Identification of Interactions Among Casein Gels Using Dissociating Chemical Agents

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ABSTRACT

To evaluate the nature of the main interactions that are involved in the formation of casein gels made by the acidification or rennet coagulation of milk, we investigated the combined effects of ultracentrifugation and specific dissociating agents on protein solubilization. The method used was based on the ability of gelled proteins to resist the dissociating action of solutions of urea, sodium dodecyl sulfate, or EDTA. Results showed that hydrophobic interactions and calcium bonds were the most important forces involved in the rennet milk gel matrix; hydrophobic, hydrogen, and electrostatic interactions were homogeneously distributed in the gel formed from acidified milk.
(**Key words**: acid coagulation, rennet coagulation, protein interactions, dissociating agents)

INTRODUCTION

Milk proteins are surface-active compounds that constitute a group of strongly interacting proteins. Most of the protein in milk exists as casein micelles, which represent spherical aggregates of casein proteins and colloidal calcium phosphate (26). Although numerous different hypotheses have been proposed for the structure of casein micelles, a model in which the casein micelle is composed of subunits of variable composition appeared to be one of the more frequently used (5, 25). Nevertheless, the exact nature of the forces involved between the subunits is unknown.

The association of casein micelles during coagulation requires a balance of interactions between proteins, interactions between protein and solvents, and attractive and repulsive forces between adjacent polypeptide chains. Hence, the curd matrix geometry, the flexibility of the gel strand, and the strength of the connections (chemical nature and extent of protein interactions) depend on the coagulation phenomenon (acid or rennet coagulation) and on experimental conditions, such as pH, temperature, and ionic strength (6). The acidified gel or particle gel consists of associated demineralized macromolecules (3, 24). Rennet action produced the formation of para-κ-casein and clusters of casein micelles. Previous works (24, 28) have shown that pH and rennet influence the resulting gel properties differently. However, very little literature was available on the type of interactive forces that are responsible for the formation and rheological behavior of acidified casein and rennet milk gels. Results of Roefs and van Vliet (23) pointed to the importance in an acidified milk gel of electrostatic interactions, hydrophobic interactions, van der Waals attractions, and steric and entropic effects related to protein conformation. The enzymatic coagulation of milk that originated with the proteolysis of κ-casein by rennet led to the formation of hydrophobic sites on the surface of para-κ-casein micelles.

The objective of this study was to provide information about the nature of the protein interactions involved in the process of acid coagulation or rennet coagulation of milk. The methodology used was based on the dissociation of skim milk gel in various specific dissociating solutions (e.g., SDS, urea, and EDTA) as has been previously described (18). Urea establishes strong hydrogen bonds with polypeptide groups of proteins and, consequently, denatures proteins by breaking intramolecular hydrogen bonds (20). Urea can also weaken hydrophobic interactions. Unlike urea, ionic detergents, such as SDS, are unable to compete for the peptide hydrogen bonds that stabilize the secondary structure of proteins. Indeed, SDS chiefly interacts with both the charged groups and the nonpolar groups of the side chains of proteins, inducing intramolecular electrostatic repulsions and the competitive breaking of hydrophobic interactions. It should be also noted that the binding of ionic detergent may disrupt peptide hydrogen bonds indirectly (19, 22). Often EDTA is the calcium chelating agent used to obtain milk that is depleted in colloidal calcium phosphate (11, 14).

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MATERIALS AND METHODS

Skim Milk

Reconstituted skim milk was made by dissolving a commercial powder that had been processed using low heat (Laiterie Matines-SILI, Plouvien, France) at 12% (wt/vol) in deionized water. Until use, this mixture was stored at 4°C for 12 h to allow the milk to equilibrate. Then the mixture was kept at 20°C for 2 h for acid coagulation and at 30°C for 2 h for rennet coagulation. To prevent bacterial growth, 0.04% (wt/vol) sodium azide was added.

Milk Coagulation

Acid coagulation. For the acid coagulation of milk, Glucono-δ-lactone (Merck, Darmstadt, Germany) was added to milk (1.84%, wt/vol), and the milk was left for about 20 h at 20°C to obtain a final pH of 4.4.

Rennet coagulation. Rennet coagulation of milk was achieved at 30°C using 30×10^{-3}% (vol/vol) commercial rennet (520 mg of chymosin/L; Chr. Hansen, Arpajon, France).

