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Preparation of hoki (*Johnius belengerii*) bone oligophosphopeptide with a high affinity to calcium by carnivorous intestine crude proteinase

Won-Kyo Jung, Pyo-Jam Park, Hee-Guk Byun, Sung-Hoon Moon, Se-Kwon Kim *

Department of Chemistry, Pukyong National University, Busan 608-737, Republic of Korea

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Abstract

In the present study, the skeletons discarded from industrial processing of hoki (*Johnius belengerii*) were digested by a heterogeneous enzyme extracted from the intestine of a carnivorous fish (also discarded from industrial processing), bluefin tuna (*Thunnus thynnus*), in order to utilize the bone in nutraceuticals with a high bioavailability of calcium. The tuna intestine crude enzyme (TICE) could effectively biodegrade the hoki bone matrices composed of collagen, non-collageneous proteins, carbohydrates and minerals. A fish bone phosphopeptide (FBP) containing 23.6% of phoshorus was isolated from the hoki bone hydrolysates degraded by TICE using HA affinity chromatography and gel permeation chromatography. After the FBP, with a molecular mass of 3.5 kDa, was interacted with calcium, 41.1 mg/l of soluble calcium were maintained at 20 mM phosphate buffer (pH 7.8) without the formation of insoluble calcium phosphate. The results provide evidence that the carnivorous fish intestine enzyme (TICE) could degrade the teleost (*J. belengerii*) bone, and the fish bone oligophosphopeptide prepared by the enzymatic degradation of the bone could be utilized as a nutraceutical with a potential calcium-binding activity.

Keywords: Teleost bone; Bone proteins; Tuna intestine crude enzyme; Fish bone oligophosphopeptide; Calcium binding activity

1. Introduction

Bone is regarded as a composite tissue, basically made up of two phases, an organic and an inorganic one. Mammalian bone is composed of a well organized extracellular matrix that contains embedded crystals of hydroxyapatite (HA). The major part, 90% of the organic matrix, is collagen (mainly type I collagen), and the remaining 10% consists of over 200 other non-collagenous proteins, such as osteocalcin, osteopontin, osteonectin, fibronectin, thrombospondin, proteoglycan I, II and growth factors (IGF-1, PDGF and TGF-ß). These components are produced by the osteocytes, and the molecules with an affinity for calcium are associated with bone remodelling and cell attachment (Garner, Anderson, & Ambrose, 1996).

Recent studies have clearly defined the similarities and differences between fish bone and mammalian bone (Huysseune, 2000), and the structure, distribution, function and activity of organic components in fish bone have been identified (Nishmoto, Waite, Nishimoto, & Kriwacki, 2003; Witten & Hall, 2002). Despite the fact that fish bone contains bioactive and nutraceutical molecules, few efforts have been made to utilize fish bones in functional materials.

Annually, more than 50% of total fishery products (over 120 million tons per year) are discarded as inedible byproducts, such as bone, skin, fins, internal organs and

* Corresponding author. Tel.: +82-51-620-6375; fax: +82-51-628-8147.
E-mail address: sknkim@mail.pknu.ac.kr (S.-K. Kim).

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head. Thus, many studies have been performed to utilize the large amounts of protein, oil, mineral, carbohydrate and nucleic acid originating from fishery byproducts, and to improve their functional properties (Kim et al., 2001, 2003; Nagai & Suzuki, 2000; Nair & Gopakumar, 1982; Rodriguez-Estrada, Chung, & Chinachoti, 1994; Shahidi & Janak Kamil, 2001). However, studies on the utilization of organic components or minerals in the fish bone are scarce (Kim et al., 2003; Larsen, Thilsted, Kongsbak, & Hansen, 1992).

In the present study, we report the digestion of hoki (*Johnius belengerii*) bones by crude enzymes extracted from bluefin tuna (*Thunnus thynnus*) intestine, the preparation of phosphopeptide with a high affinity for calcium, its chemical composition and partial characterization of its calcium binding property.

