Food Chemistry 242 (2018) 156–161

Contents lists available at ScienceDirect

Food Chemistry

journal homepage: www.elsevier.com/locate/foodchem

Blue mussel (*Mytilus edulis*) protein hydrolysate promotes mouse mesenchymal stem cell differentiation into osteoblasts through up-regulation of bone morphogenetic protein

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ARTICLE INFO

Keywords:
Blue mussel
Enzymatic hydrolysis
Osteoblast
Bone morphogenetic protein
Noggin

ABSTRACT

Seafood provides a range of health benefits due to its high-protein level. In this study, the osteogenic effect of blue mussel (*Mytilus edulis*) protein hydrolysates (BMPH) on osteoblast differentiation were examined using mouse mesenchymal stem cells (MSCs). A preparation we called BMPH < 1 kDa which showed the highest osteogenic effect in MSCs, was prepared by peptic hydrolysis. BMPH < 1 kDa treatment stimulated osteoblast differentiation with alkaline phosphatase (ALP) induction, osteocalcin and type I collagen activity as well as calcium deposition. Osteoblast differentiation stimulated by BMPH < 1 kDa treatment was achieved by expression of osteogenic lineage markers, such as bone morphogenetic protein-2 (BMP-2), and downstream signal and transcription factors, including p-Smad1/5/8, Dlx5, runt-related transcription factor 2 (Runx2), and osterix. BMPH < 1 kDa activated phosphorylation of mitogen-activated protein kinases. Adding noggin, a BMP antagonist, inhibited BMPH < 1 kDa-induced ALP activity in MSCs. Taken together, our results show that BMPH < 1 kDa promoted osteoblast differentiation by activating BMP-2.

1. Introduction

Bone is a dynamic living tissue that is tightly regulated by two dynamic processes, bone resorption and bone formation. Osteoclasts are uniquely adapted to remove bone minerals and matrices, creating an eroded cavity, whereas osteoblasts produce osteoclastogenic factors, bone matrices, and bone mineralization, filling the cavity with new bone (Kim, Lee, Ryu, & Suh, 2014; Raggatt & Partridge, 2010). Additionally, a number of growth factors, cytokines, and exogenous factors are involved in the regulation of bone mass (Matsuo & Irie, 2008; Panetta, Gupta, & Longaker, 2010). However, bone homeostasis when disrupted by an imbalance between the activity of osteoclasts and osteoblasts, results in the development of osteoporosis, hypercalcemia, tumor metastasis into bone, rheumatoid arthritis, and Paget's disease (Gu et al., 2015; Jakab, 2014; Rodan & Martin, 2000). Although anti-osteoporotic agents, such as bisphosphonates, selective estrogen receptor modulators, and calcitonin are available, the long-term use of these medications results in serious side effects (Yu et al., 2013).

Osteoblasts play an important role in bone formation. Osteoblastic differentiation from mesenchymal stem cells (MSCs) is strongly regulated by bone morphogenetic proteins (BMPs) (Yamaguchi, Komori, & Suda, 2000). BMPs phosphorylate downstream signal molecules such as Smad1/5 followed by activation of transcription factors including Runx2 and osterix, which are essential transcription factors for osteoblast differentiation and bone formation (Nohe, Keating, Knaus, & Petersen, 2004; Wan & Cao, 2005). Finally, Runx2 and osterix regulate type I collagen, ALP, and osteocalcin expression (Yamaguchi et al., 2000). Therefore, chemicals or drugs that can stimulate BMP without side effects may be useful for bone regeneration.

Accumulating evidence suggests that eating seafood provides a range of health benefits due to high protein content and the cleavage of proteins to small bioactive peptides by gastrointestinal digestion (Sarmadi & Ismail, 2010; Udenigwe & Aluko, 2012). Blue mussel (*Mytilus edulis*) is an important shellfish that is cultured in Korea. Their protein content is approximately 55% on a dry basis, some of which can be converted to bioactive peptides by enzymatic hydrolysis. Previous work demonstrated that blue mussel protein hydrolysates exhibited potent antioxidant and anti-inflammatory activities (Park, Ahn, & Je, 2014). Antioxidant peptides were also isolated from blue mussel protein hydrolysates (Park, Kim, Ahn, & Je, 2016; Wang et al., 2013). However, scant information is available for marine protein-derived food factors with beneficial effect on bone health. Therefore, the objective of this

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http://dx.doi.org/10.1016/j.foodchem.2017.09.043
Received 9 May 2016; Received in revised form 29 August 2017; Accepted 8 September 2017
Available online 12 September 2017
0308-8146/

study was to generate bioactive peptides from blue mussel by enzymatic hydrolysis and to evaluate the effect of these peptides on bone formation and osteoblast differentiation from mouse MSCs.

2. Materials and methods

2.1. Materials

Blue mussel was purchased from Yeosu Fisheries Co. (Yeosu, Korea) and the protein content was shown to be 55% (dry basis) by Kjeldahl method. Pepsin (1:10000 units) was purchased from Junsei Chemical Co. (Tokyo, Japan). Antibodies (phospho-Smad1/5, Smad1/5/8, Dlx5, Runx2, osterix, p-ERK, p-JNK, p-p38, and β-actin) were purchased from Santa Cruz Biotechnology (Santa Cruz, CA, USA). Recombinant noggin was purchased from Wako Pure Chemical Industries Ltd. (Osaka, Japan). Assay kits for BMP-2 (BMP-2 Quantikine ELISA Kit, R & D systems Inc., Minneapolis, MN, USA), type I collagen (SIRCOL Collagen Assay Kit, Biocolor, UK), and osteocalcin (Osteocalin EIA kit, Biomedical Technologies Inc., Stoughton, MA, USA) were purchased. All other chemicals were from Sigma-Aldrich (St. Louis, MO, USA).

