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Casein precipitation equilibria in the presence of calcium ions and phosphates

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Abstract

Calcium ion induced aggregation and precipitation equilibria of β-casein were studied in the presence or absence of mono- and polyphosphates at pH 5.5 and 7.5. We analyze the data by assuming ion binding at a well-defined stoichiometric ratio, leading to protein charge neutralization and formation of slightly soluble complexes. The precipitation curves are in good agreement with the model, showing the expected larger induction region and steeper slope at the lower pH. The addition of phosphates leads to better precipitation of the protein, which can be explained by a mechanism involving the formation of calcium phosphate microcrystals. These crystals provide a substrate for protein adsorption, with subsequent cross-binding of the casein micelles and formation of sturdy aggregates of co-precipitated calcium phosphate and casein. The crystallite formation leads to effective separations in commercial caseinates, which would be impossible with Ca^{2+} alone.

Keywords: Casein; Precipitation equilibria; Calcium ions

1. Introduction

Processes for production of dairy products such as cheese, processed cheese, creams and spreads are of major industrial importance. Improvement and optimization of these processes and the design of new consumer products are of significant interest to the food industry. Although much research has been done in these areas, some of the fundamental interactions among the dairy proteins and the mechanisms of their separation are not understood on a quantitative level; hence optimization is carried out empirically rather than on the basis of theoretical models. In this paper, we investigate quantitatively the equilibria and the mechanisms of precipitation of pure β-casein and caseinate mixtures in the presence of Ca^{2+} ions and mono- and polyphosphates. These precipitation processes are commonly encountered in cheese production, where calcium salts are added

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as precipitants, and (poly)phosphates as 'emulsifiers' [1], in order to modify the properties of the product; however, their mechanism of action has not been clarified.

In milk, casein micelles exist as colloidal particles 100–300 nm in diameter. They are composed of 93.3% casein and 6.6% inorganic constituents. The casein constituents, α_{s1}-, α_{s2}-, β-, and κ-casein, exist in proportions of approximately 3:0.8:3:1 by weight. The non-protein component of the micelles, expressed as low molecular weight ions, include calcium (37.5%) and phosphates (50%), and smaller amounts of citrate (7.5%) and magnesium (2%) [2–6]. Thus the main inorganic constituent of casein micelles is 'micellar' or 'colloidal' calcium phosphate [3,7]. The presence of both calcium and phosphates in the natural micelles motivates interest in the way in which the interactions between these two species mediate the protein precipitation and separation patterns.

β-Casein was chosen as a model protein because it has specific affinity to Ca^{2+} and is one of the major dairy proteins. This protein is devoid of any cysteinyl residue and is essentially structureless. It has a molecular weight of 24 000 and contains 209 amino acid residues, with a total chain length of about 720 Å in extended conformation [8,9]. The first 50 of the 209 amino acids are hydrophilic, whereas the rest are mainly hydrophobic. Due to the large hydrophobic chain, β-casein forms micelles in solution [9,10].

Three different general models of the micelle structure have been proposed in the literature [7,11–14]. The models of the first group are called coat-core models, that is, there is a coat of some types of caseins surrounding a core of the other protein constituents. Some models place the α_s- and the β-casein on the inside and the κ-casein on the outside; other models assume that the β-casein is mostly in the center and the α_s- and κ-casein on the outside. The models of the second group are the internal structure models, in which the whole micelle is assumed to be a three-dimensional network of various casein subunits: there is no specific 'inside' or 'outside' protein. The models of the third group are the sub-micelle models, where the three casein subunits are postulated to come together to form an aggregate of geometrically identical sub-micellar particles, held together by calcium and phosphate bridges.

In the present paper, we report experiments aimed at resolving the effect of casein charge and ion binding on the precipitation equilibria. The experiments are carried out at two pH values above the isoelectric point: 5.5 and 7.5. Four different casein-Ca^{2+} precipitation patterns are compared, namely, in systems without phosphate, with monophosphate, with cyclic phosphate, and with linear phosphate. The role of calcium phosphate microcrystals in the systems with phosphates is evaluated. The results with pure β-casein are compared to these for a commercial caseinate mixture.

2. Materials and methods

2.1. Materials

β-Casein (purified > 90%) was purchased from Research Organics Inc., Cleveland, OH. A commercial sodium caseinate sample (Alanate 180 from New Zealand) was provided by Kraft Foods Inc., Glenview, IL. Trisodium trimetaphosphate (cyclic polyphosphate) and pentasodium tripolyphosphate hexahydrate (linear) were bought from Sigma, St. Louis, MO. Calcium chloride dihydrate was purchased from Fisher, Pittsburgh, PA. All other reagents were products of Sigma.

