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RESEARCH ARTICLE

Isolation of a Calcium-binding Peptide from Bovine Serum Protein Hydrolysates

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Abstract A calcium-binding peptide was isolated from the hydrolysates of bovine serum protein (BSP). BSP was hydrolyzed using 3 different types of proteases, Alcalase, Flavourzyme, and Protamex, and the degree of hydrolysis was determined and monitored using trinitrobenzenesulfonic acid and SDS-PAGE. The hydrolysates of BSP using Alcalase were selected and ultra-filtered below 3 kDa. The membrane-filtered solution was then fractionated using ion exchange chromatography and normal phase HPLC to isolate a calcium-binding peptide. The calcium-binding capacity was determined by the orthophenanthroline method. The sequence of the purified calcium-binding peptide was analyzed using LC/electron spray ionization (LC/ESI)-tandem mass spectroscopy and identified to be Asp-Asn-Leu-Pro-Asn-Pro-Glu-Asp-Arg-Lys-Asn-Tyr-Glu, which has a molecular weight of 1,603 Da.

Keywords: calcium binding peptide, enzymatic hydrolysate, isolation, bovine serum protein

Introduction

Calcium is an essential mineral in the human body and plays an important role in the construct of skeletal organizations, intracellular metabolism, nerve conduction, muscle contraction, and cardiac functions (1). An adequate calcium intake can increase the bone density in children and prevents osteoporosis among middle-age people (2). Most calcium is absorbed in the small intestine, but it is often difficult to absorb calcium because of its high reactivity with other compounds, such as phosphate and oxalate (3). Therefore, many studies have been performed to improve calcium absorption and bioavailability. Casein phosphopeptides, which could be bound to minerals, increase the stability, absorption, and bioavailability of calcium (4). In particular, peptides that consist of small amino sequences have crucial biological activities and a potential for use as ingredients in functional foods (5,6). Accordingly, calcium-binding peptides may be an appropriate supplement for improving calcium absorption in the body.

The Korean cow is a hybrid of *Bos taurus* × *Bos zebu,* which has been raised in Korea since BC 4,000 (7). Although it is partly used in blood sausages, pudding, and blood curd, the blood from Korean cows is mostly discarded from slaughterhouses and not commonly consumed because of its strong color and unpleasant flavor (8). Serum proteins in bovine blood consist of albumins (3.3%) and globulins (4.2%) (9), hence they can be a good source of high-quality protein (10), and their hydrolysates can be utilized as a nutritional supplement and functional ingredient. However, few studies have been conducted on the application of bovine serum as an industrial source.

The objective of this research is to investigate the utilization of bovine serum protein (BSP) as a calcium supplement by isolating a calcium-binding peptide from its hydrolysate.

Materials and Methods

Materials Bovine serum protein (BSP) samples were obtained from Bionel Co. (Nonsan, Korea). Alcalase (from *Bacillus licheniformis*, activity 2.4 AU/g protein), Flavourzyme (from *Aspergillus oryzae*, activity 500 LAPU/g protein), and Protamex (from *B. licheniformis* and *Bacillus amyoliquefaciens*, activity 1.5 AU/g protein) were obtained

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from Novo Nordisk Co. (Bagsvaerd, Denmark). Calcium chloride, ortho-cresolphthalein complexone, 2-amino-2-methyl-1-propanol, and trinitro-benzenesulfonic acid of analytical grade were purchased from Sigma-Aldrich (St. Louis, MO, USA). The solvents of HPLC grade were purchased from J.T. Baker Inc. (Phillipsburg, NJ, USA).

Preparation of enzymatic hydrolysates Hydrolysates of BSP were prepared according to the method of a previous study (11). In the preparation of a 2%(w/v) serum protein solution, the lyophilized protein sample was dissolved in 10 mM phosphate buffer. Enzymatic hydrolysis of BSP was then performed using 3 different proteases: Alcalase, Flavourzyme, and Protamex. The BSP was hydrolyzed under the optimal hydrolysis conditions for each protease, as follows: Alcalase with a 20:1 substrate to enzyme ratio (w/v) at pH 8.0, 55°C for 12 h; Flavourzyme with a 20:1 substrate to enzyme ratio (w/w) at pH 7.0, 50°C for 12 h; and Protamex with a 20:1 substrate to enzyme ratio (w/w) at pH 7.0, 50°C for 12 h. The hydrolysates were then heated at 95°C for 10 min to inactivate the enzymes. The reaction mixtures were centrifuged at 3,500×*g* for 20 min, and the supernatant was filtered below 3 kDa using an ultrafiltration disc (Ultracel PL-3; Millipore Co. Billerica, MA, USA).

