Separation of Calcium-binding Protein Derived from Enzymatic Hydrolysates of Cheese Whey Protein

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ABSTRACT : This study was carried out to separate the calcium-binding protein derived from enzymatic hydrolysates of cheese whey protein. CWPs (cheese whey protein) heated for 10 min at 100°C were hydrolyzed by trypsin, papain W-40, protease S, neutrase 1.5 and pepsin, and then properties of hydrolysates, separation of calcium-binding protein and analysis of calcium-binding ability were investigated. The DH (degree of hydrolysis) and NPN (non protein nitrogen) of heated-CWP hydrolysates by commercial enzymes were higher in trypsin than those of other commercial enzymes. In the result of SDS-PAGE (sodium dodecyl sulphate polyacrylamide gel electrophoresis), β-LG and α-LA in trypsin hydrolysates were almost eliminated and the molecular weight of peptides derived from trypsin hydrolysates were smaller than 7 kDa. In the RP-HPLC (reverse phase HPLC) analysis, α-LA was mostly eliminated, but β-LG was not affected by heat treatment and the RP-HPLC patterns of trypsin hydrolysates were similar to those of SDS-PAGE. In ion exchange chromatography, trypsin hydrolysates were shown to peak from 0.25 M NaCl and 0.5 M NaCl, and calcium-binding ability is associated with the large peak, which was eluted at a 0.25 M NaCl gradient concentration. Based on the results of this experiment, heated-CWP hydrolysates by trypsin were shown to have calcium-binding ability. ***(Asian-Aust. J. Anim. Sci. 2004. Vol 17, No. 5 : 712-718)***

Key Words : Cheese Whey Protein, Calcium-binding Protein, Enzymatic Hydrolysates

INTRODUCTION

Milk and dairy products are widely recognized as an excellent source of calcium in the diet and an adequate calcium intake is necessary for the maintenance of bone health (Miller, 1989). It has been shown that consumption of high concentrations of calcium in early life contributes to development of maximal bone density, which in turn can prevent osteoporosis in later life (Buttriss, 1990; Renner, 1994). Milk has a functional role in growth of newborn animals, and so milk whey protein possibly have components that affect bone metabolism (Takada et al., 1997). Milk proteins have two major protein groups, about 80% casein and 20% whey protein (Fox, 1992; McIntosh et al., 1998). Whey is the soluble fraction of milk, rich in proteins, minerals and lactose that are separated from casein during the manufacture of cheese or casein (de la Fuente et al., 2002). Whey proteins in bovine milk consist of β-lactoglobulin (β-LG), α-lactalbumin (α-LA), immunoglobulin (Ig), bovine serum albumin (BSA) and other trace elements (Fox, 1992).

Proteolytic hydrolysates attracted lot of interest over several years for their potential functionality and their prospective uses as ingredients in food, pharmaceutical and cosmetic products (Chobert et al., 1988; Marshall, 1994; Anon, 1998). Enzymatic hydrolysates of whey proteins were found to influence their functional properties (McIntosh et al., 1998; FitzGerald and Meisel, 1999) and to improve their physical and chemical characteristics (Kilara, 1985; Guo et al., 1995; Bertrand-Harb et al., 2002). Several biologically active peptides were identified in the enzymatic hydrolysates of various forms of milk protein, for example, opioid peptide (Drewnowski, 1992), ACE-inhibitory peptide (Mullally et al., 1997), antithrombotic peptide (Jolles et al., 1993), immunomodulatory peptide (Ragno et al., 1993), anticarcinogenic peptide (Gallaher and Schmidl, 1998) and mineral carrier peptide (Reynolds, 1997; Oukhatar et al., 2000).

Recently, among the biologically active peptides derived from milk protein, casein phosphopeptide (CPP) are formed *in vivo* following digestion of milk casein by gastrointestinal proteinases. CPP with phosphoseryl residual, which accelerates the absorption of calcium in the intestine have already found interesting applications in functional food, dairy products and pharmaceutical products as an additive for the therapeutical formula and prevention of bone disease (Meisel and Schlimme, 1996; Reynolds, 1997; Oukhatar et al., 2000). Also, whey protein components, α-LA possesses a strong calcium-binding site at Lys79, Asp82, 84, 87 and 88 (Veprintsev et al., 1999; Noyelle and van Deal, 2002). However, the calcium-binding ability of other whey protein components has still not been identified.

