Nutrition 30 (2014) 719–725

Contents lists available at ScienceDirect

Nutrition

journal homepage: www.nutritionjrnl.com

Basic nutritional investigation

Shark protein improves bone mineral density in ovariectomized rats and inhibits osteoclast differentiation

Kazuki Uehara M.S. [a], Akira Takahashi [b], Mutsuto Watanabe M.S. [a], Yoshihiro Nomura Ph.D. [a,*]

[a] Applied Protein Chemistry, Faculty of Agriculture, Tokyo University of Agriculture and Technology, Fuchu, Tokyo, Japan
[b] Chuka Takahashi Corporation, Chuo-ku, Tokyo, Japan

ARTICLE INFO

Article history:
Received 15 April 2013
Accepted 16 November 2013

Keywords:
Postmenopausal osteoporosis
Ovariectomized rats
Osteoclast differentiation
Shark protein
Bone resorption

ABSTRACT

Objectives: Fish proteins are potential sources of natural medicines and food additives. There are many studies being performed to develop underutilized fish proteins. Therefore, the aim of this study was to determine how shark protein functions as a dietary supplement for bone health.
Methods: Three groups of ovariectomized (OVX) rats were fed different diets containing 20% casein protein, 20% shark protein, or 20% cod protein for 4 wk. Bone mineral density of the right femur was measured by dual-energy x-ray absorptiometry and quantitative computed tomography. Furthermore, we prepared low-molecular-weight peptides from shark protein using protease for in vitro studies. Calcitriol was added to bone marrow cells and the receptor activator of the nuclear factor-κB ligand was added to RAW264 cells. After 7 d, the number of tartrate-resistant acid phosphatase-positive cells was counted.
Results: In the shark protein-fed group, bone mineral density of the femur epiphysis was higher than that of the casein protein-fed group. In particular, the shark protein-fed group showed an increase in bone mineral density, represented mainly by trabecular bone. Shark protein hydrolysates inhibited osteoclast formation in bone marrow cells and RAW264 cells.
Conclusions: These results suggest that shark protein might suppress the bone loss caused by estrogen deficiency through the suppression of osteoclast formation.

Introduction

Shark protein, which is available on the world market, usually comes from sharks that are caught during tuna trawler fishing. The commercial value of the shark is represented mainly by its fin, which is used as a luxury foodstuff. Therefore, shark fins are normally obtained by the controversial practice of finning, in which a shark's fin is cut off and the rest of its body is tossed into the ocean. However, in Japan, the entire shark body is utilized. Its cartilage is used as a source of chondroitin sulfate and collagen is purified from its skin. Although shark protein is the main ingredient in fish-paste products, its commercial value remains low. In our laboratory, we examined how shark protein can be used to increase its commercial value.

Bone is an important organ that regulates mineral homeostasis. Menopause causes physiological changes that can lead to an imbalance between bone formation and bone resorption, resulting in net bone loss and osteoporosis, mainly caused by estrogen deficiency [1,2]. Current therapies for osteoporosis include estrogen replacement therapies and the use of bisphosphonates. These therapies are effective in preventing bone loss caused by menopause, but some are accompanied by adverse side effects, such as uterine bleeding, carcinogenesis, and cardiovascular disease [3–5]. Therefore, diet therapy and lifestyle changes that minimize bone loss in postmenopausal women would be very helpful in decreasing the need for drug therapy to prevent osteoporosis. Recently, hydrolyzed collagen of porcine origin [6], oil palm leaf extract [7], soy isoflavones plus vitamin D_3 [8], and lactoferrin [9] have become available as food supplements for improving bone mineral density (BMD). However, few studies have examined fish protein–derived food factors that have a beneficial effect on BMD. Therefore, the aim of this study was to determine how shark protein functions as a dietary supplement for bone health.

KU performed the experiment and drafted the manuscript. AT and MW advised on the experimental design. YN offered instruction and advice on the manuscript.

* Corresponding author: Tel.: +81 42 367 5790; fax: +81 42 367 5791.
E-mail address: ny318@cc.tuat.ac.jp (Y. Nomura).