2.2. Measurements of the extent of β-casein precipitation

In all experiments, 5 mg/ml protein solution of fixed electrolyte concentration, phosphate concentration and pH (adjusted with small amounts of 0.1 M HCl and NaOH) was separated into 10–15 equal aliquots of 5 ml in volumetric vials. A known amount of $CaCl_2$ solution was added to each vial with a micropipette under stirring. Precipitation usually occurred within a few s to min, and the milky solutions were left overnight under mild stirring to allow equilibrium conditions to be reached. The precipitate was then separated by mild centrifugation (800 g for 1 h). A sample of the clear supernatant was pipetted out and diluted,

and the residual (unprecipitated) protein concentration was determined by spectrophotometry at 280 nm. All separations and characterizations were performed at 22±2 °C.

In some samples the supernatant was still cloudy after the centrifugation step because of the formation of casein micelles. We assume that this protein resisting centrifugal separation, albeit partially aggregated, was in stable solution. In order to break the micelles and clarify the supernatant before the spectrophotometry measurements, we added 1 M urea, a strong dissociating agent, previously used to inhibit the aggregation of β-casein [2,15,16].

Dynamic light scattering measurements were performed on a Brookhaven 9000 Light Scattering Spectrometer equipped with a 2 W Ar-ion laser. Optical microscopic observations in regular and polarized light were carried out on an Olympus BH-2 microscope.

3. Results

3.1. Data analysis

The results of the precipitation experiments can be presented in any of a number of ways. Few attempts have been made previously to describe protein precipitation by quantitative models, because the underlying interactions and phase behavior of proteins in solution are much more complex than is the case for low molecular solutes. However, we show here that a model for plotting and interpreting the precipitation data based on a few simple assumptions, allows a reasonable semiquantitative characterization of the process to be achieved:

1) We assume that, similarly to isoelectric precipitation, the positive charges on the calcium ions in the precipitate fully neutralize the negative charges on the proteins, as the precipitate as a whole should not have a net charge. If we denote by k the stoichiometric ratio of Ca^{2+} to protein in the precipitated material, according to this assumption the net negative charge on the protein (P) will be $-2k$, and the 'chemical formula' of the precipitate will be $P^{2k-}Ca_k^{2+}$.
2) It is known that calcium ions adsorb relatively strongly to casein [5,8,9,17], yet we can expect that the bound ions will be in equilibrium with an even larger number of free ions in solution. If the degree of ion binding is denoted as $\alpha = k/n$, where n is the total number of calcium ions added per protein molecule (both bound and in solution), the precipitation process can be represented as

$$P^{2k-} + nCa^{2+} \rightarrow P^{2k-}Ca_k^{2+} + (1-\alpha)nCa^{2+} \quad (1)$$

3) Like any ionic precipitate, the $P^{2k-}Ca_k^{2+}$ complex will have a certain solubility product, K_{sp}, which based on the above equation can be defined as

$$K_{sp} = [P^{2k-}][Ca^{2+}]^n \quad (2)$$

The parameters that are available from our experiments are the original concentration of protein in solution, C_{p0}, the residual concentration of unprecipitated protein, C_{pf} (measured by spectrophotometry) and the concentration of added calcium ions, Ca^{2+}. Starting from Eq. (2) and the species mass balances for protein and precipitant, some re-arrangement gives

$$\frac{C_{pf}}{C_{p0}} = 1 - \frac{Ca^{2+}}{nC_{p0}} + \frac{\sqrt{K_{sp}}}{nC_{p0}\sqrt{C_{pf}}} \quad (3)$$

which is the equation used to interpret the data. By plotting the ratio C_{pf}/C_{p0} against Ca^{2+}/C_{p0}, we obtain a precipitation curve with the predicted behavior shown in Fig. 1. At first the addition of Ca^{2+} does not lead to protein separation, as the amount of $P^{2k-}Ca_k^{2+}$ is below the precipitation threshold. The initial flat region where $C_{pf}/C_{p0} = 1$ is then a measure of the solubility product, evaluated in the plot by the solubility threshold n_0, the number of calcium ions added per protein molecule before precipitation begins (Fig. 1). This plot is somewhat similar to the widely used Scatchard plots [18,19], but we do not expect it to detect the true binding events, but rather to allow us to obtain semiquantitative data on the precipitate formation. When the Ca^{2+} concentra-