2. Materials and methods

2.1. Materials

Both hoki (*J. belengerii*) frames and bluefin tuna (*T. thynnus*) intestines obtained from industrial processing were provided by Dongwon Co. (Busan, Korea), and stored at −70 °C prior to use. Commercial proteinases (α-chymotrypsin from bovine pancreas, type II; trypsin form bovine pancreas, type II; papain from papaya latex, type IV; pepsin from porcine gastric mucosa; pronase E from *Streptomyces griseus*, type XIV; collagenase from *Clostridium histolytium*, type I) were purchased from Sigma Chemical Co. (St. Louis, MO). Alcalase and Neutrase were obtained from Novo Co. (Denmark). Synthetic substrates (BAPNA, *N*-benzoyl-DL-arginine–*p*-nitroanilide; BAEE, *N*-benzoyl-L-arginine ethyl ester; BTEE, *N*-benzoyl-L-tyrosine ethyl ester; ATEE, *N*-acetyl-L-tyrosine ethyl ester) and type I collagen from bovine Achilles' tendon were purchased from Sigma Chemical Co. (St. Louis, MO).

Calcium standard solution (1 g/l) was prepared by dilution of Ca standard (Titrisol, Merck Co.) with distilled-deionized water, and the lanthanum solution (5 g/100 ml) was prepared with La_2O_3 (Merck Co.) before use. Glass and polyethylene materials were soaked in HNO_3 (sp. gr. 1.40) for 10 min, and then rinsed three times with distilled-deionized water. All other chemicals were of analytical grade or better.

2.2. Preparation of teleost bone and carnivorous intestine crude enzyme

Hoki frame and tuna intestine, as fishery processing by-products, were used for recovering a teleost bone and extracting carnivorous intestine enzyme, respectively. The teleost bone was recovered from hoki frames according to the method of Kim et al. (2003), and tuna intestine crude enzyme (TICE) was extracted according to the method of Kim, Jeon, Byun, Kim, and Lee (1997).

2.3. Assay of proteolytic activity of carnivorous intestine crude enzyme

According to the method of Kim et al. (1997), proteolytic activities of the TICE were assayed with various substrates. To identify tryptic activity of the TICE, BAPNA and BAEE were used as nitroanilide and ester synthetic substrates, respectively. In the assay for α-chymotryptic activity, BTEE and ATEE were used as ester synthetic substrates. Collagenolytic activity of the TICE was measured using type I collagen as a natural substrate, composing 90% of organic matrix in the bone (Edwards & O'Brien, 1980). One unit (U) of enzyme activity was defined as the amount of enzyme that is required for the hydrolysis of 1 μmol of substrate per 1 min.

2.4. Chemical analysis

2.4.1. Proximate composition

Crude protein, lipid, ash and moisture of fish bone were determined according to the Association of Official Analytical Chemists method (AOAC, 1995). Crude protein was estimated from the total nitrogen multiplied by 6.25.

2.4.2. Mineral composition

Fish bone was ashed at 600 °C for 4 h using an electric furnace, followed by drying. Total minerals in the ash were determined by energy dispersive X-ray spectroscopy (Hitachi model H-7500, Hitachi Co., Japan) after it was ground into a fine powder. Calcium concentration in sample solution was measured by a flame atomic absorption spectrometer (Simatzu AA-680, Simatzu Co., Japan) fitted with a hollow cathode lamp. Instrumental conditions were wavelength = 422.8 nm, slit = 0.7 nm, acetylene flow = 1.75 l/min, air flow = 14.0 l/min, nebulizer = spoiler. Lanthanum solution was added to 0.1% (w/v) sample solutions.

2.4.3. Protein and carbohydrate analysis

Protein concentration in sample solutions was determined by the method of Lowry, Rosebrough, Farr, and Randall (1951) using bovine serum albumin as a standard. After a demineralization of sample with Chelex-100 (Bio-Rad, Hercules, CA), phosphorus was determined by the colorimetric method, using a phosphoprotein phosphate assay kit (Pierce Biotechnology, Inc., Rockfold, IL), and phosvitin (Sigma Chemical Co., St. Louis, MO) was used as a standard. Collagen protein was determined by measuring hydroxyproline content according to the method of Edwards and O'Brien (1980). Carbohydrate was determined by the

phenol sulfuric acid method of Dubios, Gillies, Hamilton, Rebers, and Smith (1956), using a mixture of D-glucosamine and mannose (1:1 weight ratio) as a standard.

2.5. Preparation of fish bone hydrolysates by TICE-digestion

After being crushed into hoki bone powder, the powder was digested with the TICE under the experimental conditions (pH 9.0, 40 °C, enzyme/substrate: 1/100, substrate concentration: 1%) for 48 h according to Kim et al. (1997). Commercial enzymes (α-chymotrypsin, trypsin, papain, pepsin, pronase E, Neutrase, Alcalase, and collagenase) were used for comparing with the biodegradability of the TICE. After incubation at 100 °C for 5 min to inactivate enzyme, fish bone hydrolysates were filtered through an ashless Whatman No. 41 filter paper (Whatman International Ltd., Maidstone, UK). The filtrates were centrifuged at 3000*g* for 20 min and lyophilized. Chemical analysis of the soluble hydrolysates was performed as described previously.