0899-9007/$ - see front matter © 2014 Elsevier Inc. All rights reserved.
http://dx.doi.org/10.1016/j.nut.2013.11.005

Materials and methods

Materials

Seventeen-wk-old female Wistar rats were purchased from the Sankyo Labo Service Co. Inc. (Tokyo, Japan). Casein was purchased from the Oriental Yeast Co., Ltd. (Tokyo, Japan). Alcalase®2.4 L FG was purchased from Novozymes A/S (Bagsvaerd, Denmark). Umamizyme G was purchased from Amano Enzyme Inc. (Aichi, Japan). Murine macrophage RAW 264 cells were provided by RIKEN BRC through the National Bio-Resource Project of the MEXT (Ibaraki, Japan). Fetal bovine serum (FBS), penicillin–streptomycin–neomycin (PSN) antibiotic mixture, non-essential amino acids, and minimum essential medium (MEM) were purchased from Life Technologies Japan Ltd. (Tokyo, Japan). Receptor activator of nuclear factor-κB ligand from mouse (RANKL), 17 β-estradiol (E_2), calcitriol (1 α,25-dihydroxy vitamin D_3 [1,25$(OH)_2$ D_3]), and α-MEM were purchased from Sigma-Aldrich Inc. (St. Louis, MO, USA). Tartrate-resistant acid phosphatase (TRAP) staining kit was purchased from Primary Cell Co., Ltd. (Hokkaido, Japan). Other chemical reagents were all of special grade from Wako Pure Chemical Industries, Ltd. (Osaka, Japan)

Preparation of shark protein hydrolysates

Shark protein hydrolysates (SH) were made from fresh great blue shark (*Prionace glauca*) protein. Shark protein was heated in 80°C water for 3 min, and then compressed to remove excess water. The same quantity of distilled water was added along with proteases (Alcalase®2.4 L FG) at 3.333% (dry weight). After 30 min, the protein was further treated with 0.5% (dry weight) proteases (umamizyme G) at 50°C and pH 7.0 for 24 h. Alcalase®2.4 L FG is an exo-type peptidase and umamizyme G is a mixture of endo- and exo-type peptidases with a broad substrate specificity. The reaction was stopped by heating at 85°C for 20 min. After all hydrolysates were centrifuged to remove the precipitates, the supernatant was collected and freeze-dried. To compare with SH, cod protein hydrolysates (CH) also were prepared in the same manner.

Amino acid analysis of casein, shark protein, and cod protein using high-performance liquid chromatography

To determine the amino acid profile of casein, shark protein, and cod protein, reverse-phase chromatography was used. Amino acid analysis was performed using a previously described method [10], with slight modifications [11]. The resulting phenylthiocarbamyl amino acids were separated using an octadecylsilane column (TSKgel ODS-80 TsQA 4.6 mm × 150 mm; Tosoh Co., Tokyo, Japan) at 1.0 mL/min using a binary linear multistep solvent gradient. Solution A consisted of 50 mM sodium acetate buffer, pH 6.0, containing 3% acetonitrile. Solution B consisted of 60% acetonitrile. Gradient profile was: 0 to 15 min, 0 to 70% B; 15 to 25 min, 70 to 100% B; 25 to 26 min, 100% B; 26 to 28 min, 0% B. The column was maintained at 40°C. Elution peaks were monitored at 254 nm.

Molecular weight distribution of SH and CH by high-performance liquid chromatography

The average-molecular-weight distributions of SH and CH were analysed by gel-filtration chromatography. Ten mg of two types of peptide sample were dissolved in a mixture of 500 μl of ultrapure water and 500 μl of 45% acetonitrile in water, in the presence of 0.1% trifluoroacetic acid. After solubilization, the solutions were filtered and 50 μl injected into a silica-based column (TSKgel G2500 PW_{XL} 7.8 mm × 30 mm; Tosoh Co.) using a high-performance liquid chromatography LC-8020 Model II chromatograph (Tosoh Co.) and elution was performed with 45% acetonitrile in water in the presence of 0.1% trifluoroacetic acid at a flow rate of 0.5 mL/min. The column was maintained at 40°C. The elution peaks were monitored at 215 nm.

