A Modified Procedure for Caseinophosphopeptide Analysis

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Abstract

A modified procedure is established for analyzing caseinophosphopeptides. The sodium caseinate hydrolysate is first treated by immobilized metal ion affinity chromatography to enrich the phosphopeptides. Because of the formation of Fe^{3+}–peptide complexes, ethylenediaminetetraacetic acid is added to the bound fraction eluted with the immobilized metal ion affinity chromatography to disintegrate the complexes. Thus, the subsequent high-performance liquid chromatographic analysis is facilitated. A stepwise gradient elution is also suggested to enhance the resolution of caseinophosphopeptides during high-performance liquid chromatographic analysis.

Introduction

Caseinophosphopeptides (CPPs) formed during tryptic hydrolysis of casein increase the bioavailability of calcium (1,2), help prevent tooth enamel demineralization (3), and are promising additives for toothpaste and foods (4). Commercial CPP products are available in Japan. Because of the increasing commercial applications of CPPs, a simple and reliable analytical method is required to determine the identity and purity of the product.

Analyzing CPPs requires a prepurification step followed by high-performance liquid chromatography (HPLC) (5,6). Immobilized metal ion affinity chromatography (IMAC) is the method frequently used for prepurification (7,8). The IMAC procedure uses a metal ion (Fe^{3+}) as a ligand to bind with the phosphate groups on CPPs. Because of the strong binding between CPPs and Fe^{3+}, metal ions are often pulled from the affinity support by the CPP during elution and subsequently form a Fe^{3+}–CPP complex that causes the effluent to become slightly yellowish in color. The intensity of the yellow color is enhanced after a subsequent evaporation–concentration process. The effect of the Fe^{3+}–CPP complex on the analysis of CPPs with HPLC has not yet been reported.

After prepurification, CPPs are often separated with HPLC and then characterized by amino acid sequence analysis. Good resolution during HPLC is essential for satisfactory CPP analysis. However, simple isocratic and even linear gradients during HPLC analysis often fail to achieve a distinct resolution (5,6,8,9). A stepwise gradient is apparently required during analysis; however, to our knowledge, there is no literature on this topic.

To establish a proper procedure for CPP analysis, we attempted to more thoroughly understand the effect of the Fe^{3+}–CPP complex formed during IMAC separation on HPLC analysis and to propose a method to eliminate this problem. In addition, a proper stepwise gradient is established to obtain satisfactory resolution during HPLC analysis of CPPs.

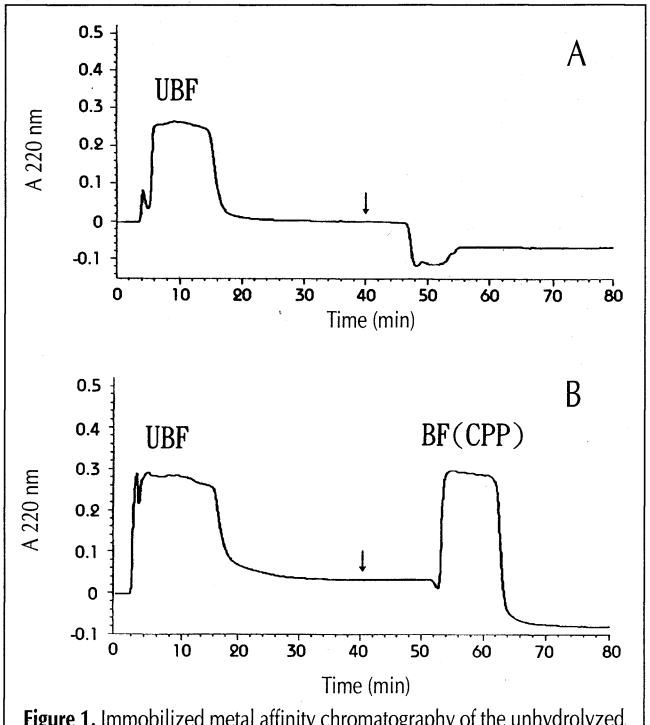

Figure 1. Immobilized metal affinity chromatography of the unhydrolyzed sodium caseinate (A) and the tryptic hydrolysate of sodium caseinate (B) on Fe^{3+}-chelating sepharose. Eluents: A, 0.1M sodium acetate (pH 5.0); B, 0.05M triethylammonium hydrogen carbonate (pH 8.0). Flow rate was 1 mL/min. The arrow indicates the time at which eluent A switches to eluent B. UBF indicates the unbound fraction and BF indicates the bound fraction, which was the caseinophosphopetide (CPP).

