Effect of Modification of Amino Groups on Crosslinking of Casein by Micellar Calcium Phosphate

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ABSTRACT

Bovine α_{s1}-CN was acetylated, succinylated, and citraconylated. Artificial casein micelles (α_{s1}-κ-CN micelles) were prepared at a casein concentration of 2.5% with 20 to 50 m*M* calcium, 17 to 27 m*M* phosphate, and 10 m*M* citrate, and crosslinking of casein by micellar calcium phosphate was examined by HPLC in the presence of 6 *M* urea. The content of casein aggregates that were crosslinked by micellar calcium phosphate in modified α_{s1}-κ-CN micelles was lower than that in intact α_{s1}-κ-CN micelles. Urea-insoluble calcium phosphate was present in succinylated α_{s1}-κ-CN micelles, suggesting that it did not participate in crosslinking. The modified α_{s1}-CN was less crosslinked by micellar calcium phosphate than intact α_{s1}-CN, even when modified α_{s1}-CN coexisted equivalently with intact α_{s1}-CN in artificial casein micelles. The amino groups also affected crosslinking of casein by micellar calcium phosphate.

(**Key words**: casein micelle, calcium phosphate, crosslinking)

Abbreviation key: **MCP** = micellar calcium phosphate, **USMUF** = urea-simulated milk ultrafiltrate.

INTRODUCTION

In bovine milk, the major part of casein occurs in the form of highly hydrated colloidal aggregates of 20 to 600 nm in diameter, commonly referred to as casein micelles (16), which contain 6.6% of inorganic materials, of which the main constituents are calcium phosphates. Calcium phosphate in casein micelles, which are associated with casein, is called micellar calcium phosphate (**MCP**) or colloidal calcium phosphate. The MCP plays an important role in maintaining the structure of casein micelles because casein micelles are disaggregated into submicelles when MCP is removed (14, 15, 17).

In some models of the structure of casein micelles (16, 22), MCP is considered to link submicelles. To provide direct experimental evidence for the linkage between casein and MCP, Aoki et al. (4) separated casein aggregates crosslinked by MCP using HPLC on a TSK-GEL G4000SW column in the presence of 6 *M* urea. Caseins also are crosslinked through their ester phosphate groups by MCP (7). At least three phosphate groups are needed for crosslinking of casein by MCP (5), and the order of crosslinking by MCP agrees with the content of phosphate groups (2).

Although several models have been proposed (12, 16, 19), the structure of MCP has not yet been sufficiently resolved. The structure of MCP and the interaction of casein with MCP need to be studied. The dissociation groups, in addition to the phosphate groups, may participate in crosslinking of casein by MCP. Possibly, MCP interacts with amino groups (20). In this study, the effect of modification of amino groups on crosslinking of casein by MCP was investigated.

MATERIALS AND METHODS

Preparation of Casein Samples

Whole bovine casein was prepared from skim milk from the university herd milk of Holstein cows by acid precipitation at pH 4.6 with .1 *M* HCl.

Bovine α_{s1}-CN was prepared by the method of Zittle and Custer (23), followed by purifica-

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tion on a DEAE-cellulose column in the presence of 3.3 *M* urea.

Bovine κ-CN was prepared by the method of Zittle and Custer (23), followed by purification on a Sephacryl S-300 column in the presence of 6 *M* urea (3).

Modification of Amino Groups of α_{s1}-CN

Succinylation was performed by the method of Hoagland (11). One gram of α_{s1}-CN was dispersed in 100 ml of 75 m*M* phosphate buffer. After the pH was adjusted to 7.5 with 1 *M* NaOH, the solution volume was adjusted to 125 ml with the phosphate buffer, and .25-g increments of succinic anhydride (Nacalai Tesque, Inc., Kyoto, Japan) were added to a total of 1 g with stirring. During succinylation, the pH was maintained at about 7.5 with 3.5 *M* NaOH. After the pH was stabilized at 7.5, the solution was dialyzed against several changes of distilled water for 3 d at 4°C to remove impurities and excess reagents. The succinylated α_{s1}-CN was recovered by lyophilization. The extent of succinylation was calculated to be 99%, based on the reaction with fluorescamine according to the method of Böhlen et al. (8).