Gel transition. The transition of milk to gel for both acid and rennet coagulations was followed by the turbidimetric method previously reported (1) (Analite Nephelometer 152; McVan Instruments, Victoria, Australia). The variation of turbidity, $\Delta\tau$, was measured as $\Delta\tau = \tau - \tau_0$, where τ_0 is the turbidity value of milk before glucono-δ-lactone or rennet addition. Stiffness (K) of both acidified and rennet gels was measured as a function of time by dynamic measurements at 10 Hz using a rheometer (Viscoprocess; Metravib, Ecully, France) as previously described by Lagoueyte et al. (17). At low strain, the stiffness is defined as the stress (Newtons per square meter) divided by the strain (dimension less number) and, consequently, in SI units, stiffness is expressed as Newtons per square meter (21).

Centrifugation Test in the Presence of Dissociating Agents

Samples of acidified or rennet milk gels (4 ml) were dispersed in 16 ml of aqueous dissociating solutions containing from 2 to 10 g/L of SDS, from 1 to 6 *M* urea, or 2 m*M* EDTA, or a combination of two of these dissociating agents. For the reference sample, 16 ml of deionized water were used instead of the dissociating solution. The resulting mixtures were rapidly homogenized (Ultra-Turrax T 25; IKA-Labortechnik, Janke and Kundel, Staufen, Germany) for 1 min at 9000 rpm. The pH value of the mixtures of samples of the acidified milk gel was adjusted to 4.4 with 1 *M* NaOH or 1 *M* HCl. Gel dispersions were then ultracentrifuged at $86{,}000 \times g$ for 32 min with a Beckman ultracentrifuge (rotor Ti 70; Beckman Instruments France S.A., Gagny, France) at the temperature of milk coagulation. After ultracentrifugation, the supernatants were carefully removed and kept at 4°C until protein analysis, and then tubes were drained.

Compositional Analysis

Soluble and insoluble proteins obtained after ultracentrifugation were quantified using the modified Lowry method (2). The proteins of the supernatant were analyzed by SDS-PAGE (16). Supernatant samples were diluted in 62.5 m*M* Tris·HCl buffer containing 100 g of glycerol/L, 20 g of SDS/L, and 50 ml of 2-mercaptoethanol/L followed by heat treatment in boiling water for 4 min. The protein content of the supernatant was analyzed by SDS-PAGE according to the method of Laemmli (16) using a 5 to 20% separating gradient gel and a 4.5% stacking gel. Protein bands were fixed in 12% (wt/vol) trichloroacetic acid for 30 min, then stained overnight in 0.05% (wt/vol) R-250 Coomassie blue (Sigma Chemical Co., St. Louis, MO), and destained with a solution of methanol, acetic acid, and water (5:7.5:87.5; vol/vol/vol). Relative amounts of individual protein components were estimated by densitometry (Scanner Densitometre GS 300; Transmittance-Hoefer Scientific Instruments, San Francisco, CA) associated with a Schimadzu C-R5 A Chromatopac (Touzart et Matignon, Vitry/Seine, France).

Statistical Analysis

Experimental data were presented as the means of triplicate measurements from five experiments and subjected to ANOVA (Stat View™; Abacus Concepts, Inc., Berkeley, CA). Fisher's protected least significant difference test was used to compare paired means, and differences between means were considered to be significant at $P < 0.05$.

RESULTS

Milk Gel Transition Studies

The curves for turbidity and pH as related to time are reported in Figure 1 for both acid and rennet coagulation of milk. The turbidity curve obtained for acid coagulation of milk could be divided into three

stages (Figure 1A): an increase in turbidity between pH 6.7 and 5.6, a significant decrease in turbidity to pH 5.2, and an increase in turbidity below pH 5.2. In contrast, the turbidimetric profile obtained at a constant pH 6.7 for enzymatic milk coagulation showed a rapid increase in turbidity (Figure 1B). Maximum turbidity was reached 20 h after glucono-δ-lactone addition for the formation of acidified milk gel and 14 h after rennet addition for the formation of the enzymatic milk gel. The time values corresponding to the turbidity plateau were taken as a reference for the beginning of the dissociation test. As shown in Figure 1, the final turbidity value of the rennet milk gel was higher than the value for the acidified milk gel. The value for stiffness of the rennet milk gel was six times higher than the value obtained for the acidified milk gel (Table 1).

