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ORIGINAL CONTRIBUTION

Identification of interactions involved in rennet gel structures using dissociating chemical agents

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Abstract The dissociation of curds by enzymic coagulation by EDTA, urea, and ionic strength was considered to study the forces involved in the curd matrix structure. Curds were obtained at 25 (C_{25}) and 38 °C (C_{38}) and the effect of temperature on these forces was studied. Curd dissociation was followed by quantifying the release of proteins, calcium, and phosphate by the curds mechanically disrupted in the presence of the dissociating agent. In all the cases, the release of whey proteins and mineral revealed the liberation of entrapped whey. At difference with C_{38}, C_{25} at low EDTA concentrations or with NaCl liberated α_S- and β-casein in similar proportions and higher amounts of minerals. Then, coagulation at low temperatures could favor the formation of weak electrostatic bonds involving Ca. Their dissociation by urea at a concentration of 4 M or higher was very effective, especially for C_{38}, suggesting that high temperature favored the formation of hydrophobic interactions.

Keywords Curd · Enzymic coagulation · EDTA · Urea · Ionic strength

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Introduction

Milk bovine proteins can be divided in two groups: caseins, which precipitate at pH 4.6, and whey proteins (β-lactoglobulin, α-lactoalbumin) that remain in solution at the same pH [1]. Casein micelles (CM) are roughly spherical casein aggregates stabilized in colloidal suspension in milk. Their stability is the result of a net negative charge and a particular structure, in which the hydrophobic α_{S1}- and α_{S2}-caseins are mainly confined in the particle core, while the amphiphilic κ-casein projects its hydrophilic moiety to the aqueous environment, thus providing additional steric stability [2].

Enzymic coagulation of milk, extensively used in cheese making, is a several-step process that starts with destabilization of the CM by rennet proteolysis of their surface κ-casein [3]. The second step involves the aggregation of the partially hydrolyzed CM [para casein micelles (pCM)] to form a continuous proteic matrix that can retain entrapped fat globules and bacteria [4]. Because the structure of such complex and heterogeneous system determines almost completely the structure, and consequently the texture and physical properties, of the final cheese, recent studies have been carried out to characterize the nature of the interactions between pCM in curds [5]. An approach frequently applied in this kind of study is based on the dissociation of curds by the action of specific dissociating chemical agents [urea, sodium dodecyl sulfate (SDS), EDTA]. These studies have shown that, in rennet milk gels, hydrophobic interactions and calcium bridges are among the most important forces involved [6].

The objective of the present study was to provide further information about the nature of the protein interactions contributing to the structure of the gel network in curds obtained by enzymic coagulation of milk. The method used

was based on the dissociation of skim milk gels by specific dissociating agents, EDTA and urea in our case, and by variation of the ionic strength with NaCl. Dissociation was followed by quantifying proteins, calcium, and phosphate (Pi) released, and the proteic fractions were characterized by urea-polyacrylamide gel electrophoresis (PAGE). Because hydrophobic and electrostatic interactions depend in different ways on temperature variations, the curds studied were obtained at two temperatures to add further evidence regarding the nature of the interactions involved.

Materials and methods

Preparation of milk samples

Milk suspensions were prepared from bovine, commercial, low-heat, nonfat dried milk (Molico, Société des Produits Nestlé S. A., Vevey, Switzerland) reconstituted to 10% w/v concentration in 5 mM $CaCl_2$, with a final pH of 6.35 by addition of 1.5 M HCl. Samples of the reconstituted milk were kept for 2 h at either 25 or 38 °C to allow the milk to equilibrate at the working temperature before rennet coagulation.

Milk enzymic coagulation

Coagulation was achieved by adding 10 μL of rennet to centrifuge tubes containing 4 mL of milk suspension maintained at working temperature by a water bath. After 1 min of gentle stirring using a Teflon stirrer for mixing, the tubes were left to coagulate into the bath for 15 min.

The rennet gels were centrifuged at 3,500 g for 10 min using a Luguimac LC-10 centrifuge, and the supernatant whey was discarded. Commercial liquid rennet, activity 0.240 rennet units (RU), was a gift from Cooperativa de Tamberos de Rosario (C.O.T.A.R.) (Argentina). One RU is defined as the activity required to coagulate 10 mL of reconstituted low-heat skim milk powder (~10.7% w/w) in 0.01 M $CaCl_2$ at pH (nonadjusted) ~6.35, in 100 s under the conditions specified in the IDF Standard 110 A [7].

