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Interaction of a Cationic Acrylate Polymer with Caseins: Biphasic Effect of Eudragit E100 on the Stability of Casein Micelles

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When whole or skim milk was incubated with the cationic acrylate polymer Eudragit E100, a biphasic effect on the stability of casein micelles was observed. A precipitation phase was observed at low polymer/casein ratios. Strikingly, a solubilization phase of the aggregates was observed when the ratios of polymer/casein were increased. Purified α_s-, β-, and κ-caseins or dephosphorylated caseins were equally precipitated and resolubilized by the cationic polymer, indicating no special selectivity for a particular protein or phosphate residue for these events. An increase in the size of the aggregates as the optimum precipitating amount of Eudragit E100 was reached suggests a crossbridging of the micelles by the polymer. The inhibition of the precipitation phase by high ionic strength indicates that electrostatic interactions play a critical role in complex formation. Furthermore, a dramatic reduction in size of the protein colloidal particles upon solubilization of the aggregates was observed by dynamic light scattering, indicating a dissociation of the micellar structure. Taken together, the results indicate that at low concentration Eudragit E100 may act as a precipitant of casein micelles, mainly by ionic interaction and at high concentration as an amphipathic agent, solubilizing casein micelles with a disruption of their internal structure.

KEYWORDS: Eudragit E100; casein micelles; Eudragit−casein interaction; aminoacryl methacrylate copolymer

INTRODUCTION

The interactions between proteins and natural or synthetic polymers has been extensively studied, in particular for the modulation of living processes, immobilization or stabilization of enzymes, modification of substrate affinity, changing properties of food products, and for the development of many pharmaceutical applications (*1*−*3*). In this context, biopolymers, particularly polysaccharides, are often added to milk and dairy products in order to attain a desired texture and viscosity. However, their addition sometimes results in phase separation when the polymer exceeds a certain concentration (*3*−*5*). The phase behavior of mixtures of neutral and anionic polysaccharides and milk or casein micelles has been extensively characterized (*2, 6, 7*). Contrarily, very little attention has been given to study the interactions between casein micelles and positively charged polymers. In this context, we have recently described that chitosan, a positively charged polysaccharide with primary and secondary amine groups, promotes a selective coagulation of caseins from bovine milk in a process that involves not only electrostatic but also hydrophobic interactions (*5*). Interestingly, despite the strong interactions involved in the coagulation process, the complexes between chitosan and caseins are very well hydrolyzed by gastrointestinal proteases releasing soluble peptides with an unmodified kinetic as compared to standard coagulation procedures with acid or rennet, while lipolytic enzymes only hydrolyze about 50% of the triglycerides present in the aggregates (*8*). To gain a better understanding of the interactions between positively charged polymers and casein micelles, we focused this study on the characterization of the effects of a cationic polymer containing a tertiary amine group, the aminoacryl methacrylate copolymer Eudragit E100 (E100) (**Figure 1**), on the stability of casein micelles. Acrylic polymers have been developed as excipients for pharmaceutical formulations (*9*, *10*). In this context, the cationic acrylate E100 has been recently used with good results in the prevention of drug crystallization in drug-in-adhesive transdermal systems (*11, 12*). In the field of protein biochemistry, many different acrylic polymers have been evaluated in affinity precipitation (*1*). Among these, a pH-sensitive acrylate commercialized as Eudragit S100 has proven to be suitable for protein purification (*13, 14*) and preparation of immobilized enzymes that can be used in soluble form (*15*).

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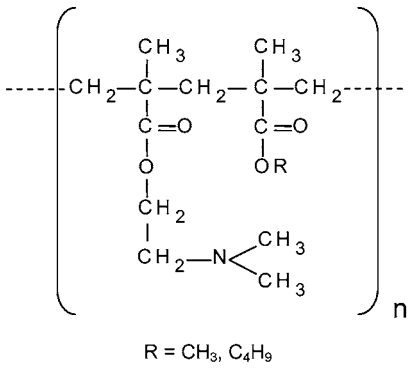

Figure 1. Chemical structure of Eudragit E100.

The results reported herein show that at low E100/milk protein ratios (***R***) the polymer causes a selective precipitation of caseins in a similar way to that described for chitosan (*5*), but at high ***R***, the excess of polymer promotes the solubilization of the precipitated E100−casein complexes with a disruption of the casein micelle structure.

MATERIALS AND METHODS

Materials. Eudragit E100, with an average molecular weight (MW) of 150 kDa, was a gift from Röhm Pharma (Darmstadt, Germany). Stock solutions of E100 were prepared in 100 mM acetate buffer at pH 6.0.

