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Fish-bone peptide increases calcium solubility and bioavailability in ovariectomised rats

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Fish-bone peptides (FBP) with a high affinity to Ca were isolated using hydroxyapatite affinity chromatography, and FBP II with a high ratio of phosphopeptide was fractionated in the range of molecular weight 5·0–1·0 kDa by ultramembrane filtration. *In vitro* study elucidated that FBP II could inhibit the formation of insoluble Ca salts in neutral pH. *In vivo* effects of FBP II on Ca bioavailability were further examined in the ovariectomised rat. During the experimental period, Ca retention was increased and loss of bone mineral was decreased by FBP II supplementation in ovariectomised rats. After the low-Ca diet, the FBP II diet, including both normal level of Ca and vitamin D, significantly decreased Ca loss in faeces and increased Ca retention compared with the control diet. The levels of femoral total Ca, bone mineral density, and strength were also significantly increased by the FBP II diet to levels similar to those of the casein phosphopeptide diet group (no difference; $P > 0.05$). In the present study, the results proved the beneficial effects of fish-meal in preventing Ca deficiency due to increased Ca bioavailability by FBP intake.

Fish-bone peptides: Calcium solubility: Ovariectomised rats: Calcium bioavailability

The major source of Ca is the diet, and the most common and trusted source of Ca is milk or other dairy products (Anderson & Garner, 1996). Dairy products contain a high content of casein. Casein phosphopeptides (CPP) derived from the intestinal digestion of casein have been shown to enhance bone calcification in rats (Lee *et al.* 1980; Tsuchita *et al.* 1993). Such CPP have the capacity to chelate Ca and to prevent the precipitation of Ca phosphate salts (Berrocal *et al.* 1989), thereby increasing the amount of soluble Ca availability for absorption across the mucosa (Yuan & Kitts, 1991, 1994).

However, some oriental people do not drink milk due to lactose indigestion and intolerance, which make them allergic to milk. Thus, there have been many studies on various Ca supplements as alternatives (for examples, soya protein isolate, fructo-oligosaccharide, fish-meal, etc), which may affect Ca bioavailability (Brouns & Vermeer, 2000; Larsen *et al.* 2000, 2003; Kumagai *et al.* 2004). As reported by Larsen *et al.* (2000), the intake of small fish with bones could increase Ca bioavailability in rats, and small fish might be an important Ca dietary supplement, especially in population groups with low intakes of milk and dairy products.

Annually, more than 50 % of total fishery products (over 120 million tons) are discarded as inedible by-products, such as bone, skin, fins, internal organs and head. Thus, many studies have been performed to utilise the large amounts of protein, oil, minerals, carbohydrate and nucleic acid originating from fishery by-products, and to improve their functional properties (Nair & Gopakumar, 1982; Rodriguez-Estrada *et al.* 1994; Nagai & Suzuki, 2000; Kim *et al.* 2001, 2003; Shahidi & Janak Kamil, 2001). However, studies on the utilisation of organic components or minerals in fish bone are scarce (Kim *et al.* 1997; Larsen *et al.* 2000, 2003). In our previous study (Jung *et al.* 2005), fish-bone phosphopeptide with the high affinity to Ca had been isolated from hoki (*Johnius belengerii*) skeletons discarded from industrial processing. The present study *in vivo* was undertaken to evaluate the beneficial effects of fish-bone peptide (FBP) as a Ca fortifier.

Materials and methods

Preparation of fish-bone peptides with calcium-binding activity

FBP with a high affinity to Ca were isolated from hoki bone-protein hydrolysates using a hydroxyapatite affinity column. Hoki bone powder was digested with *Thunnus thynnus* (bluefin tuna) intestine crude enzyme (pH 9·0; 40°C; enzyme–substrate, 1:100; substrate concentration, 1 %) for 48 h according to the method of Kim *et al.* (2003). After incubation at 100°C for 5 min to inactivate the enzyme, the tuna intestine crude enzyme-digested fish-bone hydrolysates were filtered and demineralised on a Chelex 100 resin (Bio-Rad, Richmond, CA, USA) column. Then the Ca-binding fraction was eluted throughout a hydroxyapatite affinity column (20 × 80 mm,

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Abbreviations: CPP, casein phosphopeptide; FBP, fish-bone peptide; MW, molecular weight.
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Macroprep ceramic hydroxyapatite type 1; Bio-Rad) according to a previous method (Jung *et al.* 2005). After affinity chromatography, the peptide fraction with the highest Ca affinity was collected and fractionated into three kinds of peptides with different molecular weights (MW >5 kDa, 5–1 kDa and <1 kDa) using an ultramembrane filter system with MW 5·0 and 1·0 kDa cut-off membranes (SM165; Sartorious, Göttingen, Germany). After chemical analysis, the fractions were lyophilised.

