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Study of the dissociation of β-casein from native phosphocaseinate

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Summary — Native phosphocaseinate is a relatively new product obtained from microfiltration of skim milk. The conditions used during the microfiltration of milk are believed to have only minor effects on micellar integrity. The objective of the work was to study β-casein solubilization from native phosphocaseinate in the conditions of cold storage as affected by different pH levels, calcium chelation and ionic strength. Native phosphocaseinate powder was rehydrated to 2.0% (w/w) in the presence of NaCl (3.0, 6.0 and 9.0 g/kg) and trisodium citrate (0.4, 1.0 and 1.6 g/kg) before pH adjustment (4.0, 4.6 and 5.2) followed by cold storage (0°C). The amount of soluble casein was determined by RP-HPLC of ultracentrifugation supernatants. It was found that the solubilization of β-casein was optimal in the presence of 9.0 g/kg of NaCl at pH 5.2 and that calcium chelation did not have a marked effect.

native phosphocaseinate / β-casein / solubilization / cold storage

Résumé — Étude de la dissociation de la caséine β à partir de phosphocaséinate natif. *Le phosphocaséinate natif est un produit relativement nouveau, obtenu de la microfiltration de lait écrémé. Les technologies douces des procédés à membranes sont réputées préserver l'intégrité des micelles de caséines. L'objectif de ce travail était d'étudier la solubilisation de la caséine β du phosphocaséinate natif lors de l'entreposage à froid dans des conditions de pH, de séquestration du calcium et de force ionique variables. La poudre de phosphocaséinate natif a été réhydratée à 2,0% (p/p) en présence de NaCl (3,0, 6,0 et 9,0 g/kg) et de citrate trisodique (0,4, 1,0 et 1,6 g/kg) avant ajustement du pH (4,0, 4,6 et 5,2) suivi d'un entreposage à froid (0°C). La quantité de caséines solubles a été déterminée par HPLC-RP dans les surnageants d'ultracentrifugation. Les résultats montrent que les meilleures conditions pour la solubilisation de la caséine β sont obtenues en présence de 9,0 g/kg de NaCl à pH 5,2 et que la séquestration du calcium par le citrate trisodique n'a pas d'effets marqués.*

phosphocaséinate natif / caséine β / solubilisation / entreposage à froid

INTRODUCTION

Recent developments in the membrane processing of milk have rendered possible the preparation of phosphocaseinate extract from skim milk by the use of a 0.2 μm microfiltration membrane operated under low transmembrane pressure gradient and high tangential velocity (Fauquant *et al*, 1988; Smithers and Bradford, 1991; Pierre *et al*, 1992). Analyses of the so-called native phosphocaseinate reveal a very high casein content (> 94% of the total proteins) which exhibits micellar-like behaviour as indicated by the opaqueness of the concentrate. The preparation of native phosphocaseinate offers new possibilities for protein enrichment in cheese manufacture (Pierre *et al*, 1992) and presumably provides a whey protein-depleted substrate for the production of purified β-casein. However, the physicochemical properties of native phosphocaseinates, such as soluble-colloidal equilibrium of caseins, remain yet to be defined.

The physico-chemical properties and the stability of bovine casein micelles have been the subjects of numerous studies over the past decades (reviewed by McMahon and Brown (1984), Walstra (1990) and Rollema (1992)). It is well known that casein micelles are found as complex colloidal particles ranging in diameter from 15 nm to 600 nm with a weight average diameter of 180 nm. The exact structure of casein micelles is still a matter of discussion but it is known that these colloidal particles comprise a protein moiety (93%) composed of the casein fractions α_{s1}, α_{s2}, β and κ in the approximate ratio of 3:1:3:1, and an inorganic one mainly composed of calcium phosphate complexes. The protein and inorganic salt parts of the casein micelles also show a soluble-colloidal equilibrium which is affected by physico-chemical parameters such as temperature, pH and ionic strength. The dissociation of casein micelles can be induced by removal of calcium (Lin *et al*, 1972), addition of urea (Aoki *et al*, 1986), dialysis against phosphate-free buffer (Holt *et al*, 1986), acidification (Rose, 1968) and by lowering the temperature to 4°C (Dalgleish and Law, 1988).