High molecular weight chitosan average MW 1000 kDa, having a degree of deacetylation of approximately 80%, was obtained from Aldrich (Milwaukee, WI). Stock solutions of chitosan were prepared in 100 mM acetate buffer pH 5.9.

Pasteurized whole or skim bovine milk samples were from local commercial sources. Rennet from *Mucor miehei*, sodium caseinte, phosphorylated α_s-, β-, and κ-caseins, and total dephosphorylated casein were obtained from Sigma Chemical Co. (St. Louis, MO). All other reagents used were of analytical grade.

Rennet, Acid, and Chitosan Coagulation. For rennet coagulation, milk samples were incubated for 15 min at 37 °C, with 0.01 unit/mL of rennet.

For acid coagulation, a solution of 1 M HCl was slowly added to milk at 25 °C until pH 4.6 was reached. Then, the suspension was allowed to stand for 15 min.

For chitosan coagulation, 0.3 vol of a solution of chitosan (1% w/v) at pH 5.9 were added to 1 vol of whole milk, the mixtures were shaken and allowed to coagulate at room temperature for about 10 min.

Finally, for all coagulation methods, samples were centrifuged at 5000*g* for 10 min to separate precipitate and whey.

Interaction between E100 and Milk or Caseins. The usual protocol to study the interactions between E100 and caseins was performed as follows: samples of 1 mL of whole or skim milk (30 mg/mL protein concentration) were incubated at 4, 25, or 70 °C, with 0.5 mL of different solutions of E100 to give up to 35 mg/mL (final concentration) or 0.5 mL of acetate buffer 100 mM at pH 6.0 as a control.

Total dephosphorylated caseins and α_s-, β-, and κ-caseins (5 mg/mL) were solubilized in 20 mM phosphate buffer, pH 6.8. Samples (1 mL) of caseins were incubated at room temperature with 0.5 mL of different solutions of E100 to give up to 5 mg/mL (final concentration) or 0.5 mL of acetate buffer 100 mM at pH 6.0 as a control.

In both cases, the mixtures were vigorously shaken for 30 s, to reach a complete interaction. Then, the different samples were allowed to stand for 10 min at each indicated temperature and finally centrifuged at room temperature at 5000*g* for 10 min to separate pellets from supernatants.

Sodium caseinate (5 mg/mL) was solubilized in 20 mM phosphate buffer pH 6.8. To produce model colloidal casein micelles, $CaCl_2$ was added to a final concentration of 10 mM (*16*). To study the effect of NaCl or detergents, casein micelles solution was mixed with E100 (0,5 mg/mL final concentration), or 100 mM acetate buffer pH 6.0 as a control, all containing the appropriate amount of NaCl, Tween 20, NaCl plus Tween 20 or SDS to give the final concentrations reported in **Figure 5**. The mixtures were then processed as described above to collect pellets and supernatants.

To study the effect of casein electric charge in the interaction with E100, sodium caseinate (5 mg/mL) was dissolved in 20 mM NaOH and then adjusted to pH 6.8 or 2.3 by careful addition of 1 N HCl. The interaction with E100 was performed as described above.

Determination of Protein, Triglyceride, and Calcium Concentration in Wheys. Protein concentration was determined by Biuret or by direct absorbance at 280 nm, as previously described (*5*).

The concentration of triglyceride present in whole milk or in the wheys obtained after precipitation with rennet, acid, or E100, as described above, was determined using a colorimetric enzymatic assay from Wiener Lab (Rosario, Argentina).

Ca^{2+} concentration present in whole milk or in the wheys obtained after precipitation with rennet, acid, or E100, as described above, was determined using flame atomic absorption spectrometry in a Shimadzu AAS 6501S (Kyoto, Japan). The samples were diluted in deionized water containing 1% La_2O_3 final concentration to avoid interference of matrix due to the presence of phosphate. The background correction was performed by the self-reversal method.

Polyacrylamide Gel Electrophoresis. Polyacrylamide gel electrophoresis containing SDS (SDS-PAGE) of 15% (w/v) separation gel and 4.5% (w/v) stacking gel, was performed according to Laemmli (*17*) in a vertical slab PAGE cell (Miniprotean II, Bio-Rad). Samples of milk were treated with various solutions of E100 to give 0−35 mg/mL (final concentration). Supernatants, obtained from these samples as described above, were diluted in universal sample buffer containing 2% (v/v) 2-mercaptoethanol and heated at 90 °C for 3 min. Samples containing 70−150 μg of proteins were loaded in each well. The electrophoresis were conducted for 90 min at 140 V. The proteins were stained with Coomassie Brillant Blue R-250.