Chemical analysis

Protein concentration in sample solutions was determined by the method of Lowry *et al.* (1951) using bovine serum albumin as a standard. After demineralisation of samples with Chelex-100 (Bio-Rad), P was determined by the colorimetric method, using a phosphoprotein phosphate assay kit (Pierce Biotechnology, Inc., Rockfold, IL, USA), and phosvitin (Sigma Chemical Co., St Louis, MO, USA) was used as a standard. Amino acid composition was analysed according to our previous study (Jung *et al.* 2005). Ca concentration in sample solutions was measured by a flame atomic absorption spectrometer (Simatzu AA-680; Simatzu Co., Tokyo, 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, nebuliser = spoiler. Lanthanum solution was added to 0·1 % (w/v) sample solutions.

In vitro *calcium-binding assay*

Ca-binding assays were performed according to the method of Jung *et al.* (2005). Various concentrations of FBP up to 500 mg/l 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 in the buffer system. When the pH changed, it was adjusted with 6 M-HCl or -NaOH and monitored by a pH meter (HORIBA D-51 model pH meter; HORIBA Co., Ltd, Kyoto, Japan). After removal of insoluble calcium phosphate salts and filtration using a 0·45 μm membrane, the Ca contents of the supernatant fraction were determined by flame atomic absorption spectrometry. The experiments were performed in triplicate; values are expressed as means and standard deviations.

In vivo test of calcium absorption and bone mineral density in ovariectomised rats

Experimental animals and diets. Sprague–Dawley ovariectomised rats (*n* 24; 3 months old) were obtained from Korea Research Institute of Chemical Technology (Daejeon, Korea). The rats were housed in individual shoe-box cages in a temperature- and humidity-controlled room (22 ± 2°C and 60 ± 5 % relative humidity) with a 12 h light–dark cycle in accordance with the *Guidelines on the Use of Living Animals in Scientific Investigations* (Biological Council, 1987). As shown in Table 1, all experimental diets were prepared according to the AIN-76 diet (Anonymous, 1977) with slight modification. The low-Ca diet used in the present study was made from Ca-free AIN-76 salt mix (Ralston Purina International Co., St Louis, MO, USA) with added

Table 1. Composition of the modified AIN-76 diet

	Content (g/kg)			
		Normal-Ca diet		
Ingredients	Low-Ca diet	Control	CPP	FBP
Casein	200·0	200·0	150·0	150·0
CPP	–	–	50·0	–
FBP	–	–	–	50·0
DL-Methionine	3·0	3·0	3·0	3·0
Maize starch	150·0	150·0	150·0	150·0
Sucrose	499·8	482·5	482·5	482·5
Cellulose	50·0	50·0	50·0	50·0
Maize oil	50·0	50·0	50·0	50·0
Mineral mix*	35·0	35·0	35·0	35·0
Calcium carbonate	0·175	17·5	17·5	17·5
Vitamin mix†	10·0	10·0	10·0	10·0
Choline bitartrate	2·0	2·0	2·0	2·0

CPP, casein phosphopeptide; FBP, fish-bone phosphopeptide.

*Ca-free AIN-76 mineral mix contains (g/kg): potassium phosphate monobasic, 500·00; sodium chloride, 74·00; magnesium sulfate, 36·20; magnesium oxide, 11·90; manganous carbonate, 3·50; ferric citrate, 6·00; zinc carbonate, 1·60; cupric carbonate, 0·30; potassium iodate, 0·01; sodium selenate, 0·01; chromium potassium sulfate, 0·55; finely powdered sucrose, 365·93.