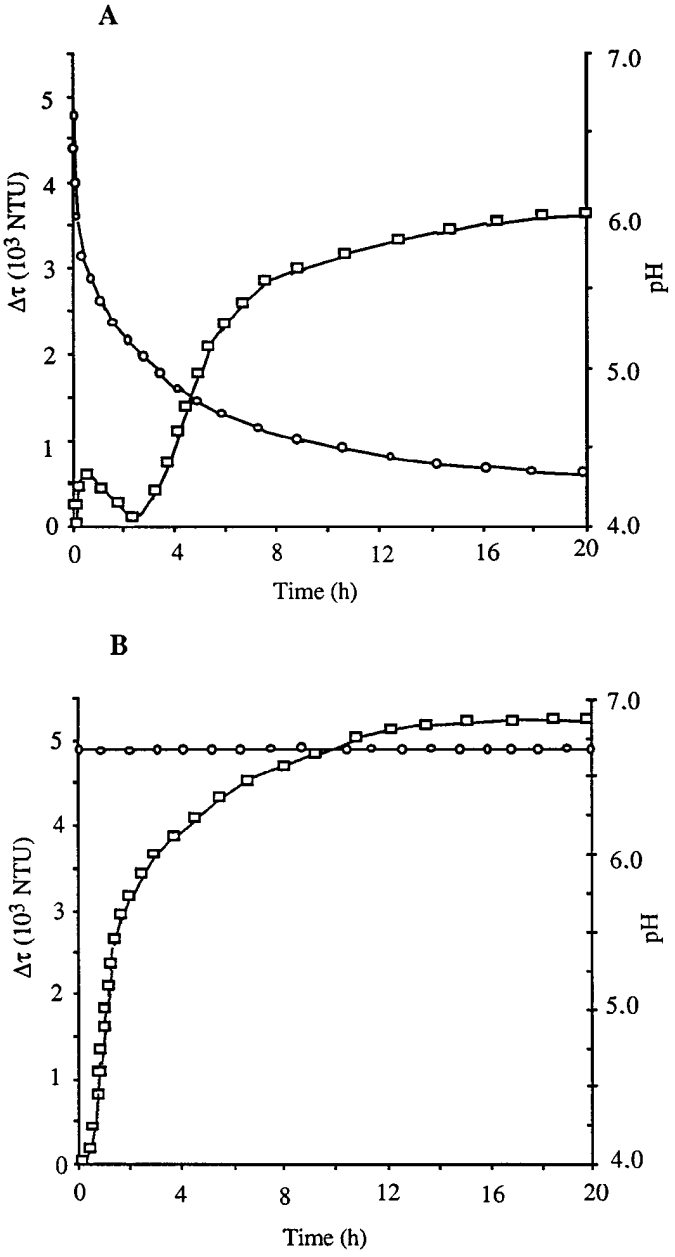

Figure 1. Changes in turbidity (Δτ; □), and pH (○) in relation to time in acid (A) and rennet (B) coagulation of milk. NTU = Nephelometric turbidimetric unit. These results are representative data from one replication.

TABLE 1. Comparison of turbidimetric and rheological parameters describing acid and rennet coagulation of milk.[1]

	Coagulation	
Parameter	Acid	Rennet
Temperature, °C	20	30
Coagulation time, h	20	20
pH	4.4	6.7
Δτ (10^3 NTU)	3.6	5.2
K, N/m^2	5550	34,600

[1]Δτ = Variation of turbidity, NTU = nephelometric turbidimetric unit, and K = stiffness.