Microscopic Analysis. Samples (1 mL) of milk treated with 0.5 mL of acetate buffer 100 mM pH 6.0 (as a control) or 0.5 mL of E100 solutions to give up to 35 mg/mL (final concentration) were analyzed at 50× and photographed without any staining in an Axiovert 135 M Karl-Zeiss microscope (Göttingen, Germany).

Particle Size Determination by Laser Light Scattering. Colloid particle sizing was performed by dynamic light scattering using a PSS-Nicomp 370 submicron particle sizer from Particle Sizing Systems (Santa Barbara, CA) with a 632.8 nm HeNe laser. A polystyrene latex standard with a mean-volume-averaged diameter of 91.4 nm was used to calibrate the instrument. Samples (0.5 mL) thermostatized at 25 °C and diluted if necessary were measured, recording the intensity of the light scattered at 90° during at least 10 min, using a Gaussian size distribution analysis.

Ion Exchange Chromatography. Soluble E100-casein mixtures were chromatographed on DEAE-Sephadex or CM-Sephadex columns previously equilibrated with 10 mM phosphate buffer pH 6.8. The elution profile was followed using a UV-detector (280 nm) and the total protein levels quantified with a Coomassie Brilliant Blue assay.

Data Presentation and Statistical Analysis. Each experiment was carried out in duplicate and was independently replicated at least twice. **Figures 2B** and **3** are from representative experiments. For the remainder of the figures and tables, the mean and standard deviation of all the determinations performed is reported. Differences among treatments were determined by the Student's *t*-test.

RESULTS

Effect of E100 on Whole Milk. When whole milk was incubated at room temperature (25 °C) with increasing concentrations of E100 at pH 6.0, an interesting biphasical process was observed. A dose-dependent aggregation and coagulation of milk was observed at concentrations of E100 up to 5 mg/mL. As with chitosan, the coagulation process observed occurred almost immediately after mixing the polymer with milk (*5*). However, as the concentration of E100 in milk was increased, a solubilization of the aggregates was observed. This solubliza-

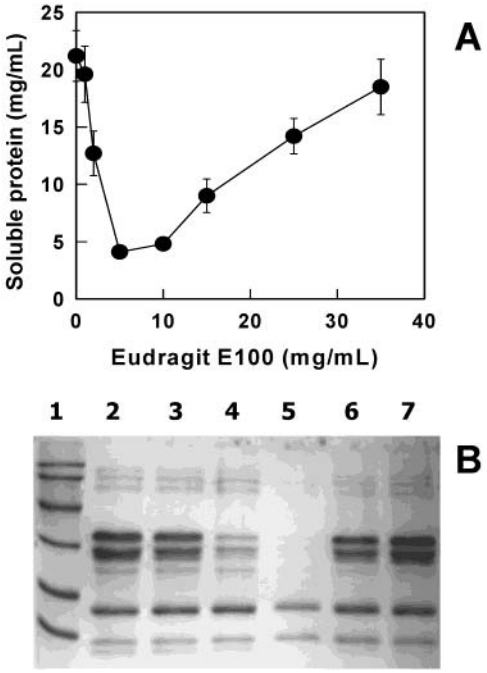

Figure 2. (A) Effect of Eudragit E100 on the stability of milk proteins. Whole milk was incubated with increasing concentrations of E100. (B) SDS–PAGE of soluble proteins after milk treatment with Eudragit E100. Samples of whole milk were treated with increasing concentrations of E100. Lane 1, molecular weight standards (from the top, molecular weights are 97, 66, 45, 31, 21, and 14 kDa). Lanes 2–7, supernatants of milk samples incubated with the following final concentrations of E100: lane 2, 0 mg/mL (control of acetate buffer); lane 3, 0.5 mg/mL; lane 4, 1.5 mg/mL; lane 5, 5 mg/mL; lane 6, 20 mg/mL; lane 7, 35 mg/mL.

tion was complete when E100 reached 35 mg/mL without aggregation or precipitation. To gain insight into the components involved in this process, a partial compositional analysis of the whey obtained using 5 mg/mL E100 was performed and compared with the wheys obtained by rennet or acid coagulation (**Table 1**). According to what could be expected from the coagulation process observed, most of the proteins were precipitated with 5 mg/mL of E100 (**Table 1**). Very little amount of triglycerides remained soluble in E100 wheys, which was similar to that obtained with rennet or acid (**Table 1**). As previously observed with chitosan-induced milk coagulation (*5*), the content of Ca^{2+} in E100 whey was lower than that present in rennet or acid wheys, indicating that most of the Ca^{2+} was retained in the precipitates. Altogether, these results suggest that casein micelles and milkfat globules are involved in the formation of the aggregates observed at low concentrations of E100.