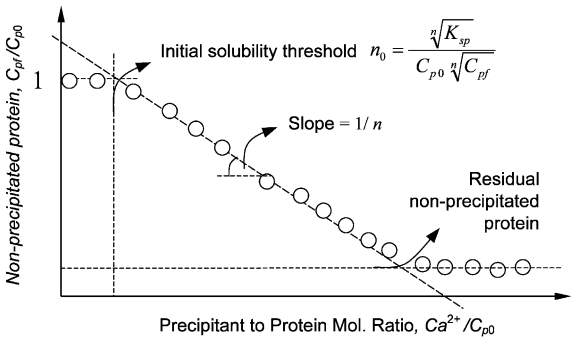

Fig. 1. Explanation of the model used for interpretation of precipitation data.

tion increases above the precipitation threshold, the protein starts precipitating. The increment n of added moles of calcium salt needed to precipitate a mole of protein can be obtained from the slope of the decreasing part of the curve. When precipitation is complete the curve levels off at the solubility of the protein-precipitant complex at an excess of the precipitant. The residual protein in solution is all bound into a complex and no separation can occur when the concentration of precipitant is increased further.

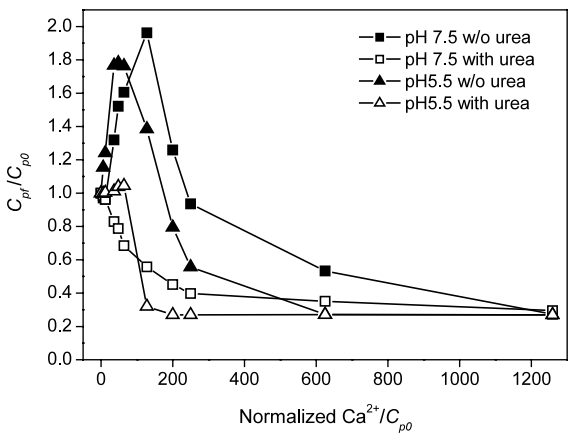

Fig. 2. Residual β-casein concentration after precipitation with increasing amounts of calcium chloride at two different pH values.

3.2. Precipitation of β-casein with Ca^{2+} alone

The precipitation curves of β-casein with calcium ions at the two pH values are plotted in Fig. 2. When a small amount of calcium chloride is initially added we observe no change in the clear solution. Above approximately 10–20 Ca^{2+} ions added per casein molecule, however, the protein solution becomes cloudy, indicating the formation of casein micelles and small stable aggregates. Concurrently, the protein begins precipitating. The turbidity of the aggregates in the supernatant affects the UV measurements and the non-precipitated protein fraction in the supernatant appears to be larger than unity, as seen in the curves without urea in Fig. 2. In order to obtain the true amount of the protein suspended in the solution, urea is added to break the casein micelles and clarify the separated supernatant solution before the measurements. The data for the clarified supernatant qualitatively follow the predictions of the simple model described above, with an initial induction region, quick decrease, and flat non-precipitated tail. All of the fitted data from this and the other precipitation curves are summarized in Table 1.

3.2.1. Effect of pH

The two solutions at different pH values show a pronounced difference in their precipitation patterns. The number of calcium ions needed to precipitate one β-casein molecule in solution can be found from the slope of the curve to be about 220 at pH 7.5 and 88 at pH 5.5 (Table 1). The finding that fewer calcium ions are required to precipitate the β-casein at the lower pH of 5.5 is consistent with the assumption that the precipitation is driven by electrostatic neutralization of the net negative charges on the protein chain, of which there are fewer at the lower pH. The precipitation at pH 5.5 starts at a higher calcium concentration; however, the final degree of precipitation is approximately the same. In both cases, almost 30% of the protein remains in solution.

3.2.2. Effect of NaCl

It should be expected that as electrostatic interactions are involved in Ca^{2+} binding to the

Table 1
Values of the initial solubility threshold n_0, the inverse slope n (number of precipitant molecules/molecule precipitated protein), and final amount of precipitated protein from the fitted data