2.6. Determination of amino acid composition

To determine amino acid composition, the soluble hydrolysates were incubated with 6 N HCl at 110 °C for 24 h in vacuum-sealed ampoules. After neutralizing and evaporation and filtering, with a glass filter, amino acid composition was determined with a Biochrom 20 amino acid analyzer (Biochrom Ltd., Cambridge, UK). Cysteine and tryptophan residues were estimated by the method of Spencer and Wold (1969) and Spande and Witkop (1967), respectively. Hydroxyproline (Hyp) was determined by the method of Edwards and O'Brien (1980).

2.7. Isolation of fish bone peptide with a high affinity for calcium

Tuna intestine crude enzyme-digested fish bone hydrolysates were filtered and demineralized on a Chelex 100 resin (Bio-Rad) column at a flow rate of 1.0 ml min^{-1}. To isolate soluble fractions with a high affinity for calcium, the demineralized fraction was applied to a HA column (20 × 80 mm, Macroprep ceramic HA type 1, Bio-Rad, Richmond, CA) pre-equilibrated with 10mM potassium phosphate buffer, pH 6.5. The separation was performed with a linear gradient of 10–200 mM phosphate buffer at a flow rate of 0.7 ml min^{-1}. The eluate was subsequently separated by a gel permeation chromatograph (GPC) column (OHpak SB-803 HQ, Shoko Co., Ltd., Japan) at a flow rate of 1.5 ml min^{-1}. All peaks eluted were monitored at 280 nm, and a soluble fraction, showing the highest Ca-binding activity, was dialyzed and lyophilized after Ca-binding assay as described below. Chemical analysis of the soluble fish bone peptide (FBP) was performed as described previously. During gel permeation chromatography, the molecular mass of FBP was examined using standard materials produced from Sigma Chemical Co. (alcohol dehydrogenase, 150.0 kDa; bovine serum albumin, 66.0 kDa; carbonic anhydrase, 29.0 kDa; cytochrome *c*, 12.4 kDa; aprotinin, 6.5 kDa; angiotensin I converting enzyme, 1.3 kDa).

2.8. Calcium binding assay

The assay was performed basically according to the method of Sato, Shindo, Gunshin, Noguchi, and Naito (1991) with the following modifications. Various concentrations, up to 500 mg/l, of FBP were mixed with 5 mM $CaCl_2$ and 20 mM sodium phosphate buffer (pH 7.8). The mixture was stirred at 22 °C for 30 min, and the pH was maintained at 7.8 with a pH meter. After removal of insoluble calcium phosphate salts and filtration using a 0.45 μm membrane, calcium contents of the supernatant were determined by flame atomic absorption spectroscopy (FAAS). The experiments was performed in triplicate, values were expressed as means ± SD.

3. Results and discussion

3.1. Chemical composition of hoki bone

Hoki (*J. belengerii*) bone was recovered from the frame (moisture: 68.10%; bone: 13.72%; flesh: 18.18%) as a byproduct of fishery processing. After air-drying and crushing into a powder, chemical compositions of hoki bone were obtained as shown in Table 1. The organic portion of hoki bone (30.54% on dry basis) was composed of 28.0% protein, 1.94% lipid and 0.56% carbohydrate. Collagen was present at 86.2% of total protein, and non-collageneous protein content was 13.8% in the bone. As reported by Garner et al. (1996), 90% of organic components in the bone matrix were a type I collagen, and the remaining 10% consisted of non-collageneous proteins, such as osteocalcin, osteopontin, osteonectin, fibronectin, thrombospondin, proteoglycan I/II and growth factors (IGF-1, PDGF and TGF-ß). These molecules were produced by cell-like osteoblasts and their functions are related to bone formation and cell attachment. Carp (*Cyprinus carpio*) osteocalcin was detected and characterized by Nishimoto et al. (2003). Lipid exists as phospholipids, and carbohydrate was detected from glycoproteins, such as hyaluronic acid and proteoglycan with side chains of chondroitin sulfate and heparin sulfate (Garner et al., 1996).