Dissociation test

The samples of rennet milk gels were dispersed at 25 °C by vigorous stirring for 1 min in 5 mL of aqueous dissociating solution containing 1 to 8 M urea, 2 to 50 mM EDTA, or 0 to 2 M NaCl. The suspensions obtained were centrifuged at 80,500×*g* for 30 min using a Beckman Coulter Optima XL 100K centrifuge. The supernatants were carefully separated and used to determine the total protein concentration using a modified Lowry's method [8]; the Pi concentration was determined by a standard colorimetric method based in the formation of phosphomolibdate complexes (UV Wiener Lab, Rosario, Argentina), and total calcium (Ca) concentration by atomic absorption spectroscopy using a Metrolab AA 250 equipment [9].

The protein composition of the supernatant was analyzed by urea-SDS-PAGE according to Laemmli's method [10]. Proteins were stained with Coomassie brillant blue R250 staining solution, and distained with 10% (v/v) methanol and 10% (v/v) acetic acid distaining solution. The relative intensity of the stained bands was determined by scanning the stained gels and analyzing the pixel densities of the digitized protein bands using specially designed software. Deconvolution of the scanning pattern curves was performed, when necessary, by means of the GRAMS program. The protein bands were identified using commercial α_S-, β-, and κ-casein (Sigma Chemical, St. Louis, MO, USA). These same standards were used to determine not only the range of protein concentrations at which the relationship pixel density concentration remains linear but also the reproductiveness of the method.

Results

Dissociation of curds by EDTA

Figure 1a shows the total amount of proteins present in the supernatants obtained by dispersion at different EDTA concentrations and posterior centrifugation of curds prepared at 25 °C (C_{25}) and 38 °C (C_{38}). Dissociation was more effective for C_{25}, producing a sharp initial increase in the amount of protein released at EDTA concentrations lower than 5 mM. The shape of the dissociation curve suggested that the release of this first protein fraction was followed by a subsequent reassociation of some of these proteins when the curds were dispersed with EDTA concentrations higher than 10 mM. On the other hand, the dissociation of proteins from the C_{38} increased steadily for increasing EDTA concentrations.

The effect of EDTA on the release of Pi and Ca from C_{25} and C_{38} is shown on Fig. 1b. In the first case, the amount of Pi and Ca dissociated increased sharply at EDTA concentrations lower than 10 mM, reaching maximal values over 20 mM for EDTA concentrations. In the case of C_{38}, the dissociating action started at EDTA concentrations higher than 10 mM, then progressed in a regular way for both ions, and finally reached 50 mM EDTA, with values lower than the half of the values obtained for C_{25} at the same concentration of dissociating agent. The simultaneity of the protein release with that of Pi and Ca for both kinds of curds strongly suggests that the released protein might have been mainly bound to the gel network through ionic bonds with the participation of calcium or calcium Pi. The

