Purification of Bovine Bone Oligophosphopeptide with High Calcium-binding Activity by *Bacillus cereus* MBL13 Collagenolytic Protease

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Abstract A demineralized bovine bone was hydrolyzed by a specific bone-degrading collagenolytic protease extracted from *Bacillus cereus* MBL13 (isolated from chopped animal bone wastes) to utilize its collagen in nutraceuticals with high calcium bioavailability. Bovine bone peptide (BBP), a novel oligophosphopeptide with a high calcium binding ability (8.25 mmol/g-protein), was isolated from bovine bone hydrolysates by Chelex 100, ultrafiltration, hydroxyapatite chromatography, gel filtration chromatography, and reverse-phase high performance liquid chromatography. The results showed that demineralization treatment can significantly increase hydrolysis ($p < 0.05$). The amino acid content of BBP showed that the Asp, Ala, Tyr, and Thr contents were remarkable increments compared to the bone hydrolysates. The molecular mass of BBP was found to be around 3.305 kDa through SDS-PAGE and MALDI-TOF mass spectrometry. FT-IR spectra showed characteristic absorption peaks. Moreover, BBP exhibited higher calcium binding activity than that of casein oligophosphopeptide (CPP). Therefore, this study demonstrated that *B. cereus* MBL13 collagenolytic protease (BCC) could degrade bovine bone collagen, and prepared oligophosphopeptide could be utilized as a nutraceutical with high calcium-binding activity.

Keywords: bovine bone, collagen, Bacillus cereus MBL13 collagenolytic protease, oligophosphopeptide, calcium binding activity

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1. Introduction

Collagens and their peptide fragments are produced in large quantities as by-products of livestock and poultry industries. Numerous studies on utilizing byproducts have been conducted [1,2,3,4]. Many studies have identified the structure, function, and biological activities of bone collagen and its peptides [5,6,7,8].

More than 5 million tons of bovine bone wastes are discarded annually in China as inedible byproducts. Prior studies have verified that animal bones contain bioactive and nutraceutical components and many studies have been conducted to utilize the proteins from animal bones. Recently, the development of environment-friendly treatment of waste animal bones for obtaining collagens and their peptide fragments has received significant attention. In fact, protein, of which most is collagen, accounts for approximately 25%–30% of the gross weight of bovine bones. Therefore, bovine bone may be a good resource for high quality collagen products. Reportedly, type I collagen has been successfully extracted from bovine bones, and the hydrolysis of collagen protein has been conducted using proteases. Collagen is a unique protein that contains a right-handed triple superhelical rod of three polypeptide chains, which forms insoluble fibers with high tensile strength and mechanical stability. The recycling of collagen from animal bone wastes has received significant research interest that mainly focuses on enzyme hydroxylation. However, only a limited number of proteases with unique characteristics can trigger bone collagen degradation [9]. Bovine bone becomes a part of solid waste due to its rigid structure. Hence, an urgent demand for developing biotechnological alternatives to such waste recycling exists.

Many peptides isolated from animals possess calcium-binding ability [10,11,12]. Casein phosphopeptides (CPPs) are well-known for their metal-binding abilities because they contain electronegative phosphoserines [13,14]. The phosphopeptides obtained from the enzymolysis of fish bones [6,7] can also chelate calcium.

The practical applications of several industrial collagenolytic proteases have limitations. Hence, knowledge of the catalytic behavior of proteases secreted from any new strain is a prerequisite for evaluating its biotechnological potential. The collagenolytic proteases are sometimes confused with collagenases, which was coined on the basis of their capacity to hydrolyze/digest native bovine achilles tendon collagen with unique

specificity. [15,16]. Many collagenolytic proteases from microorganisms have been reported [17,18,19,20]. Bacterial collagenolytic proteases in various forms have found practical applications worldwide, such as additives to laundry detergents and experimental reagents in laboratory work [21,22]. Several *B. cereus* species have been previously reported to produce collagenolytic proteases [23,24]. However, information on the specificity of collagenolytic proteases toward animal bones is limited. Studies on the isolation of strains that produce bone-degrading collagenolytic proteases and their application in degrading waste bones have not been reported.

In the present study, we report the hydrolysis of bovine bone by the collagenolytic protease secreted from *B. cereus* MBL13, the preparation of phosphopeptide with high affinity to calcium. Also, its chemical composition and the partial characterization of its calcium-binding property are studied.

2. Materials and Methods

2.1. Materials

Bovine bone frames were purchased from the local market (Luoyang, PR China), washed thoroughly using tap water, and then dried. They were stored at –4 °C prior to use. Commercial proteinases (Neutrase, Trypsin, Pepsin, Alkaline protease, and type I collagenase) were purchased from Sigma Chemical Co. (St. Louis, MO, USA). Bovine serum albumin (BSA), the dye reagent concentrate for protein determination, and the reagents for sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) were purchased from BioRad, Co. (Richmond, CA, USA). A calcium standard solution (1 g/l) was prepared by the dilution of the calcium standard (Titrisol, Merck Co.) with distilled-deionized water. All other reagents used were of the highest grade available.