Centrifugation Test in the Presence of Dissociating Agents and Calcium Chelator

The effects of different dissociating agents and the calcium chelator EDTA on the dissociation of proteins from casein micelles in acidified and rennet milk gels are reported in Table 2. Results indicated that the percentage of proteins that were dissociated by the centrifugation test varied ($P < 0.05$) with the nature of the agent added and with the kind of milk coagulation. In acid coagulation, 6 *M* urea and 10 g of SDS/L were the dissociating agents that had the most important effect on protein dissociation. Indeed, these two agents enabled dissociation of 81.5 and 97.0% of the total proteins, respectively (Table 2). No significant effect was obtained with 2 m*M* EDTA alone (dissociation of 26.7% of the total proteins). The combination of EDTA with urea or EDTA with SDS resulted in protein dissociation comparable with results that were obtained with urea or SDS alone. In rennet coagulation, these agents have nearly the same effect observed in acid coagulation, except for 2 m*M* EDTA, which induced an increase in protein dissociation. The percentage of protein that was dissociated by 6 *M* urea, 2 m*M* EDTA, or 10 g of SDS/L was 64.0, 76.0, and 98.2% of total proteins, respectively (Table 2). Ten grams of SDS/L dissociated essentially all of the protein (97 ± 3% in the acidified gel and 98.2 ± 1.2% in the rennet gel). The addition of 2 m*M* EDTA to the

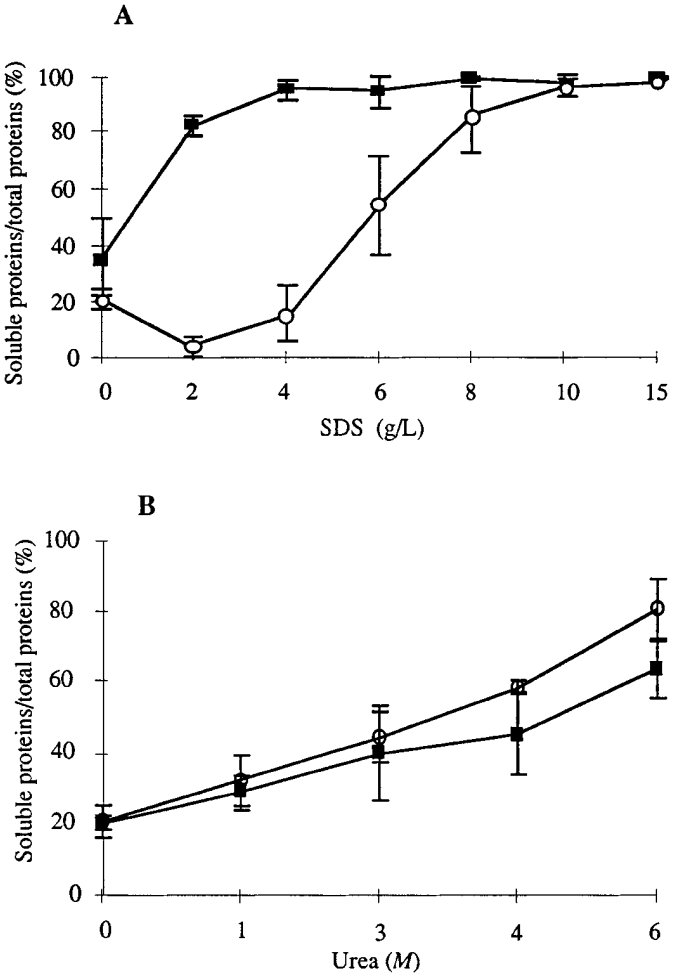

Figure 2. Effect of SDS concentration (A) and urea molarity (B) on the protein solubilization in acidified milk gel (o) and rennet milk gel (■).

dissociating solutions did not increase the percentage of protein dissociation. Statistical analysis indicated that no significant difference in protein dissociation was observed when 10 g of SDS/L were used alone or in combination with 6 *M* urea or 2 m*M* EDTA.

Figure 2 shows the effect of SDS and urea on protein dissociation at various concentrations. In acid coagulation, the effect of SDS on protein dissociation (Figure 2A) was significant ($P < 0.05$) at a concentration of 6 g/L. Total dissociation occurred at 10 g of SDS/L. In rennet coagulation, 80% of the total proteins were dissociated with 2 g of SDS/L, and 4 g of SDS/L were sufficient to dissociate all of the proteins. In both acidified and rennet milk gels, the extent of protein dissociation increased as urea molarity increased (Figure 2B). Figure 2B indicated that 81.5 and 64.0% of the total proteins were dissociated in acidified and rennet milk gels, respectively, with 6 *M* urea. In the presence of urea, the centrifugation test revealed a difference ($P < 0.05$) between the two kinds of gel when 4 *M* urea was used. The urea concentration that enabled protein dissociation appeared to be lower in the acidified milk gel (4 *M*) than in the rennet milk gel (6 *M*).