†AIN-76A vitamin mixture contains (g/kg): thiamin HCl, 0·60; riboflavin, 0·60; pyridoxine HCl, 0·70; niacin, 3·0; calcium pantothenate, 1·60; folic acid, 0·20; biotin, 0·02; vitamin B_{12}, 1·0; vitamin A palmitate 0·80; vitamin D_3 0·25; vitamin E acetate 10·00; menadione sodium bisulfite, 0·08; finely powdered sucrose, 981·15.

For details of diets and procedures, see p. 124.

$CaCO_3$ (0·175 g/kg) (Shinyo Pure Chemicals Co., Osaka, Japan) as the Ca source. After ovariectomy, rats were fed *ad libitum* with the low-Ca diet and deionised water for 6 weeks. The rats were then randomly assigned to the control and two experimental groups (eight rats per group). The control group was switched to a normal-Ca diet including $CaCO_3$ (17·5 g/kg) for 6 weeks. Rats in the experimental groups were fed on the normal-Ca diet including CPP type II (50 g/kg) produced by Meiji Seika Co. Ltd (Tokyo, Japan) and FBP II (50 g/kg).

Sampling and analytical methods

Body weight was recorded once per week throughout the 6-week experimental diet. During the 4 d metabolic balance study at the end of treatment, the amount of food and Ca intake were monitored by housing each rat individually according to the method of Zafar *et al.* (2004). Urinary Ca and faecal Ca excreted were measured by a flame atomic absorption spectrometer. Ca retention (balance) was calculated as: Ca intake − faecal Ca − urinary Ca. After 6-week feeding periods, the rats were fasted overnight and killed under pentobarbitone anaesthesia. Blood collected from carotid bleeding was centrifuged to separate serum, and serum Ca was measured by an automatic analyser (ARKRAY model SP-4410; Kyoto Daiichi Kagaku Co., Ltd, Kyoto, Japan). Right femurs were excised and connective tissues were cleared. After measuring length and weight, the breaking force of femoral centre was analysed by an INSTRON universal testing instrument (model 1011; Instron Co., Canton, MA, USA). Data were expressed as peak breaking force of femur breaking (kg unit). Broken femurs were dissolved in 3 ml 70 % HNO_3 individually. The diluted femur solution was analysed for total Ca by flame atomic absorption spectrometry. Bone mineral density of the distal region, defined as 5 % of the whole length of the left femur, was determined by dual-energy X-ray absorptiometry (HITACHI BMD-IX; Hitachi Co., Tokyo, Japan).

Statistical analysis

ANOVA was performed with Duncan's multiple range test using SAS to compare means (SAS Institute, Inc., Cary, NC, USA). The level of significance was $P < 0.05$ for all statistical tests.

Results

Chemical analysis and in vitro assay for calcium-binding activity

FBP with Ca-binding activity were isolated using hydroxyapatite affinity chromatography according to our previous method (Jung *et al.* 2005). Chemical compositions of FBP I, II and III were analysed as shown in Table 2. The FBP II fraction with the distribution of MW 5·0–1·0 kDa mainly consists of 15·1 % P (w/w) and 83·7 % protein (w/w). It consisted of 27·95 % glycine, 12·6 % threonine, 9·7 % alanine, 8·6 % serine, 8·1 % glutamate or glutamine, and 7·3 % hydroxyproline (data not shown). In the assay for Ca-binding activity (Fig. 1), FBP II showed the highest affinity to Ca as compared with other fractions, but lower than that of CPP. The solubility of Ca was dependent on the concentration of FBP II, and 26·35 mg Ca/l was obtained at a concentration of 200 mg/l at pH 7·8. In the treatment of 200 mg CPP/l, 29·64 mg Ca/l was analysed in the supernatant fraction after the formation of insoluble salts.

Body weight, food intake, calcium intake, calcium loss and retention

No significant difference in body-weight gain, food intake, and total Ca intake was found among the three groups (Table 3). Serum Ca level was slightly elevated in both the CPP and FBP II diet groups, but not significantly. The rate of Ca loss into faeces was significantly lower in the CPP and FBP II diet groups ($P < 0.05$), and higher values of Ca retention were shown in both dietary groups.