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Experimental

Materials

Sodium caseinate (Alanate 180) was obtained from New Zealand Dairy Board (Wellington, New Zealand). Porcine trypsin (EC3,4,21,4,Type IX, 13000-20000 BEAE unit/mg protein) and triethylammonium hydrogen carbonate were purchased from Sigma (St. Louis, MO). Chelating sepharose (fast flow) gel was obtained from Pharmacia (Uppsala, Sweden), and $FeCl_3$ was obtained from Ferak (Berlin, Germany).

Trifluoroacetic acid (TFA) was purchased from Pierce (Rockford, IL), and acetonitrile was obtained from Alps Chemical (Taipei, Taiwan).

Hydrolysis of sodium caseinate by trypsin

The hydrolysis was performed by mixing 50 mL of sodium caseinate (2×10^{-2} g/mL in 2mM Tris–HCl buffer, pH 7.6) with 50 mL of trypsin (2×10^{-4} g/mL in 2mM Tris-HCl buffer, pH 7.6) and incubating at 37°C for 24 h. After the reaction was complete, the mixture was heated at 70°C for 20 min to inactivate the enzyme. The mixture was adjusted to pH 4.6 and filtered through a Whatman No. 41 filter paper (Maidstone, England). The filtrate was concentrated at approximately 40°C in a rotary evaporator to one-eighth of its initial volume and later used as the sample for CPP analysis.

Prepurification of CPPs by IMAC

Purification of CPPs was conducted according to the previous studies (5–7) with modification. The chelating sepharose gel hydrated in deionized water was packed in a column (1-cm i.d.) to a height of 6.4 cm (gel volume, approximately 5.0 mL) and washed at a flow rate of 1 mL/min with 0.1M sodium acetate buffer (pH 3.0) for 20 min. A 20mM $FeCl_3$ (in 0.01N HCl) solution was then applied to the column at a flow rate of 1 mL/min until the effluent turned yellow. After unbound metal ions were washed off with sodium acetate (0.1M; pH 3.0; flow rate, 1 mL/min for 50 min), the column was equilibrated with sodium acetate (0.1M; pH 5.0; flow rate, 1 mL/min for 20 min). Then 1 mL of the concentrated hydrolysate sample was applied to the column.