Interaction of E100 with Casein Micelles. The involvement of milk proteins in the formation of the aggregates was demonstrated by analyzing the concentration that remained soluble after the addition of increasing amounts of E100 to whole milk (**Figure 2A**). Compositional analysis of the milk proteins that remained in the wheys by SDS−PAGE clearly showed that upon E100 addition from 0.5 to 5 mg/mL, the major components that precipitate from milk were the caseins, leaving a significant amount of the whey proteins in a soluble form (**Figure 2B**, lanes 2−5). However, when the E100 concentration was increased from 5 to 35 mg/mL, a solubilization of the aggregates was observed, releasing the caseins to the supernatant (**Figure 2B**, lanes 6 and 7).

Comparative analysis by optical microscopy of whole milk and different E100−casein complexes showed that the size and shape of the aggregates change at different E100/milk protein ratios (w/w) (***R***). Thus, as ***R*** was increased from 0.045 to 0.22, the aggregates increased in size and adopted more irregular shapes (**Figure 3B−D**). Remarkably, the biggest aggregates were observed at $\boldsymbol{R} = 0.22$, with the maximal precipitation of caseins from milk. Increasing the ratio of E100 to protein above 0.22, the size of the aggregates was progressively reduced, until at $\boldsymbol{R} = 1.5$, approximately 95% of the insoluble complexes were resolubilized, showing the classical highly homogeneous image of whole milk (see **Figure 3A**,**E**,**F**).

E100-Induced Casein Precipitation Does Not Depend on Milkfat. The fact that the aggregates are resolubilized by an excess of E100 suggests that despite the fact that this is a positively charged water soluble polymer, it shows some hydrophobic behavior. Furthermore, most of the casein micelles and fat globules are interacting in tight complexes in milk, and most of the triglycerides present in whole milk are precipitated by E100 (**Table 1**). Therefore, casein precipitation could be due to an unspecific trapping of the micelles within the E100-milkfat aggregates. To rule out this possibility, defatted sodium caseinate was incubated with increasing concentrations of E100. As shown in **Figure 4A**, the protein precipitation profile observed was similar to that previously found with whole milk, indicating that casein precipitation and resolubilization by E100 does not depend on the interactions of the polymer with milkfat.

E100 Interacts with α_s-, β-, and κ-Caseins Independently of the Phosphate Content. Having established that the addition of different amounts of E100 on milk can produce a precipitation and solubilization of caseins and lipids, it was of interest to examine which of the individual caseins participate in the interactions with the polymer. When E100 was added to solutions of purified α_s-, β-, and κ-caseins, a dose-dependent process of precipitation−solubilization occurred that was essentially identical to that observed for those of whole milk and sodium caseinate (**Figure 4B**).

Because caseins contain several phosphate groups that play key roles in the precipitation of these proteins by Ca^{2+}, these phosphate groups could reasonably be involved in their interaction with the positive charges of E100. As shown in **Figure 4A**, both phosphorylated and total dephosphorylated caseins interact with E100, showing a similar behavior of precipitation and solubilization. Despite the fact that these results do not rule out an involvement of casein phosphate residues in their interaction with E100 they indicate that, if present, these interactions are not essential for the observed phenomena.

Characterization of E100−Casein Interaction in the Precipitation Phase. Various types of intermolecular forces could contribute to the interaction between E100 and caseins in the precipitation phase. To gain insight into the nature of the interactions between E100 and caseins, we first explored on the relevance of hydrophilic and hydrophobic forces by studying the dependence of the observed phenomena with ionic strength, detergents, pH, and temperature.

Screening the surface charges of the interacting polymers by the addition of increasing concentrations of NaCl led to a progressive inhibition of E100−casein precipitation (**Figure 5A**). On the other hand, the inhibition of hydrophobic interactions by the addition of the nonionic detergent Tween 20, at concentrations up to 2%, was unable to prevent the precipitation of sodium caseinates by E100 (**Figure 5B**). These results indicate that the electrostatic interactions are playing an important role in the formation of complexes between E100 and caseins. In agreement with this, the aggregation of caseins