Table 2. Chemical analysis of fish-bone phosphopeptides (FBP)

FBP	P (%; w/w)	Protein (%; w/w)	Distribution of MW (kDa)†	Yield (%; w/w)
Total*	6·5	92·7	>29·0	100·0
FBP I	5·3	93·9	>5·0	59·0
FBP II	15·1	83·7	5·0–1·0	17·6
FBP III	2·9	95·6	<1·0	23·4

MW, molecular weight.
*Total FBP before fractionation. FBP I, II, and III with different MW were fractionated by ultramembrane filtration with 5·0 kDa and 1 kDa cut-off membranes.
†The MW distribution of total hydrolysates was measured by gel permeation chromatography using a Shodex Ohpak SB-803 HQ (Shodex denco; Shoko Co. Ltd; Tokyo, Japan), and compared with those of molecular markers (bovine serum albumin, 66·0 kDa; carbonic anhydrase, 29·0 kDa; cytochrome C, 12·3 kDa; aprotinin, 6·5 kDa; ACE I, 13·0 kDa).

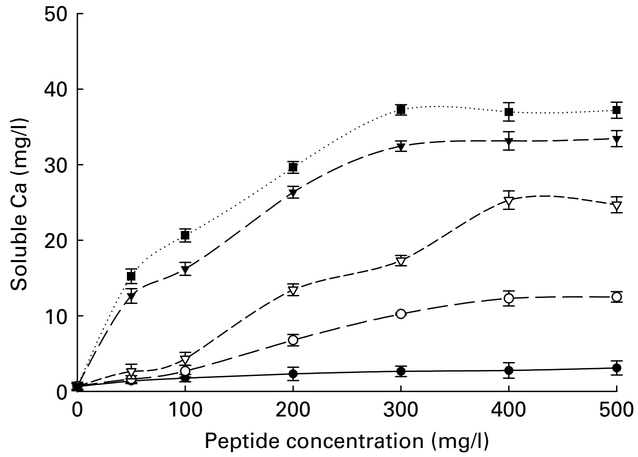

Fig. 1. *In vitro* Ca-binding activity of fish-bone peptides (FBP). Various concentrations up to 500 mg FBP/l were mixed with 5 mM-$CaCl_2$ and 20 mM-sodium phosphate buffer (pH 7·8). The mixture was stirred at 22°C for 30 min, and the pH was maintained at 7·8 in the buffer system. When the pH changed, it was adjusted with 6 M-HCl or -NaOH and monitored by a pH meter. After removal of insoluble calcium phosphate salts and filtration with a 0·45 μm membrane, Ca contents of the supernatant fraction were determined by flame atomic absorption spectrometry. The experiments were performed in triplicate. Values are means, with standard deviations represented by vertical bars. (●), Control; (○), FBP I; (▼), FBP II; (▽), FBP III; (■), casein phosphopeptide.

Femur analysis

The effects of the FBP II diet on the femur are presented in Table 4. A significant increase of the femoral weight was observed in the FBP II compared with the control ($P < 0.05$). An increase in the femoral total Ca was also found in the FBP II-fed animals. The treatment with FBP II increased the bone mineral density of the distal region ($P < 0.05$) and the breaking force of the proximal region ($P < 0.05$) in the femur.

Discussion

FBP with high Ca-binding activity were isolated using hydroxyapatite affinity chromatography according to our previous method (Jung *et al.* 2005), and the Ca-binding peptide FBP II with a high content of P (15·1 %) was fractionated in the range of MW 5·0–1·0 kDa. It was composed of high contents of 27·95 % glycine, 12·6 % threonine, 9·7 % alanine, 8·6 % serine, 8·1 % glutamate or glutamine, and 7·3 % hydroxyproline. All essential amino acids except for tryptophan (below 0·1 mg tryptophan/100 mg total amino acids) were detected in the FBP II.