Acetylation was performed by the method of Fraenkel-Conrat (10). Thirty milliliters of a solution of 3% (wt/vol) α_{s1}-CN and 30 ml of saturated aqueous sodium acetate solution were mixed at room temperature (22 to 25°C) with vigorous stirring. To the mixture, acetic anhydride (Nacalai Tesque Inc.) was added in .2-ml increments to a total of 1 ml over 1 h. The reaction solution was dialyzed against distilled water for 48 h at 4°C and lyophilized.

Citraconylation was performed by the method of Brinegar and Kinsella (9). Five hundred milligrams of α_{s1}-CN were dispersed in 50 ml of .1 *M* sodium phosphate buffer (pH 8.0) containing 6 *M* urea. To the solution were gradually added 269 mg of citraconic anhydride (Nacalai Tesque Inc.), and the pH was maintained at 7.6 to 8.0 with 1.0 *M* NaOH until the reaction was complete. The solution was dialyzed against .01 *M* Tris·HCl buffer (pH 8.0) and lyophilized.

Preparation of Artificial Casein Micelles

Artificial casein micelles were prepared according to the method of Knoop et al. (13), with minor modification, to give a final casein concentration of 2.5%. The salt solutions used were .2 *M* $CaCl_2$, .2 *M* K_2HPO_4, and 1 *M* tripotassium citrate. Final total concentrations of calcium, phosphate, and citrate were 20 to 50, 17 to 27, and 10 m*M*, respectively.

HPLC

The HPLC was carried out at room temperature (22 to 25°C) with a Shimadzu LC-5A chromatograph (Shimadzu Ltd., Kyoto, Japan) equipped with a Shimadzu SPD-2A spectrophotometric detector using a TSK-GEL G4000SW column (7.5 mm × 60 cm; Toso Ltd., Tokyo, Japan) attached to a TSK-GEL guard column (7.5 mm × 7.5 cm; Toso Ltd.). Before analysis, 6 *M* urea-simulated milk ultrafiltrate (USMUF), which was prepared by the method described by Aoki et al. (4), was passed through the system at a flow rate of .5 ml/min for 3 h. To 1 ml of artificial casein micelle solution, .5 g of solid urea (specially prepared grade) and 2-mercaptoethanol (10 m*M*) were added. The solution was allowed to stand overnight at 25°C and then was filtered through a membrane filter (pore size, .45 μm) before injection. The injection volumes were 25 and 200 μl for analytical and preparative runs, respectively.

High Performance Ion-Exchange Chromatography

High performance ion-exchange chromatography on a TSK-GEL DEAE-5PW column (7.5 mm × 7.5 cm; Toso Ltd.) was carried out with a Toso CCPE chromatograph with a Toso UV-8000 spectrophotometric detector by the method described by Aoki et al. (6). The fractions separated by HPLC were concentrated to about 1% of casein concentration through a collodion bag (Sartorius SM 13200; Sartorius Ltd., Tokyo, Japan) and dialyzed against chromatography buffer (3.3 *M* urea, 20 m*M* imidazole, and .08 *M* NaCl) containing 20 m*M* 2-mercaptoethanol. The sample solution (80 μl) was subjected to chromatography. The elution was carried out by linearly increasing the concentration gradient of NaCl at a flow rate of .5 ml/min. The concentration of NaCl was increased from .08 to .28 *M* over 70 min.

Determination of Micellar and Urea-Insoluble Calcium and Phosphate

The nonmicellar components of the artificial casein micelle solutions were separated by UF using an Amicon Diaflo YM-10 UF membrane. The UF was obtained at a rate of 1 ml/min. The first 2 ml were discarded, and the next 1 ml was retained for analysis. The salt content of the micellar components was calculated from the contents in the total and UF fractions. The urea-insoluble fraction was separated by ultracentrifugation at 100,000 × *g* for 1 h at 25°C. The urea-insoluble salt content was calculated from the contents in the total and urea-soluble fractions and represented as the concentrations in the original artificial micelle solutions because the volume changed when solid urea was added to the casein micelle solutions. Calcium was determined with a Hitachi 208 atomic absorption spectrophotometer (Hitachi Ltd., Tokyo, Japan) for a solution containing the filtrate of a mixture of 1 volume of the sample solution and 9 vol of 12% TCA, 500 ppm of 1 La, and 1 *M* HCl. Inorganic P was determined for the TCA filtrate by the method of Allen (1).