The inorganic portion, minerals (69.46% on dry basis), was mainly composed of 59.7% calcium (Ca) and 35.8% phosphorus (P) with the mole ratio of Ca/P, 1.67. The inorganic portion of vertebrate bone is

Table 1
Chemical composition of hoki bone

Chemical	Content
Protein	28.0[a]
Collagenous	86.2[b]
Non-collagenous	13.8[b]
Lipid	1.94[a]
Carbohydrate	0.56[a]
Mineral	69.5[a]
Ca	59.7[c]
P	35.8[c]
(molar ratio of Ca/P)	1.67[c]
Na	0.62[c]
Mg	0.34[c]
Cl	0.24[c]
K	0.07[c]
Zn	60[d]
Fe	14[d]
Cu	2[d]

All data were expressed as mean values ($n = 3$).
[a] g/100 g of fish bone on dry basis.
[b] g/100 g of fish bone protein on dry basis.
[c] g/100 g of fish bone mineral on dry basis.
[d] ppm of fish bone mineral on dry basis.

primarily composed of HA crystals deposited within an organic matrix of cross-linked collagen fibrils (Anderson & Garner, 1996). The HA crystals make up approximately 60–65% of bone and the HA has an extremely complicated crystalline structure $[(Ca^{2+})_{10-x}(H_3O^+)_{2x}\text{-}(PO_4{}^{3-})_6(OH^-)_2]$. In vertebrate animals, the crystals are usually organized with x value range of 0–2 (*2*, *32*). When the crystals grow at a highly organized rate in the bone matrix (x is 0), the chemical formula of HA is $Ca_{10}(PO_4)_6(OH)_2$, and the mole ratio of Ca/P is 1.67, same as that of hoki bone (Table 1). As reported by Hamada et al. (1995), the Ca/P mole ratio in the bone of marine teleosts (15 species) varied within the range 1.63–1.20, and these bones were organized as the combinational structure with HA and beta type $Ca_3(PO_4)_2$. The Ca/P ratio of hoki bone was significantly higher than that of other species (mackerel, sardine, flounder, anchovy, shark, tilefish, croaker and conger eel), but was similar to mammalian (1.68) and human (1.69) ratios. These results illustrate that hoki bone was organized with abundant Ca and organic materials.

3.2. Proteolytic activities of tuna intestine crude enzyme

Specific proteolytic activities of TICE were examined with various substrates; type I collagen as a natural substrate: BTEE and ATEE as a synthetic ester type substrate of α-chymotrypsin, BAEE as a synthetic ester type of substrate of trypsin and BAPNA as a synthetic nitroanilide-type substrate of trypsin. As shown in Fig. 1(a), the TICE could hydrolyze four kinds of synthetic substrates. The activity of the TICE was highest for the BAEE, approximately 2.5 U/mg, and the TICE had potent activity (1.6 U/mg) against the BTEE. Collagenolytic activity of the TICE was determined as 16.5 U/mg type I collagen (Fig. 1(b)). The results indicated that the TICE contained plentiful tryptic and collagenic enzymes. In our previous study (1997), the specific activities of tuna pyloric caeca crude proteinase (TPCCP) were determined using casein as a natural substrate and the same kinds of synthetic substrates. The TPCCP showed specific activity (0.54 U/mg casein), and its activity was also the highest for BAEE (2.8 U/mg). Other proteolytic activities have been elucidated from internal organs of carnivorous fishes (mackerel, carp, cod, salmon and trout) as heterogeneous or homogeneous proteinases (Gudmundsson & Hafsteinsson, 1997; Shahidi & Janak Kamil, 2001). These can be classified into two large families of serine and aspartic proteinases. Among these families, pepsin and chymosin are aspartic proteinases, and trypsin, chymotrypsin, collagenase and elastase are ser-