2.2. Preparation of blue mussel protein hydrolysates (BMPH) and membrane fractionation

Washed blue mussel was lyophilized before enzymatic hydrolysis. Firstly, BMPH was generated at enzyme (pepsin)/substrate ratios of 1:100, 1:500, and 1:1000, for 2 h, and then pepsin was inactivated by boiling at 100 °C for 10 min. The hydrolysis was performed at 37 °C and pH 2. The resultant BMPH was determined to stimulation effect on ALP activity in MSCs, and then the selected E/S ratio was applied for new digestion at different hydrolysis times of 0.5, 1, 2, 4, and 6 h.

Using the optimal conditions, BMPH was further fractionated into BMPH 3–10 kDa, BMPH 1–3 kDa and BMPH < 1 kDa peptide fractions using the Quixstand benchtop system equipped with molecular weight cut-off membranes of 1, 3, and 10 kDa.

2.3. Cell culture

MSCs were maintained in Dulbecco's Modified Eagle's Medium (DMEM) supplemented with 10% fetal bovine serum (FBS) and 1% penicillin/streptomycin in a 5% CO_2 incubator at 37 °C. After MSCs were grown to confluence they were cultured in osteogenic differentiation medium (ODM, DMEM supplemented with 50 μg/mL ascorbic acid 10 mM β-glycerolphosphate and 10^{-7} M dexamethasone). ODM was changed every 2 days

2.4. Cell cytotoxicity assay

MSCs were cultured in a 96 well-plate with BMPH for 24 h or 48 h. After incubation cells were treated with 1 mg/mL of MTT followed by incubation for 4 h. After washing with PBS DMSO was added and the absorbance of the resulting solution was recorded at 540 nm

2.5. Measurement of ALP activity and staining

MSCs were cultured in a 96 well-plate and incubated with BMPH for 7 days. Cultured MSCs were lysed with sodium carbonate buffer (25 mM pH 10) containing 0.1% triton X-100. Cell lysates were incubated with 25 mM sodium carbonate buffer containing 1.5 mM $MgCl_2$ and 3.8 mM p-nitrophenyl phosphate at 37 °C for 90 min. The optical density was recorded at 405 nm. ALP activity was calculated using the following equation in which A and A_0 were relative absorbance with and without BMPH respectively

$$\text{ALP activity } (\%) = (A - A_0)/A_0 \times 100$$

For histochemical detection of ALP, the cultured MSCs were fixed using formalin and then washed twice with PBS followed by addition of staining solution (NBT/BCIP). After incubation for 15 min at 37 °C, the images from a light microscope were captured.

2.6. Alizarin Red S staining and quantification of mineralization

Mineral accumulation in MSCs was evaluated by Alizarin Red S staining according to our previously published method (Hyung, Ahn, & Je, 2016). Cultured MSCs were washed with PBS and fixed followed by staining with 2% Alizarin Red S Solution. After washing MSCs with distilled water, mineral accumulation was examined with a microscope.

Mineralization was quantified using 10% cetylpyridinium chloride in 10 mM sodium phosphate buffer (pH 7.0). The absorbance was recorded at 562 nm. The percentage mineralization was calculated using the same equation as for ALP activity.

2.7. Measurement of BMP-2, type I collagen, and osteocalcin

MSCs were cultured in a 24 well-plate for 7 days in the presence of BMPH < 1 kDa. The culture supernatant was collected and assayed for BMP-2 type I collagen and osteocalcin content by ELISA according to the manufacture's instructions

2.8. Western blot analysis

MSCs were harvested lysed with RIPA buffer (Sigma Chemical Co.) containing protease and phosphatase inhibitors (Roche Applied Science IN USA). Proteins were separated using 10% SDS-PAGE and transferred onto PVDF membrane. The membrane was blocked with 5% skim milk or BSA at room temperature and incubated with indicated primary antibodies (overnight 4 °C). Horseradish peroxidase-conjugated secondary antibody was incubated with the membrane for 1 h at room temperature. The blot was visualized by enhanced chemiluminescence (Pierce Biotechnology IL USA).

2.9. Amino acid composition

Amino acid analyzer S433-H (Sykam GmbH, Germany) equipped with a cation separation column LCA K06/Na, was employed for amino acid analysis of BMPH < 1 kDa after acidic hydrolysis using 6 N HCl.

2.10. Statistical analysis

All experiments are conducted in triplicate and the results are expressed as means ± standard deviation. The statistical difference was analyzed by one-way analysis of variance followed by Duncan's test using PASW Statistics 19.0 software (SPSS, Chicago, IL, USA). A P-value < 0.05 was considered statistically significant.

3. Results

3.1. Preparation and osteogenic activity of BMPH in MSCs

BMPH with osteogenic activity were prepared by peptic hydrolysis. Each of the BMPHs prepared, using three different E/S ratios for 120 min of hydrolysis, showed higher ALP activity than crude extract without peptic hydrolysis and controls (without treatment). ALP stimulation by BMPHs on MSCs depended on the E/S ratio (Fig. 1A). BMPH at 1:500 generated a higher ALP activity than 1:100 and 1:1000. The cytotoxicity of BMPH at 1:500 in MSCs was evaluated for 24 or 48 h, and the MTT assay indicated no cytotoxicity in MSCs (data not shown). Therefore, hydrolysis was investigated at an E/S ratio of 1:500 for 30, 60, 120, 240, and 360 min. As shown in Fig. 1B, 120 min stimulated the greatest ALP activity. We then investigated the effect of size fractionation of BMPH using the selected conditions for hydrolysis.