2.2. Preparation of Collagenolytic Protease from *Bacillus cereus* MBL13

B. cereus MBL13 was isolated from the chopped animal bone wastes collected at Changsha in Hunan province, PR China. *B. cereus* MBL13 collagenolytic protease (BCC) was isolated and purified according to the method of Liu et al. [24].

2.3. Assay of Enzymatic Hydrolysis of *Bacillus cereus* MBL13 Collagenolytic Protease

According to the method of Jung et al. [6], the enzymatic hydrolysis of different proteases was assayed using bovine bone powder as a natural substrate. The enzymes Neutrase, Alkaline protease, Trypsin, Pepsin, type □ collagenase, and BCC (enzyme/substrate: 3/100, substrate concentration: 2%) were used at 50 °C for 7.0 h. After incubation at 95 °C for 10 min to inactivate the enzyme, the bovine bone hydrolysates were centrifuged at 10,000 ×g for 10 min and filtered with a 0.45-μm membrane. The degree of hydrolysis (DH) was determined through the *o*-phthaldialdehyde (OPA) method [25].

2.4. Preparation of Bovine Bone Hydrolysates by BCC

After being treated (removing muscle, cartilage, sponge periosteum) and degreased (121°C, 45 min), the bovine bone was demineralized (using different HCl concentrations and soaking times) and the pH value was adjusted (0.05% NaOH) to neutral state. Then, the bovine bone was dried (with moisture content of < 6%) and finely ground to bone powder. The powder was hydrolyzed by BCC under the experimental conditions (pH 8.0, 45°C, enzyme/substrate: 3/100, substrate concentration: 2%) for 6 h. After incubation at 95 °C for 10 min to inactivate the enzyme, the hydrolysate was centrifuged at 8,000 ×g for 20 min, filtered with a 0.45-μm membrane, and lyophilized. Then, the DH and the collagen polypeptide content before and after demineralization were determined. The degree of demineralization can be determined according to the method of Koga et al. [26]. The collagen polypeptide content of protein hydrolysates was determined by the biuret reagent method [3].

2.5. Isolation and Purification of Bone Peptide with High Calcium-Binding Activity

The supernatant of the bone hydrolysate was filtered and demineralized on a Chelex 100 resin (Bio-Rad, Richmond, CA, USA) column at a flow rate of 1.0 ml/min. To isolate soluble fractions with high affinity to calcium, the demineralized fraction was subsequently passed through an ultrafiltration (UF) membrane with a 4-kDa molecular weight cut-off (MWCO) (Tianjin Motian Membrance Engineering and Technology CO. Ltd., Tianjin, China) and divided into two fractions. The two fractions (< 4 kDa and > 4 kDa) were lyophilized and tested for calcium-binding activity. The higher active fraction obtained after UF was concentrated and applied to a hydroxyapatite (HA) (20 × 80 mm, Macroprep ceramic HA type I, Bio-Rad, Richmond, CA, USA) pre-equilibrated with 10 mM of potassium phosphate buffer, pH 6.5. Separation was performed with a linear gradient of 10–200 mM of phosphate buffer (pH 6.5) at a flow rate of 1.0 ml/min. All peaks eluted were monitored at 220 nm. After desalting through a Sephadex G-15 (Pharmacia Co., Uppsala, Sweden) column (16 × 300 mm, Shanghai Qingpu Huxi Instrument Factory, Shanghai, China), the eluates were tested for calcium-binding activity. The highest active fraction was fractionated by gel filtration chromatography on a column (26 × 150 mm, Shanghai Qingpu Huxi Instrument Factory, Shanghai, China) of Sephadex G-25 (Pharmacia Co., Uppsala, Sweden). The sample was dissolved in distilled water and loaded onto the column. The column was pre-equilibrated with distilled water and eluted at a flow rate of 1.0 ml/min. The absorbance at 220 nm was measured to monitor the peptide during chromatography separation. These fractions were then concentrated under reduced pressure in a rotary evaporator (Rotavapor R-114, Büchi Labortechnik AG, Flawil, Switzerland) and then lyophilized. The calcium-binding activity of each fraction was determined. The fraction that showed the highest calcium-binding activity was further purified using a semipreparative RP-HPLC column (Agilent Zorbax

SB-C18, 9.4×250 mm). Elution was performed using a linear gradient of acetonitrile (5%–25%, V/V, in 20 min) with a flow rate of 0.5 ml/min at 220 nm.

2.6. Amino Acid Analysis

The samples were hydrolyzed with 6 N HCl at 110°C for 24 h in vacuum-sealed ampoules. After neutralizing, evaporating, and filtering with a glass filter, the amino acid composition was determined with an amino acid analyzer (Hitachi 835-50, Hitachi Co. Ltd., Japan).

