RCT\(^1\) in minutes of coagulated milk as a function of adding soy protein isolate at pH 6.6, 6.3, and 6.0 and coagulated by rennet.

<table>
<thead>
<tr>
<th>Samples</th>
<th>pH</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>6.6</td>
</tr>
<tr>
<td>Skim milk</td>
<td>9.0</td>
</tr>
<tr>
<td>Control(^2)</td>
<td>9.2</td>
</tr>
<tr>
<td>SPI(_N)^3</td>
<td>16.4</td>
</tr>
<tr>
<td>SPI(_H)^4</td>
<td>17.6</td>
</tr>
</tbody>
</table>

\(^1\) LSD\(_{0.05}\) = 1.5 min.
\(^2\) skim milk (11 parts) diluted with 1 part of water.
\(^3\) skim milk containing native soy protein isolate.
\(^4\) skim milk containing heat treated soy protein isolate.
Table 4.5. Analysis of variance for rennet coagulation time (RCT) of coagulated milk as a function of adding soy protein isolate at pH (6.6 and 6.3) and calcium levels (0 and 0.05%); and coagulated by rennet.

<table>
<thead>
<tr>
<th>Source</th>
<th>df</th>
<th>MS</th>
<th>P</th>
</tr>
</thead>
<tbody>
<tr>
<td>Replicate</td>
<td>1</td>
<td>1.34</td>
<td>0.2266</td>
</tr>
<tr>
<td>Milk-blend (M)</td>
<td>3</td>
<td>50.90</td>
<td>0.0000</td>
</tr>
<tr>
<td>pH (P)</td>
<td>1</td>
<td>619.08</td>
<td>0.0000</td>
</tr>
<tr>
<td>Calcium (C)</td>
<td>1</td>
<td>306.91</td>
<td>0.0000</td>
</tr>
<tr>
<td>M x P</td>
<td>3</td>
<td>18.35</td>
<td>0.0000</td>
</tr>
<tr>
<td>M x C</td>
<td>3</td>
<td>19.36</td>
<td>0.0000</td>
</tr>
<tr>
<td>P x C</td>
<td>1</td>
<td>90.61</td>
<td>0.0000</td>
</tr>
<tr>
<td>M x P x C</td>
<td>3</td>
<td>6.59</td>
<td>0.0022</td>
</tr>
<tr>
<td>Pooled error</td>
<td>15</td>
<td>0.8410</td>
<td></td>
</tr>
<tr>
<td>Subsampling</td>
<td>32</td>
<td>0.0419</td>
<td></td>
</tr>
</tbody>
</table>

Table 4.6. Mean rennet coagulation time, RCT\(^1\) in minutes of coagulated milk as a function of adding soy protein isolate at pH (6.6 and 6.3) and calcium levels (0 and 0.05%); and coagulated by rennet.

<table>
<thead>
<tr>
<th>Samples</th>
<th>%Calcium added</th>
<th>pH 6.6</th>
<th>pH 6.3</th>
<th>pH 6.6</th>
<th>pH 6.3</th>
</tr>
</thead>
<tbody>
<tr>
<td>Skim milk</td>
<td>9.0</td>
<td>3.4</td>
<td>5.6</td>
<td>2.3</td>
<td></td>
</tr>
<tr>
<td>Control(^2)</td>
<td>9.2</td>
<td>3.5</td>
<td>5.1</td>
<td>2.1</td>
<td></td>
</tr>
<tr>
<td>SPI(_N)(^3)</td>
<td>16.4</td>
<td>5.3</td>
<td>7.0</td>
<td>2.7</td>
<td></td>
</tr>
<tr>
<td>SPI(_H)(^4)</td>
<td>17.6</td>
<td>5.7</td>
<td>7.5</td>
<td>2.7</td>
<td></td>
</tr>
</tbody>
</table>

\(^1\) LSD\(_{0.05}\) = 1.5 min.
\(^2\) skim milk (11 parts) diluted with 1 part of water.
\(^3\) skim milk containing native soy protein isolate.
\(^4\) skim milk containing heat treated soy protein isolate.
equivalent to approximately \(0.25 \times \text{RCT}\) of skim milk at pH 6.6 (Table 4.6). The reduced effect of soy proteins on the RCT of milk at lower pH's or added calcium has been observed by other researchers (1, 31).

Adding \(\beta\)-conglycinin to milk affected its RCT at pH 6.6 (Table 4.7). Adding 0.6% of \(\beta\)-conglycinin (which corresponds to 20% fortification of milk proteins with \(\beta\)-conglycinin) to milk at pH 6.6 increased its RCT to \(2.6 \times \text{control}\). On the other hand, adding 0.23% of \(\beta\)-conglycinin (equivalent to the amount present in milk-soy blend when SPI\(N\) was added to milk) increased its RCT only slightly to \(1.4 \times \text{control}\) compared to 1.8 for SPI\(N\) and 1.9 for SPI\(H\). Lowering the pH to 6.3 or adding 0.05% CaCl\(_2\) or both, reduced the RCT of \(\beta\)-conglycinin-milk blend to comparable values as for SPI\(N\).

Table 4.7. Mean rennet coagulation time (RCT) in minutes of coagulated milk as a function of \(\beta\)-conglycinin at pH (6.6 and 6.3) and calcium levels (0 and 0.05%); and coagulated by rennet.

<table>
<thead>
<tr>
<th>(\beta)-Conglycinin (%)</th>
<th>% Calcium added</th>
<th>% Calcium added</th>
<th>% Calcium added</th>
<th>% Calcium added</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>0</td>
<td>0</td>
<td>0.05</td>
<td>0.05</td>
</tr>
<tr>
<td>pH 6.6</td>
<td>0</td>
<td>0</td>
<td>0.05</td>
<td>0.05</td>
</tr>
<tr>
<td>0.23%</td>
<td>12.8</td>
<td>4.8</td>
<td>6.3</td>
<td>2.5</td>
</tr>
<tr>
<td>0.6%</td>
<td>24.4</td>
<td>8.1</td>
<td>7.6</td>
<td>2.8</td>
</tr>
</tbody>
</table>

**Curd Firming Rate, \(K_F\)**

When water was added to skim milk as in the control sample, its \(K_F\) was decreased. The presence of soy proteins, either SPI\(N\) or SPI\(H\), also significantly decreased \(K_F\) of milk at pH 6.6 (Tables 4.8 and 4.9). Heat-denatured soy proteins had a greater effect than native soy proteins on reducing \(K_F\) of milk (Table 4.9). Adding 0.005% or 0.01% CaCl\(_2\) did not increase the \(K_F\) of milk-soy blends. Increasing the CaCl\(_2\) level to 0.02% restored the \(K_F\) of milk.
Table 4.8. Analysis of variance for relative firmness at 2 x RCT of coagulated milk as a function of adding soy protein isolate at pH 6.6 with different levels of added calcium and coagulated by rennet.

<table>
<thead>
<tr>
<th>Source</th>
<th>df</th>
<th>MS</th>
<th>P</th>
</tr>
</thead>
<tbody>
<tr>
<td>Replicate</td>
<td>1</td>
<td>4.79</td>
<td>0.364</td>
</tr>
<tr>
<td>Milk-blend (M)</td>
<td>3</td>
<td>381.6</td>
<td>0.000</td>
</tr>
<tr>
<td>Calcium (C)</td>
<td>3</td>
<td>10.42</td>
<td>0.172</td>
</tr>
<tr>
<td>MxC</td>
<td>9</td>
<td>8.01</td>
<td>0.246</td>
</tr>
<tr>
<td>Error</td>
<td>15</td>
<td>5.459</td>
<td></td>
</tr>
<tr>
<td>Subsampling</td>
<td>32</td>
<td>1.804</td>
<td></td>
</tr>
</tbody>
</table>

Table 4.9. Mean relative firmness (mm) at twice the rennet clotting time\(^1\) of coagulated milk as a function of adding soy protein isolate at pH 6.6 with different levels of added calcium and coagulated by rennet.

<table>
<thead>
<tr>
<th>Samples</th>
<th>% Calcium added</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>0.0</td>
</tr>
<tr>
<td>Skim milk</td>
<td>21.3</td>
</tr>
<tr>
<td>Control(^2)</td>
<td>17.6</td>
</tr>
<tr>
<td>SPI(_N)(^3)</td>
<td>12.6</td>
</tr>
<tr>
<td>SPI(_H)(^4)</td>
<td>9.1</td>
</tr>
</tbody>
</table>

\(^1\) LSD\(_{0.05}\) = 3.5 mm.
\(^2\) skim milk (11 parts) diluted with 1 part of water.
\(^3\) skim milk containing native soy protein isolate.
\(^4\) skim milk containing heat treated soy protein isolate.
containing SPI\textsubscript{N} to that of the control but the $K_F$ of milk containing SPI\textsubscript{H} did not increase to the same extent and was still significantly different to the control (Table 4.9). However, lowering the pH to 6.3 or 6.0 (Tables 4.10 and 4.11) or adding 0.05% CaCl\textsubscript{2} (Tables 4.12 and 4.13) increased the curd firming rate of both SPI\textsubscript{N} and SPI\textsubscript{H} to that of the control.

**Relative Curd Firmness**

Diluting skim milk with water (as in the control sample) decreased the rate of curd firmness development and final curd firmness of skim milk (Figure 4.1). Adding native soy proteins (SPI\textsubscript{N}) further decreased the development of curd firmness but the final curd firmness was almost equal to that of the control at pH 6.6. Adding $\beta$-conglycinin at 0.23% level had a similar effect. On the other hand, heat-denatured soy proteins (SPI\textsubscript{H}) decreased both the rate of curd firmness development and final curd firmness under the same conditions as did $\beta$-conglycinin at 0.6% (Figure 4.1a).