Determination of available amino group concentration The degree of hydrolysis was determined using a trinitro-benzenesulfonic acid (TNBS) assay (12). Sodium borate buffer (1 M, pH 9.2) was added to the hydrolysis sample, and the mixture was reacted with 5 mM TNBS at room temperature for 30 min. The reaction mixture was terminated by adding 18 mM sodium sulfite and 2 M monobasic sodium phosphate, and the absorbance was measured at 420 nm. The TNBS assay was also used for the determination of the peptide concentration from each chromatography fraction.

SDS-PAGE SDS-PAGE was performed according to the method of Laemmli (13). Proteins were resolved on a 12% separation gel and stained with Coomassie Brilliant Blue.

Determination of calcium-binding capacity Calcium-binding capacity was measured according to the method of a previous study (3). After adding 5 mM $CaCl_2$ to the sample solution, the solution was stirred at room temperature for 1 h and centrifuged at 4,000×*g* for 20 min to remove the precipitates. The calcium concentration in the supernatant was determined using a colorimetric method (14) with the ortho-cresolphthalein complexone reagent.

Isolation of a calcium-binding peptide The hydrolysates of BSP were loaded onto a QAE-Sephadex A-25 (GE Healthcare Co., Uppsala, Sweden) column (2.5×20 cm) equilibrated with 10 mM Tris buffer (pH 8.0) and were eluted using a linear gradient of the buffer containing NaCl from 0 to 0.5 M at 1.5 mL/min. The eluate was monitored for peptides by measuring the absorbance at 214 nm. After the calcium-binding capacity of each fraction from the QAE-Sephadex A-25 chromatography was determined, the highest calcium-binding fractions were pooled and loaded onto a normal-phase HPLC (Waters, Milford, MA, USA) amino column (4.6×250 mm, Luna NH_2, Phenomenex Inc., Torrance, CA, USA). Elution was performed with A (0.1% trifluoroacetic acid in acetonitrile:water, 97:3, v/v) and B solvents (0.1% trifluoroacetic acid in acetonitrile: water, 30:70, v/v), using a gradient of 0 to 80% and 80 to 100% at 0.5 mL/min.

Peptide sequencing The molecular mass and sequence of the purified calcium-binding peptides were determined using a LC/electron spray ionization (LC/ESI)-tandem mass spectrometer (QTOF2) (Waters). An active peptide for sequencing was obtained over the *m/z* range of 200-2,000. The peak lists were generated and processed using MassLynx software version 4.1 (Waters). The resulting peak list files were searched against the SwissProt database using the Mascot search engine version 2.2.2 (Matrix Science, London, UK).

Results and Discussion

Preparation of BSP hydrolysates Enzymatic hydrolysis of protein molecules is the most common way to produce bioactive peptides (15). BSP was hydrolyzed using the commercial proteases, Alcalase, Flavourzyme, or Protamex. The available amino group concentrations of the enzymatic hydrolysates of BSP increased with the duration of the hydrolysis (Fig. 1). We found that 12 h was sufficient for the preparation of BSP hydrolysates, as the available amino group concentration did not increase after 10 h. Based on the degree of hydrolysis, Alcalase was the best protease among the enzymes used in this study.

The degree of hydrolysis of BSP was also confirmed by SDS-PAGE (Fig. 2). It is known that serum proteins mainly consist of albumin and globulin; based on the SDS-PAGE analysis in this study, the major component of BSP was albumin (66 kDa). After 2 h of hydrolysis with Alcalase, the albumin was degraded into smaller bands below 17 kDa, whereas the other proteases did not completely hydrolyze the protein until 12 h. In a previous report comparing 5 different proteases (Alcalase, Neutrase, trypsin, pepsin, and papain), Alcalase presented the highest degree of hydrolysis of bovine plasma protein (10), similar

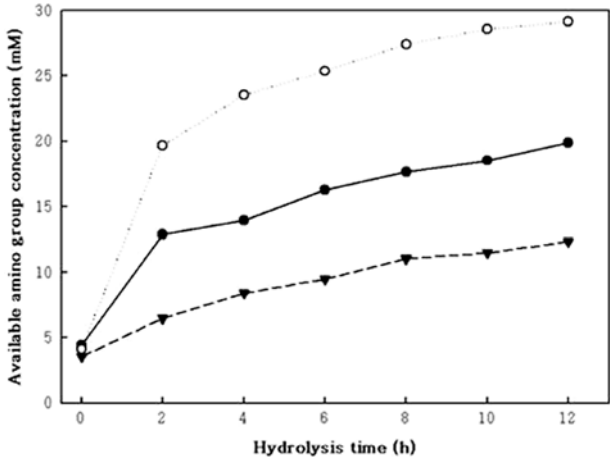

Fig. 1. Effect of hydrolysis time on the available amino group concentration of bovine serum protein hydrolysates prepared using various commercial proteases. Flavourzyme (●), Alcalase (○), Protamex (▼)