The objective of this study was to isolate the calcium-

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binding protein derived from enzymatic CWP hydrolysates.

MATERIALS AND METHODS

Enzymes and reagents

Fresh Holstein milk was obtained from a Gyeongsang National University livestock farm. Manufacture of cheddar cheese was performed according to the methods of Kosikowski (Kosikowski, 1982), and cheese whey protein (CWP) was separated in the cheese process. Defatted and demineralized CWP was prepared by ultracentrifugation (Supra 25K, Hanil Sci., Korea) and dialysis (Sigma Co., USA). After pH adjusting, CWP was heat-treated for 10 min at 100°C.

Trypsin (Bovine pancreas, activity 3.3 Anson units g^{-1} protein) and Neutrase 1.5 (*Bacillus subtilis*, activity 1.5 Anson units g^{-1} protein) were from Novo Nordisk A/S (Bagsvaerd, Denmark), Protease S (*Bacillus stearothermophilis*, activity 10,000 units g^{-1} protein) and Papain W-40 (*Carica papara* L., activity 400,000 units g^{-1} protein) were from Amano Enzymes (Japan). Pepsin (Porcine gastric mucosa, activity 0.8-2.5 units g^{-1} protein) was purchased from Sigma Chemical Co. (USA).

BSA, TNBS (trinitrobenzensulfonic acid) and calcium determination kits were purchased from Sigma Chemical Co. (USA). All other reagents were of an analytical grade.

Preparation of hydrolysates

Hydrolysis of heated-CWP was determined from methods described by Adamson and Reynolds (1996). After heating, CWP was adjusted to pH 8.0 and pH 2.0 by the addition of 0.5 N NaOH and 0.5 N HCl, respectively. Commercial food-grade enzymes preparations, dissolved in distilled water, were added to the reaction mixture at the ratio of 1:100 (enzyme:substrate, w/w, protein basis). The pH of the reaction mixture was maintained at a constant through the continuous addition of 0.5 N NaOH using a pH-stat (Metrohm Ltd., Herisan, Switzerland). During hydrolysis, samples were withdrawn after 15, 30, 60, 90, 120, 180 and 240 min and the enzyme was inactivated by heating it for 10 min at 90°C. Hydrolysates were then removed precipitate and stored at -20°C.

Determination of degree of hydrolysis (DH) and non-protein nitrogen pattern (NPN)

The DH for all enzymatic hydrolysates was determined according to Adler-Nissen (1986) and the NPN amount was determined by the Lowry et al. (1951) method.

Sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE)

SDS-PAGE was carried out according to Laemmli (1970). The separating gel was 15% (w/v) acrylamide of pH 8.8 and the stacking gel was 3% (w/v) acrylamide of pH 6.8. Gels were stained with 0.2% (w/v) commassie brilliant blue R-250 in acetic acid/methanol/H_2O (1:1:5, v/v/v) and distained in acetic acid/methanol/H_2O (1:3:6, v/v/v) solution.

Reverse phase high performance liquid chromatography (RP-HPLC)

Peptides in heated-CWP hydrolysates were analyzed by RP-HPLC on a Nucleosil (Nucleosil C_{18} 5 Micron, Alltech Associates, Inc., USA) C_{18} column (250×4.6 mm), equilibrated with solvent A [0.1% trifluoroacetic acid (TFA) in H_2O] and eluted with a linear gradient to solvent B (0.1% TFA in acetonitrile) for 40 min. Runs were conducted at room temperature using a Dionex HPLC system (ASI 100, Dionex Co., USA) at a flow rate of 1.0 ml min^{-1} and the absorbance of the column elute was monitored at 214 nm. The injection volume was generally 10 μl and the concentration of peptide material applied was approximately equivalent to 0.5 mg protein ml^{-1}. All samples were filtered through a 0.2 μm syringe filters prior to application to the C_{18} column.

Selective precipitation of calcium-binding protein

The calcium-binding protein was precipitated from the tryptic hydrolysates by the addition of 10% (w/v) calcium chloride to reach a final concentration level of 100 mM and a 50% (v/v) final concentration level for ethanol. The suspension was centrifuged (12,000×g) and the supernatant discarded. The precipitate was dried and stored at -20°C.