2.7. Tricine-SDS-PAGE of Bovine Bone Peptide (BBP)

Tricine-SDS-PAGE was carried out using the method described by Schagger et al. [27], with 10% polyacrylamide resolving gels. Protein bands were visualized by staining with Coomassie Brilliant Blue R-250.

2.8. Molecular Mass Determination

The molecular mass of the purified hypoglycemic peptide was determined using a MALDI-TOF mass spectrometer (4700 Proteomics Analyzer, Applied Biosystems, Foster City, CA, USA) equipped with a nitrogen laser (355 nm). The sample was prepared as follows. BBP was dissolved in water to a concentration of 0.5 mg/ml. Then, 5 μl of the sample solution was mixed with 5 μl of matrix solution (containing 10 mg of α-cyano-4-hydroxycinnamic acid). Subsequently, 2 μl of the mixture was directly spotted onto a MALDI target plate. MALDI-TOF MS analysis was performed directly after spotting. The spectrometer was operated in reflection mode with delayed extraction, and the acceleration voltage in the ion source was 20 kV. The accuracy of mass determinations was within 0.02%.

2.9. Raman and Fourier Transform Infrared (FT-IR) Spectroscopy

The solid BBP sample was incorporated into KBr (spectroscopic grade) and pressed into a 1.0-μm pellet. The spectra were recorded in absorbance mode from 400 cm^{-1} to 4000 cm^{-1} on a VERTEX70 Fourier transform infrared/Raman spectrometer (Bruker Co., Germany). The spectra were scanned five times.

2.10. Calcium-binding Assay

The assay was performed according to the method of Jung and Huang with the following modifications [6,28]. The various concentrations of hydrolysates and BBP of up to 500 mg/L were mixed with 5 mM of $CaCl_2$ and 20 mM of sodium phosphate buffer (pH 7.5). The mixture was stirred at 25 °C for 30 min, and the pH was maintained at 7.5. After filtration using a 0.22-μm microfiltration to remove insoluble calcium phosphate salts, the calcium contents of the supernatant were determined using a flame atomic absorption spectrometer (AA-6300C, Shimadzu Co., Japan). The instrumental conditions were as follows: wavelength = 422.8 nm, slit = 0.8 nm, acetylene flow =

1.75 L/min, and air flow = 14.0 L/min. The protein concentration in the sample solutions was determined through the method of Lowry et al. [29] using BSA as the standard.

2.11. Statistical Analysis

All data were expressed as means ± standard deviations of triplicate determinations. A least significant difference (LSD) test was performed using the SPSS software program (SPSS Inc., Chicago, IL, USA) to evaluate the mean differences between the measurements at 5% confidence level.

3. Results and Discussion

3.1. Collagenolytic Activity of *Bacillus cereus* MBL13 Collagenolytic Protease

The specific enzymatic hydrolysis of BCC was examined in comparison to various proteinases, which are commonly used in protein hydrolysis [30,31,32]. As shown in Figure 1, BCC could efficiently degrade bovine bone collagen compared with the other commercial enzymes tested ($p < 0.01$). The DH of BCC was approximately 24.80%, which was significantly higher than those of the other enzymes. In our previous study [24], the enzymatic hydrolysis of BCC was specific for type I collagen, indicating that BCC has a broad application potential in the hydrolysis of waste animal bones.
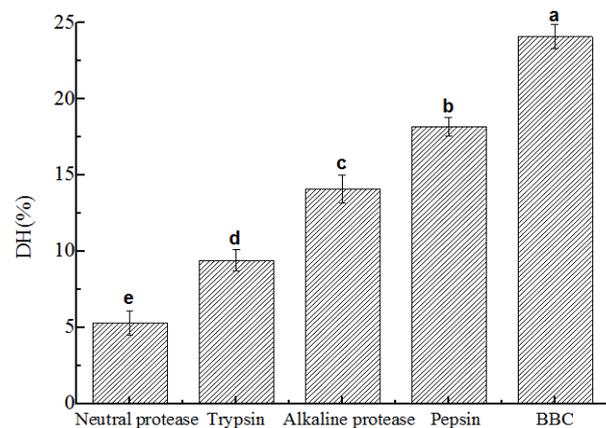

Figure 1. Comparison of collagenolytic activity between BCC and other proteases using bovine bone powder as a natural substrate. The assay was performed in triplicate; DH values are expressed as means ± SD.

3.2. Effect of Demineralized on Enzymatic Hydrolysis of Bovine Bone

The inorganic portion of vertebrate bone is primarily composed of HA crystals that are deposited within an organic matrix of cross-linked collagen fibrils [9]. HA crystals make up approximately 60%–65% of bones, and HA has an extremely complicated crystalline structure $[(Ca^{2+})_{10-\chi}(H_3O^+)_{2\chi}(PO_4^{3-})_6(OH^-)_2]$. In vertebrates, the crystals are usually organized with an χ value range of 0–2. When the crystals grow at a highly organized rate in the

bone matrix (χ is 0), the chemical formula of HA is $Ca_{10}(PO_4)_6(OH)_2$. Earlier studies have shown that demineralized bone facilitates protein hydrolysis [33,34].