Adding CaCl$_2$ at 0.005% did not improve curd firmness development of SPI\textsubscript{N} or SPI\textsubscript{H} (Figure 4.1b). However, adding CaCl$_2$ at 0.01% levels increased curd firmness development of both SPI\textsubscript{N} and SPI\textsubscript{H} (Figure 4.1c). Further, at 0.02% CaCl$_2$, curd firmness development of milk containing SPI\textsubscript{N} was slightly higher than the control with or without added calcium although it took longer to reach that firmness level (Figure 4.1d). On the other hand, the curd firmness development and final curd firmness of milk containing SPI\textsubscript{H} only increased slightly when CaCl$_2$ was added up to 0.02%. All samples had similar curd firmness development and final curd firmness when pH was lowered to 6.3 or 6.0 (Figure 4.2). Adding 0.05% CaCl$_2$ restored curd firmness development and final curd firmness of milk containing SPI\textsubscript{H} (Figure 4.3a) and $\beta$-conglycinin added at 0.6% (Figure 4.3b).
Table 4.10. Analysis of variance for relative firmness at 2 x RCT of coagulated milk as a function of adding soy protein isolate at pH 6.6, 6.3, and 6.0 and coagulated by rennet.

<table>
<thead>
<tr>
<th>Source</th>
<th>df</th>
<th>MS</th>
<th>P</th>
</tr>
</thead>
<tbody>
<tr>
<td>Replicate</td>
<td>1</td>
<td>0.12</td>
<td>0.8644</td>
</tr>
<tr>
<td>Milk-blend (M)</td>
<td>3</td>
<td>85.97</td>
<td>0.0000</td>
</tr>
<tr>
<td>pH (P)</td>
<td>2</td>
<td>68.08</td>
<td>0.0004</td>
</tr>
<tr>
<td>M x P</td>
<td>6</td>
<td>31.49</td>
<td>0.0017</td>
</tr>
<tr>
<td>Error</td>
<td>11</td>
<td>3.931</td>
<td></td>
</tr>
<tr>
<td>Subsampling</td>
<td>24</td>
<td>5.109</td>
<td></td>
</tr>
</tbody>
</table>

Table 4.11. Mean relative firmness in mm at twice the rennet clotting time\(^1\) (value in parentheses) of coagulated milk as a function of adding soy protein isolate at pH 6.6, 6.3, and 6.0 and coagulated by rennet.

<table>
<thead>
<tr>
<th>Samples</th>
<th>pH</th>
<th></th>
<th></th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>6.6</td>
<td>6.3</td>
<td>6.0</td>
</tr>
<tr>
<td>Skim milk</td>
<td>21.3</td>
<td>19.3</td>
<td>20.3</td>
</tr>
<tr>
<td>Control(^2)</td>
<td>17.6</td>
<td>16.4</td>
<td>19.6</td>
</tr>
<tr>
<td>SPI(_N)^3</td>
<td>12.6</td>
<td>13.5</td>
<td>16.9</td>
</tr>
<tr>
<td>SPI(_H)^4</td>
<td>9.1</td>
<td>17</td>
<td>20</td>
</tr>
</tbody>
</table>

\(^1\) LSD\(_{0.05}\) = 3.1 mm.
\(^2\) skim milk (11 parts) diluted with 1 part of water.
\(^3\) skim milk containing native soy protein isolate.
\(^4\) skim milk containing heat treated soy protein isolate.
Table 4.12. Analysis of variance for relative firmness at 2 x RCT of coagulated milk as a function of adding soy protein isolate at pH (6.6 and 6.3) and calcium levels (0 and 0.05%); and coagulated by rennet.

<table>
<thead>
<tr>
<th>Source</th>
<th>df</th>
<th>MS</th>
<th>P</th>
</tr>
</thead>
<tbody>
<tr>
<td>Replicate</td>
<td>1</td>
<td>0.0506</td>
<td>0.8860</td>
</tr>
<tr>
<td>Milk-blend (M)</td>
<td>3</td>
<td>65.62</td>
<td>0.0000</td>
</tr>
<tr>
<td>pH (P)</td>
<td>1</td>
<td>1.96</td>
<td>0.3784</td>
</tr>
<tr>
<td>Calcium (C)</td>
<td>1</td>
<td>177.56</td>
<td>0.0000</td>
</tr>
<tr>
<td>M x P</td>
<td>3</td>
<td>14.52</td>
<td>0.0063</td>
</tr>
<tr>
<td>M x C</td>
<td>3</td>
<td>37.02</td>
<td>0.0000</td>
</tr>
<tr>
<td>P x C</td>
<td>1</td>
<td>16.81</td>
<td>0.0178</td>
</tr>
<tr>
<td>M x P x C</td>
<td>3</td>
<td>27.66</td>
<td>0.0000</td>
</tr>
<tr>
<td>Error</td>
<td>15</td>
<td>2.379</td>
<td></td>
</tr>
<tr>
<td>Subsampling</td>
<td>32</td>
<td>5.109</td>
<td></td>
</tr>
</tbody>
</table>

Table 4.13. Mean relative firmness in mm at twice the rennet clotting time\(^1\) of coagulated milk as a function of adding soy protein isolate at pH (6.6 and 6.3) and calcium levels (0 and 0.05%); and coagulated by rennet.

<table>
<thead>
<tr>
<th>% Calcium added</th>
<th>Samples</th>
<th>pH 6.6</th>
<th>pH 6.3</th>
<th>pH 6.6</th>
<th>pH 6.3</th>
</tr>
</thead>
<tbody>
<tr>
<td>0</td>
<td>Skim milk</td>
<td>21.3</td>
<td>19.3</td>
<td>19.9</td>
<td>20.3</td>
</tr>
<tr>
<td>0.05</td>
<td>Control(^2)</td>
<td>17.6</td>
<td>16.4</td>
<td>19.2</td>
<td>18.6</td>
</tr>
<tr>
<td>0.05</td>
<td>SPI(_N)(^3)</td>
<td>12.6</td>
<td>13.5</td>
<td>18.8</td>
<td>17.8</td>
</tr>
<tr>
<td>0.05</td>
<td>SPI(_H)(^4)</td>
<td>9.1</td>
<td>17</td>
<td>20.3</td>
<td>18.7</td>
</tr>
</tbody>
</table>

\(^1\) LSD\(_{0.05}\) = 3.1 mm.
\(^2\) skim milk (11 parts) diluted with 1 part of water.
\(^3\) skim milk containing native soy protein isolate.
\(^4\) skim milk containing heat treated soy protein isolate.
Figure 4.1. Changes in gel firmness in Formagraph units (FU) after adding rennet (0.36 RU/10 g of sample, 35°C) measured using a Formagraph of samples at pH 6.6 and (a) no calcium; (b) 0.005% CaCl₂; (c) 0.01% CaCl₂; and (d) 0.02% CaCl₂. Samples include skim milk, control (11 parts skim milk + 1 part water, 11:1), SPI₁₆ (11 parts skim milk + 1 part of 8.2% undenatured soy protein isolate solution), SPI₁₆ (11 parts skim milk + 1 part of 8.2% heat-denatured soy protein isolate solution), bCG (11 parts skim milk + 1 part of 8% β-conglycinin solution), and bCG⁺ (11 parts skim milk + 1 part of 3% β-conglycinin solution).
(a) pH 6.6, no added calcium chloride

(b) pH 6.6, 0.005% added calcium chloride
(c) pH 6.6, 0.01% added calcium chloride

(d) pH 6.6, 0.02% added calcium chloride
Figure 4.2. Changes in gel firmness in Formagraph units (FU) after adding rennet (0.36 RU/10 g of sample, 35°C) measured using a Formagraph of samples at (a) pH 6.3 and (b) pH 6.0. Samples include skim milk, control (11 parts skim milk + 1 part water, 11:1), SPI_{N} (11 parts skim milk + 1 part of 8.2% undenatured soy protein isolate solution), SPI_{H} (11 parts skim milk + 1 part of 8.2% heat-denatured soy protein isolate solution), bCG (11 parts skim milk + 1 part of 8% β-conglycinin solution), and bCG* (11 parts skim milk + 1 part of 3% β-conglycinin solution).
Figure 4.3. Changes in gel firmness in Formagraph units (FU) after adding rennet (0.36 RU/10 g of sample, 35°C) measured using a Formagraph of samples with 0.05% added CaCl₂ at (a) pH 6.6 and (b) pH 6.3. Samples include skim milk, control (11 parts skim milk + 1 part water, 11:1), SPI_N (11 parts skim milk + 1 part of 8.2% undenatured soy protein isolate solution), SPI_H (11 parts skim milk + 1 part of 8.2% heat-denatured soy protein isolate solution), bCG (11 parts skim milk + 1 part of 8% β-conglycinin solution), and bCG* (11 parts skim milk + 1 part of 3% β-conglycinin solution).
(a) pH 6.6, 0.05% added calcium chloride

(b) pH 6.3, 0.05% added calcium chloride
In studies by other researchers (23, 31, 32, 33), soy protein was observed to decrease curd firmness of milk at pH 6.5. However, in contrast to results in our studies, adding CaCl₂ did not seem to improve curd firmness (hardness) of milk (31, 32). The effect of pH on curd firmness was in partial agreement to our results. A pH of ≤ 6 either restored (31) or did not restore (32) the curd firmness of milk-soy blend when compared to that of the control.