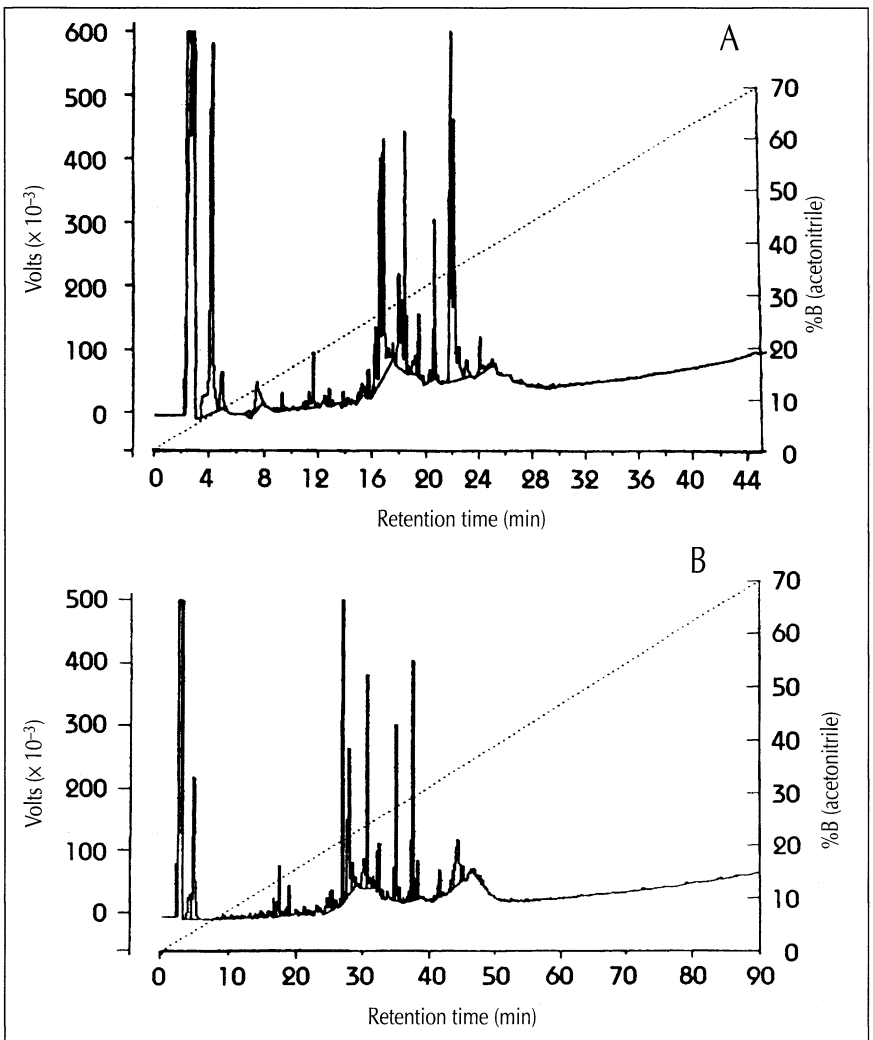

Figure 2. High-performance liquid chromatographic elution patterns of the bound fraction obtained by immobilized metal affinity chromatography of the sodium caseinate hydrolysate. Proteolysis was by trypsin (enzyme/substrate = 1/100, 24 h) and was monitored at 220 nm. The peptides were eluted at room temperature using a linear gradient from 0% A (0.1% trifluoroacetic acid [TFA] in water) to 70% B (0.1% TFA in acetonitrile) in 45 min (A) and 90 min(B). The dotted line indicates the %B gradient.

The elution of nonphosphorylated peptides (unbound fraction) was carried out with 0.1M sodium acetate buffer (pH 5.0) at a flow rate of 1 mL/min for 20 min. The phosphorylated peptides (bound fraction) were eluted with 0.05M triethylammonium hydrogen carbonate (pH 8.0) at a flow rate of 1 mL/min for 40 min. After each experiment, the chelating sepharose gel was regenerated with 100mM ethylenediaminetetraacetic acid (EDTA) that contained 1M NaCl and then washed with deionized water. The metal-free column was stored at room temperature and charged with the metal ion immediately before use.

HPLC analysis

The bound fraction (approximately 40 mL) from IMAC was concentrated to 1 mL at 40°C in a rotatory evaporator. After filtration through a 0.45-µm filter (Gelman Sciences, Ann Arbor, MI), the filtrate was analyzed by HPLC. The HPLC system (ICI Victoria, Australia) consisted of two pumps (LC 1100) and a variable wavelength UV detector (LC 1200). The separation was performed on a LiChrospher 100 RP-18 column (250 × 4-mm i.d., 5-µm particle size) from Merck (Darmstadt, Germany). Aliquots of 20 µL were injected and eluted at a flow rate of 1 mL/min with a mobile phase composed of deionized water that contained 0.1% TFA (denoted as A) and acetonitrile that contained 0.1% TFA (denoted as B). The linear gradient was from 100% A–0% B after injection to 30% A–70% B at 45 min. The CPPs were also eluted by a stepwise gradient from 100% A–0% B initially to 82.9% A–17.1% B at 9 min, 79.9% A–20.1% B at 18 min, 74.3% A–25.7%

B at 25 min, 65.8% A–34.2% B at 36 min, and 30% A–70% B at 45 min.

Peptide sequence analysis

The HPLC fractions were collected manually and dried with a Speed-Vac evaporator (Savant, France). The dried fraction was redissolved in 24 µL deionized water, injected into the HPLC system, collected, and dried again to ensure the purity of the peptide fraction's. The purified and dried sample was dissolved in 100 µL 0.1% TFA, loaded on a fibro filter (Applied Biosystems, Norwalk, CT), and dried with N_2 after the addition of 25 µL of biobrene. The sample on the fibro filter was analyzed with a protein sequencer (Model 477A, Applied Biosystems) and an amino acid analyzer (Model 120A PTH, Applied Biosystems).