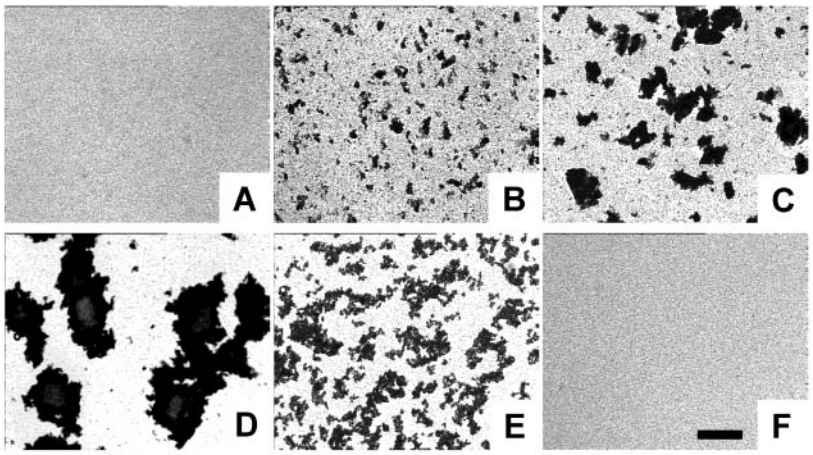

Figure 3. Microscopic analysis of Eudragit E100-milk complexes at different E100/protein ratios (***R***). (A) ***R*** = 0; (B) ***R*** = 0.045; (C) ***R*** = 0.11; (D) ***R*** = 0.22; (E) ***R*** = 0.7; (F) ***R*** = 1.5. Bar: 200 μm.

Table 1. Comparative Triglygeride, Ca^{2+}, and Protein Contents in Milk Wheys Obtained after E100, Acid, and Rennet Precipitating Procedures

coagulating agent[a]	triglyceride (mg %)[b]	Ca^{2+} (mg/L)	protein (mg/mL)
rennet	34 ± 4 (1.7%)	460 ± 20 (41.5%)	4.2 ± 0.6 (20%)
acid (1M HCl)	47 ± 9 (2.3%)	810 ± 20 (73%)	4.3 ± 0.4 (20%)
Eudragit E100 (5 mg/mL)	40 ± 10 (2.3%)	327 ± 18[c] (30%)	4.8 ± 0.5 (22%)

[a] The volume of coagulating agent used was standardized to 0.5 vol of milk used to allow an appropriate comparison. HCl was added to give a pH of 4.6. Coagulation was performed at 37 °C for rennet and at 25 °C for acid and E100. [b] Values in brackets indicate the percentage of milk content that remains soluble in whey. [c] $p < 0.05$ vs values obtained after rennet treatment.

was prevented by the addition of Tween 20 in NaCl or by SDS, both affecting not only hydrophobic but also hydrophilic interactions (**Figure 5**, parts C and D).

When both casein and E100 were solublized at pH 2.3, where most of the carboxylic groups of caseins are protonated and E100 amine groups remain positively charged, no precipitates could be detected upon mixing a casein solution with increasing concentrations of E100, with most of the protein remaining soluble after centrifugation at 5000*g* (**Figure 6**). This result emphasizes the role of electrostatic interactions in the formation of the insoluble E100−casein aggregates.

The reversibility of the aggregation process observed by an excess of E100 suggested that the electrostatic interactions involved in the formation of the aggregates would not be strong and that hydrophobic interactions would also be playing an important role in the observed phenomena. This situation should be reflected in a small standard enthalpy change of the association between E100 and casein micelles. In fact, the precipitation and resolubilization patterns were very similar over a wide range of temperatures (4−70 °C), indicating that the overall standard enthalpy change for the process would be close to zero (data not shown). These results also emphasize a role for the hydrophobic effect in the formation of the complexes between E100 and caseins.

E100 Shows Amphipathic Properties. The fact that increasing E100 concentrations in milk led to the solubilization of E100−casein aggregates raised the question about the nature of this effect. One possibility would be that the excess of positive charges on the surface of the casein micelles led to the disruption of the aggregates with the formation of water soluble complexes.

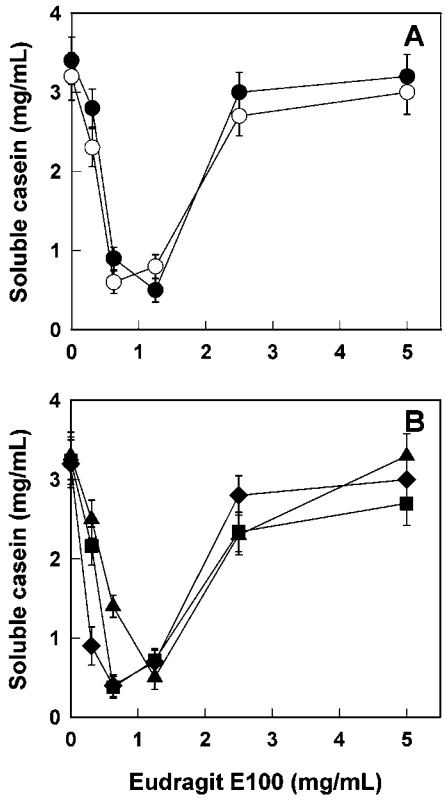

Figure 4. Interaction of Eudragit E100 with caseins. (A) Interaction with sodium caseinate (phosphorylated casein) (●) or total dephosphorylated casein (○). (B) Interaction of purified α_s- (◆), β-(▲), and κ-caseins (■) with increasing concentration of Eudragit E100.

