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Reversible Precipitation of Casein Micelles with a Cationic Hydroxyethylcellulose

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The cationic hydroxyethylcellulose Polyquaternium 10 (PQ10) was found to produce a dose-dependent destabilization of casein micelles from whole or skim milk without affecting the stability of most of the whey proteins. The anionic phosphate residues on caseins were not determinant in the observed interaction since the destabilization was also observed with dephosphorylated caseins to the same extent. However, the precipitation process was completely inhibited by rising NaCl concentration, indicating an important role of electrostatic interactions. Furthermore, the addition of 150 mM NaCl solubilized preformed PQ10−casein complexes, rendering a stable casein suspension without a disruption of the internal micellar structure as determined by dynamic light scattering. This casein preparation was found to contain most of the Ca^{2+} and only 10% of the lactose originally present in milk and remained as a stable suspension for at least 4 months at 4 °C. The final concentration of PQ10 determined both the size of the casein−polymer aggregates and the amount of milkfat that coprecipitates. The presence of PQ10 in the aggregates did not inhibit the activity of rennet or gastrointestinal proteases and lipases, nor did it affect the growth of several fermentative bacteria. The cationic cellulose PQ10 may cause a reversible electrostatic precipitation of casein micelles without disrupting their internal structure. The reversibility of the interaction described opens the possibility of using this cationic polysaccharide to concentrate and resuspend casein micelles from whole or skim milk in the production of new fiber-enriched lactose-reduced calcium−caseinate dairy products.

KEYWORDS: Cationic cellulose; casein micelles; biopolymer interaction; protein precipitation

INTRODUCTION

Among other things, milk can be considered as a stable aqueous suspension of proteins, of which the major components are supramolecular complexes of proteins (casein micelles) that are held together by hydrophobic and electrostatic interactions. Several reports have described the processes that take place in the stabilization and destabilization of these colloidal particles of caseins (*1*−*3*). In this connection, their interaction with polysaccharides has been characterized, mainly to modify the structure, texture, and stability of foods, especially in dairy products (*4*−*9*). More recently, other applications have been developed in areas outside of food processing, such as cosmetics, pharmaceuticals, and biomedicine (*10*−*13*). In this context, different physical and chemical properties of milk proteins have been used to develop procedures for their isolation and recovery (*14*). However, there are only a few studies concerning the applications of polysaccharides in the fractionation of milk constituents, most of which have been done with anionic or neutral sugars (*4*, *5*, *8*, *9*). From these studies, it is known that the addition of polymers to produce a high ratio of polymer/casein micelles sometimes results in phase separation (*15*−*17*). In the past few years, we have conducted a series of studies aimed at characterizing the interactions between cationic polymers and casein micelles (*6*, *7*, *18*−*20*). We started studying the effect of cationic polymers containing primary and secondary amine groups on casein stability (*6*). Thus, we demonstrated that the addition of chitosan to milk produces fast aggregation and coagulation of casein micelles through an associative mechanism that involves electrostatic and hydrophobic interactions (*6*). The casein−chitosan complex thus formed was shown to be well digested by gastrointestinal proteases and allowed the growth of different microorganisms used routinely in the

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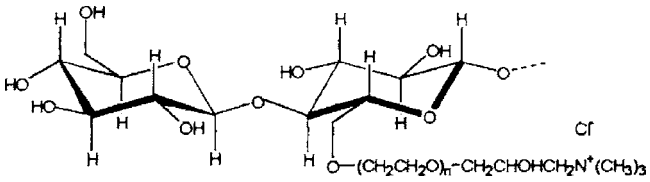

Figure 1. Molecular structure of Polyquaternium 10 (PQ10).

manufacture of fermentative dairy products (*18*, *19*). On the other hand, the studies on the interactions of caseins with a synthetic polymer containing a tertiary amine group showed biphasic behavior, with a precipitation phase at low polymer concentration and a solubilization phase of the aggregates at higher polymer/casein ratios due to a detergentlike activity of the polymer (*7*). As a logical follow-up to these studies, the aim of this work was to characterize the interactions of a cationic cellulose derivative containing a quaternary amine group, Polyquaternium-10 (PQ10), with whole milk or purified caseins. We demonstrate herein that PQ10 interacts electrostatically with casein micelles, producing their destabilization and precipitation in a process that renders aggregates that can be easily resuspended in a highly stabilized form at low ionic strength.

MATERIALS AND METHODS

Materials. Medium-viscosity cationic hydroxyethylcellulose, Polyquaternium-10 (PQ10), also known as Celquat SC-240C, was kindly provided by The National Starch and Chemical Co. (Bridgewater, NJ) (**Figure 1**). Stock solutions of PQ10 were prepared in 10 mM phosphate buffer at pH 6.8.

Pasteurized whole or skim bovine milk samples were from local commercial sources. Rennet from *Mucor miehei* and different phosphorylated and dephosphorylated caseins were obtained from Sigma Chemical Co. (St. Louis, MO).

Trypsin from porcine pancreas (EC 3.4.21.4, activity 15 000 units/mg of protein), pepsin from porcine stomach (EC 3.4.23.1, activity 210 units/mg of protein), and lipase from porcine pancreas (EC 3.1.1.3, activity 41 600 units/mg of protein) were supplied by Sigma Chemical Co. (St. Louis, MO).