Separation of calcium-binding protein

Separation of calcium-binding protein in tryptic hydrolysates was adapted from the method of Rose et al. (1969). DEAE-cellulose (Whatman DE 52) was equilibrated in 500 ml of 20 mM Tris-HCl buffer pH 7.8 and slurry of equilibrated DEAE-cellulose was packed in a glass column (20×2.5 cm). The tryptic hydrolysates was dissolved in the same buffer (pH 7.8) and loaded, and then eluted by a step gradient with the same buffer containing 0.25, 0.5, 0.75 and 1 M NaCl. The flow rate was 3 ml min^{-1}, fraction volume was 15 ml $tube^{-1}$ and elutes was monitored at 280 nm. The injection volume was 40 ml, containing about 200 mg of protein. Samples were filtered through 0.5 μm syringe filters prior to application to the column.

Determination of calcium contents and calcium-binding ability

Calcium was quantified throughout by using specific calcium colorimetric determination kits. The calcium content of fractions was determined using an o-cresolphthalein complexone calcium detection reagent (Sigma Co., USA). One mL of the reagent was added to 10

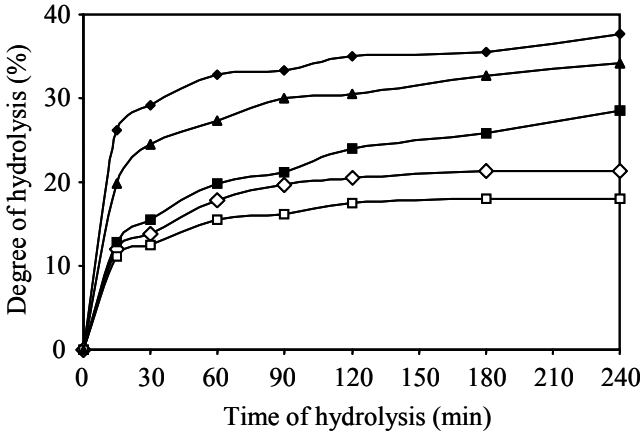

Figure 1. DH (Degree of hydrolysis) of heated-CWP by commercial proteases. Legend: trypsin (♦), Papain W-40 (◊), protease S (■), neutrase 1.5 (□) and pepsin (▲).

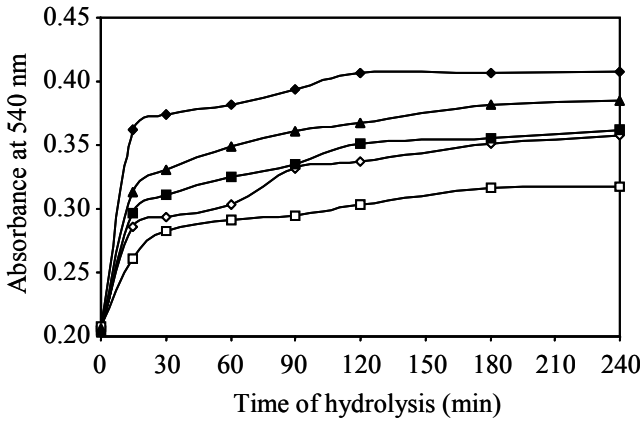

Figure 2. NPN production during hydrolysis of heated-CWP by commercial proteases. Legend: trypsin (♦), Papain W-40 (◊), protease S (■), neutrase 1.5 (□) and pepsin (▲).

μl of the sample in a disposable plastic cuvette, mixed and the absorbance of the solution was read at 575 nm on spectrophotometer (Perkin Elmer Lambda EZ 201, USA). The calcium content was determined by a calcium/phosphorous combined standard solution (0.1 mg ml^{-1}). All determinations were carried out at least five times. Calcium concentrations were expressed in mg ml^{-1}.

The calcium-binding abilities of tryptic hydrolysate fractions were determined by Ion chromatography on an IonPac CS12A column. Runs were conducted at room temperature using a Dionex IC system (DX120, Dionex Co., USA) at a flow rate of 1.0 ml min^{-1} and eluted by 18 mM of methane-based sulfuric acid. The injection volume was 25 μl and six cations (DIONEX P/N 43162) was used as a standard. All samples were filtered through a 0.2 μm syringe filters prior to application to the column. The calcium-binding abilities of tryptic hydrolysate fractions were expressed as mM of calcium bound to mg of protein.