As shown in Figure 2 (a), demineralization treatment can significantly increase the degree of hydrolysis of bovine bone ($p < 0.01$). The degree of hydrolysis of bovine bone increased constantly with the enzymolysis time. However, they began to decline at a certain time because the collagen polypeptide began further hydrolysis to amino acids. Hence, the demineralization treatment of bovine bones can improve the hydrolysis effect of protease. HCl can hydrolyze the bone structure effectively to achieve the purpose of demineralization. After hydrolysis for 4 h, the hydrolysis degree and content of polypeptides was 28.30%, which is higher than that of non-demineralized bovine bones (14.76%). The bone matrix is composed of collagen, which is attached to calcium phosphate in the form hydroxyapatite. Most of the bone mineral hydroxyapatite amorphous and the crystalline form of calcium phosphate distribution in organic matter, which enables the bone collagen not easily is resistant to be functioned by protease.
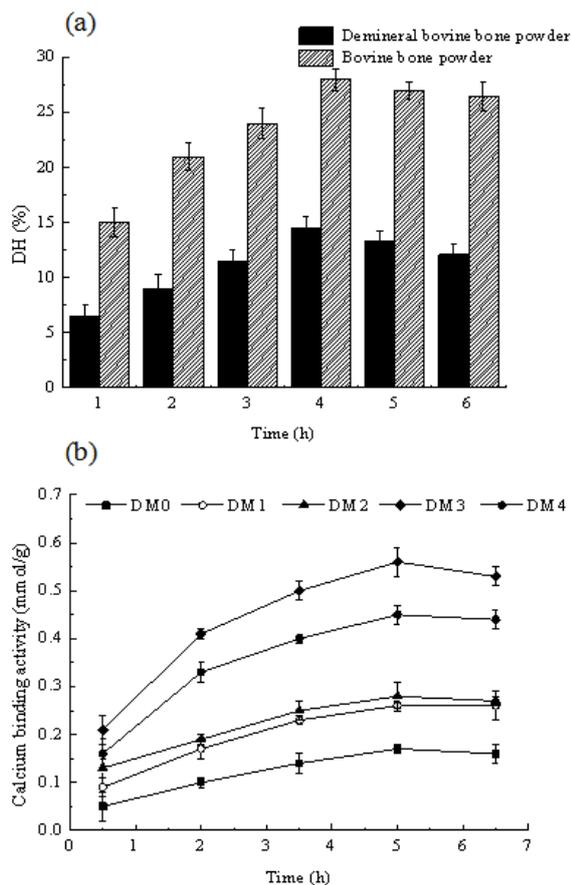

Figure 2. Effect of demineralized on enzymatic hydrolysis of bovine bone. (a) Effect of demineralization on degree of hydrolysis of bovine bone powder; (b) calcium-binding activity of bovine bone with different degrees of demineralization. DM0, bovine bone without demineralization. DM1, DM2, DM3, and DM4 represent four kinds of demineralized bovine bone with demineralization degrees of 16.12%, 33.07%, 50.78%, and 71.01% respectively. Bovine bone was digested with BCC (enzyme/substrate: 3/100, substrate concentration: 2%, pH 8.0) at 45°C for 6.0 h. The assay was performed in triplicate; values are expressed as means± SD

Table 1 shows that the degree of bovine bone demineralization increased with the HCl concentration and the demineralization soaking times. By controlling the concentration of HCl and demineralization soaking times, bovine bones with different demineralization rates can be prepared. Four kinds of bovine bone demineralization (labeled as DM1, DM2, DM3, and DM4) could be obtained, which were 16.12%, 33.07%, 50.78%, and 71.01% respectively.

Table 1. Degree of demineralization of bovine bone with HCl (mol/l)

Soaking times (Times)	Concentration of HCl (mol/l)			
	0.12	0.24	0.48	0.60
2	8.23 ±0.59	13.14 ±0.78	27.47 ±1.02	27.69 ±1.13
3	10.62 ± 1.03	22.61 ± 1.12	29.61 ± 0.92	29.70 ± 1.32
4	16.12 ± 0.65	26.94 ± 0.96	50.78 ± 2.02	51.03 ±1.87
5	20.16 ± 1.35	33.07 ± 1.91	63.38 ± 1.57	63.96 ± 1.93
6	29.02 ± 1.64	41.23 ± 2.07	70.97 ± 2.42	71.01 ± 2.14

As shown in Figure 2 (b), the hydrolysate of DM3 (demineralization rate is 50.78%) showed the strongest calcium-binding ability at 0.54 mmol/g-protein. However, excessive demineralization is disadvantageous to the calcium-binding ability of the hydrolysates. When the demineralization rate was 71.01%, the calcium-binding ability was 0.41 mmol/g-protein. The phosphorus content may be critical to the calcium-binding ability of the phosphorus peptides. Casein phosphopeptides (CPPs) could prevent the formation of insoluble calcium in the small intestines and improve calcium absorption [13]. Thus, the ability of the phosphorus peptide to bind calcium is related to its phosphorus content and molecular weight. An appropriate demineralization treatment can produce a phosphorus peptide with an appropriate phosphorus content and molecular weight. Therefore, we selected the hydrolysate of DM3 for further isolation and purification.