Protein, precipitant(s) and pH	n_0 (Ca^{2+}/C_{p0})	n (Ca^{2+}/C_{pf})	Residual protein (%)
β-casein, Ca^{2+} at pH 7.5 (Figs. 2 and 3)	5	220	29
β-casein, Ca^{2+} at pH 5.5 (Fig. 2)	64	88	27
β-casein, Ca^{2+}, 0.05 M NaCl at pH 7.5 (Fig. 3)	65	240	35
β-casein, Ca^{2+}, 0.1 M NaCl at pH 7.5 (Fig. 3)	65	226	34
β-casein, Ca^{2+}, 0.05 M monophosphate at pH 7.5 (Fig. 6)	64	172	3
β-casein, Ca^{2+}, 0.05 M monophosphate at pH 5.5	96	204	2
β-casein, Ca^{2+}, 0.05 M cyclic phosphate at pH 7.5 (Fig. 6)	6	50	6
β-casein, Ca^{2+}, 0.05 M cyclic phosphate at pH 5.5 (Fig. 7)	128	100	8
β-casein, Ca^{2+}, 0.05 M linear phosphate at pH 7.5 (Fig. 6)	64	565	5
β-casein, Ca^{2+}, 0.05 M linear phosphate at pH 5.5 (Fig. 7)	36	333	4
Caseinate, Ca^{2+} at pH 7.5 (Fig. 8)	36	236	82
Caseinate, Ca^{2+}, 0.05 M monophosphate at pH 7.5 (Fig. 9)	64	136	11
Caseinate, Ca^{2+}, 0.05 M cyclic phosphate at pH 7.5 (Fig. 9)	0	49	8
Caseinate, Ca^{2+}, 0.05 M linear phosphate at pH 7.5 (Fig. 9)	64	578	7

For explanation of the parameters see Fig. 1.

protein, the precipitation equilibria will be affected by addition of monovalent electrolyte, NaCl. However, the data show no appreciable effect of NaCl on the calcium precipitation curves for β-casein at pH 7.5 (Fig. 3 and Table 1). The number of calcium ions precipitating a single protein molecule, measured via the slope, remains essentially unchanged. The fraction of the precipitated protein in the presence of salt is slightly lower, reflecting a small 'salting-in' effect, commonly encountered in protein separations [20]. The insensitivity to monovalent electrolyte emphasizes the relatively strong and specific calcium ion binding to casein.

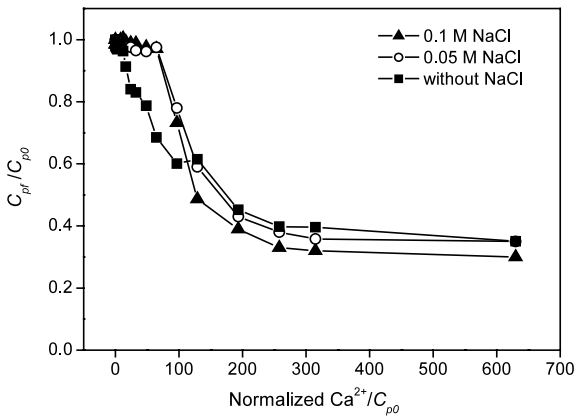

Fig. 3. Effect of monovalent electrolyte, NaCl, on the precipitation of β-casein at pH 7.5.

3.3. Precipitation of β-casein with Ca^{2+} in the presence of monophosphate

In these experiments, 0.05 M sodium monophosphate was added to the β-casein solutions, and then the precipitation curves were recorded with addition of increasing aliquots of calcium chloride. The initial region of precipitation was again accompanied by aggregation and formation of turbid milky suspensions that were difficult to clarify. The most important difference of these initially formed suspensions from the system with Ca^{2+} only is that their turbidity could not be cleared completely by addition of urea, particularly at pH 7.5.

In order to compare the precipitation patterns of the systems with and without phosphate, all the curves from the solutions clarified with urea are plotted in Fig. 4. In both cases the solutions at pH 7.5 begin precipitating first, but the initial induction region is larger for the systems with phosphates (see also Table 1). The slopes of the curves with phosphates are, however, higher, and in all four systems the precipitation is nearly complete at a molar ratio of Ca^{2+}/casein of about 250. A

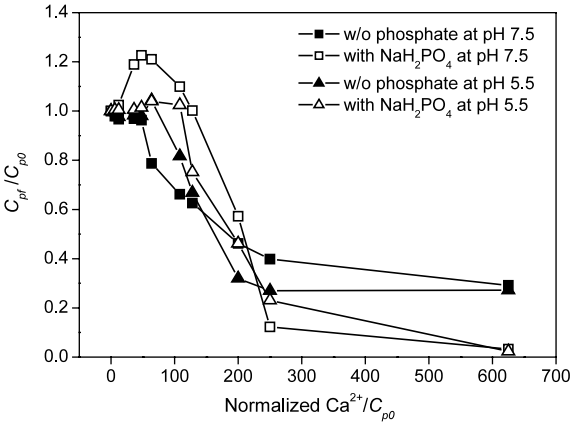

Fig. 4. Comparison of the precipitation curves for systems with and without monophosphate at pH 7.5 and 5.5.