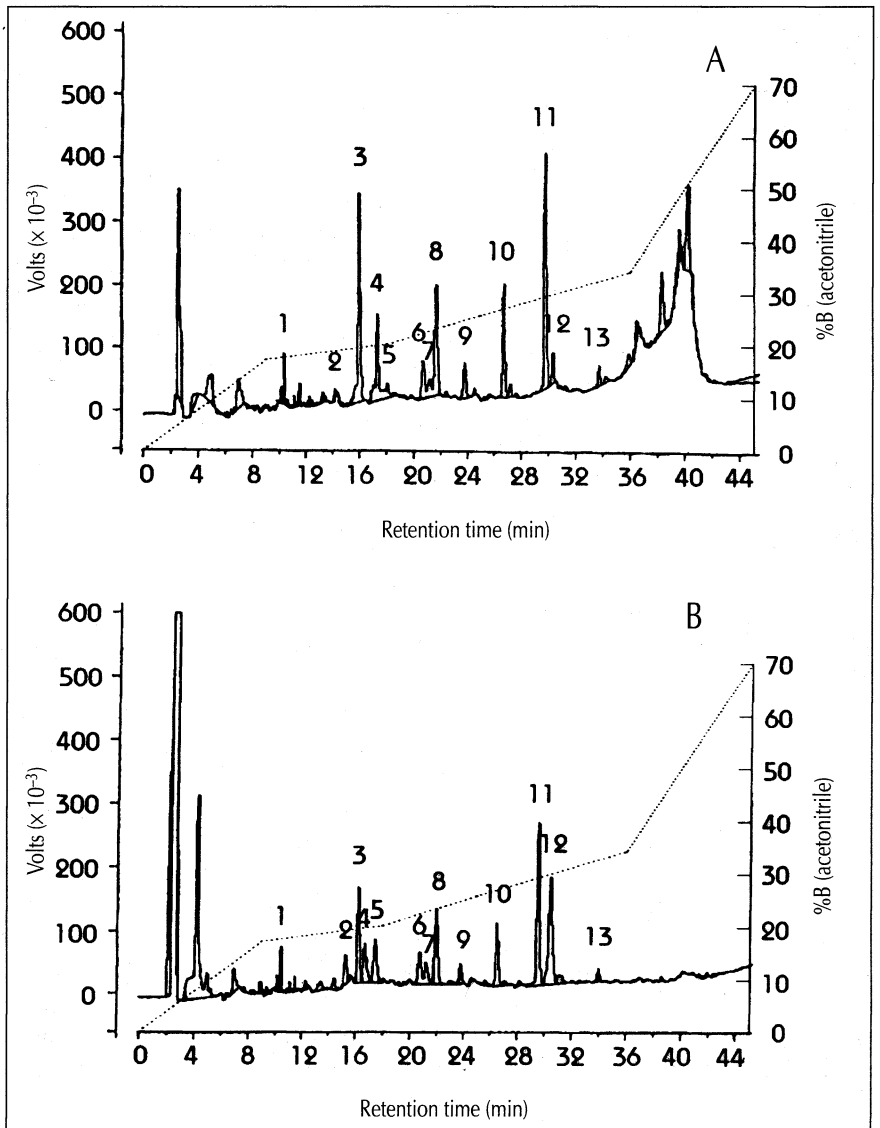

Figure 3. High-performance liquid chromatographic elution patterns of the bound fraction obtained by immobilized metal affinity chromatography of the sodium caseinate hydrolysate: A, the bound fraction without ethylenediaminetetraacetic acid (EDTA); B, the bound fraction with EDTA. Proteolysis was by trypsin (enzyme/substrate = 1/100, 24 h) and was monitored at 220 nm. The peptides were eluted at room temperature using a stepwise linear gradient from A (0.1% trifluoroacetic acid [TFA] in water) to 17.1% B (0.1% TFA in acetonitrile) in 9 min, 20.1% B at 18 min, 25.7% B at 25 min, 34.2% B at 36 min, and 70% B at 45 min.

Results and Discussion

Figure 1 shows the IMAC chromatograms of the unhydrolyzed sodium caseinate and the sodium caseinate hydrolysate. Only the hydrolysate revealed a bound fraction, which indicated that the Fe^{3+}-chelating sepharose could only interact with the peptides, presumably the phosphate groups of the CPP. It was also observed that the effluent collected from unhydrolyzed sodium caseinate was clear and colorless.

However, the effluent of the sodium caseinate hydrolysate showed a slightly yellowish color; the yellow color intensified after concentration by evaporation. This indicated that some Fe^{3+} ions were pulled off from the sepharose by the CPP. The Fe^{3+} ion in the bound fraction might interfere with the subsequent HPLC analysis.

A linear gradient is generally used for the HPLC analysis of CPPs. Figure 2A shows the typical HPLC chromatogram of CPPs obtained with a linear gradient. This figure reveals that many peaks of the CPPs were clustered. The resolution was poor, thereby affecting the identification of the CPPs. Decreasing the rate of the gradient not only prolonged the analytical time but also did not significantly improve the resolution (Figure 2B).

A stepwise linear gradient procedure was used for the HPLC analysis of CPPs. The gradient "step" could be determined as follows: (*a*) The peaks of the linear gradient HPLC chromatogram are classified into several groups, and the initial retention time (t_R) of each group is recorded. (*b*) The gradient is changed at any time during linear gradient elution by adjusting the pumping speed for solvents A and B. The time elapsed from changing the gradient to shifting the position of a certain peak as compared with its position during linear gradient elution is recorded as the response time (D_t), which is the delay between the formation of the gradient in the pump system and the elution of the sample from the column. (*c*) The point at which the concentration of acetonitrile (%B) should be shifted from a linear increment is determined by the following equation: $\%B = M(t_R - D_t)$, where M is the slope of %B versus time in the linear gradient.