The phenomena surrounding solubilization of caseins from micellar caseins during cold storage of milk have been well documented (Rose, 1968; Sharma and Randolph, 1974; Creamer *et al*, 1977; Ali *et al*, 1980; Davies and Law, 1983; Roefs *et al*, 1985; Dalgleish and Law, 1988). Creamer *et al* (1977) noted a maximum of soluble caseins at 0°C. The decrease in hydrophobic interactions (Davies and Law, 1983) and the solubilization of calcium phosphate at low temperature (Pyne, 1962) could be responsible for the solubilization of caseins. Pierre and Brulé (1981) showed that the solubilization of β-casein from micelles in milk had an optimum at a protein concentration of 30 g/l of total milk proteins. Famelart *et al* (1989) reported a plateau region in the solubilization of β-casein from sodium caseinate at a concentration of sodium caseinate of 20 g/l. Other methods for the production of β-casein also used protein concentrations in the range of 20 g/l (Terré *et al*, 1986; Murphy and Fox, 1991; LeMagnen and Maugas, 1992).

The objectives of this work were to study β-casein solubilization from native phosphocaseinate in the conditions of cold storage as affected by different pH levels, calcium chelation and ionic strength.

MATERIALS AND METHODS

Native phosphocaseinate preparation

Raw milk was skimmed at 50°C and cold pasteurized by microfiltration using a Bacto-Catch™ system (Alfa-Laval, Lund, Sweden) with 1.4 μm pore diameter Membralox ceramic membrane (SCT-Alcoa, Bazet, France) according to Trouvé *et al* (1991). The milk was then microfiltered using TECH-SEP M-14, 0.14 μm pore diameter, mineral

membrane (Miribel, France) up to 3 volume concentration ratio and diafiltered with 5 diavolumes according to the process described by Fauquant *et al* (1988) and Pierre *et al* (1992).

The native phosphocaseinate concentrate was spray dried on a NIRO atomiser as described by Schuck *et al* (1994). The composition of the native phosphocaseinate powder and the proportion of the different caseins are shown in table I and figure 1.

Table I. Phosphocaseinate powder composition. *Composition de la poudre de phosphocaséinate.*

Moisture (%)	9.0
Total nitrogen (x 6.38) g/kg	841.5
NCN	43.92
NPN	3.57
Casein	794.0
κ	127.0 (16%)
α_s	397.0 (50%)
β	270.0 (34%)
Ratio α_s/κ	3.13
Calcium (ppm)	29 000

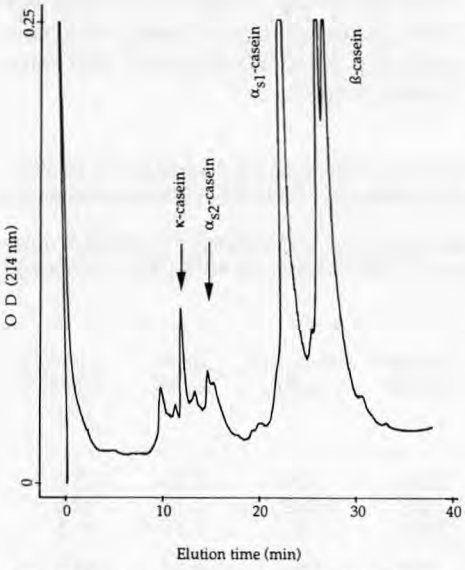

Fig 1. Native phosphocaseinate separation profile by reverse phase HPLC according to the method of Jaubert and Martin (1992).
Profil de séparation de phosphocaséinate natif par HPLC en phase inverse selon la méthode de Jaubert et Martin (1992).

Solubilization conditions

The powders were rehydrated in small glass flasks to approximately 2.5% (w/w) protein concentration. The powders were left to rehydrate at room temperature or at 50°C for 1 h in the presence of NaCl (3.0, 6.0 or 9.0 g/kg final concentration) and trisodium citrate (0.4, 1.0 or 1.6 g/kg final concentration). These suspensions were cooled to 0°C in melting ice for 1 h after which the pH was adjusted to 4.0, 4.6 or 5.2 with HCl (1.0 N); 30 min later, the pH was checked and readjusted if necessary and the solutions were completed with water to give a final concentration of 2.0% (w/w) protein. The dispersions were then stored at 0°C in melting ice placed in a cold room (4°C) overnight.