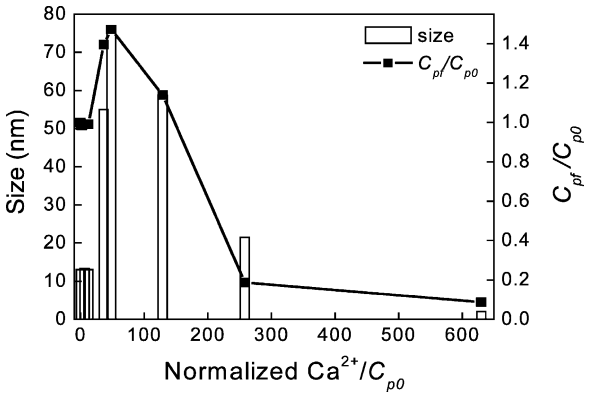

Fig. 5. Data for the effective diameter of the casein micelles in the supernatant with monophosphate measured by DLS and compared with the residual concentration of the protein.

major difference is seen in the amount of residual non-precipitated casein at high Ca^{2+} concentrations, which in the systems with phosphates is at least 25% smaller. More than 97% of the protein could be precipitated when phosphate was added (Fig. 4 and Table 1). Such complete protein separation can be desirable in practical dairy processing.

3.3.1. Size of the aggregates by dynamic light scattering

Dynamic light scattering was used to characterize the aggregated state of turbid protein supernatants before clarification with urea. The suspensions with higher turbidity were diluted with NaCl solutions with the same ionic strength as the precipitant. The effective diameters of casein aggregates, together with the spectrophotometric extinction curve indicating supernatant concentration, are plotted in Fig. 5. Before the onset of precipitation, when the protein binding sites were not saturated with calcium, β-casein existed in the solution as submicelles of size ≈ 12 nm. The size of the micelles grew with increasing Ca^{2+} concentration, reaching its highest value, 75 nm, just before the onset of precipitation. These results agree with the literature data [11,21]. The diameter of the micelles then decreased as more calcium was added and more of the protein precipitated. After the maximal precipitation extent was reached, the diameter of the species in the supernatant solution was less than 10 nm. The small residual amount of protein was present as a monomeric solution.

3.3.2. Structure of the precipitates by optical microscopy

It is known that calcium phosphates are weakly soluble and we observed formation of crystalline precipitates if we mixed the phosphate and the calcium chloride solutions at either pH 5.5 or 7.5. The amount of $Ca_3(PO_4)_2$ precipitate was visibly larger at pH 7.5. It is known that calcium phosphate microcrystals are integrated in casein micelles and dairy products [11,22–24]. Due to their strong birefringence, the calcium phosphate crystals in protein precipitates are easily detected by polarized optical microscopy. We routinely observed bright protein aggregates on a dark background in newly prepared supersaturated samples with phosphate. Large symmetrical crystals of micrometer dimensions formed in aged samples at low supersaturation (Fig. 6a). The calcium phosphate microcrystals were intermixed with the protein aggregates in the precipitates. In contrast, we did not observe any crystals in casein precipitates without phosphates, and the protein aggregates themselves were only slightly brighter than the background (Fig. 6b).

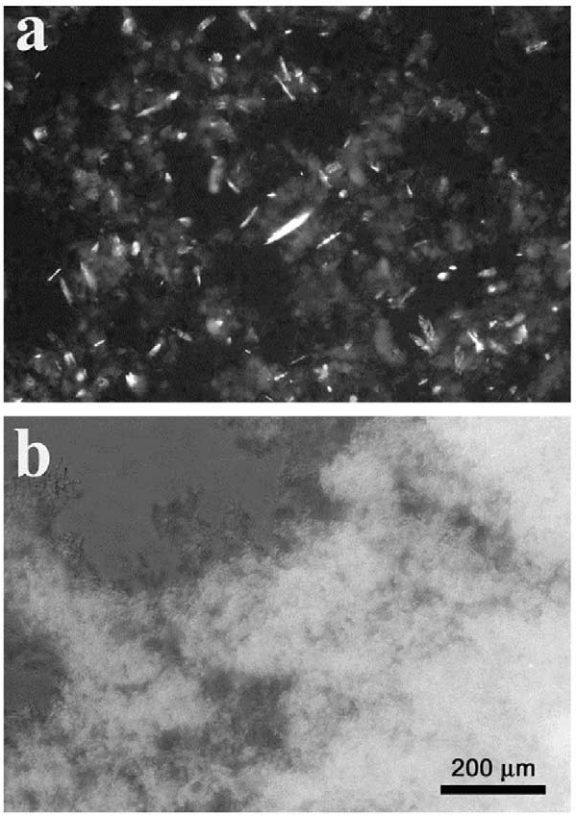

Fig. 6. Optical micrograph in crossed polarizers of casein-Ca^{2+}-monophosphate precipitate. (a) Precipitate in the presence of monophosphate; (b) precipitate without phosphate.