Lyophilized commercial cultures used were a gift from Lacteos Manfrey (Freyre, Cordoba, Argentina). For bacterial growth test, lyophilized cultures (CC7030) containing *Streptococcus thermophilus* (70%) and *Lactobacillus delbrueckii* ssp. *bulgaricus* (30%) and pure lyophilized cultures of *Propionibacterium freudenreichii* were used. All other reagents used were of analytical grade.

Interaction between Cationic Cellulose and Milk or Caseins. To study the interactions between PQ10 and milk constituents, samples of 1 mL of whole or skim milk (30 mg/mL protein concentration) were incubated with 0.1 mL of solutions containing 0−10 mg/mL PQ10. The mixtures were vigorously shaken for 30 s to reach a complete interaction. Then, the samples were allowed to stand for 10 min at 10, 25, and 70 °C and finally centrifuged at room temperature at 5000*g* for 10 min to separate pellet from supernatant.

Phosphorylated and dephosphorylated α_s-, β-, and κ-caseins (5 mg/mL) were solubilized in 20 mM phosphate buffer at pH 6.8. Samples (1 mL) of caseins were incubated at room temperature with 0.1 mL of solutions containing 0−10 mg/mL of PQ10 and then processed as described above.

To study the effect of NaCl, defatted milk was mixed with PQ10 (0.5 mg/mL final concentration), or 10 mM phosphate buffer at pH 6.8 as a control, all containing the appropriate amount of NaCl to give the final concentrations reported in **Figure 5**. The mixtures were then processed as described above to collect supernatants and pellets.

The effect of casein electric charge in the interaction with PQ10, was analyzed as follows: sodium caseinate (10 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 PQ10 was studied as described above.

Rennet and Acid Coagulation. For rennet coagulation, milk samples were incubated during 15 min at 37 °C with 0.01 unit/mL 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. Finally, for all coagulation methods, samples were centrifuged at 5000*g* for 10 min to separate the precipitated caseins from whey.

Effect of PQ10 on Rennet Activity. The effect of PQ10 on rennet activity was determined by the time required to clot reconstituted PQ10−casein complexes in 150 mM NaCl after addition of rennet. An amount of rennet (0.01 unit/mL, 15 μL) was added to test tubes containing 2 mL of reconstituted complexes or whole milk as a control preincubated at 35 °C for about 10 min. The end point was decided as being the time required for the first appearance of a grainy texture, and the results were expressed as clotting time in seconds (*21*).

Determination of Protein, Triglyceride, Calcium, and Lactose Concentration in Supernatants. Protein concentration was determined by Biuret or by direct absorbance at 280 nm as described by Bingham (*22*).

The concentration of triglyceride present in whole milk, supernatants, or reconstituted pellets was determined by a colorimetric enzymatic assay from Wiener Lab (Rosario, Argentina).

Ca^{2+} concentration present in whole milk or in the supernatants obtained after precipitation with PQ10, as described above, was determined by flame atomic absorption spectrometry (FAAS) in a Shimadzu AAS 6501S. 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 self-reversal method.

Lactose concentration was determined by a colorimetric enzymatic method as described by Alvarez et al. (*23*).

Polyacrylamide Gel Electrophoresis. Polyacrylamide gel electrophoresis containing sodium dodecyl sulfate (SDS−PAGE), of 12.5% (w/v) separation gel and 4.5% (w/v) stacking gel, was performed according to Laemmli in a vertical slab PAGE cell (Miniprotean II, Bio-Rad) (*24*). Samples of milk were treated with various concentrations of PQ10 to give 0−1.8 mg/mL. Supernatants in universal sample buffer containing 2% (v/v) 2-mercaptoethanol were heated at 90 °C for 3 min. Samples containing 70−150 μg of proteins were loaded in each well. The electrophoresis was conducted for 90 min at 140 V. The proteins were stained with Coomassie Brillant Blue R-250.

Microbial Cultures and Antibacterial Test. Microbial cultures and antibacterial tests were performed essentially as previously described (*19*). Briefly, samples of UHT milk were inoculated with a suspension containing about 2×10^4 of the microorganisms to be tested. Then, a volume of either PQ10 solution or 10 mM phosphate buffer, pH 6.8, as a control was added, and the mixtures were incubated for 30 min at 37 °C to allow for the interaction between PQ10 and bacteria. After this incubation, samples of decimal dilutions were plated in solid media for colony counting. To test the possible antibacterial effect of PQ10, solutions of the polymer were incubated with suspensions of the microorganisms in culture broth and incubated as described above.

Stability of Resuspended Casein Micelles. Milk samples (10 mL) were precipitated in graduated tubes by adding 0.5 mg/mL PQ10, and pellets were collected as described above. Then, pellets were reconstituted to the starting volume with deionized water or increasing concentrations of NaCl. To prevent microbial contamination, sodium azide was added at 0.02% (w/v). Finally, the samples were hermetically sealed and stored in a cold room (4 ± 2 °C) for at least 4 months. All samples were inspected daily for visual precipitation. In addition, aliquots of 50 μL from the upper portion of samples were sampled weekly for protein concentration determination.