Partitioning of Soy Proteins Between Curd and Whey

When milk is rennet coagulated, caseins are retained in the curd, whereas whey proteins such as β-lactoglobulin, α-lactalbumin, and bovine serum albumin are lost in the whey (lane 2, Figure 4.4). Some partitioning of different soy protein fractions between curd and whey also occurred (Figures 4.4). The whey exuded from the curds of milk-soy blends contained reduced amounts of some of the added soy proteins (compare bands corresponding to soy proteins in lanes 1 and 11, which correspond to soy proteins added to milk, with other lanes; Figure 4.4). The extent of soy proteins lost in the whey depended on the pH at which rennet coagulation was carried out. Greater loss of soy proteins occurred at pH 6.6 as compared to pH 6.0 for both SPIₐ and SPIₜ (compare lanes 3 versus 9 and 4 versus 10). More of β-conglycinin was lost in the whey as compared to glycinin. Moreover, the different components of glycinin and β-conglycinin were lost to different extents in the whey (Figure 4.4). Wheys of SPIₕ contained more acidic polypeptides of glycinin than those of SPIₐ. On the other hand, SPIₕ whey contained lesser amounts of both basic polypeptide of glycinin and β subunit of conglycinin as compared to SPIₐ whey (compare corresponding bands in lanes 3 and 4; 5 and 6; 7 and 8; Figure 4.4). When the intensity of bands for SPIₐ and SPIₕ in the wheys are compared, it seems as
Figure 4.4. SDS-PAGE analysis of rennet wheys of different samples. SPI<sub>N</sub>--native soy protein isolate. SPI<sub>H</sub>--heat-denatured soy protein isolate. Lanes 3, 5, 7 and 9--wheys of curd containing SPI<sub>N</sub>. Lanes 4, 6, 8 and 10--wheys of curd containing SPI<sub>H</sub>. Lane 1--standard SPI<sub>N</sub> (Std SPI<sub>N</sub>); Lane 2--control (C); Lane 11--standard SPI<sub>H</sub> (Std SPI<sub>H</sub>). Lanes 2, 3 and 4--pH 6.6; Lanes 5 and 6--pH 6.6 and 0.02% CaCl<sub>2</sub>; Lanes 7 and 8--pH 6.6 and 0.05% CaCl<sub>2</sub>; Lanes 9 and 10--pH 6.0. BSA--bovine serum albumin; β-LG--β-lactoglobulin; α-LA--α-lactalbumin; β-CG--β-conglycinin (made of α, α' and β subunits); G--glycinin (made of AP--acidic polypeptide and BP--basic polypeptide).
though more heat-denatured soy proteins (SPI₇) are lost as compared to native soy proteins (SPI₅). When β-conglycinin was added to milk and rennet coagulated, all the added β-conglycinin was lost in the whey (compare lane 1 versus 2 and 3; lane 7 versus 5 and 6; Figure 4.5).

**Curd Microstructure**

The para-casein micelles in renneted milk coagulum (control) appear as electron-dense spheres that have aggregated into clumps and chains (Figure 4.6). Such a structure is typical of transmission electron micrographs of milk curd before extensive curd syneresis has occurred (13). Only slight nonspecific labeling by the anti-soy antibody was observed in the control sample with no specific labeling on casein proteins. The curd containing β-conglycinin did not show any labeling (Figure 4.7) either on the micelles or between the micelles, indicating none of β-conglycinin was retained in the curd. No electron-dense material excluding casein micelles was seen in the curd.

When soy protein isolates, either native or heat-treated, were added to milk and then rennet coagulated, the soy proteins that were retained in the coagulum appeared as large aggregated particles that were less electron dense than para-casein micelles (Figures 4.8 and 4.13). Strong labeling with the anti-soy immunogold system was seen on such particles. The level of labeling on caseins was comparable to that observed for nonspecific labeling, which demonstrated the specificity of the anti-soy protein immunogold labeling system to soy proteins.

When milk-soy blends containing either native or heat-denatured soy proteins were rennet coagulated, the soy proteins existed as aggregates in the coagulum (Figures 4.8 through 4.13). The appearance of these aggregates depended on the type of soy protein added to milk, pH of the curd, and the
Figure 4.5. SDS-PAGE analysis of rennet wheys of different samples containing either 0.23% β-conglycinin (lanes 2 and 3) or 0.6% β-conglycinin (lanes 5 and 6) or no β-conglycinin (lane 4, control). Lane 1--standard 0.23% β-conglycinin (Std 0.23% β-CG). Lane 7--standard 0.6% β-conglycinin (Std 0.6% β-CG). Lanes 2, 4, and 5--pH 6.6; Lanes 3 and 6--pH 6.6 and 0.05% CaCl₂. BSA--bovine serum albumin; β-LG--β-lactoglobulin; α-LA--α-lactalbumin; β-CG--β-conglycinin (made of α, α’ and β subunits).
Figure 4.6. Transmission electron micrograph of the renneted control milk at pH 6.6 (skim milk diluted with water in the ratio 11:1). Short arrows = casein micelles. Note the smooth surface of casein micelles.
Figure 4.7. Transmission electron micrograph of rennet curd of milk containing β-conglycinin at pH 6.6 (a) 0.6% and (b) 0.23%. Short arrows = casein micelles. Note the smooth surface of casein micelles and the lack of labeling on either the casein micelles or between them.
Figure 4.8. Transmission electron micrograph of rennet curd of milk containing native soy proteins at pH 6.6 immunogold labeled for soy protein. Black dots represent the location of soy protein. Figures (a) and (b) are at different magnifications. Short arrows = casein micelles, long arrows = soy proteins, closed arrow heads = physical separation between casein micelles and soy proteins.
Figure 4.9. Transmission electron micrograph of rennet curd of milk containing native soy proteins at pH 6.6 immunogold labeled for soy protein. Black dots represent the location of soy protein. Figures (a) and (b) are at same magnifications. Short arrows = casein micelles, long arrows = soy proteins, closed arrow heads = physical separation between casein micelles and soy proteins.
Figure 4.10. Transmission electron micrograph of rennet curd of milk containing heat-denatured soy proteins at pH 6.6 immunogold labeled for soy protein. Figures (a) and (b) are at different magnifications. Black dots represent the location of soy protein. Short arrows = casein micelles, long arrows = soy proteins, short bold arrows = soy proteins attached to the surface of casein micelles.
Figure 4.11. Transmission electron micrograph of rennet curd of milk containing heat-denatured soy proteins at pH 6.6 immunogold labeled for soy protein. Figures (a) and (b) are at same magnifications. Black dots represent the location of soy protein. Short arrows = casein micelles, long arrows = soy proteins, short bold arrows = soy proteins attached to the surface of casein micelles.
Figure 4.12. Transmission electron micrograph of rennet curd of milk containing heat-denatured soy proteins at pH 6.6 and 0.05% CaCl₂ immunogold labeled for soy protein. Figures (a) and (b) are at different magnifications. Black dots represent the location of soy protein. Short arrows = casein micelles, long arrows = soy proteins, closed arrow heads = physical separation between casein micelles and soy proteins.
Figures 4.13. Transmission electron micrograph of rennet curd of milk containing heat-denatured soy proteins at pH 6.6 and 0.05% CaCl₂ immunogold labeled for soy protein. Figures (a) and (b) are at same magnifications. Black dots represent the location of soy protein. Short arrows = casein micelles, long arrows = soy proteins, closed arrow heads = physical separation between casein micelles and soy proteins.
presence of CaCl$_2$. In milk at pH 6.6, the native soy protein, SPI$_N$, aggregates (Figures 4.8 and 4.9) were bigger and more electron dense than those of heat-treated soy proteins, SPI$_H$ (Figures 4.10 and 4.11). However, the SPI$_H$ aggregates in pH 6.6 curd were distributed more uniformly throughout the curd than those of SPI$_N$. The size of SPI$_H$ soy protein aggregates in pH 6.6 curd increased in the presence of 0.05% CaCl$_2$ (Figures 4.12 and 4.13) and was much bigger than those of SPI$_H$ or SPI$_N$ at pH 6.6. Similarly, the size of soy protein aggregates in curd containing SPI$_N$ at pH 6.6 and 0.05% CaCl$_2$ or curd of either SPI$_N$ or SPI$_H$ at pH 6.3 was also very large (data not shown).

In the renneted control coagulum, the surface of para-casein micelles appeared smooth (Figure 4.6) with no proteinaceous material sticking out from their surface. These micelles were partially fused and formed chains as usually observed in renneted milk. The type of interaction occurring between soy protein aggregates and para-casein micelles in the curd depended on the type of soy proteins (SPI$_N$ or SPI$_H$), pH, and level of CaCl$_2$. The soy protein aggregates in pH 6.6 curd containing SPI$_N$ were present between the para-casein micelle chains (Figures 4.8 and 4.9). These aggregates were clearly separated from the smooth surface of para-casein micelles (Figures 4.8b, 4.9a, and 4.9b). Similarly, the soy protein aggregates in pH 6.6 curd containing SPI$_H$ in presence of 0.05% CaCl$_2$ were also clearly separated from the para-casein micelle surface (Figures 4.12b, 4.13a, and 4.13b) and present between the para-casein micelle chains. On the other hand, the SPI$_H$ aggregates in pH 6.6 curd interacted with the para-casein micelles in a totally different manner. These SPI$_H$ aggregates tended to bind to the surface of some of the para-casein micelles (Figures 4.10b, 4.11a, 4.11b) and prevented direct micelle-micelle contact although some of these aggregates were also present.
between the para-casein micelle chains.

DISCUSSION

Glycinin and β-conglycinin are the two major fractions of soy protein (8). β-Conglycinin (MW = 180,000 Da) is made up of α, α', and β subunits (8) and has an isoelectric point, pl of 4.8 (44). On the other hand, glycinin (MW = 360,000 Da) is made up of six subunits with each subunit consisting of one acidic (AP) and one basic polypeptide (BP) (8, 38). It has a pl of 6.4 (44).

When soy proteins are heated, the polypeptides that form the subunits of glycinin and β-conglycinin dissociate, unfold, and then subsequently aggregate (20). When glycinin is heated in the presence of β-conglycinin (as occurs when soy protein isolate or soy milk or soy extract is heated), they both interact with each other (20, 49). Specifically the β-subunit of conglycinin interacts with basic polypeptide of glycinin via electrostatic interaction (20). In addition, the α and α' subunits of β-conglycinin interact with acidic polypeptides of glycinin via disulfide interaction (49).

Retention of Soy Proteins in Curd

In rennet-coagulation studies using native soy protein, most of β-conglycinin is lost in the whey, whereas most of glycinin is retained in the curd (Figure 4.4). Similarly, in studies where β-conglycinin is added to milk and rennet-coagulated, all of it is lost in whey (Figure 4.5). β-Conglycinin is in a soluble form at pH 6.6 (pH > its pl). It does not seem to bind or interact with casein micelles as there is no labeling on micelles in the curd with added β-conglycinin nor is it in a form that can be physically trapped in the network (Figure 4.7). Therefore, it is lost in the whey. On the other hand, pH 6.6 is very close to the pl (6.4) of glycinin, which may exist in an aggregated form large enough to be
trapped in the coagulation casein matrix. Moreover, glycinin can aggregate in the presence of ionic calcium (38), which is present in milk. Therefore, the aggregates seen in the electron micrographs of curd containing SPI$_N$ (Figures 4.8 and 4.9) are probably those of glycinin. It can therefore be expected that β-conglycinin is lost in whey and glycinin is retained in the curd when SPI$_N$ is added to milk and rennet coagulated.