Compositional Analysis

The SDS-PAGE scanning patterns for rennet milk gels and acidified milk gels that were dispersed in water, 10 g of SDS/L, 2 m*M* EDTA, and 6 *M* urea are presented in Figure 3. Results indicated that only whey proteins (β-LG and α-LA) were present in the supernatant when acidified or rennet milk gels were

TABLE 2. Effects of different dissociating agents on protein solubilization in acidified and rennet milk gels.

	Soluble proteins/total proteins			
	Acidified		Rennet	
Agent	$\bar{X}$	SE	$\bar{X}$	SE
	(%)			
H_2O	10.5[d]	2.0	23.7[c]	6.4
SDS 10 g/L	97.0[a]	3.0	98.2[a]	1.2
EDTA 2 m*M*	26.7[d]	17.0	75.9[b]	2.5
Urea 6 *M*	81.5[b]	8.2	64.0[b]	8.4
SDS 10 g/L + urea 6 *M*	87.6[a]	8.7	97.8[a]	1.0
SDS 10 g/L + EDTA 2 m*M*	94.0[a]	0.9	97.7[a]	1.5
Urea 6 *M* + EDTA 2 m*M*	61.7[c]	1.8	76.0[b]	2.1

[a,b,c,d]Means within columns followed by no common superscript differ ($P < 0.05$).

dispersed in water (Figure 3A). The gel dispersion in 10 g of SDS/L dissociated all of the casein in rennet milk gels and in acidified milk gels (Figure 3B); with EDTA, differences were observed between the casein dissociation in the two kinds of gels (Figure 3C). As would be expected, 2 m*M* EDTA dissociated few caseins in acidified milk gels (Figure 3C), but the amount of dissociated caseins greatly increased in rennet milk gels (Figure 3C).

The addition of 6 *M* urea induced the same scanning pattern for both gels, and casein dissociation increased compared with the dissociation in water, particularly the dissociation of the β-CN (Figure 3D). The increase in protein dissociation caused by different concentrations of dispersing solutions (Table 2 and Figure 2) could be specifically correlated with the dissociation of caseins (Figure 3). Therefore, the amount of dissociated caseins increased as the concentration in the dispersing agent increased (Figure 2).

DISCUSSION

Aggregation occurs when the random diffusion of particles results in collisions or close approaches between casein particles, which lead either to linkage or to separation (10). The main interactions that are involved in casein association depend on the kind of coagulation and are generally attractive forces, such as hydrogen bonds (reinforced during cooling), hydrophobic interactions (reinforced at high temperature), electrostatic attractions, van der Waals bonding, bridges with Ca^{2+} or other divalent salts, and disulfide bridges. Steric or entropic repulsions, coulombic or electrostatic repulsions principally at pH values higher than the isoelectric pH, and interactions between protein and water act to keep polypeptide chains separated. During the formation of acidified milk gel, the decrease in pH affected the balance between the attractive and repulsive forces existing between charged groups of milk proteins and the ability of those forces to associate with water and ions (4, 9). Accordingly, pH modified the structure of interacting particles inducing great modifications in the optical, microstructural, rheological, and biochemical (mineral and casein solubilization and solvation) properties of casein particles (7, 8, 9, 27, 28). Because changes in turbidity can be related to changes in light reflection through casein particles and aggregates, turbidity changes give an indication of the