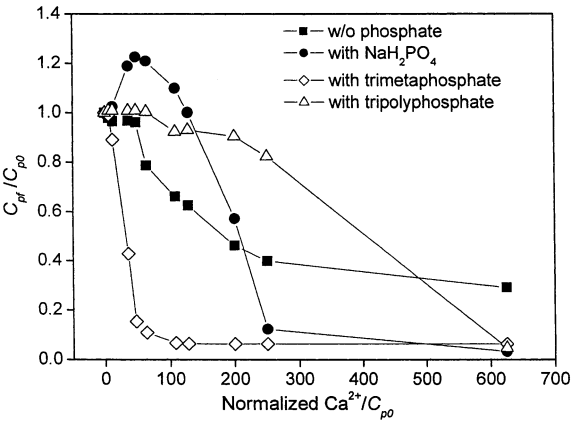

Fig. 7. Effect of monophosphate, trimetaphosphate, and tripolyphosphate on the precipitation curves of β-casein at pH 7.5 (all concentrations determined after addition of urea).

3.4. Precipitation of β-casein with Ca^{2+} in the presence of polyphosphates

The data on the precipitation of β-casein with calcium in the presence of monophosphate, trisodium tri(meta)phosphate (cyclic) or tripolyphosphate (linear) at pH 7.5 are compared in Fig. 7. Almost all of the protein ($>90\%$) is precipitated at high Ca^{2+} concentrations in the presence of all three types of phosphates. The precipitation paths for the three phosphates are, however, different. In the presence of cyclic phosphate the protein begins precipitating immediately on initial addition of Ca^{2+}, and the slope of the curve is the largest of all systems studied, requiring only 50 calcium ions per mole precipitated protein (Fig. 7 and Table 1). The linear phosphate, on the other hand, has a larger induction region and smaller slope than even the monophosphate. The trends of the three phosphates on calcium precipitation of β-casein at pH 5.5 turned out to be the same as at pH 7.5, but the effects are not nearly as significant as at pH 7.5, and the differences among the curves for different phosphates are relatively small. This can be attributed to the lessened electrostatic interactions as both the protein and the phosphates are closer to their isoelectric or deionization point.

3.5. Separations in commercial casein mixtures in the presence of Ca^{2+} and phosphates

To estimate the importance of the observed precipitation patterns in practical dairy systems containing mixed caseins, we studied Ca^{2+} induced precipitation of commercial caseinate and compared these data to those with pure β-casein. These experiments were performed at pH 7.5, where, as shown in the previous sections, the effects of the phosphates are more emphasized. The precipitation curve (Fig. 8) follows the general behavior seen previously, but only a relatively small amount ($\approx 20\%$) of protein from the caseinate could be precipitated by calcium in the absence of phosphate. The commercial caseinates

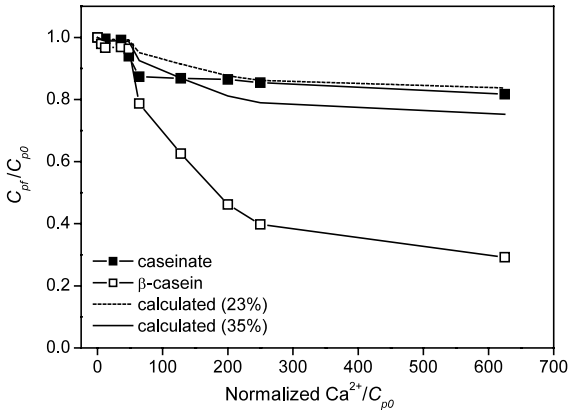

Fig. 8. Comparison of the Ca^{2+} precipitation curves of commercial caseinate and pure β-casein without phosphate at pH 7.5. The two calculated curves show the possible range of the precipitation curve for the hypothesis that only β-casein is selectively precipitated from the commercial protein mixture.

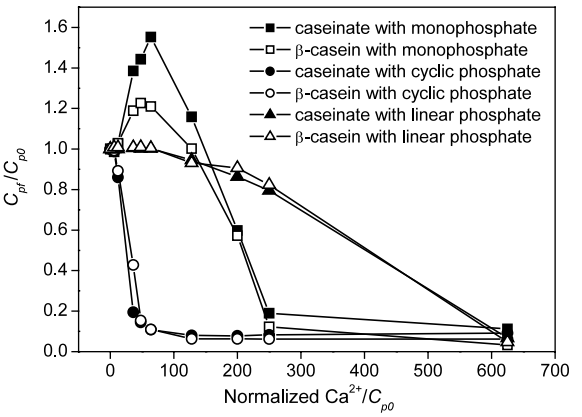

Fig. 9. Comparison of the precipitation curves of caseinate and β-casein in the presence of monophosphate, cyclic trimetaphosphate and linear tripolyphosphate ions at pH 7.5 (all concentrations determined after addition of urea).