Ultracentrifugation

After storage, the solutions were transferred to centrifuge tubes and installed in a pre-cooled Beckman 50.2 Ti rotor and centrifuged on a Beckman L8-55 ultracentrifuge at 0°C for 4 h at a mean accelerating field of 70 000 *g* (Dalgleish and Law, 1988). Supernatants were collected and frozen in test tubes at –18°C until HPLC analysis. The caseins contained in the supernatant were considered as soluble caseins.

HPLC-RP analysis

Reverse phase HPLC allowed the evaluation of the different caseins in the supernatant. The HPLC-RP system was composed of a Waters 600 multisolvent delivery system, a Waters 481 variable wavelength LC spectrophotometer set at 214 nm, a Waters 740 Data module and a Prolabo Sup-RS Stabitherm oven set at 40°C. The reverse phase column was a 15 cm Vydac C4, 214 TP 54.

The chromatography conditions were according to the method of Jaubert and Martin (1992) without reduction of the samples with dithiothreitol because there was no overlap of protein peaks.

RESULTS AND DISCUSSION

Ionic strength and ion exchange

All caseins showed an increase in solubility as the NaCl content increased (tables II and III). Pierre and Brulé (1981) reported an increase in solubility of β-casein with the addition of NaCl.

The NaCl was used to increase the ionic strength which is known to decrease the amount of calcium linked to β-casein and α_{s1}-casein (Dalgleish and Parker, 1980; Parker and Dalgleish, 1981) thereby increasing the solubility of caseins. Van Dijk (1991) reported that the addition of NaCl to milk increased the amount of Ca and Mg in the serum which would come from the divalent cations linked to the serine phosphate of the casein and not from the native micellar colloidal phosphate. This had previously been observed by Brulé *et al* (1974) in the case of UF milk retentates where NaCl addition increased the soluble calcium and magnesium but not the soluble phosphate. The interactions between the casein monomers and the phosphomicellar complex would be weakened (Pierre and Brulé, 1981) as a consequence of such an ion exchange phenomenon between calcium and sodium.

Powder solubilization conditions

Phosphocaseinate was rehydrated at two temperature levels, 25°C and 50°C. As shown in table II, the increase of the temperature of rehydration from room temperature to 50°C induced an increase in the solubilization of the total casein at 0°C for any NaCl level. It can be suggested that a higher rehydration temperature allowed a more complete solubilization of the native phosphocaseinate which in turn provided a greater content of substrate for dissociation. From these results, this phenomenon was observed for α_s, β and κ-casein fractions separately. However the effect was more pronounced for β-casein in the presence of 6.0 and 9.0 g/kg NaCl after rehydration at 50°C.

Table II. Soluble casein at 0°C from native phosphocaseinate dispersions (20 g/kg) at pH 5.2 as affected by NaCl (3.0, 6.0 and 9.0 g/kg) and temperature of rehydration (25 and 50°C) in the presence of 0.4 g/kg trisodium citrate.
Caséine soluble à 0°C dans les dispersions de phosphocaséinate natif (20 g/kg) à pH 5,2 telle qu'affectée par le NaCl (3,0, 6,0 et 9,0 g/kg) et la température de réhydratation (25 et 50°C) en présence de 0,4 g/kg de citrate trisodique.

Rehydration temperature (°C)	*NaCl (g/kg)*	*Total casein (g/kg)*	*α_s-Casein (g/kg)*	*β-Casein (g/kg)*	*κ-Casein (g/kg)*	*Ratio α_s/κ*	*β-casein soluble [a] (%)*
25	3.0	2.31	0.28	0.81	1.22	0.23	35.0
	6.0	4.10	1.08	1.53	1.49	0.72	37.4
	9.0	5.15	1.72	1.88	1.55	1.11	36.5
50	3.0	4.00	1.22	1.23	1.55	0.79	30.8
	6.0	7.37	1.94	3.34	2.09	0.93	45.3
	9.0	9.85	2.80	4.72	2.33	1.20	47.9

[a] Soluble β-casein/total soluble caseins x 100.
[a] *β-caséine soluble/total caséines solubles x 100.*

Table III. Soluble casein at 0°C from native phosphocaseinate dispersions (20 g/kg) at pH 5.2 as affected by trisodium citrate (0.4, 1.0 and 1.6 g/kg) and NaCl (3.0, 6.0 and 9.0 g/kg) rehydrated at 50°C.
Caséine soluble à 0°C dans les dispersions de phosphocaséinate natif (20 g/kg) at pH 5,2 telle qu'affectée par le citrate trisodique (0,4, 1,0 et 1,6 g/kg) et le NaCl (3,0, 6,0 et 9,0 g/kg) réhydratée à 50°C.