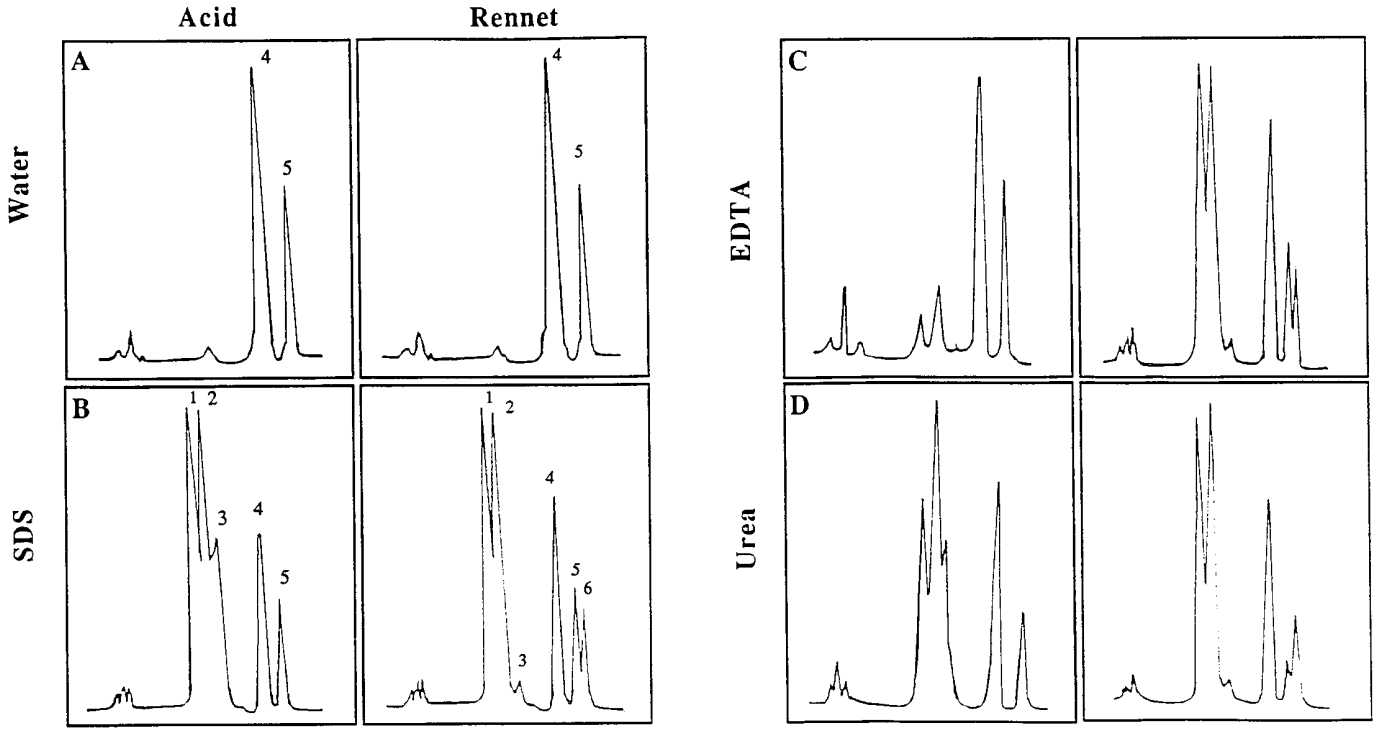

Figure 3. The SDS-PAGE scanning patterns of supernatants obtained from acidified gel (a) and rennet gel (b) samples dispersed in dissociating solutions: water (A), 10 g of SDS/L (B), 2 m*M* EDTA (C), and 6 *M* urea (D). Peak 1, α_S-CN; peak 2, β-CN; peak 3, κ-CN; peak 4, β-LG; peak 5, α-LA, and peak 6, para-κ-CN.

interactions established between casein particles during the formation of milk gels (aggregation and sometimes partial disaggregation). Results obtained for turbidity changes during the formation of acidified milk gel underlined the complexity of the mechanism involved in this type of coagulation (1, 9). Differently, in rennet milk gel formation, the profile of the curve obtained for the changes in turbidity is more simple and could be related to an aggregation of casein micelles, leading to dense clusters of particles that reflect more light and, consequently, result in increased turbidity. Although the formation of a continuous network in both acid and rennet milk coagulation occurred before turbidity reached a maximum, it could be assumed that the network of the gel does not evolve from the turbidity plateau.

To evaluate the role of various forces in acid and rennet coagulation of milk, a dissociating approach was investigated and compared with the predicted results. The utilization of dispersing agents allowed us to make general assumptions about interactions between proteins in the casein micelles in both acidified and rennet milk gels on the basis of the difference in protein dissociation after ultracentrifugation. Indeed, the amount of dissociated proteins after a specific dispersing action and ultracentrifugation reflected the action of the added agent on the gel network and, consequently, the presence of specific interactions. Hydrophobic interactions were disrupted by SDS (19, 22); hydrogen bonds and, indirectly, hydrophobic interactions were disrupted by urea (20); and ionic bonds with calcium salts were broken after complexation with EDTA. As a result, the remaining precipitate represented the casein molecules that were still interacting despite dissociation.