On the other hand, the components of heat-denatured soy proteins, SPI$_H$, behave differently when added to milk. There is a greater loss of soy proteins in the whey. The heat-induced complex formed between β-subunit of β-conglycinin and basic polypeptide of glycinin is retained in the curd, whereas acidic polypeptide of glycinin and α, α’ subunits of β-conglycinin are lost in the whey (Figure 4.4). Acidic polypeptides of glycinin with pI in the range 4.6 to 5.4 (8, 44) may exist in a soluble form in milk and will be lost in the whey if it does not complex with micelles or get physically trapped in the micelle network. A similar explanation may explain the behavior of α and α’ subunits of β-conglycinin. The soy protein aggregates seen in the electron micrographs of the curd containing SPI$_H$, therefore, may be those of a heat-induced complex containing β-subunits of β-conglycinin and basic polypeptides of glycinin.

**Milk Coagulation**

Rennet initiates coagulation by removing the glycomacropeptide portion of κ-casein on casein micelle surfaces, forming active sites. These para-casein micelles collide with similar micelles in random motion, and attach with other micelles via these active sites. This is successful only if they orient correctly (a successful collision). The number of successful collisions increases with time and eventually these micelles form chains. The micelles in adjacent chains form more cross links and form strands up to 5 micelles thick. This process of
crosslinking continues, and eventually a three-dimensional gel that fills the space is formed (13, 29).

Electrostatic and hydrophobic interactions between para-casein micelles are important in the aggregation and gel formation of renneted casein micelles (7, 10, 28, 48). In addition, the presence of Ca++ is necessary for aggregation and gel formation of para-casein micelles. It decreases the surface charge of casein as well as para-casein micelles and reduces intermicellar repulsion (10). Moreover, Ca++ increases the effective hydrophobicity of the micelles (26) by exposing buried tyrosine and tryptophan residues (37). The decreased intermicellar repulsion and increased hydrophobicity (7) result in a much stronger and faster interaction between casein micelles. Moreover, calcium can participate in crosslinking negative groups in two adjacent protein chains via bridging.

Additives such as SDS, poly-L-glutamate (26), fatty acids, trisodium citrate, phytic acid, and disodium EDTA (17) increase RCT, decrease firming rate, and decrease curd firmness by binding Ca++. Similarly, nile red and 1,8-anilinonapthalenesulfonate bind to hydrophobic regions of casein micelles and subsequently decrease the rate of aggregation of renneted casein micelles (12). Thus, additives that chelate Ca++ or bind to hydrophobic sites of caseins can drastically affect milk coagulation.

Effect of soy protein at pH 6.6. Soy proteins, native (SPI$_N$) and heat-denatured (SPI$_H$), had the greatest effect on milk coagulation at pH 6.6 with SPI$_H$ having a much greater effect than SPI$_N$. Heat-denatured soy proteins increased RCT, delayed curd firming rate, and decreased final curd firmness at pH 6.6. Undenatured soy proteins, on the other hand, increased RCT of milk but did not decrease final curd firmness at pH 6.6. Several authors have
observed a lower curd firmness in the presence of soy proteins (23, 31, 32, 33), based on which several theories have been put forward to explain the effects of soy protein on milk coagulation.

Lee and Marshall (23, 25) believe that soy proteins either form a gel network independent of casein micelles or bind to caseins, thereby chemically affecting both the electrostatic and hydrophobic interactions between casein micelles. These authors used unheated soy proteins in their study. Others (31, 40) believe that soy proteins interact chemically with milk proteins via disulfide bonds and interfere with milk coagulation in a way similar to whey proteins. On the other hand, some authors (32, 33) believe that soy proteins affect milk coagulation physically without binding to the caseins. By being present physically between the micelles, soy proteins may progressively restrict their movement and prevent them from coming into close contact thus affecting coagulum structure and firmness. They also believed that the size of soy proteins and their high water-holding capacity may play a role in the way they interfere with milk coagulation. These authors used alkali-modified commercial soy proteins in their study.

In our studies, undenatured or native soy proteins (SPI_{N}) did not bind to casein micelles (Figures 4.8 and 4.9) but existed in curd as aggregates that are larger than casein micelles. This is contrary to the view held by other researchers (24, 33) who could neither detect nor identify soy proteins in curd using scanning electron microscopy. These researchers (24, 33) therefore suggested that soy proteins are intermingled with casein micelles and are present in an unaggregated form that is too small to be seen in the electron micrographs. The native soy protein aggregates, as indicated in this study, seem to be physically trapped between the casein micelles. Moreover, the
number of casein micelles seems to be much greater than the number of soy protein aggregates. This suggests that native soy proteins do not increase RCT by decreasing the number of collisions between the casein micelles by being physically present in milk. It therefore seems that native soy proteins do not directly interfere with the electrostatic and hydrophobic interaction between the casein micelles by binding to them, contrary to that suggested by Lee and Marshall (23, 25).

An alternative explanation is that soy proteins can bind calcium (4, 22, 38) and it is known that compounds that chelate calcium hinder milk coagulation. Native soy proteins, SPIₙ, can affect milk coagulation by binding calcium because adding calcium back to the system restored RCT and curd firming rate of skim milk in our studies. Metwalli et al. (31), on the other hand, reported that adding calcium to milk-soy blend helped to decrease RCT but did not restore coagulum firmness. In their study, the soy-fortified milk had 20% less casein than the control, which in itself would decrease curd firmness. So the decrease in curd firmness in their study could be due to either lower casein content or be due to soy proteins or both. However, it is more likely to be due to lower casein content because final curd firmness of milk containing SPIₙ was the same as the control in which casein content was made comparable to experimental milks containing soy protein.

β-Conglycinin at higher levels (0.6%) affected milk coagulation (RCT and curd firmness) although it did not bind to caseins (as seen by electron microscopy in Figure 4.7) and was lost in the whey as seen by electrophoresis (Figure 4.5). Soy protein isolates usually contain 1.75 to 2% of phytic acid and more than 96% of it is associated with β-conglycinin (35). Phytic acid is a strong chelating agent. It can chelate monovalent or divalent metal ions (11) such as
calcium (42) or form phytate-calcium-soy protein complex (14). Phytic acid, when added to milk, has been observed to increase RCT and decrease curd firmness by binding chelating calcium (17). Therefore, phytic acid associated with β-conglycinin rather than β-conglycinin itself may be responsible for its effect on milk coagulation.

When β-conglycinin was added at the 0.23% level (approximately equivalent to 20% fortification of milk with soy proteins), it increased RCT and decreased curd firmness development without affecting final curd firmness of milk similar to when SPI-N was added (Figure 4.1a). The effect of 0.23% β-conglycinin was much lower than that exerted by SPI-N. Therefore, β-conglycinin present in SPI-N or phytic acid associated with it appears primarily responsible for increasing the RCT of skim milk and delaying curd firmness development at pH 6.6. Lowering the pH (which increases ionic calcium concentration in milk) or adding CaCl₂ to milk–β-conglycinin blend restored the RCT of milk and the curd firming rate although β-conglycinin was lost completely in the whey (data not shown). This indirectly confirms the mode of action of β-conglycinin in affecting rennet coagulation. Glycinin is responsible for the rest of the effect of SPI-N on milk coagulation at pH 6.6 because it can also bind to calcium (4) even though insignificant phytic acid is associated with it (35). Therefore, the results in our studies suggest that native soy proteins affect milk coagulation indirectly by binding calcium.

Heat-denatured soy proteins seem to affect milk coagulation differently than undenatured soy proteins as suggested by our study. They have a tendency to bind to para-casein micelles. Partly covering the surface of para-casein micelles would limit and restrict direct micelle-micelle contact during aggregation. This would, consequently, decrease the number of successful
collisions between renneted micelles resulting in a slower aggregation rate. Subsequently, it takes longer for a gel of particular consistency to form, resulting in a longer RCT. Moreover, the presence of soy proteins on the surface of casein micelles would decrease the number of micelles in direct contact and would further interfere with the electrostatic and hydrophobic interaction between micelles in close proximity, lowering gel firmness. In comparison, whey proteins that bind to casein micelles during UHT-heat treatment restrict direct micelle-micelle contact (30), delay milk coagulation, and lower gel firmness (30). Moreover, compounds that bind to hydrophobic sites on casein micelles delay aggregation and gelation, as mentioned earlier. Thus, it seems that heat-denatured soy proteins affect milk coagulation and prevent aggregation by chemically binding to casein micelles rather than by being physically present (unattached to micelles) in the whey or by binding water as suggested by others (32, 33). Although it is not directly evident from the electron micrograph as to which constituents of soy proteins bind to caseins, these aggregates may be the heat-induced complex as suggested earlier. The nature of interaction of this complex with casein micelles is unclear although it may be of non-covalent type.

Effect of adding CaCl₂ or lowering the pH of milk-soy blend. Adding CaCl₂ (≥ 0.02%) to milk-soy blend or lowering the milk-soy blend pH to either 6.3 or 6.0 restored the RCT and curd development rate of SPI₉ to that of the control. But in the case of milk-SPI₉ blend, adding even up to 0.02% CaCl₂ did not improve curd firmness. However, adding 0.05% CaCl₂ or lowering the pH to 6.3 or 6.0 restored coagulum properties, including final curd firmness. As discussed earlier, β-conglycinin and SPI₉ seem to affect milk coagulation by binding calcium. Adding calcium or lowering the pH increases the
concentration of ionic calcium (10), which nullifies the effect of SPI\textsubscript{N} or β-conglycinin on coagulum properties. On the other hand, SPI\textsubscript{H} seems to affect milk coagulation at pH 6.6 by binding to casein micelles. In the presence of 0.05% CaCl\textsubscript{2} at pH 6.6 (Figures 4.12 and 4.13) or pH 6.3 (data not shown), however, soy proteins in curd containing SPI\textsubscript{H} exist as aggregates that are much larger in size than casein micelles. Moreover, these aggregates did not bind to casein micelles, which allowed unhindered aggregation and gel formation of casein micelles similar to the control. The self-aggregation of heat-denatured soy proteins in the presence of CaCl\textsubscript{2} or at lower pH’s, therefore, prevented its interaction with casein micelles and milk coagulation remained unaffected.