3.3. Isolation and Purification of Calcium Binding Peptides

First, the bovine bone hydrolysates were filtered and demineralized on a Chelex 100 resin column at a flow rate of 1.0 ml/min. Four fractions (C1, C2, C3, C4) were isolated. To determine their calcium-binding activity, the yield and calcium-binding ability of C3 was the greatest among the four (Table 2). Therefore, C3 was subjected to ultrafiltration. After ultrafiltration classification, two fractions were obtained from C3: U1 (< 4 kDa) and U2 (> 4 kDa). The yield and calcium-binding ability of U1 were greater than those of U2 (Table 2), which shows that after hydrolysis, the bovine bone collagen decomposed into small molecule peptides with high calcium-binding ability. Many studies have shown that biologically active peptides usually contain 3–20 amino acid residues, and the molecular weight of the discovered peptides with calcium-binding capacity were mostly from 1 kDa to 3.5 kDa [6,7,11,35].

Table 2. Calcium binding activity of fractions after Chelex 100 and ultrafiltration

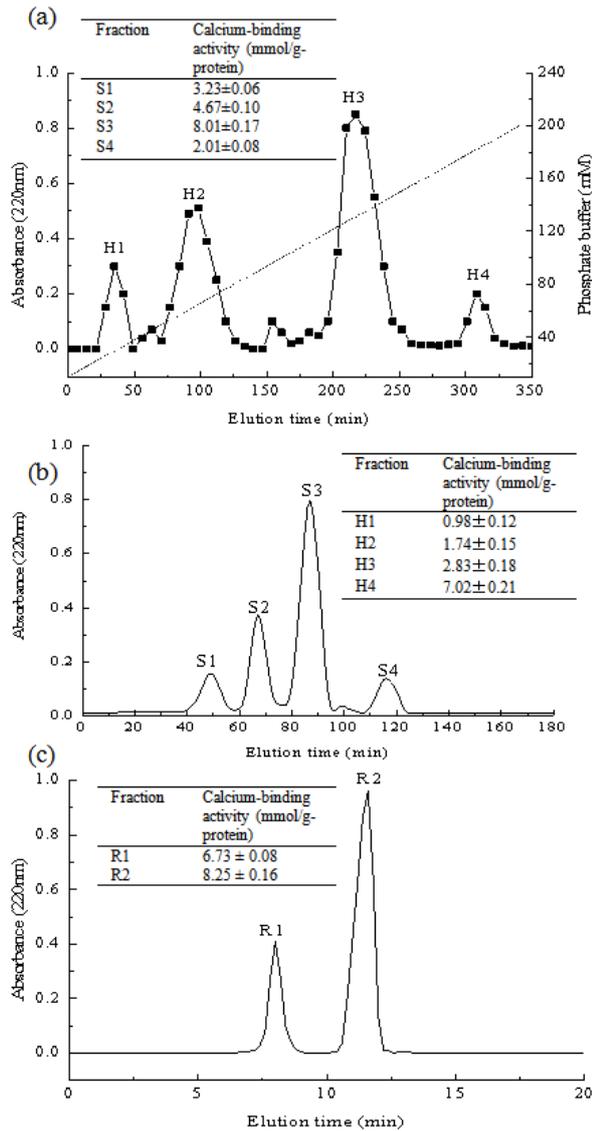|	Fraction	Calcium binding activity (mmol/g-protein)	Content (%)
Chelex 100	C1	0.03 ± 0.01	5.68
	C2	0.05 ± 0.02	7.32
	C3	0.73 ± 0.04	78.21
	C4	0.08 ± 0.03	8.79
Ultrafiltration	U1 (< 4 kDa)	0.89 ± 0.03	68.75
	U2 (> 4 kDa)	0.08 ± 0.01	31.25

Figure 3. Isolation and purification of calcium binding peptides. (a) Elution profile of BCC hydrolysate by hydroxyapatite chromatography. HA column (20 × 80 mm) was pre-equilibrated with 10 mM of potassium phosphate buffer, pH 6.5. The separation was performed with a linear gradient of 10–200 mM of phosphate buffer at a flow rate of 1.0 ml/min; (b) Elution profile of the active fraction (H4) from HA chromatography further purified by Sephadex G-25 chromatography. The column (26×150 mm) equilibrated and eluted with distilled water at a flow rate of 1.0 ml/min. (c) Elution profile of the active fraction (S3) from Sephadex G-25 chromatography further purified by RP-HPLC. The semipreparative RP-HPLC column (9.4 × 250 mm, Agilent Zorbax SB-C18) was eluted with a linear gradient of 5%–25% acetonitrile in water at a flow rate of 0.5 ml/min. All peaks eluted were monitored at 220 nm and collected for the calcium-binding activity analysis. All data were expressed as mean values (mean ± SD, n = 3).