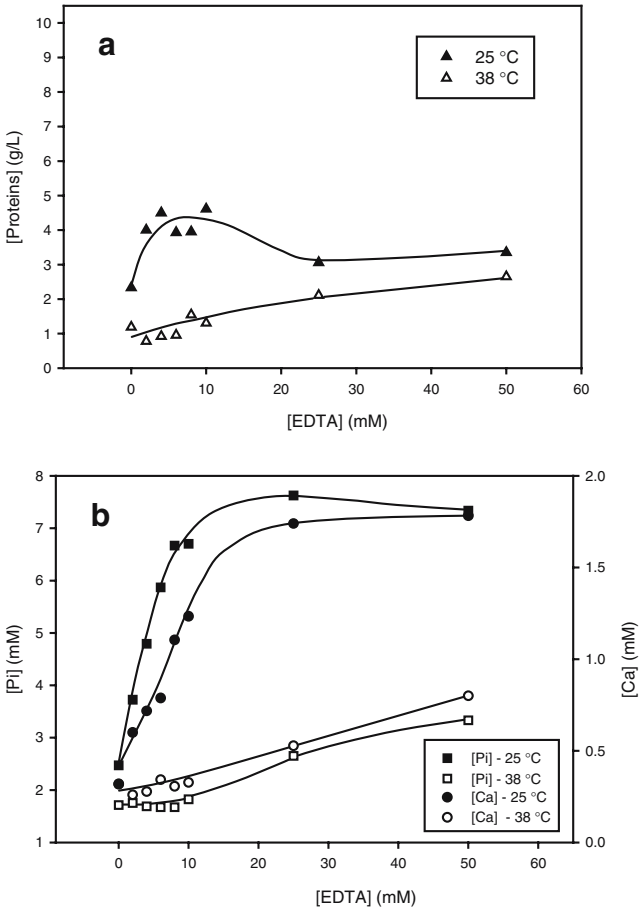

Fig. 1 Total amount of proteins (**a**) and calcium and Pi (**b**) present in the supernatants obtained by dispersion at different EDTA concentrations and subsequent centrifugation of curds prepared at two temperatures. Each *point* is the mean of at least five determinations; maximum SD, 0.1 (protein), 0.2 (Pi), and 0.02 (calcium). *Lines* between the *points* are trend lines and do not fit the data

decrease of Ca^{2+} activity by complexation with EDTA may lead to the rupture of this kind of bond, with a release of the proteins and an increase in the Pi activity. To prove if the liberation of Pi and the complexation of Ca were related, the Pi/Ca molar ratio in the supernatants was determined at the different concentrations of EDTA used. The Pi/Ca molar ratio was clearly higher than the values of 0.6 usually assigned to the stoichiometric relationship for the colloidal calcium Pi in the CM, presenting initial values of about 7 [11]. At increasing EDTA concentrations, this ratio decreased, tending to reach a value of about 4. The initially high amount of Pi found in the supernatants could be attributed to the release of whey entrapped in the curd [12], which was released when the gel network was mechanically disrupted and dissociated by EDTA action. As regards this, it is interesting to note that the initial release of Pi was higher for C_{25}, which were the looser and more hydrated curds [13].

Although the replicates of urea-PAGE patterns obtained for the supernatants of curds treated with the dissociating agents and mechanical disruption showed some quantitative differences between them, a general tendency can be observed for each of the dissociating agents used. In the case of EDTA, the supernatants from C_{25} contained β-lactoglobulin in high proportion together with a minor casein fraction composed of β- and α_S-caseins. Low concentrations of α-lactalbumin were also detected. All the mentioned proteins were identified in the correspondent urea-PAGE pattern using the adequate markers as it has been shown in Fig. 4. The presence of whey proteins can be interpreted as another evidence that suggests the rupture of the curd matrix by EDTA and the mechanical disruption liberated entrapped whey, in accordance with the observations of the release of mineral components.

In the case of C_{38}, the composition of the liberated protein fractions was similar to that observed for C_{25}, but almost only β-casein appeared in the casein fraction, and β-lactoglobulin was present in an apparently lower proportion.

Dissociation of curds by NaCl

Protein dissociation from the curds by action of NaCl (Fig. 2a) presented similar features to the dissociation induced by EDTA on C_{25}. Either for C_{25} or C_{38}, the amount of protein released reached maximal values at NaCl concentrations relatively low, decreasing after at higher values of ionic strength. This behavior can also be attributed, as in the case of the dissociation by EDTA, to a reassociation of some the released proteins with the remaining curd structure, probably by interactions in the absence of Ca, or nonelectrostatic interactions. The release of proteins by ionic strength increase, however, differed from the release caused by EDTA in two ways. In the first place, the amount of released and reassociated protein at relatively high NaCl concentrations was lower than that obtained from C_{25} at low EDTA concentrations, and, in the second place, the liberation and further reassociation produced by NaCl could be observed also for C_{38}.

In the case of Pi and Ca (Fig. 2b), C_{25} and C_{38} behaved in a similar way. There was an increasing liberation of both ions tending to reach maximal values that were lower for C_{25}. The Pi/Ca molar ratio was initially higher than the stoichiometric relationship in CM (~8 for C_{25} and ~15 for C_{38}) and decreased at increasing NaCl concentrations, reaching a value of about 6 for both curds, which suggests that one of the first effects induced by NaCl was the release of entrapped whey.