The results presented in Table 2 and Figure 2 enabled us to classify the action of each dispersing agent on the dissociation of milk proteins in both acidified and rennet milk gels. Ten grams of SDS/L induced the dissociation of proteins in both acidified and rennet milk gels, which indicated the presence of an important number of hydrophobic interactions in these two kinds of gel. The variation in SDS concentration (Figure 2A) indicated that casein dissociation in each milk gel was different for low SDS concentrations. Two grams of SDS/L did not result in casein dissociation in acidified milk gel, whereas 80% of the total protein was dissociated in rennet milk gel at this concentration. These results suggest that hydrophobic bonds were not the major interactions stabilizing the gel structure of the acidified milk gels. A concentration of 8 g of SDS/L was required to solubilize 80% of the total proteins, which indicated that a cooperative phenomenon between protein interactions existed in the acidified milk gels. Indeed, during the acid coagulation of milk, the decrease in pH reduced both the negative electrostatic energy barrier and the hydration repulsions, allowing the formation of electrostatic interactions among particles. This creation of new interactions could further facilitate other intermolecular bonds, such as hydrophobic and hydrogen interactions between contiguous proteins or polypeptides in the gel matrix.

Conversely, the strong effect of SDS on protein dissociation in the rennet milk gel reflected the important contribution of hydrophobic interactions to the formation and maintenance of this milk gel. Iametti et al. (15) showed similar results with fluorescent hydrophobic probes used to observe milk protein interactions during enzymatic coagulation. Indeed, the para-κ-CN formed at the surface of the micelle after rennet action induced a global reduction of hydrophilicity and charge of the micelle. A similar action of urea on protein dissociation in both acidified and rennet milk gels (Figure 2B) suggested the presence of hydrogen interactions in the stabilization of the structure that seemed to have the same contribution in each network. It should be noted that urea can also disrupt hydrophobic interactions. The action of 2 m*M* EDTA induced a great increase in casein dissociation in rennet gel but had no effect on casein dissociation in the acidified milk gel (Figure 3). Indeed, in the rennet milk gel at pH 6.7, the casein micelle was affected by interactions among calcium, carboxylic groups, and phosphoserine; when at pH near 4.6, almost all of the calcium was solubilized. These results led us to conclude that hydrophobic interactions and Ca^{2+} bonds are important for the stabilization of the structure of the rennet milk gel. Other weak interactions, such as hydrogen bonds, contributed less to gel structure.

Therefore, the significant difference between the network structure of acid and rennet milk gels resulted in the nature of the majority of protein interactions. Although both acid and rennet milk gels would seem to have the same hydrogen interactions, contributions differed in hydrophobic interactions, electrostatic interactions, and calcium bonds. In fact, hydrophobic interactions would be principally involved in the formation of the rennet milk gel structure, and electrostatic interactions would strongly participate in the stabilization of the gel structure of the acidified milk. This hypothesis is in agreement with results of Heertje et al. (13) and Roefs and van Vliet (23), who observed that electrostatic interactions controlled the coagulation of acidified milk. Indeed, milk coagulation was achieved at the isoelectric pH of casein (pH 4.6 at 25°C), which suggested a substantial contribution by electrostatic interactions.

Hayakawa and Nakai (12) indicated that proteins did not coagulate at the isoelectric pH when hydrophobic forces were too weak. Consequently, the participation of the other interactions was also important and confirmed that the acid coagulation of casein micelles was controlled by a balance between attractive and repulsive hydration forces (4). In fact, these interactions contributed to the stabilization of the gel network as a cooperative phenomenon (18).

CONCLUSIONS

In conclusion, the general principle that hydrophobic interactions are the major driving force for interactions among proteins was consistent with the mechanism of the enzymatic coagulation of casein micelles. However, the calcium and hydrogen bonds contribute to the specificity and stability of the protein interactions, and these interactions are fairly critical to the gel network of the acidified milk. The results of our study, in agreement with work of various other researchers, contributed to an understanding of the nature of protein interactions in both acidified and rennet milk gels.

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