The protein or peptide with calcium-binding activity could identify calcium on the surface of HA [36]. As shown in Figure 3 (a), the results showed that after HA chromatography, U1 was separated into four fractions marked as H1, H2, H3, and H4 by stepwise elution with the concentration of phosphate eluent increasing from 10 mM to 200 mM. The calcium-binding activity increased significantly. H4 was eluted with the maximum concentration of phosphate buffer (150 mM) and exhibited the highest calcium-binding ability of 7.02 mmol/g-protein, which showed that HA chromatography was very effective in separating peptide with the calcium-binding activity. The oligophosphopeptides with calcium-binding activity have been successfully separated and obtained from fish bone and carp egg [7,28] using HA affinity chromatography.

The pooled fraction (H4) was subsequently applied to Sephadex G-25 column, the fraction with the highest calcium-binding activity was pooled and isolated. As shown in Figure 3 (b), four fractions (S1, S2, S3, S4) were eluted at the various retention times corresponding to their molecular masses. Each fraction was pooled, lyophilized, and measured for their calcium-binding activities (adjusted to 50 mg/L of concentration). The results showed that fraction S3 showed the highest activity of 8.01 mmol/g-protein.

Then, S3 was subjected to RP-HPLC analysis. As shown in Figure 3 (c), two major fractions (R1, R2) were separated through the semi-preparative C18 column. The calcium-binding activity of an oligophosphopeptide from carp egg hydrolysate and pollack backbone peptide were 5.0, 7.0, and 7.85 mmol/g-protein when the protein concentration was 50 mg/L [6,7,28,35]. Compared with the peptides above, the peptide derived from bovine bone showed that R2 (BBP) had the highest calcium-binding activity of 8.25 mmol/g-protein. Therefore, the amino acid composition and identification of R2 (BBP) were further determined.

3.4. Amino Acid Composition Analysis

Table 3. Amino acid composition of the soluble hydrolysates liberated by BCC digestion and BBP

Amino acid	Amino acid Content (mg/100 mg of all amino acids)	
	Bovine bone hydrolysates	BBP
Asp	5.03 ±0.31	8.85 ± 0.58
Pro	10.29 ± 0.73	7.23 ± 0.49
Gly	20.24 ± 1.56	9.38 ± 0.61
Ala	8.12 ± 0.68	11.94 ± 0.98
Thr	12.30 ± 0.90	14.31 ± 1.01
Ser	2.70 ± 0.15	4.42 ± 0.11
Glu	11.24 ± 0.82	12.01 ± 0.92
Met	0.25 ± 0.01	0.78 ± 0.02
Ile	1.01 ± 0.02	2.05 ± 0.06
Tyr	1.58 ± 0.03	4.42 ± 0.09
Cys	0.35 ± 0.01	2.02 ± 0.04
Val	1.81 ± 0.04	2.34 ± 0.07
Phe	1.54 ± 0.03	2.56 ± 0.06
Trp	NF	NF
Leu	2.09 ± 0.06	1.97 ± 0.10
Arg	8.11 ± 0.55	8.73 ± 0.72
His	0.35 ± 0.01	0.97 ± 0.03
Lys	3.54 ± 0.08	5.21 ± 0.12
Hyp	8.51 ± 0.61	2.13 ± 0.08

NF, not found. All data were expressed as mean values (n = 3).

Table 3 illustrates the amino acid compositions of the soluble bovine bone hydrolysates and BBP. The relative Gly, Pro, and Hyp contents in the bovine bone hydrolysates, known as typical collagenous amino acids, were significantly higher than those of BBP. However, the Asp, Ala, Tyr, Thr, Ser, and Lys contents in BBP showed remarkable increments compared to the bovine bone hydrolysates. Ser, Thr, Ala, and Tyr residues are known sites of phosphorylation modification in protein, which is probably necessary for the calcium-binding ability of phosphorus peptide [35]. Jung [6,7] separated and obtained two calcium binding active peptides from the hoki (Johnius belengerii) bone and the Alaska Pollack backbone and found that they contain abundant Thr, Tyr, and Ser. Nishimoto [37] purified carp bone and obtained osteocalcin, which has high ratios of Ala, Tyr, Thr, Gln, and Asp. Jiang [35] prepared phosphopeptides from hen egg yolk phosvitin, which is composed mainly of Ser, Asx, Glx, and Arg. They reported that the phosphoseryl groups in oligophosphopeptide play an essential role in Ca^{2+}-phosphopeptide interaction.