Another possibility would be that the overall interaction between E100 and caseins involves not only the dissociation of the aggregates but also the disruption of the micellar structure and the solubilization of small positively charged hydrophilic complexes. This possibility arose from the reduction in turbidity of milk or casein solution that was observed upon the aggregation of an excess of E100 (i.e., 35 mg/mL final concentration for undiluted milk). To disclose which of these mechanisms is taking place at high concentrations of E100, we analyzed the changes in particle size distribution on casein micelles from skim milk as a function of E100 concentration by dynamic light scattering (**Figure 7**). The addition of an excess of E100 (***R*** = 1.5) produces a dramatic reduction in the particle size distribu-

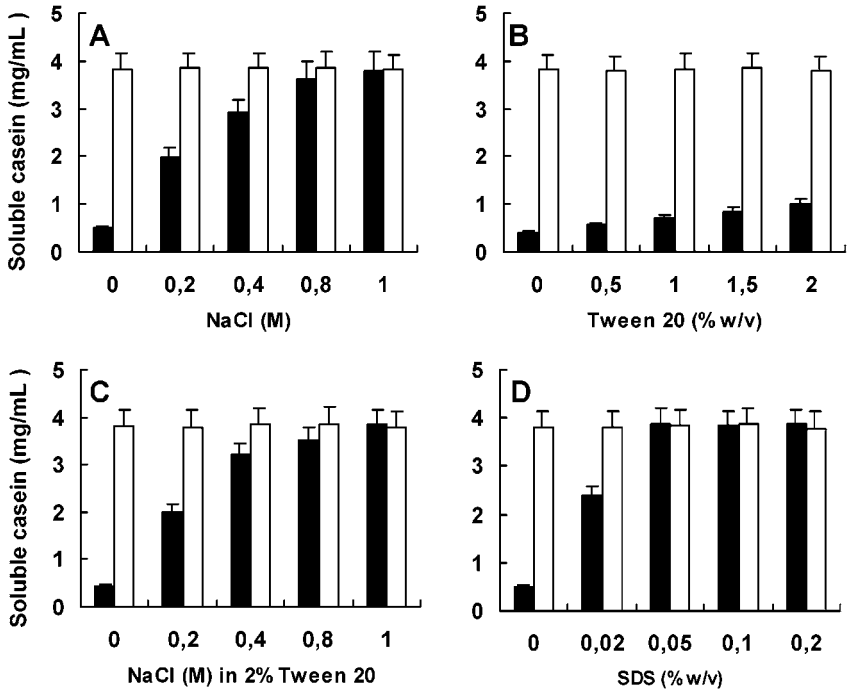

Figure 5. Effect of ionic strength and detergents on Eudragit E100–casein interaction. Sodium caseinate was incubated with E100 ($R = 0.22$) (black bar), or acetate buffer 100 mM pH 6.0 as a control (white bar) in the presence of increasing concentration of (A) NaCl, (B) Tween 20, (C) NaCl plus 2% Tween 20, and (D) SDS.

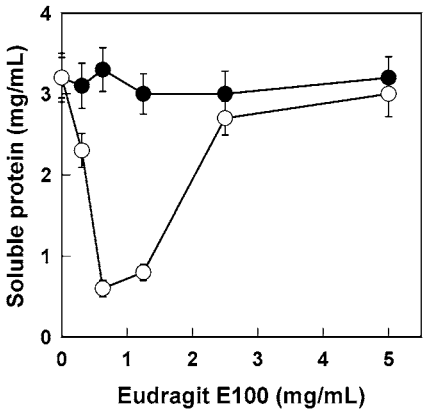

Figure 6. Effect of net charge of casein in the interaction with E100. Sodium caseinates below their isoelectric points at pH 2.3 (●) or above their isoelectric points at pH 6.8 (○) were incubated with increasing concentrations of E100.

tion. These results indicate that E100 is behaving like a "detergent", because it is dissociating not only the supra-aggregates of casein micelles with E100 but also casein micelles into submicelles or casein oligomers.