Table 1
Composition of the diets*

	Casein†	Shark‡	Cod§
Casein	20	0	0
Shark protein	0	20	0
Cod protein	0	0	20
α-cornstarch	13.2	13.2	13.2
β-cornstarch	40.0486	40.0486	40.0486
Sucrose	10	10	10
Soybean oil	7	7	7
Cellulose powder	5	5	5
Mineral mix (93G-MX)	3.5	3.5	3.5
Vitamin mix (93-VX)	1	1	1
Choline bitartrate	0.25	0.25	0.25
tert-Buthylhydroquinone	0.0014	0.0014	0.0014
Total (%)	100	100	100

* Animals were fed a modified AIN-93 G diet.
† 20% casein diet.
‡ 20% shark protein diet.
§ 20% cod protein diet.

Table 2
Amino acid concentrations in casein, shark protein, and cod protein

Amino acid weight ratio (%)	Casein	Shark protein	Cod protein
Aspartic acid/asparagine	8.2	10.4	11.9
Glutamic acid/glutamine	24.3	16.3	17.4
Hydroxyproline	0.0	0.3	0.2
Serine	4.6	3.6	4.5
Glycine	1.8	5.4	4.5
Histidine	3.2	2.9	2.4
Arginine	3.7	7.0	6.8
Threonine	4.3	5.2	5.1
Alanine	3.1	7.8	6.5
Proline	6.8	2.8	3.0
Tyrosine	5.3	3.0	3.9
Valine	7.0	5.4	5.1
Methonine	1.8	3.7	3.7
Cysteine	0.2	0.5	0.3
Isoleucine	4.5	5.3	4.0
Leucine	8.7	9.0	7.7
Hydroxylysine	0.0	0.0	0.0
Phenylalanine	5.1	4.1	3.8
Lysine	7.5	7.2	9.2

Animals and diets

The study was conducted in accordance with the guidelines of the Committee for Animal Research at Tokyo University of Agriculture and Technology. Seventeen-wk-old female Wistar rats (n = 28) were given a modified AIN-93 G diet containing 20% casein for 3 d of acclimatization ad libitum. The rats were housed in individual cages at 22 ± 1°C, 50 ± 5% humidity, on a 12-h light–12-h dark cycle, with water ad libitum. On day 7 of the experiment, 22 rats were ovariectomized (OVX) and 6 rats were sham-operated (sham). OVX rats were then divided into three groups of six or eight rats. They were fed ad libitum for 4 wk on a diet consisting of AIN-93 G with 200 g/kg casein protein as the protein source; the casein being replaced with shark protein (OVX shark) or cod protein (OVX cod); the composition of this diet is presented in Table 1. Weight was recorded every 4 d, and food intake measured every second day of the experiment. After sacrifice, the right femur was excised from each rat to determine bone density after all muscles and connective tissues had been removed.

Measurement of BMD dual-energy X-ray absorptiometry

The right femur was dissected and preserved in 70% ethanol at 4°C. BMD was measured by dual-energy x-ray absorptiometry with a Dichrom Scan PCS-600 instrument (Hitachi Aloka Medical, Ltd., Tokyo, Japan), starting scans in the most proximal area and ending in the most distal. During data analysis, the femur was divided into 20 equal segments along its major axis [12,13].

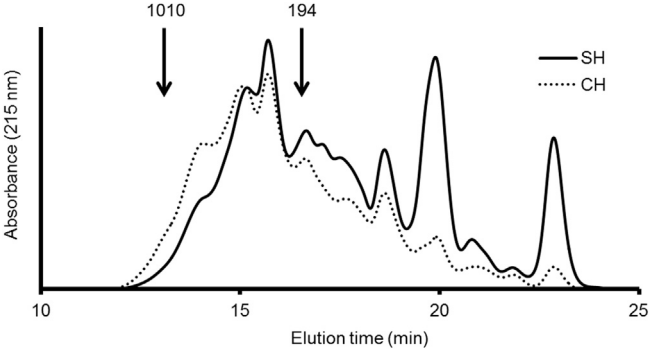

Fig. 1. Elution pattern of shark protein hydrolysates (SH) and cod protein hydrolysates (CH) by gel-filtration chromatography. SH prepared from shark protein and CH prepared from cod protein were analysed using a silica-based column. Elution was monitored by absorbance at 215 nm. Arrows indicate elution positions of standard molecular weight.