RESULTS AND DISCUSSION

To determine the content of casein aggregates crosslinked by MCP, intact and modified α_{s1}-κ-CN micelles were prepared, and HPLC on a TSK-GEL G4000SW column was performed by using USMUF as the eluent. Figure 1 (A and B) shows the elution patterns of artificial casein micelles of intact and acetylated α_{s1}-κ-CN disaggregated by 6 *M* urea and 10 m*M* 2-mercaptoethanol, respectively. As described previously by Aoki et al. (4), fraction 1 consisted of casein aggregates crosslinked by MCP, but fraction 2 was composed of monomers of individual casein constituents. The first peak of fraction 1 of acetylated α_{s1}-κ-CN micelles eluted at the void volume of the column and remained on addition of EDTA, but, in the case of bovine native casein micelles, fraction 1 disappeared on addition of EDTA, as described previously by Aoki et al. (4). The acetylated α_{s1}-CN fraction obtained by the method of Fraenkel-Conrat (10) may contain the polymer formed by side reaction during amino group modification process. The relative percentages of fractions 1 and 2 were determined from the peak areas of the chromatogram. The content of casein aggregates crosslinked by MCP of acetylated α_{s1}-κ-CN micelles was calculated by subtracting the proportion of the first peak of fraction 1 obtained after addition of EDTA from that obtained before addition of EDTA.

Figure 2 shows the elution patterns of artificial casein micelles of succinylated α_{s1}-κ-CN and citraconylated α_{s1}-κ-CN. The peak at the void volume of the column, which is eluted before the fraction 1 of succinylated α_{s1}-κ-CN micelles, was not found after ultracentrifugation (Figure 2B). This peak was possibly caused by fine particles of calcium phosphate, because no casein was present in this peak. The content of casein aggregates that were crosslinked by MCP of succinylated α_{s1}-κ-CN micelles was calculated from fraction 1 after ultracentrifugation. No polymer was caused by side reaction during the processes of amino group modification in the succinylated and citraconylated caseins (Figure 2D).

The percentages of casein aggregates crosslinked by MCP of artificial casein micelles are shown in Table 1. The content of casein aggregates crosslinked by MCP in modified α_{s1}-κ-CN micelles was lower than those in intact α_{s1}-κ-CN micelles. As shown in Table 2, the amounts of casein aggregates crosslinked by MCP in succinylated α_{s1}-κ-CN micelles increased as the ratio of calcium to phosphate increased.

To determine the amounts of micellar calcium and phosphate, the artificial casein micelles were UF. The results are shown in Table 3. The amounts of micellar calcium and

TABLE 1. The content of fraction 1 (casein aggregates crosslinked by MCP) in artificial casein micelles of intact and modified α_{s1}-CN.

	Crosslinked casein	
α_{s1}-CN	20 m*M* Ca 17 m*M* P_i[1]	30 m*M* Ca 22 m*M* P_i
	(%)	
Intact	15.7	46.5
Acetylated	14.0	30.1
Succinylated	8.1	17.8
Citraconylated	5.1	17.5

[1]P_i = Inorganic phosphate.

phosphate in the artificial casein micelles that were formed by calcium and phosphate increased as calcium and phosphate concentrations increased. However, a part of calcium and phosphate in the succinylated α_{s1}-κ-CN micelles was insoluble in the urea solution, suggesting that urea-insoluble calcium phosphate did not participate in crosslinking the casein molecules. The amounts of micellar calcium and phosphate in the succinylated α_{s1}-κ-CN micelles were calculated by subtracting the amounts of urea-insoluble calcium and phosphate in the succinylated α_{s1}-κ-CN micelles. The amounts of micellar calcium and phos-