The proteins liberated by the action of NaCl on C_{25}, identified by urea-PAGE patterns using adequate markers as is shown in Fig. 4, were whey proteins mainly

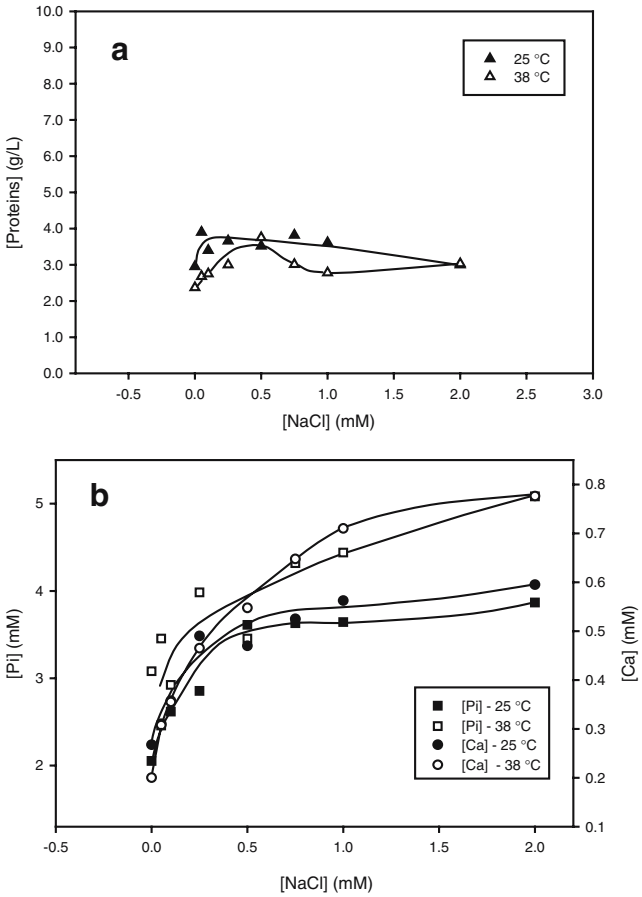

Fig. 2 Total amount of proteins (**a**) and calcium and Pi (**b**) present in the supernatants obtained by dispersion at different NaCl concentrations and subsequent centrifugation of curds prepared at two temperatures. Each *point* is the mean of at least five determinations; maximum SD, 0.2 (protein), 0.1 (Pi), and 0.01 (calcium). *Lines* between the *points* are trend lines and do not fit the data

accompanied by β-casein in relatively higher proportions than in the case of the proteins liberated by EDTA. The electrophoretic patterns obtained for C_{38} also showed the presence of whey proteins and β-casein as the principal components. However, small amounts of α_S-caseins were also detected in this case.

Dissociation of curds by urea

Figure 3a shows the protein content in the supernatants when the curds were dispersed with different urea concentrations. A similar increasing protein release was observed for both C_{25} and C_{38} below 4 M urea. Pi and Ca were found in the supernatants at almost constant concentrations from 0 to 4 M urea (Fig. 3b), showing a progressive increase for both ions, specially marked for C_{38}. The Pi/Ca molar ratio, unlike the behavior observed for the dissociation induced by EDTA or NaCl, increased for increasing urea concentrations. The values obtained for C_{25} varied from ~5 to ~9, and from ~7 to ~10 in the case of C_{38}.

Figure 4 shows some examples of the urea-PAGE patterns correspondent to curds dispersed with the action of urea. The results obtained in the absence of urea showed mainly the presence of whey proteins, either for C_{25} or C_{38}. Quite similar results were obtained at urea concentrations lower than 4 M. At higher urea concentrations, however, a clear change can be observed with an increase of the typical bands due to α_S- and β-caseins, especially in the case of C_{38}. Nonidentified fractions of low molecular weight, which were probably the result of partial proteolysis, were also observed in almost all the cases.