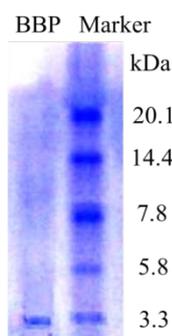## 3.5. Identification of Bovine Bone Peptide (BBP)

Figure 4. Tricine-SDS-PAGE electrophoresis of BBP obtained from bovine bone hydrolysates, with 10% polyacrylamide resolving gels. Lane 1: BBP isolated from bovine bone, lane 2: molecular mass markers (20.1, 14.4, 7.8, 5.8, and 3.3 kDa). Protein was visualized by Commassie Brilliant Blue R 250 staining of the gel. The picture is a representative of three similar experiments

BBP was identified by Tricine-SDS-PAGE, FT-IR and Raman, and mass spectrometry. Tricine-SDS-PAGE confirmed the BBP purity and mass determination (Figure 4), which was carried out using the method described by Schagger et al. [27]. This method can separate the range of the molecular weight polypeptide of 1–100 kDa. The corresponding Tricine-SDS-PAGE showed a single band at 3.3 kDa, which is an indication of its high purity.

The main conformation of protein is mainly determined by the characteristic peaks of amide I and amide III. From the known protein conformation of the Raman spectrum, we can derive the peak position: α-helical structures are 1645–1658 cm^{-1} and 1264–13l0 cm^{-1}, β-folding structures are l665–l680 cm^{-1} and 1230–1245 cm^{-1}, β-folded back structures are l663–1678 cm^{-1} and 1254–1300 cm^{-1}, and random coil structures are 1660–l666 cm^{-1} and 1242–1250cm^{-1} [38]. The Raman peak of BBP amide I and amide III were at 1666.29 cm^{-1} and 1259.86 cm^{-1} (Figure 5 (a)). The result showed that BBP consisted of β-folding and random coil structures, and the former was the main part. The

stretching bands of the two sulfur bonds of natural proteins were mostly at 510 cm^{-1}. If the band decreases, the protein is denatured. The characteristic bands of various polypeptides and amino acid residues appeared in the spetrum.
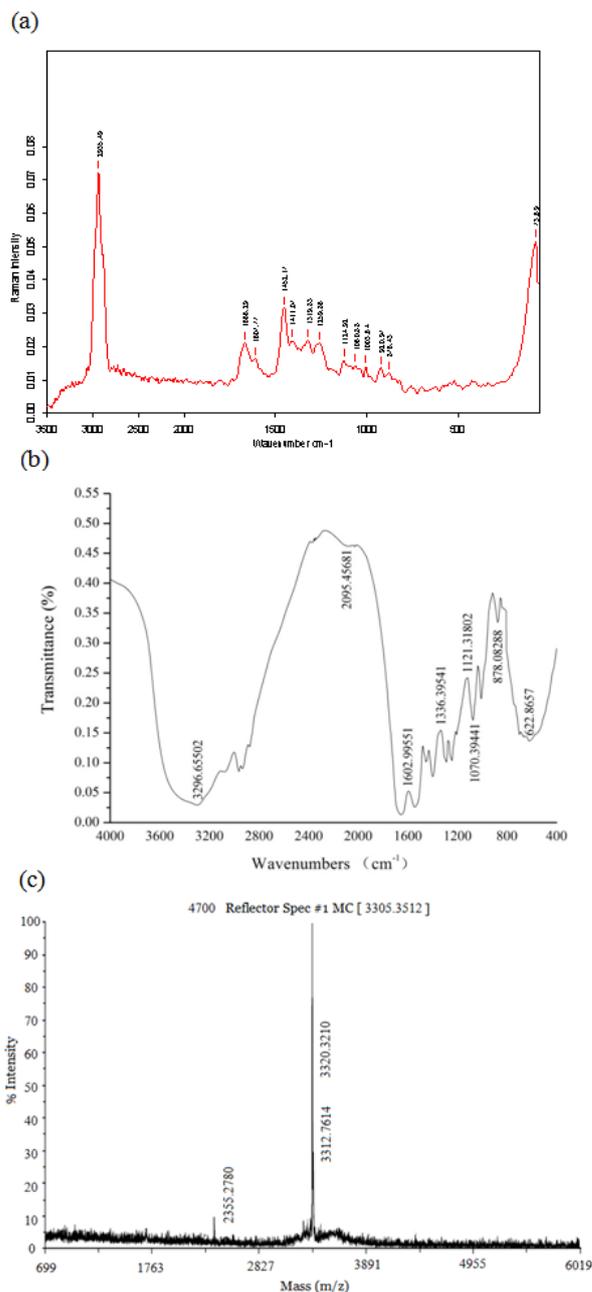

Figure 5. The Raman spectra (a), FT-IR spectra (b) and MALDI-TOF mass spectra (c) of BBP (the purified peptide with high calcium binding activity from bovine bone)