In contrast to the nullifying effects of CaCl\textsubscript{2} or low pH on milk coagulation of milk-soy blends reported in our study, others (31, 32) observed that adding up to 0.1% CaCl\textsubscript{2} or lowering the pH to ≤ 6.3 did not improve coagulum firmness (31, 32) although curd tension improved (32). Increasing CaCl\textsubscript{2} level to 0.06% seemed to increase the curd tension (which was defined differently than curd firmness by these authors) of the coagulum compared to that of the control. Lowering the pH of the milk-soy blend either helped to improve curd firmness (31) or did not improve it (32).

The experimental conditions, the type of soy protein, and the apparatus used to measure curd firmness may play a role in giving such contradictory results. Some probable reasons are discussed below.

In studies conducted by both Metwalli et al. (31) and Mohamed et al. (32), curd containing soy protein had a lower casein content, which, as discussed earlier, would reduce firmness. Mohamed et al. (32) used alkali-modified commercial soy protein isolate in their studies. The physicochemical behavior
of such soy proteins in milk or during milk coagulation may be different than either native (S\textsubscript{P}\textsubscript{IN}) or heat-denatured soy protein (S\textsubscript{PH}) used in our study. Soy proteins unfold and dissociate into subunits when treated with alkali (15, 16, 19, 45) and subsequently its dispersibility improves (34). The dissociated soy proteins may behave similarly to heat-treated soy proteins (which also dissociate after heating), and bind to caseins, resulting in a weak coagulum. However, these authors (34) did not find such an evidence using scanning electron microscopy (SEM). It is possible that at the magnification used in their study, it may have been difficult to observe such a behavior of soy proteins using SEM. Alkali-treated soy proteins can also bind more calcium (22), thereby affecting milk coagulation, but Mohamed et al. (32) observed very little changes in ionic calcium.

Changes in the pH of milk-soy blend during milk coagulation may also result in a weak curd. In the study by Mohamed et al. (32), the pH of milk-soy blend was adjusted only once to the required pH by adding HCl. When a similar one-time pH adjustment of milk-soy blend was done in some of our studies, the pH of the system increased during subsequent rennet milk coagulation and resulted in a weak curd. A higher pH decreases chymosin activity, which slows down the production of active para-casein micelle species and results in a slow rate of aggregation. Moreover, this may also affect the calcium equilibria between ionic and colloidal calcium, thereby affecting casein micelle aggregation and gelation. Another factor contributing to difference in results may be the instruments used. We used a Formagraph (utilizing the oscillation of an immersed wire loop) in our study while these authors (32, 33) used an Instron universal testing machine (which measures force required to penetrate a certain distance into the coagulum) to measure firmness and other related
parameters. Different instruments measure different parameters of the gel and, therefore, may not give comparable outputs; for example, an Instron requires a much stronger gel to be formed in order to give a signal (13).

**Implications**

Although under some conditions the deleterious effects of soy proteins on milk coagulation are overcome, we would like to bring up some points in the context of using soy proteins to partially replace caseins in cheese-like products. At lower pH's or in the presence of CaCl₂ (when the effect of soy proteins was minimized), soy proteins existed as aggregates that are much larger than the size of casein micelles. Such large particles may be prone to sedimentation in the cheese vat, which may lead to nonuniform distribution of soy proteins in the curd. Retention of soy proteins in the curd or their distribution in the curd may thus depend on the rate of coagulation versus the rate of sedimentation.

Not all soy proteins added to milk were retained in the curd as observed by electrophoresis. Soy protein fractions that remain soluble in milk are lost in the whey, whereas those that form aggregates large enough to be trapped in the curd are retained. Crosslinking both fractions of soy proteins to each other so that they form large aggregates that do not sediment may help to retain more soy proteins in the curd. Adding soy proteins to milk along with rennet may help to physically capture more soy proteins in the curd because the time available for soy proteins to sediment is decreased before aggregation begins after rennet addition. Heat-denatured whey protein concentrates have been added along with rennet to increase yield of cheese (9).

Another factor to consider is that the soy protein addition to milk may induce pH changes if soy protein isolate being used had been neutralized during its
manufacture. Soy proteins are usually acid-precipitated during manufacture of soy protein isolates from defatted soy meal, and then neutralized to pH \( \geq 7 \) to increase their dispersibility. Therefore, adding such isolates may require pH readjustment of milk-soy blend, which would then increase the total cheese-making time. An alternative approach could be to use soy protein isolates that have been adjusted to pH \( \leq 6.4-6.5 \), which may not cause pH changes in milk.

Soy proteins have been developed commercially to substitute the role of meat proteins or milk proteins in products where emulsification or foaming or water binding or heat gelation is required as a functionality. In traditional cheese making using milk, casein forms a gel (non-heat type) due to enzymic action. Soy proteins are not chymosin sensitive and therefore cannot form such chymosin-induced gels. Moreover, they do not participate in such a casein gel in a constructive manner (may not structurally contribute to the gel). Neither do they form a gel of their own in the casein curd (as they do in tofu – a curd containing soy protein coagulated by heat and calcium). Therefore, the functionality required of soy proteins may be to be inert during milk coagulation and subsequent steps, and get trapped in the curd.

**CONCLUSIONS**

Soy proteins had the greatest effect on milk coagulation at pH 6.6, with heat-denatured soy proteins (SPI\(_H\)) having a greater effect than native soy proteins (SPI\(_N\)). Both SPI\(_H\) and SPI\(_N\) increased RCT of milk. While SPI\(_H\) decreased both firming rate and final curd firmness, SPI\(_N\) decreased the firming rate without affecting final curd firmness. Both SPI\(_N\) and SPI\(_H\) behaved differently even though some of their fractions existed as aggregates in the curd. More soy proteins were lost in SPI\(_H\) whey than that of SPI\(_N\). In studies using SPI\(_N\),
glycinin was retained in curd as aggregates that were physically trapped in the curd (did not bind to caseins), whereas β-conglycinin was lost in the whey. In the case of SPIH, on the other hand, it appears that the heat-induced complex between the β subunit of β-conglycinin and the basic polypeptide of glycinin is retained in the curd by attaching to casein micelles while the rest of the soy proteins (α, α' subunit of β-conglycinin and acidic polypeptide of glycinin) are lost in the whey. The SPIH delayed milk coagulation probably by decreasing calcium activity (glycinin, β-conglycinin, and associated phytic acid can bind calcium), whereas SPIH affected milk coagulation by binding to casein micelles. This delayed aggregation (increased RCT), prevented para-casein micelles from coming into direct contact, and reduced the number and strength of links between casein micelles, thereby lowering gel strength.

Adding CaCl$_2$ or lowering the pH to 6.3 or 6.0 decreased the effect of soy proteins on RCT and curd firmness. Under these conditions, soy proteins (in both SPI and SPIH) existed as aggregates much bigger than the size of casein micelles and were physically trapped in the curd. The aggregates of SPIH did not bind to casein micelles and therefore did not affect milk coagulation. β-Conglycinin affected milk coagulation when added at a higher level (0.6%). Calcium binding by phytic acid (usually associated with β-conglycinin fraction) may have been responsible for its effect because β-conglycinin did not bind to caseins and was completely lost in the whey. Immunogold labeling can be successfully used to detect the presence of soy proteins added to milk and to study their interaction with caseins in the curd. Glycinin and β-conglycinin behave differently in milk and their behavior can further change if soy proteins are pretreated.
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CHAPTER 5
AN ELECTROPHORETIC STUDY OF THE HEAT INTERACTION BETWEEN BOVINE $\kappa$-CASEIN AND SOY GLYCININ IN A MODEL SYSTEM

ABSTRACT

Heat-induced interaction between soy glycinin and $\kappa$-casein was studied in a model system. A 0.5% solution of glycinin or $\kappa$-casein, or their mixture (0.5% each), was heated at 80°C for 10 min in the presence or absence of N-ethylmaleimide or $\beta$-mercaptoethanol and analyzed by electrophoresis. This included native PAGE in the first dimension and SDS-PAGE under reducing and nonreducing conditions in the second dimension; and SDS-PAGE in the first dimension and SDS-PAGE under reducing conditions in the second dimension. Heating resulted in an interaction between glycinin and $\kappa$-casein with two kinds of complexes being formed. One complex consisted of acidic polypeptides of glycinin and $\kappa$-casein linked by disulfide bonds. The other was a ternary complex consisting of $\kappa$-casein, and acidic and basic polypeptides of glycinin, all bound by non-covalent forces. By forming such a ternary complex when the glycinin-$\kappa$-casein mixture was heated in the presence of $\beta$-mercaptoethanol, $\kappa$-casein prevented the heat precipitation of glycinin.

INTRODUCTION

Soy proteins offer excellent functional properties, which can be tailored to meet specific needs such as to partially replace proteins from animal sources. Their use to replace milk and other animal proteins is increasing (15). Attempts have been made to add soy protein to milk in order to make cheese. Soy
protein has been added in various forms such as heat-treated soy milk (1, 2, 3, 13, 22) or soy protein isolate (14, 15, 16, 17) or soy protein extracts or heat-denatured soy proteins (11, 12). Preliminary work in our laboratory indicated that heat-denatured soy proteins affect milk coagulation differently than undenatured soy proteins (unpublished data). Heat-denatured soy proteins were more detrimental to milk coagulum properties such as rennet coagulation time and curd firmness than native soy proteins. The heat-treated proteins had a tendency to attach to the surface of para-casein micelles as observed by transmission electron microscopy (unpublished data), thereby affecting casein micelle aggregation and gelation.

Soybean protein is heterogeneous and consists of 2S, 7S, 11S and 15S protein components (19, 20). Glycinin (11S fraction), an oligomeric protein (molecular weight – 350,000 Da) is made up of 12 to 14 heterogeneous polypeptides termed acidic and basic polypeptides (19, 20). An acidic and a basic polypeptide (MW: 40,000 and 20,000 Da) are held together by a disulfide bond (19, 20) to form a subunit of 60,000 Da. Three such subunits combine by hydrophobic forces to form a glycinin monomer. Two monomers are then held together by electrostatic or hydrogen bonds or both to form the glycinin dimer (20). On the other hand, β-conglycinin (the main 7S component) is made up of α, α′, and β subunits with an approximate molecular weight of 180,000 Da (19).