typically contain 23–35% of β-casein [25,26]. If we assume that only this protein is precipitated by the Ca^{2+} added, we can calculate, on the basis of the data in Fig. 2, how much of the β-casein will be precipitated from the mixture for each calcium concentration. The results of these estimates based on the lowest and highest possible concentrations of β-casein in the caseinate are shown in Fig. 8 as dotted and solid lines, respectively. The experimentally measured caseinate precipitation curve lies between these two lines, so we hypothesize that only the β-casein, which has a specific affinity for calcium, is precipitated under these conditions. This hypothesis is indirectly supported by the approximately equal increment n found for the caseinate as in the pure β-casein samples (Table 1).

In strong contrast, when monophosphate, linear phosphate or cyclic phosphate was added during the caseinate precipitation, the precipitation profiles of pure β-casein and commercial caseinate were very similar (Fig. 9). The addition of phosphate leads to full indiscriminate precipitation of all proteins in the commercial mixture. This suggests that the precipitation mechanism in this case is different from that with Ca^{2+} only, and is probably a result of the incorporation of inorganic crystals within the structure of the precipitate. The possible origins of these mechanisms and their practical implications are discussed below.

4. Discussion

The data for β-casein precipitation in the presence of Ca^{2+} without phosphates indicate that precipitation of the protein occurs when the negative charges on the protein molecules are offset by the positive charges on calcium ions. The number of calcium ions required to precipitate a molecule of protein is much higher than the possible number of negatively charged binding sites on the protein, indicating that binding is weak, i.e. that only a small fraction of the Ca^{2+} ions are adsorbed on the molecule and in equilibrium with dissolved ions at any time. The electrostatic nature of Ca^{2+} adsorption is seen via the influence of pH, as the casein carries different charges at pH 7.5 and pH 5.5. The net charge of β-casein was calculated from the amino acid sequence by the Henderson–Hasselbalch equation (for the theory and the constants used see, e.g. [27]). The casein charge is approximately −12 charges per molecule at pH 7.5 and −7 charges per molecule at pH 5.5. At lower negative charge, fewer calcium ions will be needed to neutralize the oppositely charged groups on the β-casein chain and aggregation and precipitation will start at lower Ca^{2+} concentration. This is indeed evidenced in Fig. 2, where the higher slope of the curve at pH 5.5 indicates a lower number of

Ca^{2+} ions required for precipitation of a mole of protein (see also Table 1).

In light of this finding of relatively weak Ca^{2+} electrostatic adsorption, the data showing very little effect of NaCl on the β-casein precipitation curves (Fig. 3) are somewhat surprising, but explicable. They suggest that the adsorption of the divalent Ca^{2+} ions, although weak, is still specific and favored over the nonspecific monovalent Na^{+}. Casein, due to its specific affinity to Ca^{2+}, is the natural protein carrier for calcium in bovine milk. It is known that calcium ions bind strongly to six Ser-P sites in β-casein [12,17]. The precipitation effects that we studied probably take place after the phosphorylated sites are saturated; the divalent Ca^{2+} ions adsorb on the other negative groups on the protein surface preferentially to monovalent electrolytes.

One notable finding is that the final concentration of non-precipitated protein is not dependent on pH and only weakly dependent on electrolyte. This finding is in good agreement with the model that relates the amount of non-precipitated protein to the solubility of the presumably neutral protein–Ca^{2+} complex. It can be expected that once the protein charges are neutralized, the solubility of the neutral complex will be constant, regardless of the number of charges that have been neutralized in the process.

Addition of phosphates to the system not only influences the adsorption equilibria [28], but introduces a new mechanism of casein precipitation. As mentioned, mixing calcium and phosphate ions alone precipitated crystalline calcium phosphate. In the presence of β-casein sturdy casein micelles were formed under these conditions, which do not disassemble after addition of urea. It is known that caseins are able to inhibit the growth of calcium phosphate crystals and thereby stabilize the amorphous form of the colloidal calcium phosphate naturally occurring in casein micelles in milk [29,30]. This property accounts for the high concentrations and high bioavailability of calcium and phosphates in milk [15].