Hydrolysis of Caseins and Lipids in the PQ10−Milk Aggregates by Pepsin, Trypsin, and Pancreatic Lipase. The proteolytic activity of pepsin and trypsin and the lipolytic activity of pancreatic lipase were determined as described previously (*18*). Briefly, milk aggregates were resuspended in 50 mM HCl−KCl, pH 2.0, for pepsin assay and in 50 mM phosphate, pH 8.0, for trypsin assay. At different times of incubation at 25 °C, the hydrolysis reactions were stopped by the addition of one volume of trichloroacetic acid (10% w/v) and UV absorption was measured in supernatants obtained after centrifugation at 10000*g* for 10 min. Lipase activity was measured by the titration of free fatty acids (FFA) released from the aggregates as described (*18*).

Particle Size Determination by Dynamic Light Scattering. Colloid particle sizing was performed by dynamic light scattering with a PSS-Nicomp 370 submicrometer particle sizer from Particle Sizing Systems (Santa Barbara, CA) with a 632.8 nm HeNe laser. A Dow polystyrene latex standard with a mean volume-averaged diameter of 91.4 nm was used to calibrate the instrument. Samples (0.5 mL) of defatted milk were incubated with PQ10 at final concentrations of 0.1, 0.25, 0.5, and 1.0 mg/mL and thermostated at 25 °C for 10 min. Then, samples were diluted $^1/_{100}$ with water and measured, recording the intensity of the light scattered at 90° for at least 10 min, by use of a Gaussian size distribution analysis. The mean volume-averaged diameter and standard deviation of the determinations performed are reported.

Microscopic Analysis. Samples of whole milk and PQ10−casein complexes obtained with increasing PQ10 concentrations were analyzed and photographed without any staining in an Axiovert 135 M Karl-Zeiss microscope at 50×..

RESULTS

Effect of PQ10 on Whole Milk Stability. In previous reports, we described that polymers containing primary, secondary, and tertiary amine groups such as chitosan and Eudragit E100 (E100) produce a drastic destabilization and coagulation of casein micelles that occurs almost immediately after the polymers are mixed with milk (*6*, *7*). On the contrary, the addition of increasing concentrations of PQ10, a cationic hydroxyethylcellulose containing quaternary amine groups (**Figure 1**), did not produce an immediate visible change in the appearance of milk. However, when the mixtures of milk with PQ10 were centrifuged at 3000*g* for 5 min, a white pellet was observed. As PQ10 concentrations were increased up to 0.5 mg/mL, the volume of precipitated material also increased, reaching approximately 20% of the initial milk volume. The quantitative analysis of proteins in the supernatants showed that, at concentrations of PQ10 above 0.5 mg/mL, approximately 15% of total proteins remained soluble (**Figure 2A**). The amount of PQ10 needed to obtain the maximal precipitation of proteins was found to be substantially lower (on a weight percent basis) than that required when other cationic polymers such as chitosan and Eudragit E100 were used (*6*, *7*). As observed with the other cationic polymers, the precipitation process took place without changes in milk pH.

PQ10 Precipitates Casein Micelles. The high amount of protein collected by centrifugation suggested that, as with chitosan and E100, caseins would be the main components involved in the interaction with PQ10. An analysis by SDS−PAGE of the proteins that remained soluble showed that as the polymer concentration was increased, the casein fraction gradually disappeared from the supernatants obtained after centrifugation of the samples at 3000*g* for 10 min (**Figure 2B**). These results indicate that caseins are the main components that precipitate upon PQ10 addition to milk, while nearly all of the whey proteins remain soluble. However, a very low molecular mass protein (<14 kDa), probably a protease peptone fraction, was also precipitated by PQ10 (see lane 5 in **Figure 2B**). The precipitation profile was very similar over a wide range of temperatures, from 25 to 75 °C, suggesting that despite the formation ot complexes between caseins and PQ10, the overall process does not involve a high enthalpy change (data not shown).

Size Determination of Casein Aggregates. A comparative analysis by optical microscopy of different aggregation states of milk after addition of increasing concentrations of PQ10 is illustrated in **Figure 3**. The analysis of PQ10−casein complexes showed that the size of the aggregates increases with an increment in polymer concentration from 0.5 to 2 mg/mL. PQ10

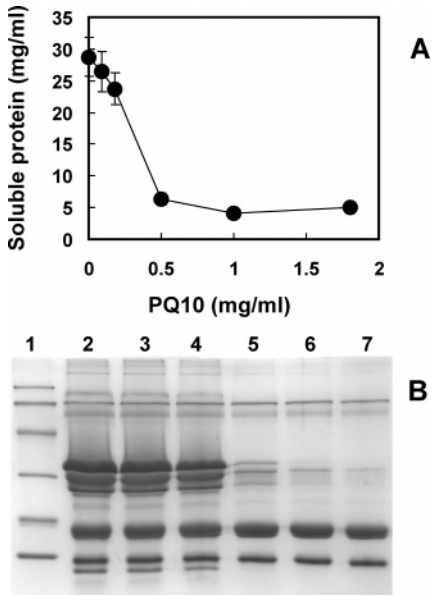

Figure 2. Effect of PQ10 on the stability of milk proteins. Whole milk was incubated with increasing concentrations of PQ10 and after centrifugation, the soluble protein was determined (**A**) or analyzed by SDS–PAGE (**B**). (Lane 1) Molecular mass standards (from the top, molecular masses are 97, 66, 45, 31, 21, and 14 kDa). (Lanes 2–7) Supernatants of milk samples incubated with the following final concentrations of PQ10: lane 2, 0 mg/mL (control phosphate buffer); lane 3, 0.1 mg/mL; lane 4, 0.2 mg/mL; lane 5, 0.5 mg/mL; lane 6, 1.0 mg/mL; lane 7,1.8 mg/mL. All results are expressed as mean ± standard deviation ($n = 4$).