**Heat Interactions of Glycinin**

When glycinin is heated alone or in the presence of β-mercaptoethanol, it forms smaller soluble polypeptides consisting of acidic polypeptides and large aggregates consisting of basic polypeptides (6, 8, 9, 10, 30, 31, 33, 35, 36). Glycinin has been observed to interact upon heating with β-conglycinin, the major protein of the soybean 7S fraction (6, 8, 28, 29), and meat protein myosin
The glycinin basic polypeptides interact with β subunits of β-conglycinin mainly via electrostatic forces (6, 28). The acidic polypeptides interact with α and α' subunits of β-conglycinin via disulfide bonds (33). However, the basic polypeptides of glycinin interact with meat proteins, specifically with the heavy chains of myosin via hydrophobic forces (20). Glycinin interacts with myosin and β-conglycinin only after it dissociates into constituent polypeptides (6, 20).

**κ-Casein and Milk Coagulation**

Caseins exist in milk as large colloidal protein aggregates called casein micelles. There are four types of caseins: α_s1, α_s2, β, and κ. κ-Casein is found predominantly on the periphery of the micelles and stabilizes them against coalescence (26). Hydrolysis of micellar κ-casein by rennet is crucial in initiating milk coagulation (5). Moreover, the para-κ-casein remaining on the casein micelles after rennet hydrolysis is hydrophobic and takes part in aggregation of renneted casein micelles (5). Additives that bind with κ-casein or para-κ-casein can affect milk coagulation. β-Lactoglobulin, which interacts with micellar κ-casein upon heating (24), affects chymosin hydrolysis of κ-casein and coagulation properties of milk (23).

Our objective was to see whether glycinin, after dissociating into respective polypeptides upon heating, could interact with κ-casein in a model system. This would partially help to explain why the heat-treated soy proteins have a tendency to stick to casein micelles. Therefore, as part of the study, we added β-mercaptoethanol to accelerate the heat-induced dissociation of glycinin into acidic and basic polypeptides. Heat-induced interactions were studied using native PAGE, two-dimensional SDS-PAGE (under reducing and nonreducing conditions), and SDS-PAGE under nonreducing conditions.
MATERIALS AND METHODS

Soy Glycinin

Crude soy glycinin was prepared from defatted soy flour (Protein Technologies International, St. Louis, MO) by the method of Nagano et al. (18). It was further purified by gel filtration on Sepharose 6B (Pharmacia, Uppsala, Sweden) using a 140 x 2.6 cm column according to Thanh et al. (27). Purity of this protein preparation was >95% determined by SDS-PAGE.

κ-Casein

κ-Casein was purified by the method of Zittle and Custer (37) as modified by Doi et al. (7). Purity of this protein preparation was >95% determined by SDS-PAGE.

Preparation of Protein Solutions

Soy glycinin and κ-casein solutions in 35 mM sodium phosphate buffer, pH 7.0, were made using approximately 15 mg/ml freeze-dried protein. The pH of the protein solution was readjusted to 7.00±0.01 and centrifuged at 15,000 rpm (20,000 x g) for 20 min at 20°C. Protein concentration was measured at 280 nm using $E_{1%}^{1cm}$ of 8.04 for soy glycinin (20) and 9.5 for κ-casein (26).

Heat Treatment

One-milliliter portions of protein solutions—0.5% glycinin, 0.5% κ-casein, or 0.5% glycinin + 0.5% κ-casein (GK-mixture)—in phosphate buffer were placed in 12 x 75 mm disposable glass test tubes, covered with aluminum foil and parafilm, and heated in a 80°C water bath for 10 min. The test tubes were then cooled in tap water (25°C). Standard conditions hereafter refer to heating the protein solutions in the absence of β-mercaptoethanol (ME) or
N-ethylmaleimide (NEM). Protein solutions were also heated under the same conditions in the presence of 50 mM ME (ME-heated) or 10 mM NEM (NEM-heated). This was replicated three times and analyzed by electrophoresis each time to confirm the electrophoretic pattern.

**First Dimension Native PAGE and Second Dimension SDS-PAGE**

Unheated and heated samples were analyzed by native PAGE in the first dimension using PhastGel Gradient 4-15% Gels (Pharmacia, Uppsala, Sweden) in a PhastSystem according to Pharmacia LKB Biotechnology (21). A small aliquot (100 µl) of unheated or heated sample was diluted with 200 µl of Tris (10 mM)-EDTA (1 mM) buffer, pH 8.0. Bromophenol blue (1.0 µl of 4.5%, wt/vol, solution) was added as tracking dye and 4.0 µl of sample was loaded on the gel. Samples were loaded at 10 Vh at the anodic end of gel with the conditions being 400 V, 1.0 mA, 2.5 W, and 15°C and run until 192 Vh, the final conditions being 400 V, 10.0 mA, 2.5 W, and 15°C. The gels were stained with Coomassie blue, destained in the PhastSystem development unit (Pharmacia LKB Biotechnology), then fixed in 10% glycerol and 10% acetic acid solution, and air dried at 25°C.

For running gels in the second dimension, the first dimension native PAGE gel resulting from the procedure described above was not stained. Instead, each lane (corresponding to one sample [about 3-4 mm wide]) was cut along the length of the gel and second dimension separation was performed according to Pharmacia LKB Biotechnology (21) using a PhastGel Homogeneous 20% Gel. The cut lane was immersed in Tris (0.112 M)-acetate (0.112 M) buffer, pH 6.40, containing 2.5% SDS (plus 5% ME if reducing conditions required) and 0.1% Bromophenol Blue for 3.0 min, placed in the
sample loading area of the gel and run until 90 Vh; the final conditions were 250 V, 10.0 mA, 3.0 W, and 15°C. The gels were then stained and destained as described above.

**First Dimension SDS-PAGE (Nonreducing) and Second Dimension SDS-PAGE (Reducing)**

SDS-PAGE in the first dimension of unheated and heated protein sample solutions was done in a PhastGel Homogeneous 12.5% gel according to Pharmacia LKB Biotechnology (21). Sample solutions (150 µl) were diluted with 150 µl of Tris (20 mM)-EDTA (2 mM) buffer, pH 8.0, containing 5% SDS, then heated in a boiling water bath for 3 min and cooled. Bromophenol blue tracking dye was added and 4 µl of sample was loaded on the gel. Samples were loaded at the anodic end of the gel at 250 V, 1.0 mA, 3.0 W, and 15°C at 0 Vh and run until 95 Vh; the final conditions were 250 V, 10.0 mA, 3.0 W, and 15°C. The gel was stained, destained, fixed, and dried as described above. Second dimension SDS-PAGE under reducing conditions was done as described above.

**RESULTS**

**Visual Observation**

When glycinin was heated at 80°C for 10 min in the presence of β-mercaptoethanol (ME), a precipitate was formed. However, when the glycinin–κ-casein mixture was ME-heated similarly, there was no such precipitate formed.
Gel Electrophoresis of Protein
Solutions Heated Under
Standard Conditions

First dimension native-PAGE and second dimension SDS-PAGE. Native-PAGE physically separates the proteins into separate components based on size and charge and this pattern of various heated protein solutions is shown in Figure 5.1. Unheated glycinin (lane 1) showed six bands (G1, G2, G3, G4, G5, and G6), all of which were later shown to consist of both acidic and basic polypeptides. When heated, glycinin (lane 2) formed some large aggregates that did not enter the stacking gel (B1) or resolving gel (B2) with some proteins having the same mobility as bands G4, G5, and G6 of unheated glycinin. The heated GK-mixture (lane 3) contained bands similar to heated glycinin as well as a new band (C3) with the same mobility as unheated κ-casein. It also had the most material remaining at the sample well and less intense bands corresponding to unreacted glycinin as in G4. Heated κ-casein (lane 4) consisted of only large aggregates that entered neither the stacking nor resolving gel.

The two large aggregates of heated glycinin (B1 and B2) each separated into acidic and basic polypeptides, when analyzed by second dimension SDS-PAGE under reducing conditions (Figure 5.2a). Bands corresponding to G4 and G5 were also composed of acidic and basic polypeptides and represent unreacted glycinin. The band with the highest mobility (G6) seem to consist largely of acidic polypeptides (Figure 5.2a) although the separation in the second dimension is not a clear single dark band. Interestingly, in unheated glycinin this band contained both acidic and basic polypeptides (data not shown).

Lane K and G (Figure 5.2b) are the control standards for pure κ-casein and
Figure 5.1. Native PAGE of various protein solutions (without additives) in gradient 4-15% gels. 1--unheated glycinin; 2--heated glycinin; 3--heated glycinin + κ-casein; 4--heated κ-casein; 5--unheated κ-casein.
Figure 5.2. Two-dimensional SDS-PAGE gels of (a)--heated glycinin, gel run under reducing conditions; (b)--heated glycinin + κ-casein, gel run under reducing conditions. K and G refer to standard κ-casein and glycinin respectively run under similar conditions; and (c)--heated glycinin + κ-casein, gel run under nonreducing conditions, the first dimension being the corresponding native PAGE gel. AP--acidic polypeptide; BP--basic polypeptide; κCN--κ-casein and SU--subunit.
glycinin run on a separate gel as compared to samples under similar conditions. The bands corresponding to acidic polypeptides and basic polypeptides are very broad, and so is the band corresponding to \( \kappa \)-casein. In the heated GK-mixture, the large aggregates (C1 and C2) contained \( \kappa \)-casein as well as acidic and basic polypeptides of glycinin (Figure 5.2b). The slow-moving band (C3) contained only acidic polypeptides and \( \kappa \)-casein (Figure 5.2b). Only some of the C1 material, none of the C2 material, and very little of the C3 material entered into the second dimension gel when nonreducing conditions were used (Figure 5.2c), even when the stacking gel concentration was reduced from 7.5% to 6% acrylamide. There was a streak observed in lanes corresponding to all the bands with no clear separation into discrete bands (Figure 5.2c). The fastest band (G6) consisted mainly of acidic polypeptides without any detectable \( \kappa \)-casein (Figure 5.2b).