Discussion and conclusions

The main interactions that are usually considered to be involved in maintaining the caseins in the curd matrix obtained by enzymic coagulation are attractive forces of low energy such as hydrophobic interactions, electrostatic attractions, and Ca bridges [5, 6]. Information about the role played by such forces can be derived from the study of

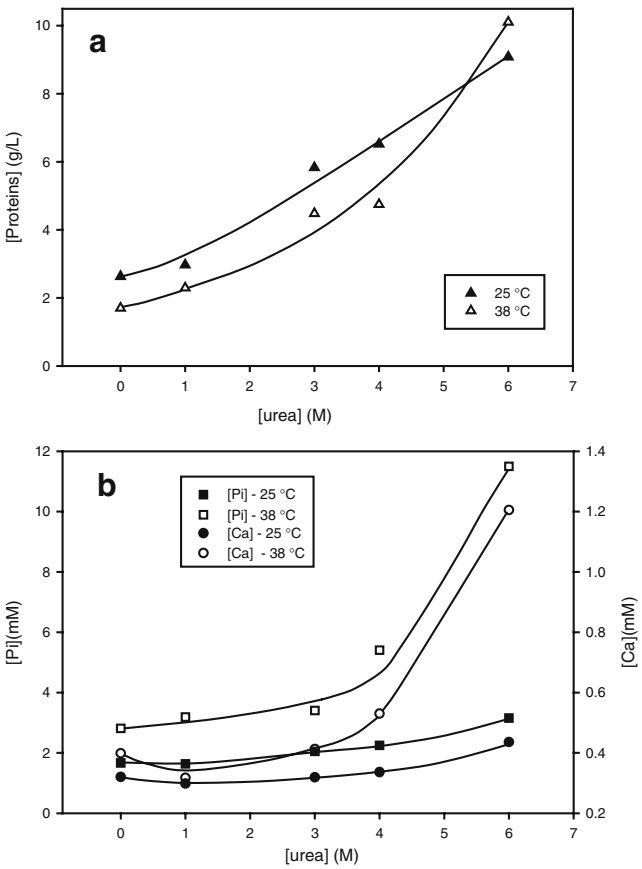

Fig. 3 Total amount of proteins (**a**) and calcium and Pi (**b**) present in the supernatants obtained by dispersion at different urea concentrations and subsequent centrifugation of curds prepared at two temperatures. Each *point* is the mean of at least five determinations; maximum SD, 0.1 (protein), 0.2 (Pi), and 0.01 (calcium). *Lines* between the *points* are trend lines and do not fit the data

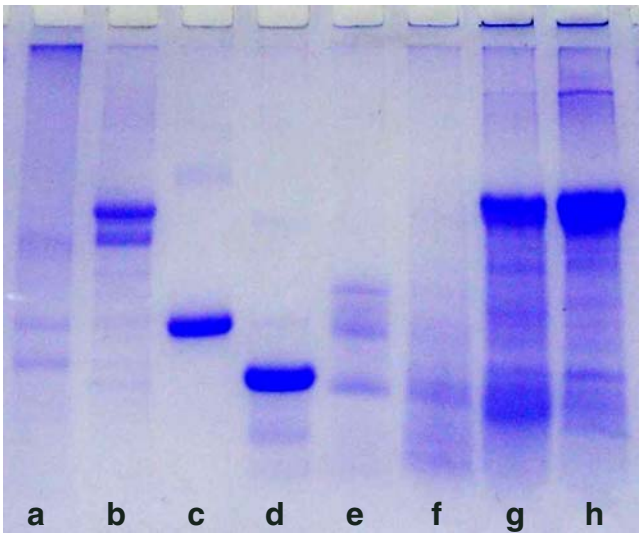

Fig. 4 Urea-PAGE patterns (pixel densities vs R_f) obtained at 25 °C for the supernatants of curd treated with different concentrations of the dissociating agent (urea) and mechanical disruption. *a–d* weight markers: *a* κ-casein, *b* α and β-casein, *c* β-lactoglobulin, *d* α-lactalbumin; *e–h* supernatants of curd treated with different concentrations of urea: *e* without urea, *f* 1 M, *g* 4 M, *h* 6 M

the action of different dissociating agents on the integrity of the curds obtained in different conditions.