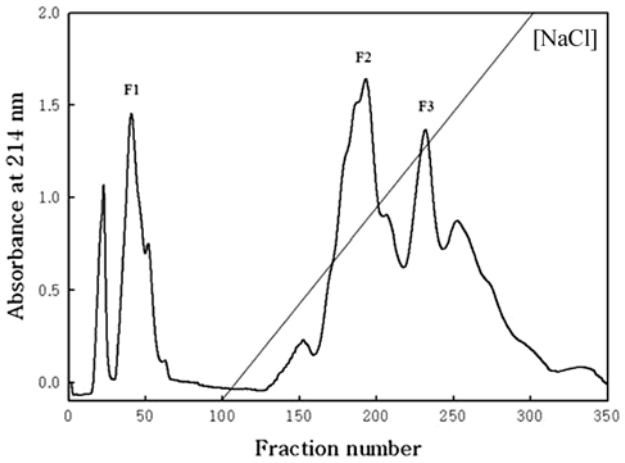

Fig. 3. Elution profile of bovine serum protein hydrolysates from ion exchange chromatography. Absorbance was measured at 214 nm.

to our results. Therefore, Alcalase was selected as the most suitable protease based on the results of the TNBS assay and SDS-PAGE; the Alcalase-hydrolysate of BSP was used for the isolation of a calcium-binding peptide.

Isolation of a calcium-binding peptide from BSP

Because small-molecular-weight peptides can be suitable for intestinal absorption (16), the calcium-binding peptides were separated from the hydrolysates of BSP. Those peptides having a high affinity for calcium were fractionated in the molecular mass range of 1-5 kDa, according to previous reports (3,17), and the BSP hydrolysates were filtered to isolate molecules with a molecular mass below 3 kDa.

Because calcium-binding peptides may have negative charges that interact with the divalent cations, the membrane-filtered BSP hydrolysate was separated using anion exchange chromatography (18). Figure 3 shows that there are 3 major peaks fractionated from the chromatography; the contents of calcium and peptide were determined for each chromatographic peak (Table 1). Among the 3 eluted fractions, the F3 fraction had the highest calcium content per peptide concentration. Therefore, the F3 fraction was pooled and subjected to normal-phase LC using an amino column for further purification. Figure 4 indicates that there are 2 major peaks separated during the gradient elution; the F31 fraction had a higher calcium binding capacity than that of the F32 fraction (Table 1). Consequently, the F31 fraction was pooled and analyzed to determine its molecular weight, and the peptide was sequenced using a Nano-LC/ESI MS. The calcium-binding peptide from the BSP hydrolysates was identified as Asp-Asn-Leu-Pro-Asn-Pro-Glu-Asp-Arg-Lys-Asn-Tyr-Glu, having a molecular weight of 1,603 Da. Thus, the F31 fraction was turned out to be homogeneous, and the identity was confirmed by comparison with the data in the National Center for Biotechnology Information (NCBI) database. The calcium-binding peptide corresponds to the 235 to 247th amino acid sequence of serotransferrin, an

