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Effect of calcium-binding peptide from Pacific cod (*Gadus macrocephalus*) bone on calcium bioavailability in rats

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ABSTRACT

Bone collagen peptide with high affinity to Ca was extracted from Pacific cod (*Gadus macrocephalus*) bone. FTIR spectra of calcium-binding bone collagen peptide showed that band at 3381 cm^{-1} shifted to 3361 cm^{-1}, 1455 cm^{-1} moved to 1411 cm^{-1}, and amide II became deeper valley, compared with that of bone collagen peptide. This peptide was sequenced by Q-TOF-MS and sequences of Gly-Pro-Glu-Gly, Gly-Glu-Lys, Gly-Pro-Leu-Gly and Gly-Leu-Pro-Gly appeared repeatedly in some peptides. From SEM, after chelated with calcium, the loose and porous structure turned into granular structure. From the animal experiment, Ca apparent absorption rate, Ca retention rate and femur Ca content of calcium-binding bone collagen peptide group were significantly higher than those of model and $CaCO_3$ groups ($P < 0.05$), while serum ALP was significantly lower than model group ($P < 0.05$) and similar to control group. The results suggested that calcium-binding bone collagen peptide could improve bioavailability of Ca and thus prevented Ca deficiency.

1. Introduction

Calcium is an essential mineral for living organisms to maintain good health. In human body, Ca accounts for about 1–2% of body weight (about 1200 g in adult). About 99% of Ca is found in bones and teeth in the form of phosphate, contributing to rigidity and structure (Nordin, 1997). Calcium has many biological functions such as intracellular metabolism, coagulation, nerve conduction, blood muscle contraction, cardiac functions, bone growth and structural support of the skeleton (Bass & Chan, 2006; Miller, Jarvis, & McBean, 2001). For adults, the average recommended dietary allowance (RDA) of calcium in China is about 800 mg per day, and for adolescents and the elderly, it raises to 1200 mg per day. Adequate Ca intake is essential to maintain bone density, offer maximum protection against a negative calcium balance, especially for the elderly (Cashman, 2002). Calcium deficiency will increase bone resorption and decrease the bone mass (Weaver & Liebman, 2002), hence, results in rickets and osteoporosis (Guénguen & Pointillart, 2000).

However, calcium is difficult to be absorbed from diet directly because of the precipitation of insoluble calcium salts formed in a basic environment such as in the intestine (Jin, Fu, & Ma, 2011). Therefore, there has been a great deal of interest in the study of calcium supplementary products which can be absorbed effectively. Studies found that some food ingredients, especially protein (Saito, Lee, & Kimura, 1998), had positive effects on the calcium absorption. Recently, it has been studied that peptides from some dietary proteins have the capacity of chelating calcium and can increase the absorption and bioavailability of calcium. For instance, peptides from whey protein (Zhao, Huang, Cai, Hong, & Wang, 2014), soybean protein (Lv, Liu, Ren, Li, & Guo, 2013), flesh of clam (Wang, Chen, Liu, & Wu, 2012) and tilapia protein (Charoenphun, Cheirsilp, Sirinupong, & Youravong, 2013). In addition, it has been reported that peptides derived from fish bone also have high ability in chelating calcium (Jung, Park, Byun, Moon, & Kim, 2005) and can increase the absorption and bioavailability of calcium (Jung, Lee, & Kim, 2006). It is well known that fish bone is often discarded as a kind of processing byproduct and possesses low economic value. Whereas, 30% of the organic component of fish bone is collagen (Nagai, Izumi, & Ishii, 2004), which makes fish bone to be considered as a good source of collagen and peptide. Thus, many studies have been performed to utilize the large amounts of proteins from fish bone, particularly utilize their ability to bind calcium (Jung & Kim, 2007; Jung et al., 2006). However, studies on the bioavailability of fish bone peptides chelated with calcium are scarce, as most of them only focused on the bioavailability of peptides (Jung, Karawita et al., 2006).

It will be interesting to study whether the peptide chelated with calcium is more effective than peptide in the bioavailability of cal-

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cium. In this study, the Pacific cod (*Gadus macrocephalus*) bone, which contains 35.7% protein (higher than oily fish like salmon) (Toppe, Albrektsen, Hope, & Aksnes, 2007), was used to produce hydrolytic peptides for chelating calcium. The structure of peptide and calcium-binding peptide were analyzed to study the binding site, and the peptide sequences were detected. Finally, the calcium bioavailability of peptide and calcium-binding peptide were evaluated in low calcium model rats.

2. Materials and methods

2.1. Materials

Pacific cod (*Gadus macrocephalus*) bone were obtained from Qingdao Fusheng Foods Co., LTD (Qingdao, China), and stored at −20 °C until use. All solvents used for sample extraction were of reagent grade.

Neutral protease, alkaline protease and trypsin were obtained from Guangxi Nanning Pangbo Biological Engineering Co., Ltd., China. Neutral protease and alkaline protease are bacterial endoprotease, which are fermented by Bacillus subtilis 1398 and Bacillus licheniformis 2709, respectively. Trypsin is produced by the pancreas. The protease activity of neutral protease, alkaline protease and trypsin were 2.53×10^5, 1.82×10^5 and 2.40×10^5 U/g, respectively. One unit of protease activity (U) was defined as the activity that under 40 °C, releases 1 μg of tyrosine from a specified casein substance per minute.