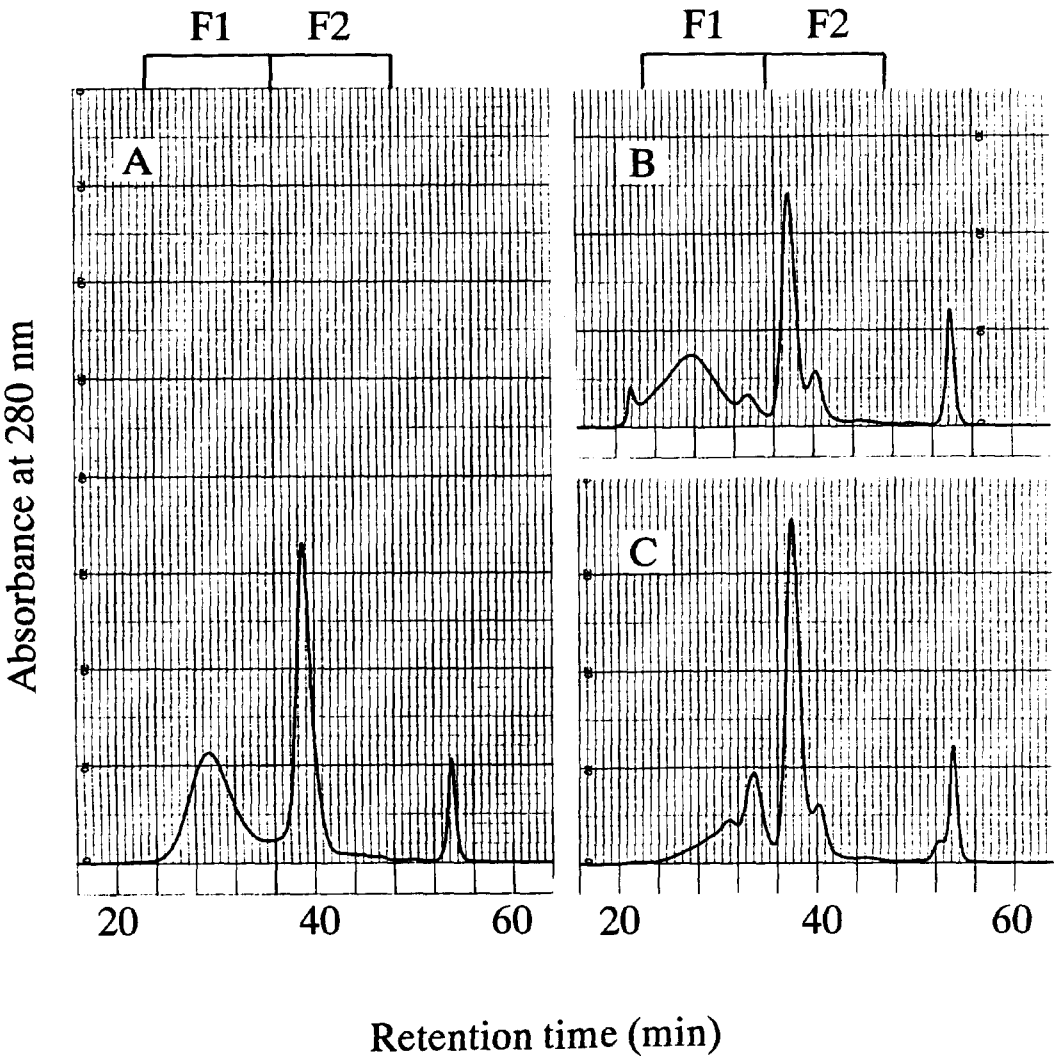

Figure 1. The HPLC patterns of artificial micelles of intact and acetylated α_{s1}-κ-CN disaggregated in 6 *M* urea. The weight ratio of α_{s1}- and κ-CN was 9:1. Calcium and inorganic phosphate concentrations were 30 and 22 *mM*. A) Intact α_{s1}-κ-CN micelles, B) acetylated α_{s1}-κ-CN micelles, and C) EDTA-treated acetylated α_{s1}-κ-CN micelles. F1, Fraction 1 consisted of casein aggregates crosslinked by MCP; F2, fraction 2 was composed of monomers of individual casein constituents. Column, TSK-GEL G4000SW (7.5 mm × 60 cm); flow rate, .5 ml/min; and eluent, 6 *M* urea-simulated milk ultrafiltrate.