The finding of an amphipathic character of E100 prompted us to evaluate the possibility that E100 could also disrupt the integrity and dissolve other casein coagula. For this purpose, casein aggregates obtained by rennet or high molecular weight chitosan (HMWC), as described under materials and methods, were mixed with an excess of E100. It is known that rennet-induced casein aggregates are a consecuence of strong hydrophobic interactions after enzymatic removal of casein glycomacropeptide (*18*, *19*). Otherwise, it was previously shown that HMWC−casein aggregates involve both electrostatic and hydrophobic interactions (*5*). In our experimental conditions, the addition of increasing concentrations of E100 to rennet and chitosan−caseins aggregates resulted in a solubilization of both

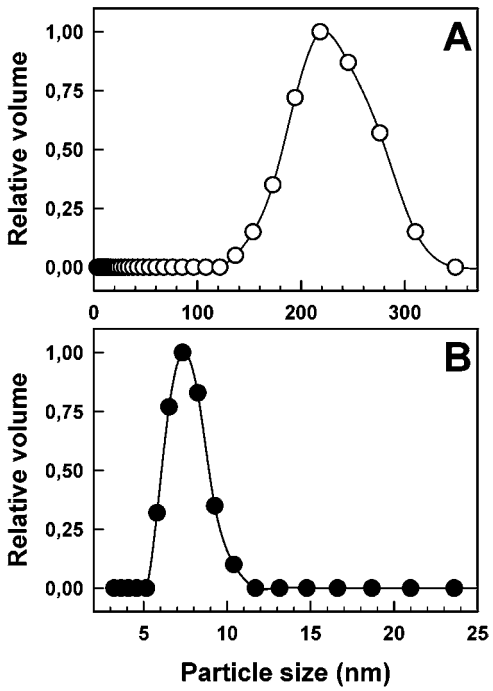

Figure 7. Casein micelles size distribution for (A) skim milk or (B) skim milk treated with Eudragit E100 at $R = 1.5$.

aggregates at 80 and 60%, respectively (data not shown). In agreement to what was observed for E100−casein aggregates, the maximum solubilization was reached at $R = 1.5$.

An Excess of E100 Results in the Formation of Soluble Cationic E100−Casein Complexes. When the experimental control of casein micelles or E100−casein soluble complexes ($R = 1.5$) were subjected to anion exchange chromatography onto DEAE-Sephadex, it was observed that casein was completely retained in DEAE-Sephadex, while soluble E100−casein complexes eluted without any interaction with the column matrix

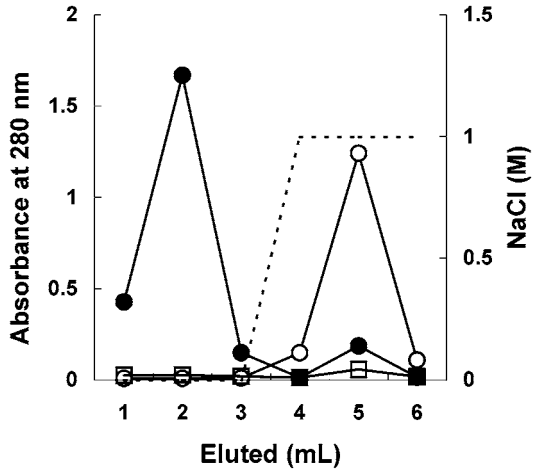

Figure 8. Ion exchange chromatography on DEAE-Sephadex of casein (○), E100 (□), and E100–casein soluble complexes ($R = 1.5$) (●). Salt gradient (dotted line).

(**Figure 8**). On the other hand, the incubation of soluble E100−casein with CM-Sephadex resulted in a precipitation of caseins, probably as a result of the removal of the excess of E100 by the resin with the formation of hydrophobic E100−casein complexes (data not shown). These results indicate that E100−casein complexes should have a net electropositive charge to remain soluble.

DISCUSSION

We have previously shown that chitosan, a positively charged polysaccharide with primary and secondary amine groups in its structure, causes the precipitation of milkfat and caseins from bovine milk (*5*). In the current study, we show that E100, a positively charged acrylate polymer with a tertiary amine group in its structure, displays a biphasic coagulating-resolubilizing effect of caseins and milkfat from bovine milk. This work also shows that, from the various intermolecular forces that could be involved in complex formation, electrostatic and hydrophobic interactions play a critical role between E100 and caseins. This is strikingly different from the situation in the chitosan−casein complex formation, where despite the fact that a combination of hydrophobic and electrostatic interactions have been involved in the coagulation process observed, no resolubilization of the aggregates was observed with an excess of chitosan (*5*).

As with chitosan (*5*), a direct association between the polymer and casein was observed, with a precipitation phase that was independent of the phosphate content of the caseins and did not show selectivity for any special casein, because purified α_s-, β-, and κ-caseins interact with E100, showing a similar precipitation pattern to milk.