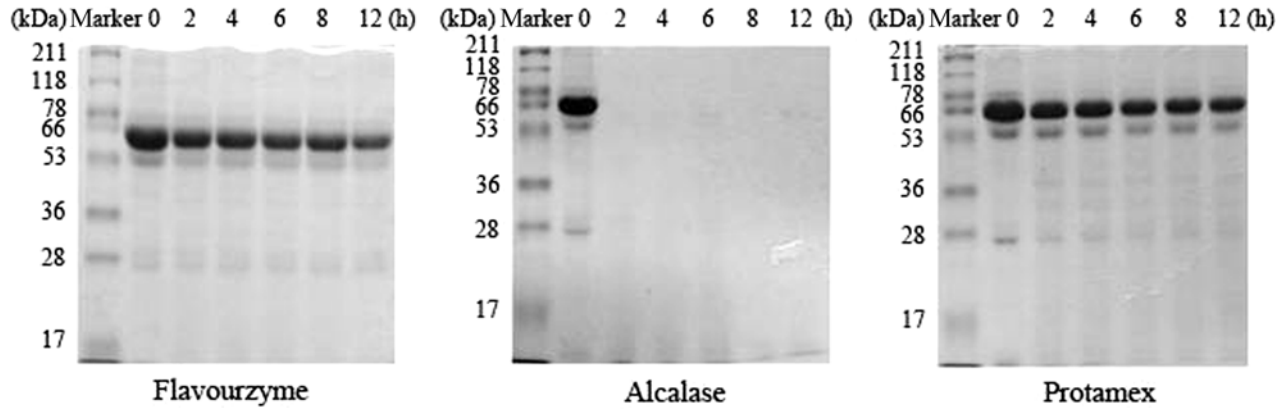

Fig. 2. Effect of hydrolysis time on SDS-PAGE profile of bovine serum protein hydrolysates.

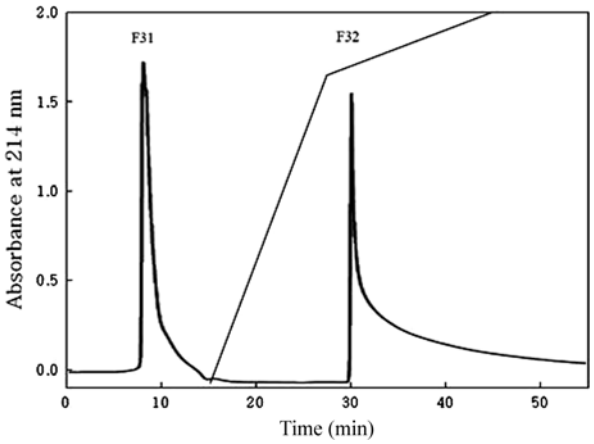

Fig. 4. Elution profile of the F3 fraction (Fig. 3) from normal-phase HPLC. Absorbance was measured at 214 nm.

Table 1. Calcium-binding activity for each fraction from chromatography

Chromatography	Fraction	Ca concentration (mM)	Peptide concentration (mM)
IEC	F1	0.0220	0.2064
	F2	0.0256	0.0139
	F3	0.0257	0.0122
HPLC	F31	0.1108	0.1950
	F32	0.0822	0.3849

iron-binding protein, from bovine serum. In addition, Sun *et al*. (19) reported that only approximately 30% of the transferrin in human serum is saturated with iron and that it has the capacity to bind to other metals. Therefore, our results suggest that the peptide isolated from transferrin in Korean cow serum is able to bind a calcium ion.

Korhonen and Pihlanto (20) reported that the amino acid composition and sequence of bioactive peptides are related to the stability, absorption, and bioavailability of calcium. In addition, considering that peptides with biological activity usually comprise 3-20 amino acid residues, it is reasonable that the calcium-binding peptide isolated in this study has 13 amino acids. In particular, the calcium-binding peptide from the BSP hydrolysates contains acidic residues: 2 Asp and 2 Glu. Similarly, Lee and Song (3) reported that a calcium-binding peptide isolated form porcine blood plasma protein hydrolysates has 8 amino acids, including Asp and Glu. The major amino acid composition of calcium-binding peptides from cheese whey protein includes Asp, Glu, Lys, Leu, and Pro and, in particular, has the highest amount of Glu (21). According to the report of Cariolou *et al*. (22), the Asp in abalone shell peptides has a role in the calcium binding during shell formation. The overall negative charge of the peptide due to the Asp and Glu plays an important role in calcium binding, and it appears that acidic amino acid residues have a high affinity for calcium. In addition, there are 3 Asn residues in the peptide isolated in this study. According to the report of Nemirovskiy and Gross (23), peptides having 1 Asp and 2 Asn residues had a higher calcium-binding activity than peptides with 2 Asp and 1 Asn because the Asn side chain is a better ligand than Asp for calcium. It should also be noted that the calcium-binding peptide from the BSP hydrolysates also contains Lys and Arg. Tuan *et al*. (24) reported that a calcium-binding protein from the chick chorioallantoic membrane contains acidic amino acids and is also abundant in basic amino acids. It is suggested that basic amino acids may be involved in calcium binding. Along with these results, it is suggested that Asp, Glu, Asn, Arg, and Lys may be the amino acids responsible for the ability of the peptide to bind calcium.

In conclusion, a calcium-binding peptide from BSP hydrolysate was isolated and analyzed using ion exchange chromatography, normal-phase HPLC, and LC/ESI MS. The calcium-binding peptide derived from bovine serum in this study can be utilized as a functional food ingredient and calcium supplement.

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