Trisodium citrate (g/kg)	*NaCl (g/kg)*	*Total casein (g/kg)*	*α_s-Casein (g/kg)*	*β-Casein (g/kg)*	*κ-Casein (g/kg)*	*Ratio α_s/κ*	*β-casein soluble [a] (%)*
0.4	3.0	4.00	1.22	1.23	1.55	0.79	30.8
	6.0	7.37	1.94	3.34	2.09	0.93	45.3
	9.0	9.85	2.80	4.72	2.33	1.20	47.9
1.0	3.0	5.31	1.13	2.60	1.58	0.72	49.0
	6.0	6.71	1.71	2.93	2.07	0.83	43.5
	9.0	7.89	2.42	3.20	2.27	1.07	40.6
1.6	3.0	4.86	1.43	1.66	1.77	0.81	34.2
	6.0	5.33	1.45	2.13	1.75	0.83	40.0
	9.0	13.14	4.09	6.20	2.85	1.44	47.5

[a] Soluble β-casein / total soluble caseins x 100.
[a] β-caséine soluble/total caséines solubles x 100.

Calcium chelation

A minimal level of 0.4 g/kg of trisodium citrate was used in order to chelate calcium as suggested by the work of Brulé and Fauquant (1981). It is known that a decrease in free calcium can cause solubilization of colloidal phosphate with loss in micellar integrity (Pyne, 1962; Morr, 1967) leading to the solubilization of β-casein, κ-casein (Ono *et al,* 1978) and ultimately to that of α_s caseins (Lin *et al,* 1972).

It is known (Payens, 1982) that the sensitivity of the casein fractions to calcium is related to their phosphoserine (Ser-P) content and it increases in the following order: α_{s2} (10–13 Ser-P/molecule) > α_{s1}(8–9 Ser-P/molecule) > β(4–5 Ser P/molecule) > κ(1 Ser-P/molecule). The sensitivity to calcium of the four caseins explains the effect observed in table III.

The combined effect of adding citrate and NaCl presumably decreases the ionic calcium content while ion exchange phenomena between calcium and sodium favour the solubilization of caseins from the micelles. Surprisingly, a minimum in the solubilization of β-casein was obtained with 1.0 g/kg citrate and 9.0 g/kg NaCl. There is no satisfactory explanation for this observation.

β-Casein did not show change in solubility when trisodium citrate content was increased from 0.4 to 1.0 g/kg. The lower amount of calcium that β-casein can bind is related to its lower content in phosphoserine residues in its primary sequence compared to the α_s-caseins (Dalgleish and Parker, 1980; Swaisgood, 1982). This calcium binding property explains the increase in solubility of α_s-caseins upon depletion of calcium of the native micellar phosphocaseinate. κ-Casein exhibits the lowest phosphoserine content among the four fractions and its calcium binding potential is low. This was reflected by slight changes in soluble κ-casein upon calcium chelation.

The results of this study are in agreement with those reported by Holt *et al* (1986) in milk. As they reported, β- and κ-casein are slightly affected and show the same behaviour towards calcium phosphate chelation; α_s-caseins are greatly affected toward calcium phosphate solubilization mainly because of their high level of basic and acidic amino acid residues which can be linked with Ca and micellar calcium phosphate.

pH conditions

Three pH levels were tested: below the isoelectric point of all caseins (4.0), at the isoelectric pH of precipitation of the casein micelle (4.6) and at the isoionic point of β-casein (5.2). As the pH increased from 4.0 to 5.2, the solubility of β- and α_s-caseins increased while κ-casein was only slightly affected by pH change (table IV).

For β-casein the solubility is much more important at its isoelectric point. α_{s1}-Casein showed the highest increase in solubility at pH 5.2 where it can be 10 times more soluble than at the other pHs. Walstra (1990) reported on several changes occurring in casein micelles of milk in the pH range 5.2–5.3 and concluded that the bonds responsible for casein micelle integrity are weakest or fewest at pH 5.2 or 5.3. At lower pH, increasing electrostatic interactions between casein molecules keep the micelles together and at higher pH, the increasing quantity of colloidal phosphate has the same effect.