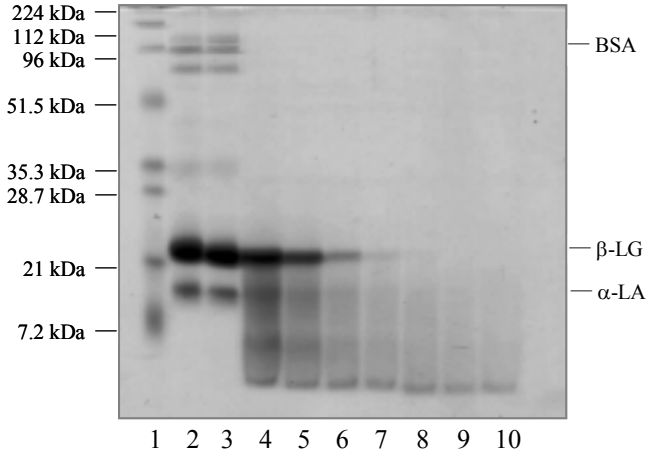

Figure 3. SDS-PAGE patterns of heated-CWP hydrolysates by trypsin at 50℃ for 15, 30, 60, 90, 120, 180 and 240 min (lanes 4-10). Lane 1, Standard broad range marker (Bio-Rad, USA): myosin (224 kDa), β-galactosidase (112 kDa), bovine serum albumin (96 kDa), ovalbumin (51.5 kDa), carbonic anhydrase (35.3 kDa), soybean trypsin inhibitor (28.7 kDa), lysozyme (21 kDa) and aprotinin (7.2 kDa); lane 2, Standard bovine whey (Sigma Co., USA); lane 3, Heated-CWP (10 min at 100°C).

Protein determination

Protein concentration in the commercial enzyme preparations, hydrolysates and fractions were determined by the dye-binding method of Bradford (1976). BSA was used as the standard.

RESULTS AND DISCUSSION

DH and NPN

The degree of hydrolysis of heated-CWP by commercial enzymes is shown in Figure 1. The DH of heated-CWP enzymatic hydrolysates ranged from 18.0 to 37.7%, depending on the specificity of enzymes. The DH of all enzymes were rapidly increased during the 30 min of beginning, and then gradually increased in the latter reaction time. Of all the hydrolyzed enzymatic hydrolysates, trypsin showed the highest DH, 37.7%, by 240 min. More explanation is needed for that CWP has more trypsin specific site than other enzymes' site (Monti and Jost, 1978).

Figure 2 shows the NPN amount of heated-CWP during the hydrolysis by commercial enzymes. Trypsin showed the highest NPN amount. The NPN patterns of commercial enzymes were similar to those of DH patterns.

Change of CWP during digestion in SDS-PAGE

The electrophoretic patterns of heated-CWP by commercial enzymes were shown in Figure 3 and 4. Figure 3 shows electrophoretic pattern of heated-CWP by trypsin. The three major bands of native CWP were observed as BSA, β-LG and α-LA (Figure 3, lane 2). These three bands were still visible after heat treatment, but they were weakly