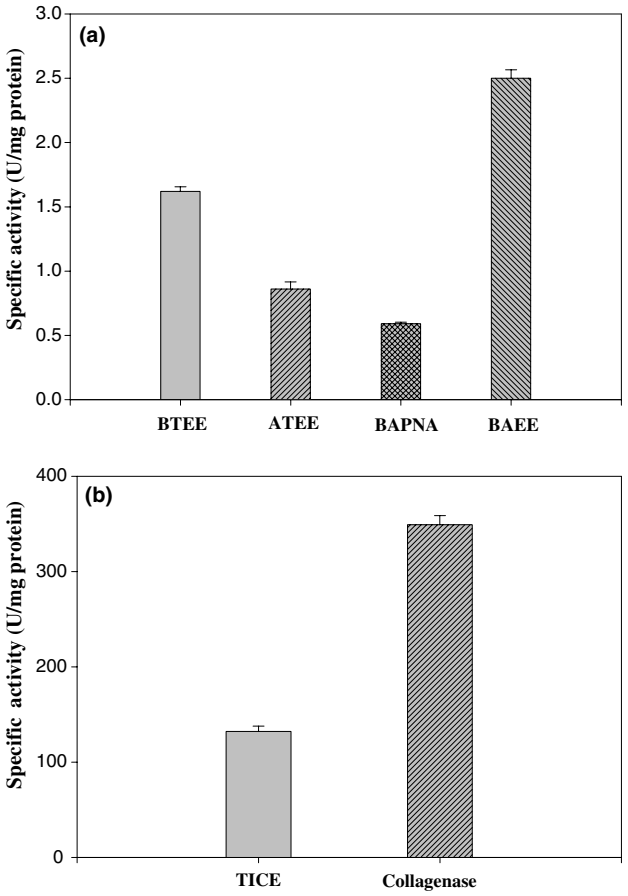

Fig. 1. Activities of TICE on natural and synthetic substrates. One unit (U) of enzyme activity is defined as the amount of enzyme that is required for the hydrolysis of 1 μmole of substrate per 1 min. (a) Activities of TICE on synthetic substrates (BAPNA, *N*-benzoyl-DL-arginine-*p*-nitroanilide; BAEE, *N*-benzoyl-L-arginine ethyl ester; BTEE, *N*-benzoyl-L-tyrosine ethyl ester; ATEE, *N*-acetyl-L-tyrosine ethyl ester). (b) Collagenolytic activity of TICE using type I collagen as a natural substrate. The assay was performed in triplicate; values are expressed as means ± SD.

Table 2
Chemical composition of the soluble bone hydrolysates liberated by various enzymes

Enzyme	Content (mg/100 mg hoki bone on dry basis)				
	Total[a]	Protein		Calcium	Trace elements
		Collagen	Non-collagen		
TICE	32.1	13.4	10.3	6.55	1.96
Alcalase	24.6	13.9	4.62	4.15	1.92
Pepsin	23.9	11.5	5.23	5.72	1.47
Collegenase	32.7	23.7	2.13	5.77	1.15
Neutrase	24.1	14.0	4.68	4.32	1.16
Papain	25.7	16.8	3.45	3.64	1.83
α-Chymotrypsin	21.4	12.7	3.06	4.04	1.56
Pronase E	21.6	13.0	2.95	4.03	1.66
Trypsin	20.4	12.2	3.33	3.92	0.89

All data were expressed as mean values ($n = 3$).
[a] Total, total content of soluble hydrolysates liberated from hoki bone.

ine proteinases. Ramakrishna, Hultin, and Atallah (1987) reported that the dogfish enzyme, in general, could hydrolyze collagen molecules more efficiently than bovine enzyme, no matter whether the substrates were soluble or insoluble. Bezerra, Santos, Lino, Vieira, and Carvalho (2000) reported that the highest proteolytic activity was found in the stomach, and that the alkaline activity was greatest in the pyloric caeca in the tambaqui (*Clolssoma macropomum*) digestive tract.

3.3. Enzymatic hydrolysis of fish bone by the TICE

As shown in Table 2, the TICE could efficiently degraded fish bone in comparison with other commercial enzymes tested. Total bone hydrolysates liberated by TICE were 32.12% of total bone. Protein content was 21.9% of the total soluble hydrolysates, which consisted of 13.4% collagen and 10.3% non-collagenous protein. Phophoprotein was determined as 16.65% of non-collagenous protein, and the content of soluble calcium liberated by the TICE was 6.55%. Among commercial enzymes tested, collagenase liberated the largest amounts of hoki bone hydrolystes (32.7%) by destroyting the cross-linked structure of the organic matrix. In the soluble hydrolysates prepared by collagenase, 23.7% collagen was liberated, but non-collagenous hydrolysates were not markably liberated. Table 3 illustrates the amino acid composition in the soluble hydrolysates liberated by the TICE digestion. Proteins in the hydrolysates consisted of Gly, Thr, Glx, Ala, Asx, Ser, Hyp and Arg. As reported by Jiang and Mine (2002), Ca-binding phosphoproteins, such as osteocalcin, phosvitin and casein phosphoprotein, mainly consist of Ser, Thr, Ala and Tyr residues phosphorylated or bound to Ca (Houben et al., 1999). Pro, Gly, Pro and Hyp residues are known as typical amino acids of collagen (Edwards & O'Brien, 1980). Calcium binding phosphoproteins derived from non-collagenous materials in the bone have a high affinity for Ca^{2+} ion on the surface of HA, and function in cell signalling and the recruitment of osteoclasts and osteoblasts, which have active roles in bone resorption and deposition, respectively (Hoang, Sicheri, Howard, & Yang, 2003).