The infrared spectra showed that the BBP had the characteristic absorption peaks of the peptide in the spectral region 400–4000 cm^{-1} (Figure 5 (b)). The collagen peptide at the 3180 cm^{-1} and 3020 cm^{-1} protein belongs to the infrared spectra of the amide A and amide B bands and are caused by the stretching vibration of N-H. The characteristic absorption of collagen polypeptide (-CONH-) is generally believed to be near 3080 cm^{-1}, the absorption peak at 2962 cm^{-1} indicated that it was the collagen polypeptide. The protein amide I band (polypeptide C=O stretching) was observed as a band at

1641 cm^{-1}, while a band at 1455 cm^{-1} was attributed to the amide II (C-N stretching and N-H bending modes) vibrations [39,40]. The characteristic absorptions derived from phosphate groups in the infrared spectrum at 1244, 1107, and 907 cm^{-1} were assigned to P=O, P-O-C, and P-O-binding to alkyl groups, respectively. The collagen polypeptide contains high content of glycine, hydroxyproline and proline (the distinctive sequence: Gly-Pro-Hyp), which produces the collagen peptide with infrared spectral characteristics from 1200 cm^{-1} to 1400 cm^{-1}. Then, the Hyp of absorption peaks at 1107 cm^{-1} and 847 cm^{-1} was also characterized.

The molecular mass of BBP was determined to be 3.3053512 kDa by MALDI-TOF-MS (Figure 5 (c)). The small peaks at m/z 2355.2780 may be due to impurities or peptide fragments. However, the separation of peptides is known to be very difficult due to their complexity. Jung et al. [6] reported the molecular mass of fish bone phosphopeptide to be 3.5 kDa.
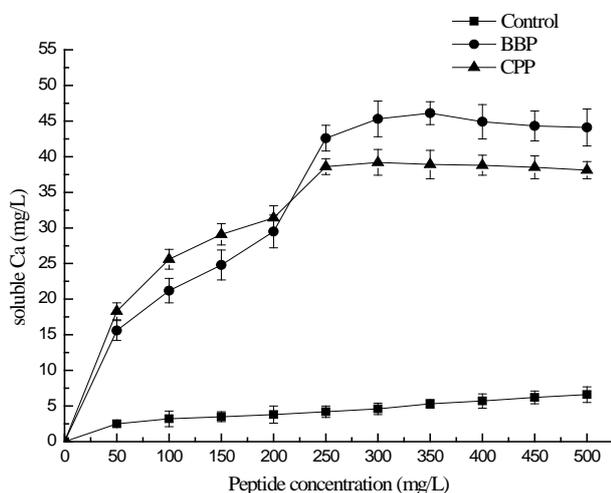
3.6. Calcium Binding Activity of BBP

Figure 6. Calcium binding activity of BBP. Various concentrations of BBP (up to 500 mg/l) were mixed with 5 mM $CaCl_2$ and 20 mM sodium phosphate buffer (pH 7.5). The mixture was stirred at 25oC for 30 min, pH 7.5. After with 0.22 μm microfiltration to remove insoluble calcium phosphate salts, calcium contents of the supernatant were determined by FAAS (AA-6300C, Shimadzu Co., Japan). The experiments were performed in triplicate, values are expressed as means ± SD

By determining the calcium contents of the supernatant, the BBP could inhibit the formation of insoluble calcium phosphate (Figure 6). Calcium binding activity of the BBP was higher than that of casein oligophosphopeptide (CPP). The solubility of Ca was dependent on the concentration of BBP, and 43.02 mg/l of calcium was obtained at a concentration of 350 mg/l. As reported by Jiang and Mine [35], the solubility of 36.3 mg/l of calcium was obtained at 200 mg/l of the FBP, prepared from hoki (Johnius belengerii) bone by Jung at al. [6], and the solubility of 41.1 mg/l of calcium was obtained at a concentration of 250 mg/l.

4. Conclusion

The calcium-binding ability of enzymatic hydrolysates of bovine bone was influenced by demineralization treatment. Approximately, bovine bone with 30% demineralization rate can be effectively hydrolyzed by BCC. After Chelex 100 and ultrafiltration, 68.75% of hydrolysates was the fraction of U1 with MW < 4 kDa, which showed a better calcium-binding ability. H3 eluted with the maximum concentration of phosphate buffer exhibited the highest calcium binding ability of 2.83 mmol/g-protein after HA chromatography. Further purification was performed using size exclusion chromatography and RP-HPLC to obtain BBP, an oligophosphopeptide with a high calcium binding ability (8.25 mmol/g-protein). BBP was rich in Asp, Ala, Tyr, Thr, Ser, and Lys, which are probably necessary for the calcium-binding ability of phosphorus peptide, indicating that BBP is an oligophosphopeptide with a molecular mass of 3.3053512 kDa. After the BBP was interacted with calcium, 43.02 mg/l of soluble calcium were maintained at 20-mM phosphate buffer (pH 7.0) without the formation of insoluble calcium phosphate, which was higher than that of CPP. Thus, a novel nutraceutical with high calcium bioavailability can be provided for the food industry through further studies of its calcium-binding and bioavailability mechanism in vitro and in vivo assays.

Acknowledgements

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