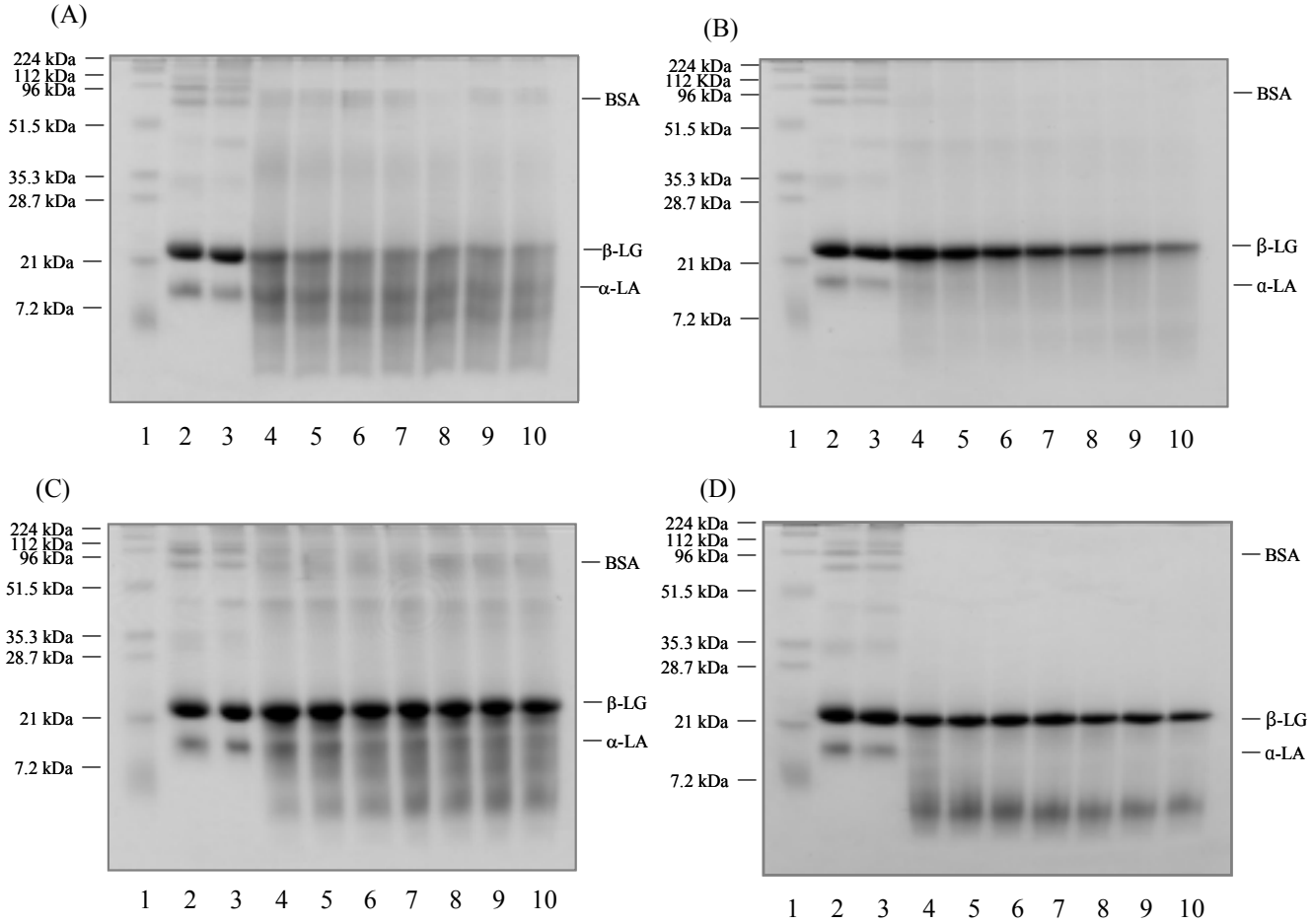

Figure 4. SDS-PAGE patterns of heated-CWP hydrolysates by commercial enzymes at 50°C for 15, 30, 60, 90, 120, 180 and 240 min (lanes 4-10). Lane 1, Standard broad range marker (Bio-Rad, USA): myosin (224 kDa), β-galactosidase (112 kDa), bovine serum albumin (96 kDa), ovalbumin (51.5 kDa), carbonic anhydrase (35.3 kDa), soybean trypsin inhibitor (28.7 kDa), lysozyme (21 kDa) and aprotinin (7.2 kDa); lane 2, Standard bovine whey (Sigma Co., USA); lane 3, Heated-CWP (10 min at 100°C). (A) papain W-40, (B) protease S, (C) neutrase 1.5 and (D) pepsin.

stained (Figure 3, lane 3). This is in accordance with the observations of Guo et al. (1995) who reported that β-LG heated for 5-10 min at 90-100°C enhanced the extent of proteolysis by trypsin. After 120 min of hydrolysis by trypsin, these three bands almost disappeared (Figure 3, lane 8). Also, the optimum reaction time was 180 min and the molecular weight of peptides derived from tryptic hydrolysates was smaller than 7 kDa (Figure 3, lane 9). This result was in accordance with those of Jost and Monti (1977) who reported an effective increase in the percentage of water-soluble nitrogen in whey protein by trypsin hydrolysis.