When EDTA was used as a dissociating agent, it can be assumed that curd dissociation was mainly based on its complexing action on Ca. Although this action is accompanied by an increase in the ionic strength because of the concomitant increase in Pi activity [12], it must be assumed that the amount of protein released by EDTA was higher than the values obtained when NaCl was used as a dissociating agent at clearly higher ionic strength, thus indicating that the effect of the ionic strength increase is not so important in the case of EDTA. The effective initial protein dissociation produced by low concentrations of EDTA on C_{25} suggested the presence of a group of relatively weak bonds involving Ca in this kind of curd. These bonds maintain a fraction of α_S- and β-casein loosely held in the curd matrix, and their disruption produced the liberation of a part of the whey trapped in the gel structure. Because this initial behavior was not observed in the case of C_{38}, it can be assumed that it was the coagulation at low temperature that favored the formation of these electrostatic interactions involving Ca and Pi, relatively easy to dissociate by EDTA action. This appears to be consistent with the exothermic nature of that kind of interaction and with the physical characteristics of less compact and more hydrated gels [13, 14]. Moreover, the release of proteins and minerals was higher for C_{25} even in the absence of EDTA, when the curds were mechanically dispersed with water alone, suggesting the presence of a fraction of bonds weak enough to be broken by shear forces (initial points in Fig. 1a,b).

In the case of NaCl, its dissociating action lies mainly on the increase of ionic strength that acts by weakening coulombic interactions. However, it must be taken into account that Na^+ can displace Ca^{2+} from its bonds, as it has been postulated for CM [15]. This could be the reason why the action of NaCl as a dissociating agent presents certain similarities with the stronger action of EDTA. In fact, the action of NaCl on C_{25} followed a pattern similar to that observed when EDTA was used as a dissociating agent, with an initial release of proteins that subsequently reassociated to the rest of the curd matrix. Dissociation of C_{38} also showed an initial release of a protein fraction, probably followed by further reassociation, but this behavior was observed at a higher ionic strength than in the case of C_{25}, suggesting the presence of stronger electrostatic interactions in the gels obtained at higher temperature. Entrapped whey release, shown by the liberation of Pi, Ca, and whey proteins, can also be observed by NaCl action. These results suggest, in the same way that EDTA results did, the presence of a group of electrostatic interactions, some of them with Ca participation. These interactions loosely retain a casein fraction mainly integrated by β-casein, with a minor participation of α_S-caseins, especially in the case of C_{25}.

Urea, as a dissociating agent, showed a quite different action. The results lower than 4 M obtained at urea concentrations were mainly related to the release of whey proteins, i.e., to the release of entrapped whey. Until 4 M urea, the amount of released proteins was similar to the amounts obtained by EDTA or NaCl actions. For higher urea concentrations, there was a faster increase in protein release, showing even a sharp increase in the case of C_{38}. It is well known that urea can act weakening and disrupting hydrophobic interactions. Because these kinds of interactions are usually of endothermic nature, higher temperatures could favor them, as in the case of C_{38}. The presence of more and stronger hydrophobic interactions in this kind of gel could be coherent not only with the higher release of proteins at urea concentrations higher than 4 M, but also with the more compact and less hydrated nature of these curds [13]. Unlike the other dissociating agents, the action of urea released important amounts of α_S-caseins and β-casein, accompanied by increasing amounts of Pi and Ca. This release of increased mineral fractions could be attributed to the increasing presence of the phosphorilated caseins in the supernatants. Because samples for urea-PAGE were previously treated with SDS, reduction, and strong heating, it is impossible to determine if the released protein fraction contained either complex particles or isolated proteins and minerals. However, the first possibility is based on the turbidimetric aspect that the supernatants presented before being submitted to electrophoretic analysis. Probably, the results obtained here show that the rupture

of hydrophobic bonds caused by urea may lead to the disruption of the micellar structure itself, with liberation of the components of the "core" of the CM as complex phosphocasein particles.

In summary, comparison of the effects produced by EDTA, NaCl, and urea on the integrity of C_{25} and C_{38} suggests that higher temperatures favored the participation of hydrophobic interactions in the structure of the curd during enzymic coagulation. On the other hand, lower temperatures induced the formation of weak electrostatic bonds, some of them with Ca participation, which could be capable of retaining a fraction of the caseins bound to the gel matrix.

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