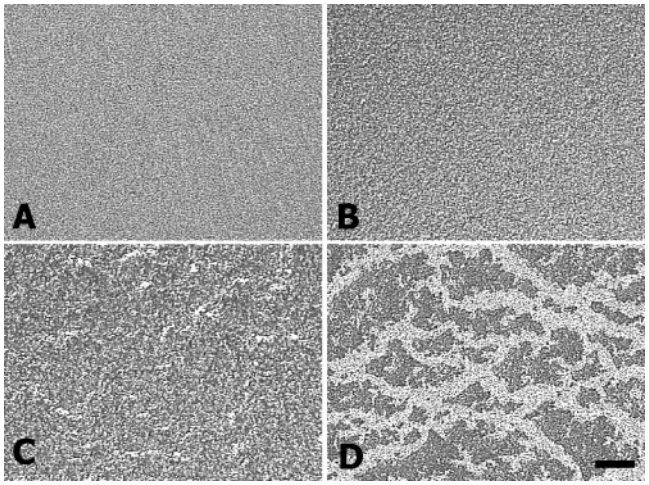

Figure 3. Microscopic analysis of PQ10–milk complexes obtained at different PQ10 concentrations. (**A**) Control phosphate buffer, pH 6.8; (**B**) PQ10, 0.5 mg/mL final concentration; (**C**) PQ10, 1.0 mg/mL final concentration; (**D**) PQ10, 2.0 mg/mL final concentration. Bar = 200 μm.

at 0.5 mg/mL was the minimum concentration needed to produce the complete precipitation of casein micelles (see **Figure 2**). However, an increment in PQ10 concentration, a condition that did not increase the amount of precipitated caseins, produced an increment in the size of casein−PQ10 aggregates, suggesting that some cross-bridging could be occurring. To obtain more information about the interaction of PQ10 with casein, we analyzed the changes in particle size distribution of PQ10−casein micelles complexes as a function of PQ10 concentration by dynamic light scattering (**Table 1**). The addition to milk of 0.1 and 0.25 mg/mL PQ10, concentrations that produce 5% and 10% casein precipitation, induced minor changes in the size of

Table 1. Effect of PQ10 on Casein Micelles Size[a]

PQ10 (mg/mL)	particle diameter[b] (nm)	volume[c] (μm^3)
0	280 ± 140	0.011
0.10	310 ± 174	0.015
0.25	328 ± 176	0.018
0.50	1050 ± 175	0.52
1.00	2100 ± 1800	5.56

[a] Data are reported as mean ± standard deviation (SD) of two experiments performed in triplicate ($n = 6$). [b] As determined by DLS; given as mean ± SD. [c] Estimated from Vrij's model.

Table 2. Comparative Triglygeride, Ca^{2+}, and Protein Contents in Milk Supernatants Obtained after PQ10, Acid, and Rennet Precipitating Procedures

coagulating agent	triglyceride (mg %)	Ca^{2+} (mg/L)	protein (mg/mL)
rennet	34 ± 4 (1.7)[a]	460 ± 20 (41.5)	4.2 ± 0.6 (15)
acid (1 M HCl)	47 ± 9 (2.3)	810 ± 20 (73)	4.3 ± 0.4 (16)
PQ10 (0.5 mg/mL)	2050 ± 100 (68)	327 ± 18 (30)	4.8 ± 0.5 (18)
PQ10 (1 mg/mL)	380 ± 20 (13)	336 ± 22 (32)	4.6 ± 0.4 (17)

[a] Values in parentheses indicate the percentage of milk content that remains soluble in whey. All results are expressed as mean ± standard deviation ($n = 3$).

the aggregates (**Table 1**). The addition of 0.5 mg/mL PQ10, the lowest concentration needed to precipitate most of the casein micelles, produced a noticeable increase in the size of aggregates (**Table 1**). Furthermore, the addition of an excess of PQ10 (1 mg/ mL) produced aggregates with even higher sizes (**Table 1**). By use of Vrij's model for the analysis of the interactions of colloid−polymer mixtures, where the colloids are modeled as hard spheres, the volume of casein micelles and each of the PQ10−casein aggregates was estimated (*25*). The addition of 0.1 and 0.25 mg/mL PQ10 produced only a slight increase in the volumes, while PQ10 at 0.5 and 1 mg/ mL produced aggregates with volumes 47 and 500 times higher than control casein micelles (**Table 1**). These results indicate that PQ10 not only can bind onto individual casein micelles but also produces cross-bridging, forming aggregates that include other components of milk like fat globules.