Table 3
Amino acid composition of the soluble hydrolysates liberated by MICE digestion and FBP isolated

Amino acid	Content (mg/100 mg of all amino acids)	
	Hoki bone hydrolysates	Fish bone phosphopeptide
Asx[a]	7.15	10.4
Thr	12.0	13.3
Ser	7.01	7.75
Glx[b]	10.0	11.6
Pro	5.92	4.21
Gly	21.3	9.61
Ala	9.16	12.0
Cys	0.22	1.62
Val	1.95	2.97
Met	1.98	2.10
Ile	0.98	1.96
Leu	1.89	1.79
Tyr	2.01	4.94
Phe	2.21	4.26
Trp	NF[d]	0.19
His	0.16	0.96
Lys	2.66	4.11
Arg	6.16	6.27
Hyp[c]	6.87	NF[d]

All data were expressed as mean values ($n = 3$).
[a] Asx, Asp + Asn.
[b] Glx, Glu + Gln.
[c] Hyp, hydroxyproline.
[d] NF, not found.

3.4. Isolation and biochemical properties of fish bone oligophosphopeptide

As shown in Fig. 2, the isolation of fish bone phosphopeptide (FBP) with a calcium binding activity was

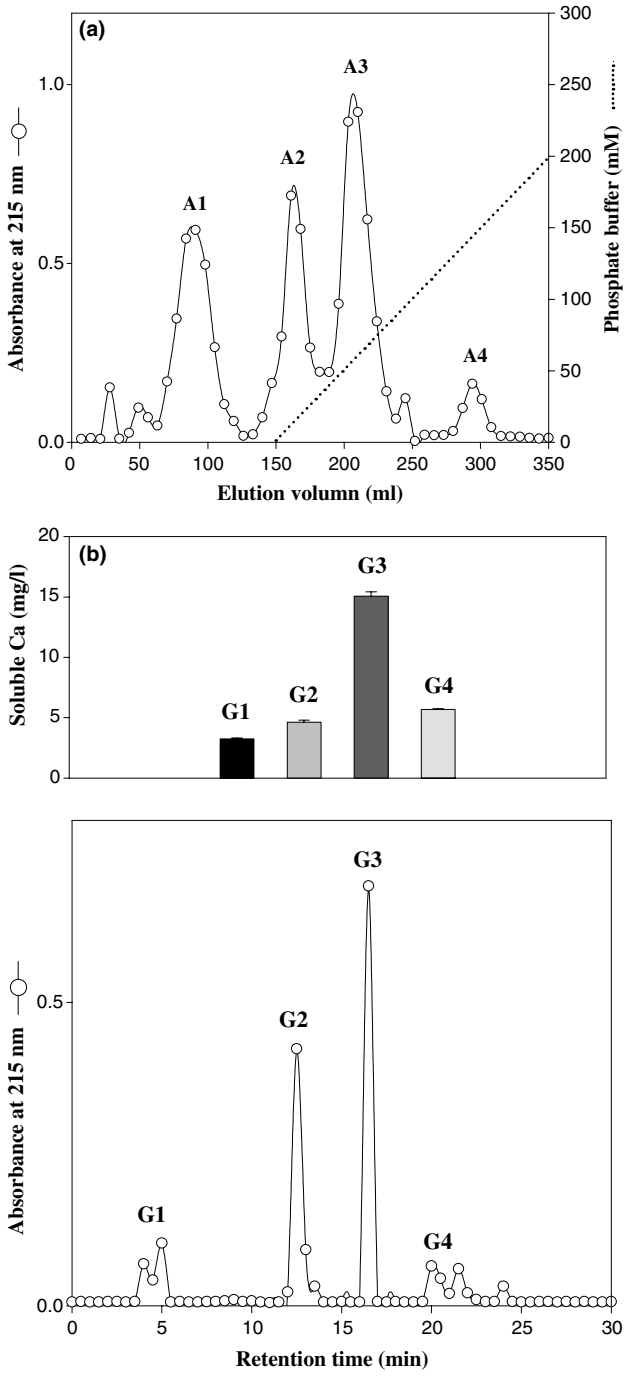

Fig. 2. Isolation of FBP from hoki bone hydrolysates. (a) Affinity chromatography on the HA column (20 × 80 mm, Macroprep ceramic HA type 1) pre-equilibrated with 10 mM potassium phosphate buffer, pH 6.5. The separation was performed with a linear gradient of 10–200 mM phosphate buffer at a flow rate of 0.7 ml min^{-1}. (b) Further isolation of A3 fraction by gel permeation chromatography on OHpak SB-803 HQ column (lower panel) and the calcium binding activity (upper panel). The assay was performed in triplicate; values are expressed as means ± SD.