As reported by Jiang & Mine (2000), the solubility of 36·3 mg Ca/l was obtained at 200 mg oligophosphopeptide from egg yolk phosvitin/l, with 35 % phosphate retention, and the solubility was higher than that of commercial CPP II. As reported by Hoang *et al.* (2003), Ca-binding phosphoproteins, such as osteocalcin, can recognise Ca on the surface of hydroxyapatite. Dohi *et al.* (1987) isolated two Ca-binding proteins with the γ-carboxyglutamic acid (gla protein) domain from bullfrog *Rana catesbiana* using hydroxyapatite affinity chromatography. The present study *in vitro* elucidated that FBP with the high affinity to Ca was produced from enzymic

In vivo effects of fish-bone peptides

Table 3. Effects of fish-bone peptide (FBP) II intake on body weight, calcium intake and calcium retention in ovariectomised rats

(Mean values and standard deviations)

	Control (*n* 8)		CPP (*n* 8)		FBP II (*n* 8)	
Experimental groups...	Mean	SD	Mean	SD	Mean	SD
Body-weight gain (g/d)	14·4	1·5	14·2	2·5	13·9	1·9
Food intake (g/d)	12·9	0·9	13·0	1·3	12·5	1·5
Ca intake (mg/d)	54·8	5·9	54·2	4·8	54·4	5·5
Faecal Ca (mg/d)	53·3^{a}	3·4	44·8^{b}	4·2	46·5^{a,b}	3·6
Urinary Ca (mg/d)	0·9	0·4	1·4	0·5	1·5	0·5
Ca retention (mg/d)*	0·2^{a}	0·5	8·0^{b}	1·2	6·4^{b}	1·7
Serum Ca (mg/l)	106·7	12·9	119·7	20·9	120·6	18·6

CPP, casein phosphopeptide.

[a,b]Mean values within a row with unlike superscript letters were significantly different ($P<0{\cdot}05$).

*Ca retention (balance) was calculated as: Ca intake − faecal Ca − urinary Ca (Zafar *et al.* 2004).

For details of diets and procedures, see p. 124.

Table 4. Effects of fish-bone peptide (FBP) II intake on femur in ovariectomised rats

(Mean values and standard deviations)

	Control (*n* 8)		CPP (*n* 8)		FBP II (*n* 8)	
Experimental groups...	Mean	SD	Mean	SD	Mean	SD
Femoral total Ca (mg)	143^{a}	7	153^{b}	9	155^{b}	10
Femur length (mm)	33·9	0·5	34·8	0·4	34·3	0·6
Femur wet weight (g)	1·12^{a}	0·06	1·32^{b}	0·05	1·22^{a,b}	0·06
Bone mineral density of the distal femur (g/cm^2)	0·161^{a}	0·018	0·229^{b}	0·029	0·213^{b}	0·025
Breaking force (kg)	3·96^{a}	0·57	8·97^{b}	1·03	8·48^{b}	0·97

CPP, casein phosphopeptide.

[a,b]Mean values within a row with unlike superscript letters were significantly different ($P<0{\cdot}05$).

For details of diets and procedures, see p. 124.

hydrolysates using the hydroxyapatite affinity column and the MW cut-off ultramembrane filtration, and could increase Ca solubility in the presence of phosphate under the neutral pH.

In vivo effects of FBP II on Ca bioavailability were further studied in the ovariectomised rats. Menopause is a time when oestrogen deficiency leads to accelerated bone resorption and negative bone balance. The present study was undertaken to evaluate the beneficial effects of FBP as a Ca fortifier in osteoporosis induced by ovariectomy and a concurrent low-Ca diet. During the experimental period corresponding to the menopause with osteoporosis disease, the loss of bone mineral (Ca) was decreased by FBP II supplementation in the ovariectomised rats. After the low-Ca diet, the FBP II diet, including both normal levels of Ca and vitamin D, significantly decreased Ca loss in faeces and increased Ca retention as compared with the control. The levels of femoral total Ca, bone mineral density, and breaking strength were also significantly increased by the FBP II diet to a level similar to those of the CPP diet group (no difference; $P>0{\cdot}05$). It illustrates that the increased Ca retention by FBP II intake led to the prevention of mineral loss in the osteoporosis-modelling rats.

As reported by Larsen *et al.* (2000), the intake of small fish with bones can increase Ca bioavailability, and the small fish may be an important source of Ca, especially in population groups with low intakes of milk and dairy products. In the present study, the results proved the beneficial effects of fish-meal in preventing Ca deficiency due to increased Ca bioavailability by FBP intake. Furthermore, it is possible to provide a novel nutraceutical with a high bioavailability for Ca to oriental people with lactose indigestion and intolerance and Ca-fortified supplements, such as fruit juice or Ca-rich foods, as alternatives to dairy products.

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