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

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(Received 27 January 2005 – Revised 2 September 2005 – Accepted 2 September 2005)

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 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 belenguerii) 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 x 80 mm,
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., Rockford, 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-CaCl2 and 20 mM-sodium chloride solutions. after 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 using a 0.45 mm cell, 1.75 l/min, air flow 14.0 l/min, nebuliser = spoiler. 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 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 ± 3°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 CaCO3 (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 CaCO3 (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 Daichi 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% HNO3 individually. The diluted femur solution was analysed for total Ca by flame atomic absorption spectrometry.

Table 1. Composition of the modified AIN-76 diet

<table>
<thead>
<tr>
<th>Ingredients</th>
<th>Low-Ca diet</th>
<th>Control</th>
<th>CPP</th>
<th>FBP</th>
</tr>
</thead>
<tbody>
<tr>
<td>Casein</td>
<td>200.0</td>
<td>200.0</td>
<td>150.0</td>
<td>150.0</td>
</tr>
<tr>
<td>CPP</td>
<td>–</td>
<td>–</td>
<td>50.0</td>
<td>–</td>
</tr>
<tr>
<td>FBP</td>
<td>–</td>
<td>–</td>
<td>50.0</td>
<td>–</td>
</tr>
<tr>
<td>l-Methionine</td>
<td>3.0</td>
<td>3.0</td>
<td>3.0</td>
<td>3.0</td>
</tr>
<tr>
<td>Maize starch</td>
<td>150.0</td>
<td>150.0</td>
<td>150.0</td>
<td>150.0</td>
</tr>
<tr>
<td>Sucrose</td>
<td>499.8</td>
<td>482.5</td>
<td>482.5</td>
<td>482.5</td>
</tr>
<tr>
<td>Cellulose</td>
<td>50.0</td>
<td>50.0</td>
<td>50.0</td>
<td>50.0</td>
</tr>
<tr>
<td>Maize oil</td>
<td>50.0</td>
<td>50.0</td>
<td>50.0</td>
<td>50.0</td>
</tr>
<tr>
<td>Mineral mix*</td>
<td>35.0</td>
<td>35.0</td>
<td>35.0</td>
<td>35.0</td>
</tr>
<tr>
<td>Calcium carbonate</td>
<td>0.175</td>
<td>1.75</td>
<td>17.5</td>
<td>17.5</td>
</tr>
<tr>
<td>Vitamin mix†</td>
<td>10.0</td>
<td>10.0</td>
<td>10.0</td>
<td>10.0</td>
</tr>
<tr>
<td>Choline bitartrate</td>
<td>2.0</td>
<td>2.0</td>
<td>2.0</td>
<td>2.0</td>
</tr>
</tbody>
</table>

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

*Ca-free AIN-76 mineral mixes contain (g/kg): potassium phosphate monobasic, 500.00; sodium chloride, 74.00; magnesium sulfate, 36.20; magnesium oxide, 11.90; manganese carbonate, 3.50; ferric citrate, 6.00; zinc carbonate, 1.60; copper 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 B12, 1.0; vitamin A palmitate 0.80; vitamin D3 0.25; vitamin E acetate 1.00; menadione sodium bisulfite, 0.08; finely powdered sucrose, 981.15.

For details of diets and procedures, see p. 124.
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 ($P<0.05$), and higher values of Ca retention were shown in both dietary groups.

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

<table>
<thead>
<tr>
<th>FBP</th>
<th>P (%: w/w)</th>
<th>Protein (%: w/w)</th>
<th>Distribution of MW (kDa)†</th>
<th>Yield (%: w/w)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Total*</td>
<td>6.5</td>
<td>92.7</td>
<td>&gt;29.0</td>
<td>100.0</td>
</tr>
<tr>
<td>FBP I</td>
<td>5.3</td>
<td>93.9</td>
<td>&gt;5.0</td>
<td>59.0</td>
</tr>
<tr>
<td>FBP II</td>
<td>15.1</td>
<td>83.7</td>
<td>5.0–1.0</td>
<td>17.6</td>
</tr>
<tr>
<td>FBP III</td>
<td>2.9</td>
<td>95.6</td>
<td>&lt;1.0</td>
<td>23.4</td>
</tr>
</tbody>
</table>

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 1.0 kDa cut-off membranes.