2.2. Preparation of peptide and calcium-binding peptide from Pacific cod bone

The Pacific cod bones were washed and hydrolyzed by 0.5% neutral protease and alkaline protease (1:1) at 50 °C for 2 h to rinse the bone residues. After autoclaved at 120 °C for 30 min to be softened, they were homogenized in the ratio of 1:3 (bone: water) and incubated at 70 °C for 4 h, then centrifuged at 4000×*g* for 20 min and the supernatant was obtained. Trypsin and neutral protease (1:1, enzyme concentration: 1%) was used to treat the supernatant under 50 °C for 2.5 h and the hydrolysate was terminated by heating in boiling water for 5 min to inactivate the enzyme. After lyophilization, the Pacific cod bone collagen peptide (BCP) was obtained.

The lyophilized BCP was dissolved in deionized water to a final concentration of 5% (w/v). The BCP solution was adjusted pH value to 5.4 and mixed with $CaCl_2$ (BCP: Ca ratio was 2:1). The solution was stirred at 55 °C for 1.5 h and absolute ethanol (6 times volume of the solution) was added to the solution in order to remove free calcium. Then the mixture was centrifuged at 10,000×*g* for 10 min, and the precipitate, referred to as calcium-binding bone collagen peptide (BCP-Ca), was lyophilized.

2.3. Fourier transform infrared spectroscopy (FTIR)

Fourier transform infrared spectroscopic (FTIR) spectra were obtained from discs containing 1 mg lyophilized samples (BCP and BCP-Ca) in about 100 mg potassium bromide (KBr) under dry conditions. The FTIR spectra were recorded by an infrared spectrophotometer (Nicolet 200SXV, Thermo-Nicolet Co., Madison, WI, USA) from 4000 to 500 cm^{-1} at a data acquisition rate of 2 cm^{-1} per point. Background was subtracted and the spectra were analyzed by the Omnic 6.0 software (Thermo-Nicolet, Madison, Wisconsin).

2.4. Mass spectrometry identification and sequence analysis of BCP

Agilent G6540 Q-ToF (Agilent Technologies, Santa Clara, CA, USA) high-resolution mass spectrometer with an electrospray ionization (ESI) interface was used to identify BCP. The MS analysis was performed on mass spectrometer set from 700 to 900 *m*/*z*. Parameters of mass spectrometer were as follows: 5 L/min dry gas flow at 280 °C, 3.5 kV ion spray voltage of the capillary pressure, 2.1×10^5 Pa spray pressure, 11 L/min sheath gas flow, 5 L/min a nebulizer gas flow, 500 V nozzle voltage and 135 V decomposition voltage.

2.5. Scanning Electron Microscope (SEM)

The samples were smeared on to an aluminum plate followed by gold sputtering, then the scanning electron microscopy (JSM-840, JEOL Ltd., Japan) was used to get images at a voltage of 15 kV.

2.6. In vivo test of calcium absorption in rats

2.6.1. Animals and diets

Male Wistar rats (1 month old) weighing 100–110 g were obtained from Jinan Pengyue Laboratory Animal Breeding Co., LTD (Jinan, Shandong, China). The rats were housed in individual cages under controlled temperature, humidity and light (22 ± 2 °C, 60 ± 5% relative humidity and 12 h light/dark, respectively) with free access to distilled water. All diets were prepared according to the AIN-93 diet (Reeves, Nielsen, & Fahey, 1993). The diets were adjusted to either normal calcium (17.5 g $CaCO_3$/kg diet, normal diet) or low calcium (0.175 g $CaCO_3$/kg diet, low-Ca diet) with the addition of $CaCO_3$. All procedures were carried out according to the PR China legislation on the use and care of laboratory animals and were approved by the College of Food Science and Engineering, Ocean University of China.

2.6.2. Feeding procedures

Sixty Wistar rats were used in this experiment. The rats were randomly segregated into control, model, $CaCO_3$ group and three experimental groups (ten rats per group). The control and model groups were fed *ad libitum* with the normal diet and low-Ca diet for 4 weeks, respectively, meanwhile they were fed saline by gavage once a day. Three experimental groups were fed by gavage daily with doses of 50 (BCP-Ca-L group), 100 (BCP-Ca-M group), and 200 (BCP-Ca-H group) mg/kg body wt/day BCP-Ca for 4 weeks with low-Ca diet simultaneously. The $CaCO_3$ group was fed by gavage daily with $CaCO_3$ (the content of Ca^{2+} was equal with that of Medium BCP-Ca group).

2.6.3. Sampling and analytical methods

Daily animal feed intakes (corrected for food spilled) and weekly body-weights were recorded routinely.

At the last 3 days of the 4-week treatment, rats were placed in individual metabolic cages immediately after dosed for calcium balance experiment. Urine and feces were weighed and collected every 24 h in the 3 day. During this period, feed intake was monitored. Excreted urinary Ca and fecal Ca were measured by the flame atomic absorption spectrometer (Shimazu AA-6880, Japan). The indices of Ca calculated from data on Ca intake, fecal and urinary calcium excretion were as follows: Ca apparent absorption = Ca intake − fecal Ca; Ca retention = Ca apparent absorption − urinary Ca; Ca apparent absorption rate (%) = Ca apparent absorption/Ca intake × 100%; Ca retention rate (%) = Ca retention/Ca intake × 100%.

After the 4-week feeding period, all rats were fasted overnight and anaesthetized with diethyl ether. Blood collected from abdominal aorta was centrifuged to separate serum, then serum Ca,