According to these procedures, the peaks of the HPLC chromatogram that resulted from linear gradient elution were divided into three groups: t_{R1}, which was 15 to approximately 16.9 min; t_{R2}, which was 16.9 to approximately 20.5 min; and t_{R3}, which was 20.5 to approximately 26 min. The experimental results indicated that D_t was equal to 4 min under our HPLC operating conditions. Therefore, based on the original

Table I. Amino Acid Sequences of Caseinophosphopeptides (CPPs)

Peak no.*	Primary structure of the CPP
1	α s2 (126–136) casein: Glu-Gln-Leu-***SerP***-Thr-*SerP*-Glu-Glu-Asn-Ser-Lys
3	β (33)–48) casein: Phe-Gln-***SerP***-Glu-Glu-Gln-Gln-Gln-Thr-Glu-Asp-Glu-Leu-Gln-Asp-Lys
8	α s1 (43–58) casein: Asp-Ile-Gly-***SerP***-Glu-***SerP***-Thr-Glu-Asp-Gln-Ala-Met-Glu-Asp-Ile-Lys
9	α s2 (138–149) casein: Thr-Val-Asp-Met-Glu-***SerP***-Thr-Glu-Vla-Phe-Thr-Lys
10	α s1 (106–119) casein: Val-Pro-Gln-Leu-Glu-Ile-Val-Pro-Asn-***SerP***-Ala-Glu-Glu-Arg
11	α s1 (104–119) casein: Tyr-Lys-Val-Pro-Gln-Leu-Glu-Ile-Val-Pro-Asn-***SerP***-Ala-Glu-Glu-Arg
12	β (2–25) casein: Glu-Leu-Glu-Glu-Leu-Asn-Val-Pro-Gly-Glu-Ile-Val-Glu-***SerP***-Leu-***SerP-SerP-SerP***-Glu-Glu-Ser-Ile-Thr-Arg

* Peaks as seen in Figure 3.

linear gradient (slope = 70% B/45 min), the stepwise gradient should be started at %B = 70/45 × (15 – 4) = 17.1%, 70/45 × (16.9 – 4) = 20.1%, and 70/45 × (20.5 – 4) = 25.7%. The third period of the gradient should be ended at 26 min, that is, %B = 70/45 × (26 – 4) = 34.2%. After the third period of gradient elution, all of the CPP peaks should be resolved. The gradient could be increased from 34.2% B to 70% B in a short period of time (e.g., 9 min) to remove the non–CPP components from the column. Some preliminary experiments are necessary to determine the time interval required for completing each step of the gradient. According to the stepwise gradient conditions already described, Figure 3A shows the HPLC chromatogram for the CPPs. Overall the resolution has improved markedly, thereby making the identification of various CPPs much easier. In addition, collecting each HPLC fraction for further analysis would be much easier.

Figure 3A, however, showed that at a retention time of approximately 40 min, a large cluster of compounds appeared that could not be resolved. This low polarity compound was suspected to be a group of peptides complexed with the Fe^{3+} ion. To inhibit this possible Fe^{3+}–peptide complex formation, we added 2.5 mL of 0.1M EDTA to the bound fraction (approximately 20 mL) from IMAC before concentration. When the EDTA-added bound fraction was analyzed by stepwise gradient HPLC, not only did the cluster around t_R = 40 min disappear but the peaks 2, 5, 7, and 12 also became more distinguishable. Part of the CPPs seemed to form a complex with Fe^{3+} and made the HPLC separation difficult. Adding EDTA disintegrated the complex, thereby facilitating the HPLC analysis significantly.

Table I shows the identities of some CPPs on the HPLC chromatogram. It was found that the CPPs were produced from α s1, α s2, and β-casein by mapping the amino acid sequence. Both multiphosphorylated peptides (peaks 1, 8, and 12) and monophosphorylated peptides (peaks 3, 9, 10, and 11) were recovered by IMAC and separated by HPLC.

Conclusion

CPPs produced from tryptic hydrolysis of sodium caseinate can be analyzed by IMAC and then HPLC. The CPPs in the hydrolysate first should be enriched by IMAC. Because the CPPs may pull the Fe^{3+} ions from the Fe^{3+}-chelated sepharose gel and form Fe^{3+}–CPP complexes, EDTA should be added to the sample solution to disintegrate the complexes before HPLC analysis. A proper stepwise gradient is required to obtain satisfactory separation of CPPs during HPLC analysis.

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