accomplished using HA affinity chromatography and gel filtration chromatography (GPC). The results of affinity chromatography are illustrated in Fig. 2(a). Of the peaks (A1, A2, A3, A4) corresponding to four fractions, A4 was eluted at high phosphate concentration (150 mM phosphate buffer), and this indicates that A4 has the highest affinity for the HA (as compared to other fractions). As reported by Hoang et al. (2003), Ca-binding proteins, such as osteocalcin, can recognize calcium on the surface of HA. Dohi, Iwami, Yonemasu, and Moriyama (1987) isolated two Ca-binding proteins with γ-carboxyglutamic acid (gla protein) from bull frog *Rana catesbiana* using HA affinity chromatography. The pooled fraction (A4) was subsequently applied to the GPC column. As shown in Fig. 2(b), four fractions (G1, G2, G3, G4) were eluted at the various retention times corresponding to their molecular masses, and the G3 fraction (FBP), among the fractions adjusted to 50 mg/l of concentration, showed the highest Ca-binding activity (approximately 15 mg/l). The molecular mass of FBP was estimated to be 3.5 kDa (Fig. 3). Phosphate content of the FBP was measured as 23.6%, and characteristic absorptions, derived from phosphate groups in the infrared spectrum (Shimadzu IR-408, Shimadzu Co., Japan) at 1250, 1150 and 1000 cm^{-1} were assigned to P═O, P–O–C, and P–O-binding to alkyl groups, respectively (Fig. 4). Other components, such as carbohydrate and lipid, were not detected in the FBP.

As shown in Fig. 5, the FBP could inhibit the formation of insoluble calcium phosphate, as measured by determining the calcium contents of the supernatant after the formation of Ca–CPP complex. Calcium binding

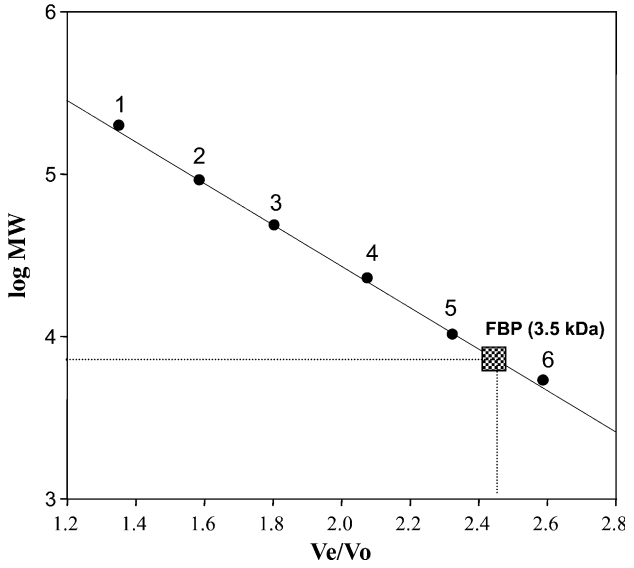

Fig. 3. Determination of molecular mass of FBP. Molecular weight of FBP was measured during the gel permeation chromatography in Fig. 2(b). under the same conditions. Ve/Vo (Ve, volume of eluted protein; Vo, volume of eluted Blue dextran). • (standard materials) 1, alcohol dehydrogenase (150.0 kDa); 2, bovine serum albumin (66.0 kDa); 3, carbonic anhydrase (29.0 kDa); 4, cytochrome *c* (12.4 kDa); 5, aprotinin (6.5 kDa); 6, angiotensin I converting enzyme, 1.3 kDa; ▩, intact KPL.