Effects of PQ10 on Milk Lipids and Calcium. It is well-known that most of the casein micelles and fat globules are interacting in tight complexes in milk. Our previous results have shown that most of the triglycerides present in whole milk are precipitated by the catoinic polymers chitosan and E100 (*6*, *7*). Analyzing the amount of tryglyceride that remains in the supernatant after casein precipitation by the addition of PQ10, we found that at 0.5 mg/mL (the lowest concentration needed to obtain an almost complete precipitation of casein micelles from milk) approximately 65−70% of total triglycerides remained in the supernatant fraction (**Table 2**). However, when the concentration of PQ10 was increased above 1 mg/mL, most of the lipids coprecipitated with caseins in a way similar to that observed for rennet- or acid-induced casein precipitation (**Table 2**). On the other hand, after casein precipitation with 0.5 mg/mL PQ10, most of the Ca^{2+} remained associated with precipitated casein micelles (**Table 2**). These results are in agreement with those previously observed for other cationic polymers studied, chitosan and Eudragit E100 (*6*, *7*).

Interaction of PQ10 with Purified Caseins. It is well-known that the anionic superficial glycomacropeptide portion of κ-casein is one of the most important components that contributes to the stability of casein micelles. To know whether PQ10 interacts selectively with κ-casein or also with other casein

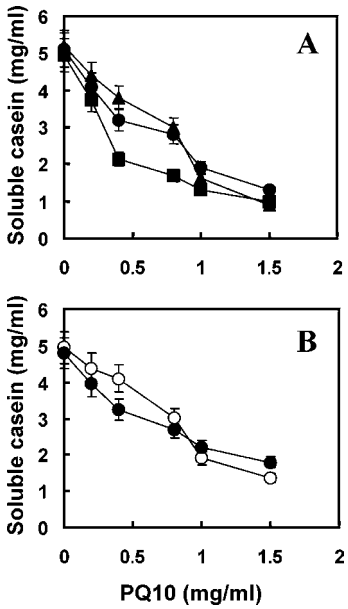

Figure 4. Interaction of PQ10 with caseins. (**A**) Interaction of PQ10 with purified α_s- (●), β- (■), and κ- (▲) caseins. (**B**) Interaction of PQ10 with sodium caseinate (phosphorylated casein) (●) or total dephosphorylated casein (○). All results are expressed as mean ± standard deviation ($n = 4$).

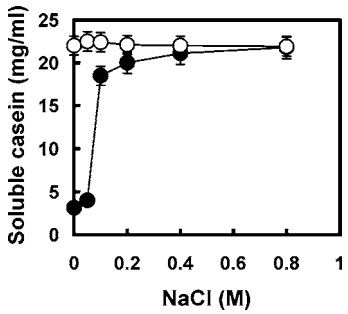

Figure 5. Effect of ionic strength on PQ10–casein interaction. Skim milk in the presence of increasing concentrations of NaCl was incubated with phosphate buffer, pH 6.8, as a control (○), or with PQ10 (0.5 mg/mL final concentration) (●) and then centrifuged to precipitate the PQ10–casein complex. All results are expressed as mean ± standard deviation ($n = 4$).

molecules, we studied its interactions with purified caseins. **Figure 4A** shows that PQ10 interacted and precipitated individual α_s-, β-, and κ-caseins approximately to a similar extent.

The phosphate groups are the strongest acidic components of casein micelles. Therefore, we studied how the presence of these residues affected the interaction with PQ10. As shown in **Figure 4B**, both phosphorylated and dephosphorylated caseins were similarly precipitated by PQ10, suggesting that phosphate groups are not playing an essential role in the interaction and destabilization of casein micelles by the polymer.

Characterization of PQ10−Casein Interaction. To gain insight into the nature of this interaction and considering the opposite net electric charges of these macromolecules, we studied the role of electrostatic interactions by two different approaches: (1) evaluation of the effect of increasing ionic strength and (2) modification of the net charge of caseins.

Increasing the ionic strength in milk by raising the concentration of NaCl before the addition of PQ10 led to a progressive inhibition of PQ10−casein precipitation (**Figure 5**). As shown in **Figure 5**, at concentrations of NaCl above 150 mM, most of

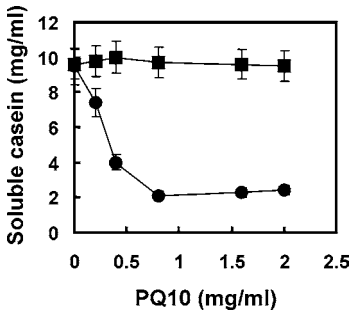

Figure 6. Effect of net electric charge of casein in the interaction with PQ10. Sodium caseinate below their isoelectric point at pH 2.3 (■) or above their isoelectric point at pH 6.8 (●) were incubated with increasing concentrations of PQ10 and centrifuged to collect the PQ10–casein complex. All results are expressed as mean ± standard deviation (n = 4).

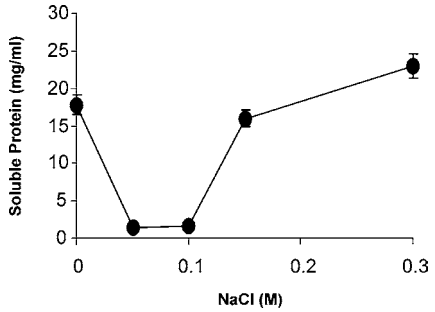

Figure 7. Effect of ionic strength on the stability of resuspended PQ10–casein complex. PQ10–casein complexes were obtained as described under Materials and Methods and then resuspended to the initial volume with water or increasing concentrations of NaCl. Then, the samples were centrifuged at 5000*g* for 5 min to remove the insoluble complex, and the protein content was determined in the supernatant. All results are expressed as mean ± standard deviation (n = 4).

the casein micelles remained soluble, suggesting that their interaction with PQ10 was inhibited. As with the other positively charged polymer studied, when casein was incubated with PQ10 at pH 2.3, no precipitates could be detected (**Figure 6**).Taken together, these results indicate that the association between PQ10 and caseins is mainly determined by electrostatic interactions.