These results agree well with those reported previously for milk. Rose (1968) reported that in milk at 4°C (pH 5.3) there was the maximum concentration in serum caseins. At pH 5.4, Van Dijk (1990a) reported that all the inorganic phosphate and a part of the cations associated with the caseins were dissociated and that between pH 5.4 to 4.5 all the rest of the associated cations also dissociated. An important part of the β-casein is stabilized in the micelle *via* hydrophobic interactions, the

Table IV. Soluble casein at 0°C from native phosphocaseinate dispersions (20 g/kg) as affected by pH (4.0, 4.6 and 5.2) and NaCl (3.0, 6.0 and 9.0 g/kg) rehydrated at 25°C in 1.6 g/kg trisodium citrate. *Caséine soluble à 0°C dans les dispersions de phosphocaséinate natif (20 g/kg) telle qu'affectée par le pH (4,0, 4,6 et 5,2) et le NaCl (3,0, 6,0 et 9,0 g/kg) réhydratée à 25°C dans le citrate trisodique à 1,6 g/kg.*

pH	*NaCl (g/kg)*	*Total casein (g/kg)*	*α_s-Casein (g/kg)*	*β-Casein (g/kg)*	*κ-Casein (g/kg)*	*Ratio α_s/κ*	*β-Casein soluble [a] (%)*
4.0	3.0	2.53	0.79	0.84	0.90	0.88	33.2
	6.0	2.13	0.43	0.81	0.89	0.48	38.0
	9.0	1.35	nd	0.69	0.66	–	51.1
4.6	3.0	1.49	0.11	0.85	0.53	0.21	57.1
	6.0	2.23	0.37	0.80	1.06	0.35	35.9
	9.0	1.70	0.01	0.74	0.95	0.01	43.5
5.2	3.0	3.72	0.86	1.41	1.45	0.59	37.9
	6.0	5.05	1.54	1.88	1.63	0.95	37.2
	9.0	5.28	1.73	1.77	1.78	0.97	33.5

nd: not detected. [a] Soluble β-casein / total soluble caseins x 100.
nd : non déterminé. [a] β-caséine soluble / total caséines solubles x 100.

rest of the β-casein is probably linked in the micelle by its phosphoserine residues and by the micellar calcium phosphate (Van Dijk, 1990b). The acidification of native phosphocaseinate solutions limited the interactions of the caseins with the divalent cations and permitted their solubilization. In accordance with Van Dijk (1990b) who reported that β-casein solubilization was enhanced by acidification of milk and that it became important below pH 5.9, Roefs *et al* (1985) reported that for low heat skim milk powder at pH 4.6, 5.4 and 6.6, 100%, 45% and 87% of the caseins were sedimented by ultracentrifugation at 5°C and that for fresh milk, at 4°C (pH 5.4), 65%, 60% and 40% of β-, κ- and α_s-casein respectively, were soluble. Dalgleish and Law (1988) also reported a maximum solubility of β-, κ- and α_{s1}-caseins at 4°C (pH 5.2).

The experiments of β-casein solubilization from sodium caseinate have usually been performed at pH 4.0 to 4.6 (Famelart *et al*, 1989 ; LeMagnen and Maugas, 1992). At pH 4.57, Davies and White (1960) reported that in milk at 20°C, 96% of the magnesium, 97% of the calcium and 73% of the total phosphorus was soluble. Pyne (1962) reported that between 0 and 2°C at pH 4.6 there was complete demineralisation of the caseins that leads to the solubilization of the caseins.

CONCLUSION

The behaviour of the native micellar phosphocaseinate prepared by microfiltration is close to that of native casein micelles. It appears that the microfiltration process did not have an important disturbing effect on the casein micelles.

Native micellar phosphocaseinate thus represents a convenient substrate for the preparation of casein fractions. The present study showed that it was possible to produce a β-casein rich fraction that contained ≈90% of the β-casein of the native micellar phosphocaseinate with a relative purity of ≈48%. The best conditions for that are: 0°C, pH 5.2, 9.0 g/kg of NaCl and 1.6 g/kg of trisodium citrate.

The native micellar phosphocaseinate offers new possibilities for the production of casein products of different ratios of caseins that ultimately could be designed for specific applications.

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