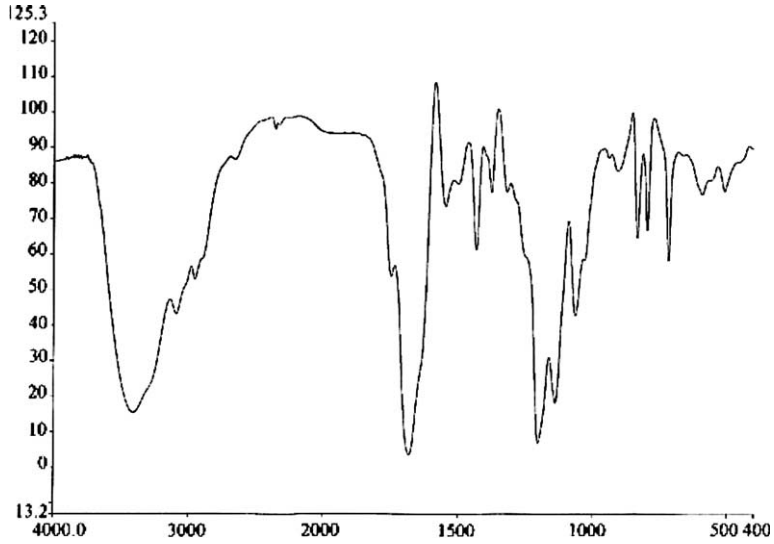

Fig. 4. FT-IR spectrum of FBP.

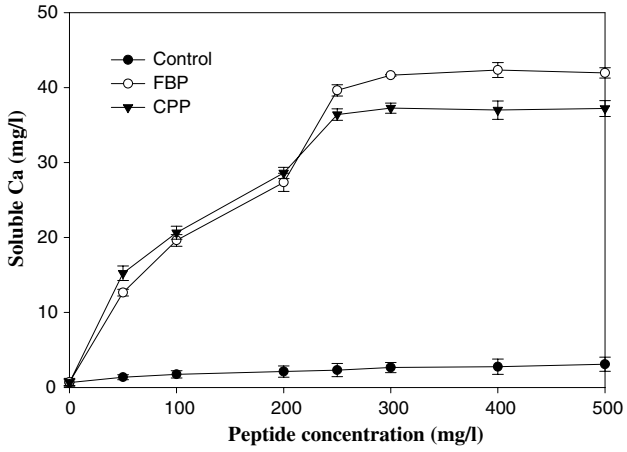

Fig. 5. Calcium binding activity of FBP. Various concentrations, up to 500 mg/l, of FBP were mixed with 5 mM $CaCl_2$ and 20 mM sodium phosphate buffer (pH 7.8). The mixture was stirred at 22 °C for 30 min, and the pH was maintained at 7.8 with a pH meter. After removal of insoluble calcium phosphate salts and filtration with 0.45 m membrane, calcium contents of the supernatant were determined by FAAS. The experiments was performed in triplicate; values are expressed as means ± SD.

activity of the FBP was higher than that of casein oligophosphopeptide (CPP). The solubility of Ca was dependent on the concentration of FBP, and 41.1 mg/l of Ca was obtained at a concentration of 250 mg/l. The pH of the reaction system was maintained at 7.8 because low pH could increase the solubility of insoluble calcium salt. As reported by Jiang and Mine (2000), the solubility of 36.3 mg/l of Ca was obtained at 200 mg/l of the oligophosphopeptide from egg yolk phosvitin with 35% phosphate retention, and the solubility was higher than that of commercial CPPII (Meiji Seika Co., Ltd., Tokyo, Japan).

In the amino acid composition of the FBP, the relative contents of Gly, Pro and Hyp, knowntypical collagenous amino acids, were significantly lower than those of hoki bone hydrolysates. However, the contents of Thr, Ser, Glx and Ala, which are phosphorylated or Ca^{2+}-binding, showed remarkable increments as compared to the bone hydrolysates (Table 3). Nishimoto et al. (2003) isolated and characterized an osteocalcin from carp *Cyprinus carpio*, and carp osteocalcin has high ratios of Ala, Tyr, Thr, Gln and Asp. The calcium binding oligophosphopeptide, prepared from hen egg yolk phosvitin by Jiang and Mine (2000), mainly consisted of Ser, Asx, Glx and Arg, and they reported that phosphoseryl groups in the oligophosphopeptide played an essential role in Ca^{2+}-phosphopeptide interaction.

In conclusion, fish bone phosphopeptide was isolated from *J. belengerii* bone by carnivorous fish intestine heterogeneous enzyme using HA affinity chromatography. It showed that fish bone phosphopeptide was an oligophosphopeptide with 23.6% phosphorus and a molecular mass of 3.5 kDa, and that the phosphopeptide could solublize more calcium than CPP. Thus, it is possible to provide a novel nutraceutical with a high bioavailability for calcium through further studies on its molecular structure and in vivo assays.

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