Analysis of Solubilized Casein−PQ10 Complex. The inhibition of the precipitation of caseins with PQ10 by a rise in NaCl prompted us to study whether the concentrated PQ10−casein complexes obtained by centrifugation could be resuspended in a stabilized condition similar to that observed for caseins in untreated milk. Thus, concentrated PQ10−casein complexes were resuspended to the initial volume with water or NaCl concentrations up to 200 mM and then centrifuged for 5 min at 5000*g* to evaluate the resultant stability of the suspensions formed (**Figure 7**). In the presence of water, PQ10−casein complexes showed an unexpected stability in solution remaining as a homogeneous suspension even after centrifugation. However, this suspension of PQ10−casein complexes remained stable in water only for 2 days at 4 °C. On the contrary, when complexes were resuspended with 50−100 mM NaCl, no stable suspensions were observed, with most of the caseins precipitating after the centrifugation step. However, with 150 mM NaCl, most of the caseins remained soluble for at least 4 months at 4 °C.

The fact that untreated and reconstituted casein micelles after PQ10 treatment show similar sizes of approximately 250−300 nm (data not shown) strongly suggests that caseins resuspended from PQ10−casein complexes recover structures similar to those present in untreated milk.

Table 3. Comparative Triglygeride, Protein, and Lactose Contents in Supernatants Obtained after Resuspension of PQ10–Casein Precipitates in 150 mM NaCl

PQ10 used to precipitate caseins (mg/mL)	triglyceride (mg %)	protein (mg/mL)	lactose (g %)
0.5	980 ± 70 (33)[a]	23 ± 2 (85)	0.41 ± 0.02 (10)
1.0	2600 ± 90 (87)	23 ± 2 (85)	0.40 ± 0.03 (10)
1.5	2800 ± 200 (94)	23 ± 2 (85)	0.41 ± 0.03 (10)

[a] Values in parentheses indicate the percentage of milk content that is resolubilized with one volume of 150 mM NaCl. All results are expressed as mean ± standard deviation (n = 4).

A comparative analysis of protein, lipids, and lactose present in milk and resolubilized PQ10−casein complexes is shown in **Table 3**. While the amount of protein recovered was approximately 15% lower than that of untreated milk, due to the lack of the fraction of whey proteins, the amounts of triglycerides and lactose that remained associated with PQ10-treated casein micelles were 70% and 90% lower than those of untreated milk when 0.5 mg/mL PQ10 was used for the precipitation of caseins. In agreement with what was observed for the precipitation, the amount of milkfat depended on the concentration of PQ10 used.

Effect of PQ10 on the Activity of Proteases and Lipase. With the possibility that PQ10 could be used to develop new casein-enriched dairy products, we studied the effect of PQ10 on the activity of rennet, a well-known protease used in cheesemaking. On the other hand, to asses the nutritional profile of PQ10-induced casein aggregates, we evaluated how its presence could affect the hydrolytic activity of different gastrointestinal proteases (pepsin and trypsin) and pancreatic lipase.

When PQ10-treated milk was incubated with rennet, the time to induce milk coagulation was reduced, either with or without 10 mM Ca^{2+} (**Table 4**). These results demonstrate that not only does PQ10 not inhibit the proteolytic activity of rennet, but it could even activate the enzyme. The studies about the effect of PQ10 on the activity of gastrointestinal proteases, showed that casein alone as well as PQ10−casein aggregates were similarly hydrolyzed by pepsin and trypsin as evaluated by UV absorbance of trichloroacetic acid- (TCA-) soluble peptides (**Table 4**). These results show not only that the caseins that form part of the complexes with PQ10 are accessible to the enzyme but also that, under our assay conditions, their activity was relatively independent of the physical state of the caseins (soluble in milk or aggregated with PQ10).

It was also observed that the lipolytic activity of pancreatic lipase for triglycerides associated with the PQ10−casein complex was similar to that observed for the hydrolysis of the lipids present in milk alone (**Table 4**). These results suggest that PQ10 did not affect substantially either the substrate or the enzyme activity.

Effect of PQ10 on Growth of Milk Fermentative Bacteria. Previous reports have indicated that cationic polymers exhibit antimicrobial activity (*19*). The potential for using PQ10 as a natural fiber for the development of new fermentative dairy products requires that bacteria could grow in its presence. Therefore, we studied the effect of PQ10 on the viability of different bacterial strains used in the production of fermentative dairy products. We analyzed the effect of different concentrations of PQ10 on (a) *Lactobacillus delbrueckii* ssp. *bulgaricus*,

Table 4. Effect of PQ10 on the Activity of Proteases and Lipase[a]

substrate	rennet(s)	pepsin (ΔA_{280nm}/min)	trypsin (ΔA_{280nm}/min)	pancreatic lipase (mequiv L^{-1+} min^{-1}
milk (control)	4176 ± 162	0.021 ± 0.005	0.033 ± 0.004	0.40 ± 0.06
milk + 10 mM Ca^{2+}	701 ± 69	nd	nd	nd
PQ10 (0.5 mg/mL)–milk	276 ± 47	0.019 ± 0.002	0.031 ± 0.005	0.38 ± 0.05
PQ10 (0.5 mg/mL)–milk + 10 mM Ca^{2+}	76 ± 4	nd	nd	nd

[a] All results are expressed as mean ± standard deviation ($n = 4$). nd, not determined.