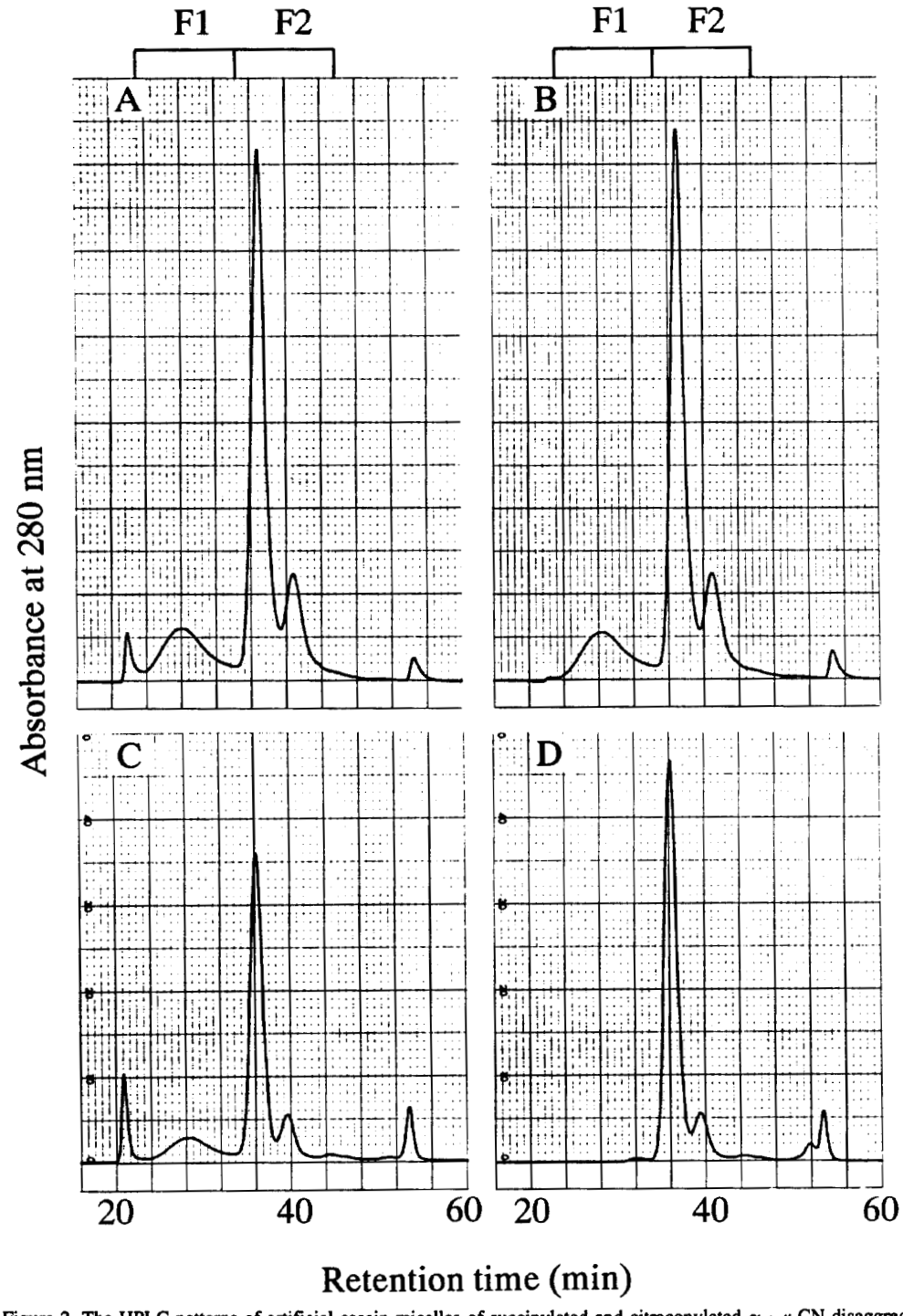

Figure 2. The HPLC patterns of artificial casein micelles of succinylated and citraconylated α_{s1}-κ-CN disaggregated in 6 *M* urea. The micelles were formed by 30 m*M* calcium and 22 m*M* inorganic phosphate. A) Succinylated α_{s1}-κ-CN micelles, B) succinylated α_{s1}-κ-CN micelles after ultracentrifugation, C) citraconylated α_{s1}-κ-CN micelles, and D) EDTA-treated citraconylated α_{s1}-κ-CN micelles. F1, Fraction 1 consisted of casein aggregates crosslinked by MCP; F2, fraction 2 was composed of monomers of individual casein constituents. Column, TSK-GEL G4000SW (7.5 mm × 60 cm); flow rate, .5 ml/min; and eluent, 6 *M* urea-simulated milk ultrafiltrate.