Figure 4 shows the electrophoretic patterns of heated-CWP by other commercial enzymes except trypsin. As illustrated in Figure 4A, three major bands of heated-CWP were slightly hydrolyzed by papain W-40. These results do not agree with those of Otte et al. (1997) who reported rapid hydrolysis of β-LG by papain. By the use of protease S, BSA and α-LA were rapidly hydrolyzed, and β-LG was gradually hydrolyzed during reaction times (Figure 4B). In the hydrolysis by neutrase 1.5, BSA and β-LG were mostly not hydrolyzed, but α-LA was hydrolyzed (Figure 4C). BSA was quite susceptible to pepsin and was completely degraded within the first 15 min of hydrolysis. α-LA was also no longer visible in the gel after about 30 min of hydrolysis. But β-LG was much less affected by pepsin, showing only a small reduction during the hydrolysis (Figure 4D). These results was agreed with those of Schmidt and van Markwijk (1993).

From the results of mentioned above, trypsin was observed as the most suitable enzyme to produce the hydrolysates for separation of calcium-binding protein from CWP.

Reverse phase HPLC of native CWP and heated-CWP

Figure 5 shows the chromatograms of native CWP and heated-CWP. In the chromatogram of native CWP, three major peaks were identified by means of the retention time

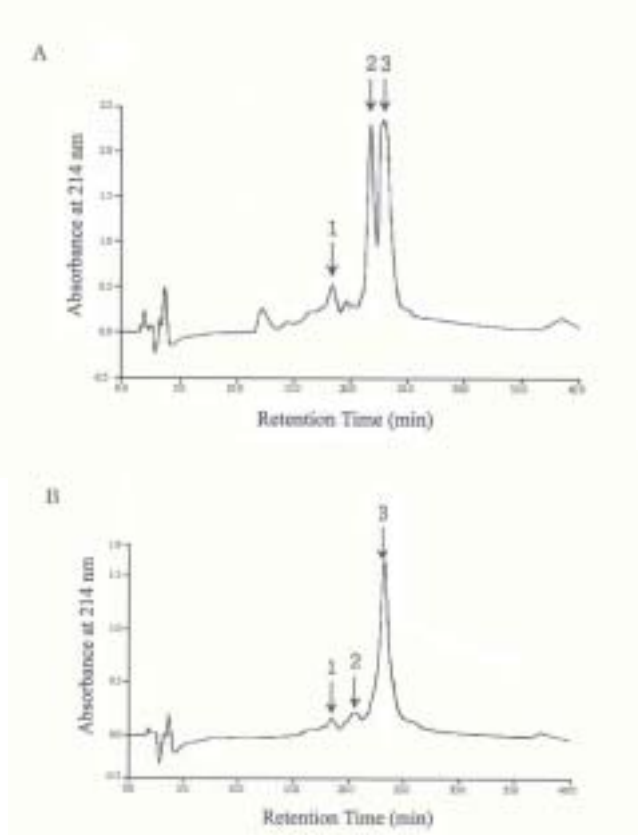

Figure 5. RP-HPLC chromatograms of native CWP (A) and heated-CWP (B). Legend: BSA (1), α-LA (2) and β-LG (3).

of the standard as BSA (retention time=18.2 min), α-LA (retention time=22 min) and β-LG (retention time=23 min) (Figure 5A). After heat treatment (10 min at 100°C), one large peak and two small peaks were detected as β-LG and BSA and α-LA, respectively. The peaks corresponding to BSA and α-LA were mostly affected by heat treatment, but β-LG was affected a little by heat treatment (Figure 5B). These results show that, β-LG is resistant to heat due to the strong structure (two disulfide bonds and a free sulfhydryl group) in native β-LG.

Figure 6 shows the chromatogram of tryptic hydrolysates from heated-CWP. The peaks corresponding to BSA, β-LG and α-LA were mostly hydrolyzed by trypsin. These results agreed with the SDS-PAGE results.

Separation and Identification of calcium-binding protein

The separation and identification of the calcium-binding protein from tryptic hydrolysates were shown in Figure 7 and 8, respectively.

Ion exchange chromatography of tryptic hydrolysates from heated-CWP on DEAE-cellulose formed the elution profiles seen in Figure 7. The first peak, a large fraction, came out a NaCl gradient concentrate of 0.25 M. The latter,