Table 5. Effect of PQ10 on the Growth of Milk Fermentative Bacteria

	L. delbrueckii ssp. *bulgaricus* (CFU/mL)	*S. thermophilus* (CFU/mL)	*P. freudenreichii* (CFU/mL)
control (10 mM phosphate, pH 6.8)	1.5×10^4	2.5×10^4	3.5×10^4
PQ10 (5 mg/mL)[a] + culture medium	1.0×10^4	2.0×10^4	2.0×10^4
PQ10 (5 mg/mL)[a] + milk	1.5×10^4	1.5×10^4	4.0×10^4

[a] Final concentration of PQ10.

(b) *Streptococcus thermophilus*, and (c) *Propionibacterium freudenreichii*. The addition of PQ10 up to concentrations of 10 mg/mL (10 times higher than that needed to precipitate caseins) into culture medium or whole milk loaded with approximately 10^4 colony-forming units (CFU)/mL of each strain, did not show any inhibitory effects on the bacterial growth (**Table 5**). Similar results were observed when the initial bacterial load was around 10^9 CFU/mL (data not shown). These results indicate that PQ10, either free or complexed to caseins, does not affect the viability and growth potential of milk fermentative bacteria.

DISCUSSION

In previous reports we described that cationic polymers containing primary amine groups (chitosan) and tertiary amine groups (Eudragit E100) can be used to isolate casein micelles from whey proteins by a fast aggregation and coagulation process (*6*, *7*). Electrostatic and hydrophobic forces are involved in the interactions between casein and both polymers (*6*, *7*). In this paper, we demonstrate that PQ10, a positively charged polysaccharide containing quaternary ammonium groups, interacts in a selective way with casein micelles. However, contrary to the fast coagulating effect on milk previously observed with chitosan and Eudragit E100, the addition of PQ10 produced a slow aggregation of casein micelles. As expected, the electrostatic interaction is the major driving force in the association between PQ10 and caseins, as shown by the impairment of complex formation by screening of the electric charges by NaCl or a change in pH to 2.3. In agreement with these results, a key role of electrostatic interaction of cationic cellulose hydrogels containing quaternary amine groups with anti-inflammatory drugs has been recently reported (*26*).

The interaction of PQ10 with purified individual caseins showed the same profile as that found for chitosan and Eudragit E100, showing no selectivity for purified α_s-, β-, or κ-caseins. As with the other cationic polymers, the carboxylates of caseins give them enough negative charge to attain a full interaction since PQ10 was able to precipitate phosphorylated and dephosphorylated caseins to a similar extent.

The addition of low concentrations of NaCl (150 mM) not only prevented the interactions, as mentioned above, but also was able to dissociate the PQ10−casein aggregates, restoring a protein suspension with a size similar to that of untreated micelles. These results are clearly different than those observed in previous studies with chitosan and Eudragit E100 (*6*, *7*). The cationic aminoethyl acrylate E100 also formed aggregates with caseins that involve electrostatic interactions. However, 1 M NaCl was necessary to dissociate them (*7*). On the other side, the primary amine-containing polysaccharide chitosan formed aggregates with caseins through electrostatic and hydrophobic interactions. Therefore, to dissociate these aggregates, the simultaneous combination of two drastic conditions was necessary, such as high ionic strength (1 M NaCl) with 2% nonionic detergent (*6*).

Our results also showed that the interaction of PQ10 with caseins could affect their interaction with milkfats. It was found that the addition of 0.5 mg/mL PQ10 produced a precipitation of most of the caseins and around 35−40% of the lipids. This effect is clearly different than that observed for the coagulation of caseins with rennet, acid, or the cationic polymers chitosan and E100 (*1*, *6*, *7*). These methods usually produce bigger casein aggregates than PQ10, including the fat globules that coprecipitate with caseins (*6*, *7*).

The treatment with rennet, which removes the hydrophilic glycomacropeptide of κ-casein, and the acid pH both induce the expression of a new hydrophobic entity at its overall isoelectric point, with a high tendency to aggregate that also tends to remain associated with the fat globules. On the other side, the addition of high molecular weight cationic polymers has been found to induce the cross-bridging of casein micelles that are partially bound with fat globules. Therefore, we speculate that the coprecipitation of milkfat with caseins is in this case due to the precipitation of this tertiary complex as a supramolecular aggregate.

In the case of PQ10, around 0.5 mg/mL represents the lowest amount of polymer that is required to precipitate most of the caseins. These aggregates have a diameter of approximately 1 μm with a volume of around 0.5 μm^3 (**Table 1**). According to Vrij's model, these aggregates could be formed by around 50 casein micelles of 0.001 μm^3 each. With these results in mind, it is tempting to speculate that the aggregate of 50 casein micelles could represent the minimum aggregation number for their precipitation. Thus formed, and being the smallest aggregates that precipitate, these exclude an important proportion of the fat globules that remain in the supernatant. However, at higher concentration, PQ10 induces a cross-bridging of casein micelles, thus forming bigger aggregates that entrap lipids that co-precipitate. The main difference from the coprecipitation

observed with the cationic polymers chitosan and E100 is that in this case the interaction is mainly electrostatic, but with chitosan and E100 there is a partial hydrophobic association between the polymers and the casein micelles, and a direct interaction with fat globules has been also observed (*6*, *7*).