TABLE 2. The content of fraction 1 in artificial casein micelles of intact and succinylated α_{s1}-CN.

Ca	P_i[1]	Crosslinked casein	
		Intact	Succinylated
(m*M*)		(%)	
30	22	46.5	17.8
40	22	57.5	36.1
50	22	58.1	53.1

[1]P_i = Inorganic phosphate.

phate in the intact α_{s1}-κ-CN micelles were somewhat higher than those in the succinylated α_{s1}-κ-CN micelles. The amounts of urea-insoluble calcium and phosphate in the succinylated α_{s1}-κ-CN micelles decreased as the ratio of calcium and phosphate increased. The amounts of micellar phosphate in the intact α_{s1}-κ-CN micelles, formed by 30 m*M* calcium and 22 m*M* phosphate, and succinylated α_{s1}-κ-CN micelles, formed by 40 m*M* calcium and 22 m*M* phosphate, were 11.7 and 11.1 m*M*, respectively (Table 3). However, the contents of casein aggregates crosslinked by MCP in the intact α_{s1}-κ-CN micelles, formed by 30 m*M* calcium and 22 m*M* phosphates, and succinylated α_{s1}-κ-CN micelles, formed by 40 m*M* calcium and 22 m*M* phosphates, were 46.5 and 36.1%, respectively (Table 2). Thus, crosslinking of succinylated α_{s1}-CN by MCP needs larger amounts of calcium and phosphate than that of intact α_{s1}-CN.

To obtain further evidence for a role of amino groups in crosslinking by MCP, artificial casein micelles were prepared at 30 m*M* calcium and 22 m*M* phosphate with intact α_{s1}-, modified α_{s1}-, and κ-CN, of which the weight ratio was 4.5:4.5:1, and then HPLC was carried out. The contents of casein aggregates crosslinked by MCP were 39.1, 24.6, and 22.9% for intact α_{s1}-succinylated α_{s1}-κ-CN micelles, intact α_{s1}-acetylated α_{s1}-κ-CN micelles, and intact α_{s1}-citraconylated α_{s1}-κ-CN micelles, respectively.

To determine the relative casein composition of the casein aggregates crosslinked by MCP, high performance ion-exchange chromatography was carried out on a TSK-GEL DEAE 5PW column. Figure 3 shows the elution patterns of caseins from whole micelles and casein aggregates formed by crosslinking by MCP of intact α_{s1}-succinylated α_{s1}-κ-CN micelles. Quantitative determination of casein constituents was made by peak area measurement of the chromatogram. The proportions of the intact and modified caseins that were crosslinked by MCP were calculated from the content and casein composition of the crosslinked casein aggregates. As shown in Table 4, the proportions of the modified caseins crosslinked by MCP were lower than those of intact casein. The modified caseins were only slightly crosslinked by MCP, even in the intact α_{s1}-modified α_{s1}-κ-CN (4.5:4.5:1) micelles. These results indicate that amino group modified α_{s1}-CN is less crosslinked than the intact α_{s1}-CN.

Ter Horst (18) proposed the formation of a complex between the ϵ-NH_3^+ group of lysine in casein with calcium and phosphate; calcium is also bound to the carboxyl and organic phosphates in casein. Visser et al. (21) suggested

TABLE 3. Distribution of Ca and P_i[1] in intact and succinylated α_{s1}-κ-CN micelles.