†The MW distribution of total hydrolysates was measured by gel permeation chromatography using a Shodex Ohpak SB-603 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; apro- tinin, 6.5 kDa; ACE I, 13.0 kDa).

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 phosphovitin/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 phosphopeptides, 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...
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 osteo-
porosis-induced by ovariectomy and a concurrent low-Ca diet.
During the experimental period corresponding to the meno-
pause with osteoporosis disease, the loss of bone mineral
(Ca) was decreased by FBP II supplementation in the ovari-
ectomised rats. After the low-Ca diet, the FBP II diet, includ-
ing both normal levels of Ca and vitamin D, significantly
decreased Ca loss in faeces and increased Ca retention as com-
pared 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* > 0.05). It illustrates that the
increased Ca retention (balance) was calculated as: Ca intake – faecal Ca – urinary Ca (Zafar et al. 2004).

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)

<table>
<thead>
<tr>
<th>Experimental groups…</th>
<th>Control (n 8)</th>
<th>CPP (n 8)</th>
<th>FBP II (n 8)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Mean</td>
<td>SD</td>
<td>Mean</td>
</tr>
<tr>
<td>Body-weight gain (g/d)</td>
<td>14·4±1·5</td>
<td>14·2±2·5</td>
<td>13·9±1·9</td>
</tr>
<tr>
<td>Food intake (g/d)</td>
<td>12·9±0·9</td>
<td>13·9±1·3</td>
<td>12·5±1·5</td>
</tr>
<tr>
<td>Ca intake (mg/d)</td>
<td>54·8±5·9</td>
<td>54·2±4·8</td>
<td>54·4±5·5</td>
</tr>
<tr>
<td>Faecal Ca (mg/d)</td>
<td>53·3±3·4</td>
<td>44·8±4·2</td>
<td>46·5±3·6</td>
</tr>
<tr>
<td>Urinary Ca (mg/d)</td>
<td>0·9±0·4</td>
<td>1·4±0·5</td>
<td>1·5±0·5</td>
</tr>
<tr>
<td>Ca retention (mg/d)*</td>
<td>0·2±0·5</td>
<td>0·5±1·2</td>
<td>0·4±0·6</td>
</tr>
<tr>
<td>Serum Ca (mg/l)</td>
<td>106·7±12·9</td>
<td>119·7±20·9</td>
<td>120·6±18·6</td>
</tr>
</tbody>
</table>

CPP, casein phosphopeptide.

*Mean values within a row with unlike superscript letters were significantly different (*P* < 0.05).

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)

<table>
<thead>
<tr>
<th>Experimental groups…</th>
<th>Control (n 8)</th>
<th>CPP (n 8)</th>
<th>FBP II (n 8)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Mean</td>
<td>SD</td>
<td>Mean</td>
</tr>
<tr>
<td>Femoral total Ca (mg)</td>
<td>143±7</td>
<td>153±9</td>
<td>9±3</td>
</tr>
<tr>
<td>Femur length (mm)</td>
<td>33·9±0·5</td>
<td>34·8±0·4</td>
<td>34·3±0·6</td>
</tr>
<tr>
<td>Femur wet weight (g)</td>
<td>1·12±0·06</td>
<td>1·32±0·05</td>
<td>1·22±0·06</td>
</tr>
<tr>
<td>Bone mineral density of the distal femur (g/cm²)</td>
<td>0·16±0·018</td>
<td>0·22±0·029</td>
<td>0·21±0·025</td>
</tr>
<tr>
<td>Breaking force (kg)</td>
<td>3·96±0·57</td>
<td>8·97±1·03</td>
<td>8·48±0·97</td>
</tr>
</tbody>
</table>

CPP, casein phosphopeptide.

*Mean values within a row with unlike superscript letters were significantly different (*P* < 0.05).

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

Acknowledgements

This research was supported by a grant (p-2004–01) from the
Marine Bioprocess Research Center of the Marine Bio 21
Center funded by the Ministry of Maritime Affairs and Fish-
eries, Republic of Korea.

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