The results that E100−casein precipitation was prevented with high salt concentration or by protonation (pH 2.3) of acid groups emphasize the importance of the electrostatic interactions in the formation of insoluble complexes between E100 and caseins. Thus, the precipitation by E100 appears to be a consequence of the neutralization of the negative charges of carboxylates and phosphates present in caseins. The increase in size of E100−casein precipitates as E100 reaches an optimum precipitating concentration suggests that E100 is able to produce a cross-bridging between casein micelles. Unexpectedly, exceeding the optimum precipitating concentration of E100 led to a complete solubilization of the aggregates. One possibility for this would be that with an excess of E100 casein micelles become covered by positively charged "hairs" of E100, thus leading to the formation of a highly polar and water soluble complex. However, the reduction in turbidity and the light scattering data clearly show that the solubilization of E100−casein aggregates by an excess of E100 is due to the disruption of the micellar structure into submicelles or caseins oligomers. In this context, it has been shown that electrostatic interaction, hydrophobic bonding, and calcium phosphate-mediated links contibute to the cohesive interactions responsible for maintaining casein micellar integrity (*2*, *22*, *23*). On the other side, the molecular structure of E100 shows polar and nonpolar moieties (**Figure 1**). For these reasons, electrostatic and hydrophobic interactions would both be participating in the dissociation of the micellar structure by E100.

The concept that water soluble positively charged complexes are formed between an excess of E100 and caseins was further confirmed, using ionic exchange resins. Moreover, the results with DEAE and CM-Sephadex demonstrate that E100−casein complexes remain soluble only as cationic complexes, because removing the excess of E100 led to the formation of hydrophobic aggregates that re-precipitate.

Several chemical agents have been used for the dissociation of casein micelles, which include acidification, calcium chelating agents such as EDTA or citrate, anionic detergents such as SDS, or chaotropic agents such as urea or guanidine hydrochloride (*20*−*23*). However, a biphasic effect of precipitation and solubilization−dissociation of milk casein micelles or calcium-induced micelles, like that observed for E100, was only demonstrated by treatments with hot ethanol or 2,2,2-trifluoroethanol (TFE) (*24*−*27*). It is well known that ethanol is able to reduce the colloidal stability of casein micelles and coagulate milk. However, when a mixture (1/1, v/v) of milk and 65% ethanol is heated at 65 °C, a reversible dissociation of casein micelles takes place. This heat-induced dissociation of casein micelles by heating in the presence of alcohols was suggested to be due to an increase in the repulsive forces between caseins, probably as a consequence of a decrease in the dielectric constant of the milk and a reduction of phosphoseryl cross-linking that increase the protein hydrophilicity (*25*, *26*). On the other side, TFE produces a precipitation and solubilization of milk casein at low temperature in a concentration-dependent manner that resembles the effects observed with E100. Thus, the addition of low levels of TFE on milk produce a casein precipitate, but at higher concentrations, casein precipitates are solubilized. This occurs with dissociation of micelles, probably due to the reduction in the dielectric constant of the aqueous system by the presence of TFE. Our results of E100 activity on casein micelles support the idea that micelle dissociation is due to an amphipathic behaviour of E100 polymer. The hypothesis of a cationic detergent behavior of E100 was further confirmed by its ability to solubilize casein aggregated by other treatments such as rennet or chitosan.

The fact that E100 precipitation and dissociation−solubilization of casein micelles occurs with the same concentration dependence in a broad range of temperature, between 4 and 75 °C, suggests that the standard enthalpy change of the overall interactions involved in both processes would be close to zero. This reflects rather weak interactions among E100 and caseins and is probably the reason for which it is relatively easy to remove the excess of E100.

Remarkably, chitosan, a cationic polymer with primary and secondary amine groups that coagulates casein micelles through electrostatic and hydrophobic interactions, was unable to induce solubilization or dissociation of casein aggregates, no matter how much excess of it was added to the mixture (*5*). These

results emphasize the importance of the functional groups distribution within the cationic polymer for its final properties upon interaction with anionic colloidal protein micelles.

Altogether, the results suggest that at low E100 concentrations the polymer would interact with the negative surface charges of casein micelles by electrostatic forces. As E100 concentration is increased, this would lead to the neutralization of the charges in a sort of titration process, turning the casein-E100 complex hydrophobic and precipitating. The absence of charges in the surface of the aggregates would allow the excess of E100, due to its amphipathic character, to access the inside of the structure, where its positive charges could interact electrostatically with inner negative charges and its hydrophobic groups would disrupt the internal hydrophobic interactions. The presence of an excess positive charge would thus lead to a dissociation of the micelle structure, with the formation of positively charged casein-E100 complexes. Further experiments are in progress to gain a deeper insight into the molecular details of these events.

Finally, the finding that E100 is able to interact in a broad range of temperatures, with milk proteins with a special selectively for caseins, and in a biphasic process that involves protein aggregation-coagulation and a further dissociation−resolubilization and in a technically simple process opens the possibility to further explore these findings for the development of new industrial applications of this acrylate polymer.

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