Ca	P_i	Micellar Ca		Micellar P_i		Urea-insoluble P_i	
		Int[2]	Suc[3]	Int	Suc	Int	Suc
(m*M*)							
20	17	10.8	8.7	3.4	2.0	0	.8
30	22	20.8	14.3	11.7	5.0	0	4.4
40	22	29.2	24.3	14.8	11.1	0	2.2
50	22	37.1	33.3	18.0	15.3	0	1.2

[1]P_i = Inorganic phosphate.

[2]Int = Intact.

[3]Suc = Succinylated.

that the interaction between phosphate ions and α_s- or κ-CN or poly-L-lysine took place only when Ca^{2+} was present. Aoki et al. (7) confirmed that caseins were crosslinked through their ester phosphate groups by MCP. Phosphate groups are essential for crosslinking by MCP. However, when the amino groups of casein were modified, the content of the casein aggregates crosslinked by MCP became lower (Table 1). Furthermore, the content of casein aggregates crosslinked by MCP of succinylated α_{s1}-κ-CN micelles was lower than those of acetylated α_{s1}-κ-CN micelles (Table 1). Acetylation replaces the positively charged amino groups of lysine by neutral acetyl groups; succinylation changed the positively charged groups to negatively charged succinyl anionic groups. Succinylation causes larger changes in the electrostatic nature of proteins because of the elimination of some positively charged groups in conjunction with the introduction of the negatively charged succinyl groups. The content of casein aggregates crosslinked by MCP of succinylated α_{s1}-κ-CN micelles was lower probably because of increased negative repulsive forces. Our results suggest that amino groups also participate indirectly in crosslinking of casein by MCP.

TABLE 4. Partition of intact and modified α_{s1}-CN in micellar calcium phosphate-crosslinked casein.

Type of micelles	Intact	Modified
	(%)	
Intact α_{s1}-acetylated α_{s1}-κ-CN	28.2	21.0
Intact α_{s1}-succinylated α_{s1}-κ-CN	47.9	21.8
Intact α_{s1}-citraconylated α_{s1}-κ-CN	34.5	20.4

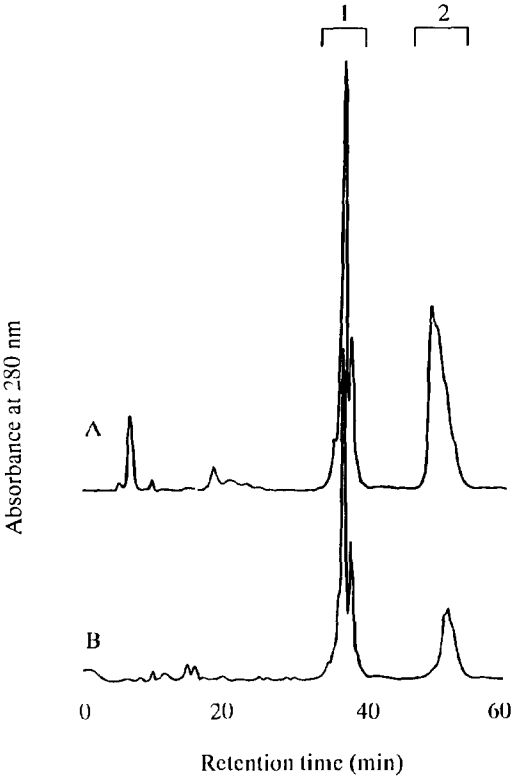

Figure 3. High performance ion-exchange chromatographic patterns of intact α_{s1}-succinylated α_{s1}-κ-CN micelles and casein aggregates crosslinked by MCP separated by HPLC of intact α_{s1}-succinylated α_{s1}-κ-CN micelles. The weight ratio of intact and succinylated α_{s1}-CN and κ-CN was 4.5:4.5:1. Calcium and inorganic phosphate concentrations were 30 and 22 m*M*. A) Intact and succinylated α_{s1}-κ-CN micelles, and B) casein aggregates crosslinked by MCP. 1) Intact α_{s1}-CN and 2) succinylated α_{s1}-CN. Column, TSK-GEL DEAE-5PW (7.5 mm × 7.5 cm); flow rate, .5 ml/min; buffer, 20 m*M* imidazole (pH 8.0), 3.3 *M* urea, and .08 *M* NaCl; and gradient, .08 to .28 *M* NaCl for 70 min.

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