It was also found that, after centrifugation of PQ10-treated milk to remove the supernatant containing whey proteins, the pellet of PQ10−casein complex may be resuspended at the initial volume by the addition of distilled water. In this condition a suspension optically similar to untreated milk was obtained. However, the suspension thus formed is unstable and a proteinaceous sediment was observed when it was allowed to stand for 24−48 h. However, when the aggregates were resuspended with 150 mM NaCl, the caseins remained as an optically stable suspension for at least 4 months. The different behavior observed under the two conditions could be explained because, in the presence of water, the electrostatic association of PQ10 with caseins cannot be dissociated, and therefore an unstable suspension was observed. On the other side, with 150 mM NaCl the PQ10−casein complex is dissociated, rendering free casein micelles that can recover their physicochemical properties and remain stable in suspension.

Taken together, these results suggest that the electrostatic interactions are the major driving forces for the destabilization and precipitation of casein micelles by the cationic hydroxyethylcellulose PQ10. This behavior is clearly different than that found for the other cationic polymers previously studied. Although the chemical structure of the three cationic polymers studied so far is different, it is noticeable that a general trend is observed from interactions that are dependent on both electrostatic and hydrophobic interactions to almost completely electrostatic associations. Thus, it was found that both electrostatic and hydrophobic interactions contribute energetically to the association between casein micelles with the primary and secondary amine-containing polymer chitosan (*6*). On the other hand, the tertiary amine-containing cationic polymer E100 may act as a precipitant of casein micelles mainly by ionic interactions that could be dissociated by high ionic strength (i.e., 1 M NaCl) (*7*). However, its intrinsic amphipathicity leads to a disruption of their internal structure (*7*). In the case of the cationic cellulose derivative containing a quaternary amine group (PQ10), its association with casein micelles was found to be fully dependent on electrostatic interactions that could be dissociated by a relatively low ionic strength (i.e., 150 mM NaCl). It is also interesting to remark that the use of PQ10 for precipitation and resuspension allows one to obtain a casein suspension that retains most of the initial calcium content of milk. Altogether, these results suggest that the association and dissociation by PQ10 of casein micelles does not produce substantial changes in the internal structure of the micelles.

To explore further the possibility of using PQ10 to induce milk aggregates that could be used in the development of new dairy products, it was necessary to know if the aggregates of caseins with this polymer could be digested by proteolytic and lipolytic gastrointestinal enzymes. The results reported herein demonstrate that PQ10−casein complexes are hydrolyzed to a similar extent as casein alone by proteolytic enzymes such as pepsin and trypsin, suggesting that the caseins are not buried inside the polymer structure in an inaccessible condition. Moreover, in agreement with this hypothesis, our results also show that PQ10−casein complexes are proteolyticaly cleaved by rennet even better than casein alone (**Table 4**). On the other hand, similar results were observed for the hydrolytic activity of the lipids present in milk and in the PQ10−casein complexes by pancreatic lipase. As the assays employed to estimate proteases or lipase activity are based on the measurement of the release of soluble products, these results suggest that PQ10 would not significantly affect the bioavailability of proteins and lipids.

The use of a polymer as a natural fiber in the potential development of fermentative dairy products specifically requires that different strains of lactic bacteria could growth in its presence. In a previous report we described that the addition of chitosan to culture media containing different strains of lactic bacteria produced the inhibition of bacterial growth (*19*). However, when bacteria were previously added into milk, the addition of chitosan did not affect their growth (*19*). The stronger association of chitosan with caseins left no free chitosan to inhibit bacterial growth. Contrarily, the results reported herein indicate that concentrations of PQ10 10 times higher than those necessary to precipitate all the casein micelles did not affect the viability of different strains of bacteria.

In conclusion, we have described here a method to destabilize, fractionate, and partially purify milk proteins by the addition of a cationic cellulose derivative containing quaternary amine groups that shows the following characteristics: (1) PQ10 interacts selectively with casein micelles without affecting the solubility of most of the whey proteins and does not affect the pH of milk; (2) the PQ10−casein complexes can be concentrated up to 5 times by a simple low-speed centrifugation step; (3) the amount of lipids that remain associated with these aggregates can be regulated by varying the concentrations of PQ10 used in the precipitation of caseins; (4) the precipitated micelles can be resuspended by the addition of a low ionic strength solution (i.e., 150 mM NaCl) rendering a suspension optically stable for at least for 4 months; (5) the resuspended micelles retain most of the calcium present in untreated milk but only around 10% of the lactose; (6) the presence of this biopolymer does not affect the activity of gastrointestinal proteases and lipase; (7) PQ10 does not affect the growth of milk fermentative bacteria when present in milk; and (8) the cationic cellulose PQ10 can be considered relatively safe as its LD_{50} in rats is >5 g/kg (according to its safety data sheet as provided by The National Starch and Chemical Co.). With all these properties, it seems quite likely that PQ10 could be used soon in the manufacturing of new casein-enriched lactose-reduced dairy products.

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