The sturdiness of the micelles in the presence of phosphate is probably caused by the colloidal calcium phosphate crystals, which 'glue' the casein monomers to the micelles and precipitates. The calcium phosphate microcrystals form in the same calcium concentration range as the casein aggregates. Due to the natural affinity of the protein for the crystal surfaces [22,23], the crystals and the protein are co-precipitated in a structure that is sturdy enough to resist disassembly by urea. The formation of the co-precipitated phases of inorganic microcrystals and casein leads to drastically improved separation efficiency. The co-precipitation patterns vary depending on the type of phosphate used and the affinity of protein binding to the crystals, but as expected, the cyclic polyphosphates that precipitate Ca^{2+} most strongly are also most active in protein co-precipitation (Fig. 7). Again, the differences in the precipitation rates become unimportant at high calcium concentrations where almost all of the protein is precipitated by the combination of charge neutralization and binding to the surfaces of the microcrystals.

The most important practical effect is seen in the separations of the caseinate protein mixture—the formation of mixed co-precipitates of strongly-bound calcium phosphate crystals and casein. Whereas Ca^{2+} ions precipitate only the β-casein component, the Ca^{2+}-phosphate microcrystals precipitate all of the caseins present, thus ensuring much better separation (Fig. 9). We hypothesize that this is due to strong adsorption of all caseins on the surfaces of the inorganic crystals, instead of the selective calcium precipitation of β-casein alone (Fig. 10). The addition of phosphates before the precipitation process changes the separation mechanism from precipitation of single-phase casein aggregates to the formation of a complex multiphase precipitate of proteins and inorganic crystals. This finding explains the important role of phosphates in cheese-making, where they are empirically known to be strong modifiers and enhancers of dairy casein separations and rheology [1].

5. Summary

We obtained quantitative data on the precipitation patterns of casein with calcium ions and phosphates at pH 7.5 and 5.5. The major findings

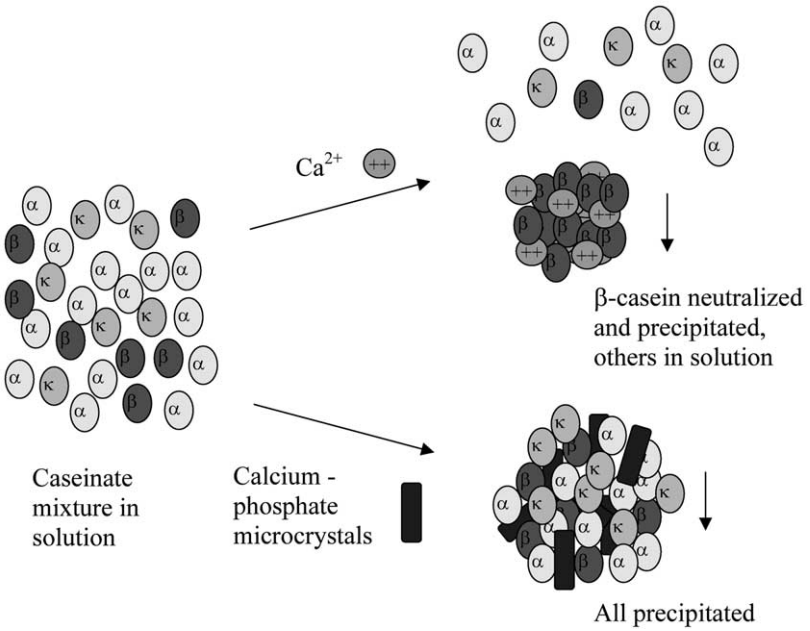

Fig. 10. Schematics of the hypothesis of the origin of two types of Ca^{2+} induced separation in the caseinate solutions with and without phosphates (species are not drawn to scale).

related to the micellization/precipitation equilibria, and of interest to practical fabrication of dairy products are:

- The most important factor for β-casein precipitation in the absence of phosphate ions is the effective neutralization of the negative charges on the protein molecules by weak Ca^{2+} adsorption. As the calcium ions exhibit specific binding to the casein, the addition of monovalent electrolyte has no discernible effect on casein precipitation.
- The presence of phosphates leads to more complete protein precipitation. The mechanism of separation changes to co-precipitation of protein and inorganic microcrystals.
- The activity of the phosphates in the casein separations decreases in the order cyclic phosphate > monophosphate > linear phosphate. The phosphate effects are more pronounced at pH 7.5 than at pH 5.5.
- The most drastic effect of phosphates is observed in commercial caseinate mixtures where only a small part of the protein is precipitated by Ca^{2+} alone, but nearly all of the proteins are separated in the presence of phosphates. We hypothesize that this pronouncedly improved separation is caused by indiscriminate co-precipitation of the proteins by the inorganic microcrystals as compared with the selective precipitation of β-casein by calcium ions (Fig. 10).

The simple experimental technique and interpretation developed have proven useful in understanding the complex precipitation phenomena and will be used in various further studies on protein separations.

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