First dimension nonreducing SDS-PAGE and second dimension reducing SDS-PAGE. SDS breaks all types of forces holding proteins together (in quaternary structures or other aggregates) except covalent bonds. In the presence of SDS, unheated glycinin (lane 1, Figure 5.3) dissociated to a subunit band (SU, \( \approx 60,000 \) daltons), acidic polypeptide \( A_4 \) (which is held in the glycinin subunit \( A_5A_4B_3 \) by non-covalent forces), and the remaining \( A_5B_3 \) part of that subunit as described by Staswick et al. (25). In addition there were a few bands of faint intensity that could not be identified. Heated glycinin (lane 2) produced similar bands, plus a large aggregate (S1) that did not enter the stacking gel, a streaking pattern (S), and a new band S2. When glycinin was heated with \( \kappa \)-casein (lane 3), it had less intense S1 and SU bands, more streaking (S), more intense S2, \( A_4 \), and S3 bands, and a intense band of similar mobility to \( \kappa \)-casein.
Figure 5.3. SDS-PAGE gels of protein solutions in 12.5% gels run under nonreducing conditions of 1--unheated glycinin; 2--heated glycinin; 3--heated glycinin + κ-casein; 4--heated κ-casein and 5--unheated κ-casein. SU--subunit and κCN--κ-casein.
The electrophoretic patterns of heated glycinin and GK-mixture in second dimension SDS-PAGE under reducing conditions are shown in Figure 5.4. The large aggregate (S1) in heated glycinin consisted of both acidic and basic polypeptides (Figure 5.4a). In contrast, S1 in heated GK-mixture contained predominantly acidic polypeptides (Figure 5.4b). The streak (S) consisted of acidic polypeptides and also contained κ-casein in heated GK-mixture. The resolution of bands S2, A4, and S3 into their respective polypeptides is hard to decipher from the second dimension gel because the bands are broad and they overlap.

Proteins Solutions Heated with NEM or ME

Heating glycinin, κ-casein, or their mixture with NEM did not prevent the formation of large protein aggregates. All had bands that would not enter the stacking or resolving gels in first dimension native PAGE (Figure 5.5). Except for band B3, which was only slightly less mobile than G6, the first dimension electrophoretic pattern of heated glycinin was the same with or without NEM. In contrast, more κ-casein remained unaggregated when it was NEM-heated (compare lane 4 in Figures 5.1 and 5.5). When the GK-mixture was NEM-heated, it produced more of the aggregate C3 (compare lane 3 in Figures 5.5 and 5.1). Bands B1, B2, G4, and G5 in NEM-heated glycinin had the same composition as standard-heated glycinin (compare Figures 5.6a and 5.2a). However, band G6, in contrast to standard heated glycinin, contained basic as well as acidic polypeptides in NEM-heated glycinin (Figure 5.6a).

In the NEM-heated GK-mixture, protein aggregates (C1 and C2) contained κ-casein as well as acidic and basic polypeptides of glycinin (Figure 5.6b). Band C3 contained mainly κ-casein and very little acidic polypeptides, whereas
Figure 5.4. Two-dimensional SDS-PAGE gels run under reducing conditions of (a)—heated glycinin and (b)—heated glycinin + \(\kappa\)-casein, first dimension being the corresponding SDS-PAGE gel run under nonreducing conditions. AP—acidic polypeptide; BP—basic polypeptide; \(\kappa\)CN—\(\kappa\)-casein and SU—subunit.
Figure 5.5. Native PAGE of various protein solutions (with additives) in gradient 4-15% gels of 1--unheated glycinin; 2--heated glycinin + NEM; 3--heated glycinin + κ-casein + NEM; 4--heated κ-casein + NEM; 5--heated glycinin + κ-casein + ME; and 6--unheated κ-casein.
Figure 5.6. Two-dimensional SDS-PAGE gels under reducing conditions of (a)--heated glycinin + NEM; (b)--heated glycinin + κ-casein + NEM; and (c)--heated glycinin + κ-casein + ME, the first dimension being the corresponding native PAGE gel. AP--acidic polypeptide; BP--basic polypeptide and κCN--κ-casein.
in standard heated GK-mixture, it contained both κ-casein and acidic polypeptides (compare Figures 5.6b and 5.2b).

The precipitate formed in ME-heated glycinin consisted mainly of basic polypeptides, whereas the supernatant contained only acidic polypeptides (Figure 5.7). This agrees with the observations made by other researchers (6, 8, 9, 35). Native PAGE of the supernatant showed a band whose electrophoretic mobility was similar to that of G6 (data not shown). In contrast, there was no precipitate formed in ME-heated GK-mixture, and the heated solution (lane 5, Figure 5.5) consisted of soluble aggregate (C1) and some unreacted glycinin (G4 and G6), but no C2 or C3 aggregates, which was observed for NEM-heated GK-mixture heated without reducing/blocking agents. There were also very few intact subunits present in the ME-heated GK-mixture when it was analyzed by SDS-PAGE (data not shown). Aggregate C1 contained κ-casein as well as acidic and basic polypeptides of glycinin (Figure 5.6c) similar to when the GK-mixture was heated alone or in the presence of NEM. The band G4 seemed to contain both acidic and basic polypeptides similar to heated glycinin or GK-mixture. Band G6, however, seems to contain mainly κ-casein and acidic polypeptides.

**DISCUSSION**

**Unheated Glycinin**

When unheated glycinin was analyzed by PAGE, it produced multiple bands, which were assumed to be various associated/dissociated forms of glycinin. Glycinin is present as a dimer at ionic strength ≥ 0.5 (20, 30, 32) but undergoes reversible and irreversible association/dissociation reactions at lower ionic strengths and pH, and during freezing, freeze drying, and storage (4,
Figure 5.7. SDS-PAGE under reducing conditions of 1--standard glycinin; 2--supernatant formed when glycinin was heated in presence of ME and 3--precipitate formed when glycinin was heated in presence of ME. AP--acidic polypeptide and BP--basic polypeptide.
Therefore, it was not unexpected to find multiple forms of glycinin on the native PAGE gel when used under our experimental conditions (35 mM phosphate buffer, ionic strength 0.07).

**Interaction Between Glycinin and \( \kappa \)-Casein**

Visual observation (\( \kappa \)-casein prevents precipitation of ME-heated glycinin), and changes in electrophoretic patterns indicate that an interaction occurs between glycinin and \( \kappa \)-casein when they are heated together. Glycinin, when heated under nonreducing conditions, dissociates into subunits, which then undergo sulphydryl-disulfide exchange reactions to form aggregates and lower molecular weight compounds (9, 34, 35, 36). Therefore, less intact glycinin (band G4 in Figure 5.1) or intact subunits (band SU in Figure 5.3) were observed after heating, and a considerable amount of protein became too large to enter the native PAGE gel (lane 2, Figure 5.1). Adding \( \kappa \)-casein further decreased the amount of intact glycinin and its subunits, but on the other hand, it increased the amount of lower molecular weight compounds formed (bands S2, S3, and A4, Figure 5.3) as well as the extent of streaking (compare lanes 2 and 3, Figure 5.3). This suggests that \( \kappa \)-casein promotes greater dissociation of the glycinin molecule and its subunits, and also increases subsequent covalent crosslinking.

**Interaction between acidic polypeptides of glycinin and \( \kappa \)-casein.** Glycinin, on dissociation, releases both acidic and basic polypeptides (9, 34, 35, 36). There is some indication that some of the released acidic polypeptides seem to form polymeric covalent linkages with \( \kappa \)-casein. In the heated GK-mixture (without ME or NEM), the new band (C3) in native PAGE (lane 3, Figure 5.1) seems to consist of acidic polypeptides of glycinin and \( \kappa \)-casein (Figure 5.2b).
This C3 band is absent in heated glycinin (lane 2, Figure 5.1) or heated \( \kappa \)-casein (lane 4, Figure 5.1). When the free sulfhydryl groups were blocked by heating in the presence of NEM, this band in heated GK-mixture consisted predominantly of \( \kappa \)-casein (Figure 5.6b). Moreover, this C3 band was absent when the GK-mixture was ME-heated. Because ME breaks disulfide linkages and NEM blocks formation of disulfide bond, it can probably be concluded that the complex C3 is held together by disulfide bonds. This covalently held C3 complex (observed in native PAGE) could be appearing as a streak in the SDS-PAGE gel (Figure 5.3) because this streak resolved into acidic polypeptides and \( \kappa \)-casein on a second dimension gel (Figure 5.4b). This also suggests that the type of interaction between \( \kappa \)-casein and acidic polypeptides of glycinin is via covalent disulfide linkage because SDS breaks non-covalent linkages in proteins.

Interaction between basic polypeptides of glycinin and \( \kappa \)-casein. The ability of \( \kappa \)-casein to stabilize the basic polypeptides of glycinin against precipitation suggests that there is an interaction between them. When glycinin was ME-heated, a precipitate consisting of only basic polypeptides (as observed by electrophoresis, Figure 5.7) was formed with the acidic polypeptides remaining in the supernatant (Figure 5.7). In contrast, the aggregate formed in ME-heated GK-mixture did not precipitate and was most likely the top band in lane 5 of Figure 5.5. The protein aggregate was too large to enter the stacking gel and seems to made up of \( \kappa \)-casein as well as acidic and basic polypeptides of glycinin (Figure 5.6c). Evidently by forming such an aggregate, \( \kappa \)-casein prevented self-aggregation of the basic polypeptides.

This non-covalently bound aggregate (formed in ME-heated GK-mixture) thus appears to be a ternary complex between \( \kappa \)-casein, acidic polypeptides of
glycinin, and basic polypeptides of glycinin. Such a non-covalently linked complex can form even when the two proteins are heated together in normal conditions (i.e., without adding a reducing agent) because heating releases polypeptides from the glycinin subunit. Such a release of basic polypeptides from glycinin, heated in the presence or absence of ME, has been shown to occur by Utsumi et al. (28) and Utsumi and Kinsella (29) in their studies related to the interaction between glycinin and β-conglycinin. However, there is no direct electrophoretic evidence from our studies to suggest that glycinin and κ-casein form non-covalent complexes with each other when heated in the absence of ME. Although the protein aggregate C1 (Figure 5.1) seems to consist of AP and BP of glycinin, and κ-casein (Figure 5.2b), it could also be individual aggregates of glycinin and κ-casein that were themselves too large to enter the gel.