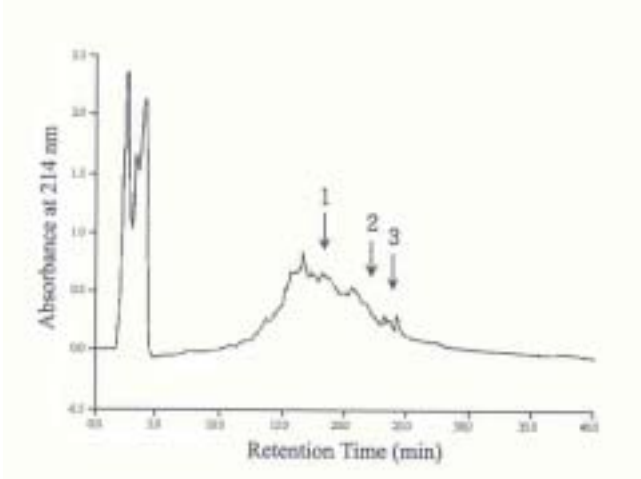

Figure 6. RP-HPLC chromatograms of heated-CWP hydrolysates by trypsin. Legend: BSA (1), α-LA (2) and β-LG (3).

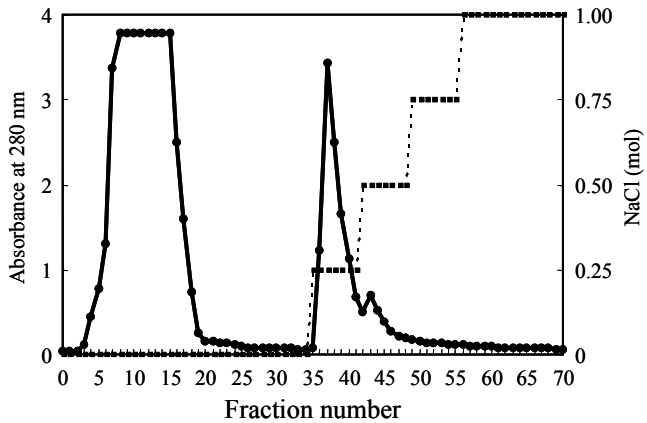

Figure 7. Ion exchange chromatograms of heated-CWP hydrolysates by trypsin.

small peak, was eluted to be at a NaCl gradient concentrate of 0.5 M. As a result of the calcium content analysis of two fractions, calcium-binding activity is associated with the large fraction peak, which occurs at a NaCl gradient concentration of 0.25 M. These results confirm previous observations (Friedlander and Norman, 1980).

To confirm the calcium-binding ability of eluted fractions from ion exchange chromatography, the fractions were analyzed via ion chromatography in a calcium affinity column (Figure 8). In terms of calcium-binding ability, the large fraction peak bound $CaCl_2$ much more than the small fraction peak was gradually increased.

CONCLUSION

To provide the basic information for the effect application conditions of CWP and the development possibility of calcium-binding protein derived from CWP hydrolysates, we hydrolyzed CWP by various commercial enzymes, and studied the properties of CWP hydrolysates,

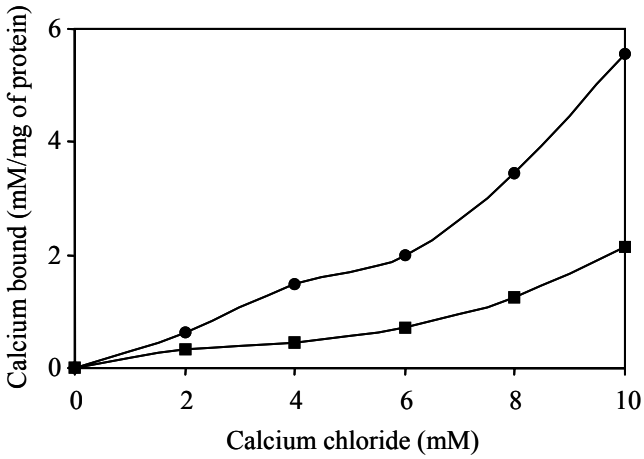

Figure 8. Calcium-binding ability of tryptic hydrolysates fractions eluted from ion exchange chromatography. Legend: large fraction peak (●) and small fraction peak (■).

then, we separated and confirmed the calcium-binding protein derived from CWP hydrolysates. The DH and NPN amounts of tryptic hydrolysate were higher than those of other enzymatic hydrolysates. SDS-PAGE and RP-HPLC patterns showed that trypsin almost hydrolyzed BSA, β-lactoglobulin and α-lactalbumin. The calcium-binding protein was isolated from the tryptic hydrolysates fraction, which eluted at a 0.25 M NaCl gradient concentration as a result of ion exchange chromatography. The results of the present study demonstrate that calcium-binding proteins can be produced by trypsin hydrolysates derived from CWP.

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