Some of the gels run in the second dimension did not show good resolution of the bands into acidic polypeptide, basic polypeptide, and κ-casein. Moreover, the standard glycinin and κ-casein were run on a separate gel (but under similar conditions). The standards, glycinin and κ-casein (Figure 5.2b), seem to be overloaded and hence they resolved into very thick bands. The basic polypeptide of glycinin had the greatest mobility, acidic polypeptide of glycinin had the least mobility, and κ-casein was in-between acidic and basic polypeptides. However, band C1 (Figures 5.6b and 5.6c) did resolve into three distinct bands corresponding to acidic polypeptide, κ-casein, and basic polypeptide. Therefore, from these two gels and visual observation, there is evidence that glycinin and κ-casein interact when they are heated in the presence of β-mercaptoethanol. Moreover, band C3 (only present in heated GK-mixture) did resolve into acidic polypeptide and κ-casein, which also seems
to suggest that acidic polypeptide of glycinin and κ-casein interact with each other.

Interaction and its relation to milk coagulation. When acidic polypeptide and basic polypeptides have already been released from the glycinin subunit (as when ME-heated), they can non-covalently interact with κ-casein. When soy protein isolate (which consists of both glycinin and β-conglycinin) is heated, the polypeptides from their subunits are also released as discussed earlier. Subsequently, when such a heated soy protein is added to milk, these polypeptides can potentially interact with milk casein micelles. The chances of interacting with κ-casein are higher because most of the κ-casein is present on the surface of casein micelles (26). The potential interaction between soy glycinin and κ-casein (or para-κ-casein) could explain why the heat-treated soy proteins had a tendency to attach to the surface of casein micelles as observed by transmission electron microscopy, thereby affecting casein micelle aggregation and gelation.

CONCLUSIONS

When glycinin and κ-casein are heated together, some acidic polypeptides of glycinin interact with κ-casein via disulfide linkages. When they are heated in the presence of β-mercaptoethanol, κ-casein prevents the heat precipitation of glycinin. Both acidic and basic polypeptides of glycinin seem to associate non-covalently with κ-casein. By forming such a ternary complex, κ-casein prevents the heat precipitation of glycinin. The acidic and basic polypeptides of glycinin can potentially interact with κ-casein after they are released from the subunits.
REFERENCES


36 Yamagishi, T., F. Yamauchi, and K. Shibasaki. 1982. Effect of sulfhydryl and disulfide compounds on the formation and quality of thermal

CHAPTER 6
GENERAL SUMMARY

Native (unmodified) soy proteins (SPI_N) affected milk coagulation to the greatest at pH 6.6. It increased rennet coagulation time (RCT) of milk and decreased curd firming rate (development of curd firmness), but did not affect final curd firmness. Adding 0.02% CaCl_2 restored RCT and curd firmness development rate of milk-SPI_N blend to that of the control. Lowering the pH to 6.3 or 6.0 also helped to restore RCT and rate of curd firmness development. Some of the soy proteins that were added to the milk were lost in the whey. All of β-conglycinin and part of glycinin are lost in the SPI_N-whey at pH 6.6. Adding CaCl_2 did not help to retain more soy proteins; however, lowering the pH to 6.0 helped to retain more glycinin in the curd. Soy proteins helped to increase moisture retention in curds. Commercial soy protein isolate, SPI_M (heat-denatured, partially hydrolyzed and partially deamidated soy protein), increased moisture retention significantly greater than SPI_N.

Both glycinin and β-conglycinin present in SPI_N seem to affect milk coagulation at pH 6.6. Both fractions were almost equally responsible for increasing the RCT and decreasing the rate of curd firmness development. However, phytic acid associated with β-conglycinin may be responsible to a greater extent than β-conglycinin itself.

Heat-denatured soy proteins (SPI_H) impaired milk coagulation to a greater extent than undenatured soy proteins at pH 6.6. SPI_H decreased the curd firming rate further although the effect on RCT was not significantly different than that of SPI_N. Moreover, SPI_H decreased final curd firmness, which did not improve when 0.02% CaCl_2 was added. However, adding 0.05% CaCl_2 or...
lowering the pH to 6.3 or 6.0 restored RCT and curd firmness to that of the control. There was a greater loss of soy proteins in SPI_H-whey as compared to SPI_N-whey. More basic polypeptide of glycinin and β-subunit of β-conglycinin were retained in the curd although more acidic polypeptides of glycinin were lost in the whey.

Immunogold-labeling was used successfully to localize either undenatured or heat-denatured or commercial soy proteins in the curd. When SPI_N is added to milk, β-conglycinin (pI ~ 4.8) remains in a soluble form, whereas glycinin (pI ~ 6.4 and calcium sensitive) aggregates. These glycinin aggregates are retained in the curd, whereas β-conglycinin is lost in the whey. Moreover, the curd prepared from milk to which β-conglycinin was added did not show any labeling, indicating that β-conglycinin is not trapped in the curd. The glycinin aggregates (which appear less electron-dense than casein micelles) did not bind to the casein micelles but instead were physically trapped in the curd. Therefore, when native soy proteins are added to milk and rennet coagulated, only glycinin is retained in the curd as an aggregate.

Soy proteins in curd containing heat-denatured (SPI_H) or commercially manufactured soy protein isolates (SPI_M), on the other hand, had a different appearance although they too existed as aggregates. The SPI_M aggregates (which had a more open structure than SPI_N) were much larger than aggregates of SPI_N. However, the aggregates of SPI_H (which had a more fibrous appearance) were smaller than those of SPI_N in curd prepared at pH 6.6. Both the SPI_H and the SPI_M aggregates had a tendency to adhere to the micelles in the curd, whereas those of SPI_N were physically trapped in the curd. In presence of CaCl_2, however, the size of SPI_H aggregate was much larger. It did not bind to the casein micelles but was physically trapped in the curd. The
exact constituents of SPI\textsubscript{M} aggregates are not known but the aggregate in SPI\textsubscript{H} may be the heat-induced complex between basic polypeptide of glycinin and β-subunit of β-conglycinin.

The SPI\textsubscript{H} and SPI\textsubscript{N} soy proteins affect milk coagulation via different mechanisms. The SPI\textsubscript{H} proteins bind to the surface of casein micelles. This decreases the number of successful between para-casein micelles and decreases the rate of aggregation rate (increased RCT). Moreover, soy proteins (by being on the surface of casein micelles) not only decrease the number of micelles in direct contact, but they also interfere with electrostatic and hydrophobic interaction between micelles in close proximity. Thus both the number and strength of linkages between micelles would decrease, resulting in a gel with lower firmness. On the other hand, the SPI\textsubscript{N} proteins just lower the rate of aggregation without affecting final gel firmness. They do not bind to casein micelles and therefore they do not directly impede caseins from aggregating. Soy proteins (glycinin, β-conglycinin and phytic acid associated with it) can bind to calcium ions. They probably delay the rate of aggregation by binding calcium because compounds that chelate calcium affect milk coagulation. Moreover, adding calcium did restore milk coagulation. Under conditions where SPI\textsubscript{H} did not affect milk coagulation, it existed as large aggregates that did not bind to casein micelles but were physically trapped in the curd.

Glycinin and κ-casein can interact when they are heated together. Both acidic and basic polypeptides of glycinin interact non-covalently with κ-casein. This complex helps to prevent the heat precipitation of glycinin when heated in the presence of β-mercaptoethanol. Some acidic polypeptides of glycinin can also interact with κ-casein via disulfide linkages. The acidic and basic
polypeptides of glycinin can interact with κ-casein after they are released from the subunits. This may partially explain why heat-treated soy proteins have a tendency to attach to the casein-micelle surface (where most of κ-casein is present) as observed in TEM of milk curd containing heated soy proteins.
VITA

NAME :
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PERSONAL BACKGROUND :
Date of Birth : August 4, 1966.
Place of Birth : Madras (Tamil Nadu), India.
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EDUCATION :
M.S. (Nutrition and Food Science) Utah State University 1989-1992
B.S. (Food Technology) University of Bombay 1986-1989
B.S. (Chemistry) University of Bombay 1983-1986

EXPERIENCE

Food Scientist, General Mills Inc., Minneapolis, MN. 05/1996-present.

Research Assistant, Utah State University, Logan, UT. 1989-05/1996.
• Studied how soy proteins affect rennet coagulation of milk.
• Studied the effect of lactose, raw milk microbial quality, and level of concentration on the storage stability of UHT-processed concentrated skim milk.
• Prepared dairy products like cheese, yogurt, and ice cream on pilot plant scale.
• Operated pilot scale Ultra-High-Temperature (direct and indirect heat) and membrane processing (Ultrafiltration and Reverse Osmosis).
• Proficient in protein purification, chromatography, and electrophoresis techniques, electron microscopy and differential scanning calorimetry.

Teaching Assistant, Utah State University, Logan, UT. Fall 1992.
• Taught a graduate/senior level laboratory course in Food Chemistry.

Laboratory Assistant, Department of Chemical Technology, University of Bombay. 1988-1989
• Developed process for canning typical Indian vegetables & snack food.
• Supervised mango pulp preparation and canning operations.

HONORS
• "Gandhi Scholarship", Utah State University, 1994-95.
• "Vice President’s Research Fellowship Award", Utah State University, 1993-94, 1992-93 and 1990-91.
• Member of "Phi Kappa Phi" honor society.
• "Gold Medalist" (First in University of Bombay), B.Sc.(Tech), Food Technology.
• Several awards and scholarships, B.Sc.(Tech), University of Bombay, 1987-89.
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MEMBERSHIPS
The Institute of Food Technologists.
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PUBLICATIONS


N. Venkatachalam and Donald J. McMahon. "Interaction between soy proteins and milk proteins during rennet milk coagulation", ready for submission.

PRESENTATIONS


OTHER INTERESTS

Tennis, Table Tennis, Cricket, Badminton, Hiking, Camping, Stamp and Coin Collecting, Photography.