Thesis for the Degree of Master of Science

A novel peptide purified from Low molecular weight Ark shell (*Scapharca subcrenata*) protein hydrolysates enhances osteogenic differentiation through bone morphogenetic protein signaling

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August 2016

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낮은 분자량의 꼬막 가수분해물로부터 정제된 새로운 펩타이드가 BMP-2 기작을 기반으로 한 뼈형성 세포분화 촉진에 기여

Advisor: Prof. Jae-Young Je

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A dissertation

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Abstract

Aging of human bone is characterized by decreased bone formation and bone mass. These changes demolish a balance between bone formation by osteoblast and bone resorption by osteoclast in bone tissues. The imbalance causes bone-related diseases such as osteoporosis, hypercalcemia, and Paget's disease. Recently, various therapeutic approaches to the diseases are investigated, but the conventional drugs have side effects because most of the remedies are principally based on anti-resorptive agents (*e.g.* bisphosphonates, raloxifen).

A number of natural bioactive peptides have diverse biological activity and certain products have shown beneficial effects on osteoblast differentiation unlike previously other studies. Thus, these peptides can be useful as alternative agents to treat bone-related diseases. In this study, it was suggested that ark shell protein hydrolysates (ASPHs) with low molecular weight contributed to osteoblast differentiation and the effects of ASPHs through modulating bone morphogenetic protein (BMP) signaling were investigated by measuring osteoblastic biomarkers such as BMP-2, p-Smad1/5, runt-related transcription factor-2 (Runx-2), Dlx5, osterix, and MAPKs in mouse

mesenchymal stem cells (MSCs, D1 cells). ASPHs were generated by pepsin at E/S ratio of 1:500 with 2 h hydrolysis indicated the highest ALP activity. Further ASPHs were separated according to molecular weight using molecular weight cut-off (MWCO) membrane with 10, 3, and 1 kDa. Low molecular weight peptide fraction (ASPH<1 kDa) exhibited the highest stimulation effects among the others in a dose-dependent manner. Also ASPH<1 kDa significantly increased activity of alkaline phosphatase (ALP) and amount of hydroxyapatite related bone mineralization in MSCs as indicated by ALP staining and Alizarin red S staining. The most bioactive peptide fraction (ASPH<1 kDa) was fractionated by ion exchange chromatography using Sephadex C-25 and purified by RP-HPLC on Hypersil GOLD column. At each stage of isolation, one of potential peaks was selected by measuring ALP activity and finally the single most active peak was identified. The purified peptide enhanced osteoblast diff-erentiation by regulating BMP signaling. Thus, Ark shell peptides offer the possibility to be highly valuable application as therapeutic substances.

Key words : bone remodeling, osteoporosis, osteoblast, BMP-2, Runx2, Dlx5, Smad.

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Introduction

Bone is an osseous tissue that supports and protects intrenal organs of the human body. This tissue has many functions: hematosis in bone marrow, storage of minerals, and secretion of osteocalcin, which increases insulin secretion and sensitivity (BIOMED RES INT, 2015). It consists of a layered structure called bone matrix, which is mixed with inorganic components such as hydroxyapatite ($Ca_{10}(PO_4)_6(OH)_2$) and organic materials, collagen, which protect against fracture resistance using flexible properties (Weiner,S. 1992). Commonly, the skeleton structure is made into a cartilage, switched to bone, and remodeled to conserve bone density and mass (Hutmacher, Dietmar W., 2000; Manolagas,S.C., 1995). This process is associated with a number of signals, factors, and several types of cells.

Osteoblasts are the major cell linked with osteogenesis. These cells produce bone matrix proteins like type I collagen, release osteoid just before mineralized matrix, and modulate osteoclastogenesis by secretion of Osteoprotegerin (OPG), which is inhibitor of bone resorption (Raggatt.L.J., 2010; Bosetti, Michela 2003). Differentiation into osteoblasts from mesenchymal stem cells is determined by several growing factors. Among them, BMPs are the most important materials to decide extent to replace cartilage with bone.

Particularly, BMP-2 has a crucial role in osteoblast formation(Rickard, DJ, 1994; Dragoo, Jason L, 2003). It generally intervene in differentiating from

precursor cells to mature osteoblasts. When the signal commanding bone formation arrives in two kinds of receptors, BMPR-Ⅰand BMPR-Ⅱ, the receptors are activated and phosphorylated receptor-activated Smads (R-Smads, Smad1/5) (Takase,H., 2009; Wu, Chiu-Jou, 2008). Phosphorylated R-Smads binding with common-mediator Smad (Co-Smad) for protecting signal contents against translation errors are translocated to nucleus (Moustakas,A., 2001). As a result of the signal transmission, several transcription factors such as Runx-2, osterix, distal-less homeobox 5 (Dlx) are manufactured for expression of target genes. Runx-2 has important role in differentiation from precursor cells to pre-osteoblasts (Komori,T. 2008; Lee, 2000). In other words, modulation of Runx-2 expression can determine to initiate the process of osteoblast differentiation. Resveratrol, natural phenol and phytoalexin, as anti-oxidant produced in plants when the invaders hurt them, investigated that this material has an effect on osteoblast differentiation by controlling levels of Runx-2 in human stem cells (Tseng, Pei-Chi, 2011). Decreasing levels of Runx-2 via Smad ubiquitin regulatory factor (Smurf) 1, which occurs targeting degradation of Runx-2, leads to inhibiting osteoblast differentiation by secretion of parathyroid hormone (PTH) (Bellido,T., 2003). In many other studies, content of osteoblastogenesis is demonstrated by measuring Runx-2 expressions, too. Thus, it is a criterion of research concerning bone formation.

Osterix, zinc finger-containing transcription factor or SP7, is associated with differentiation from pre-osteoblast to mature osteoblast (Zhu, Fengchang, 2012; Nakashima, Kazuhisa 2002). It is the downstream transcription factors in BMP signaling pathway, demonstrated that deficiency of osterix completely

lose the ability of osteogenesis, but the expression of Runx2 is maintained (Wu, Chiu-Jou, 2008). In addition, Dlx is a homeobox protein as the upstream transcription factor gene of Runx-2 and osterix. It is released in terminal step of bone formation, and induces expression of osteocalcin and mineralized matrix (Ryoo,Hyun-Mo, 1997). The lack of Dlx leads to abnormal development of cochlear structures and craniofacial growth (Depew,M.J., 1999). Thus, induction of the above factors contributes to osteogenic differentiation from early stage to terminal stage.

For bone remodeling, it is need to bone resorption by osteoclasts, which play a role breaking down bone tissue. This process is necessary for the repair, maintenance of bones, and regulation of calcium concentration in blood (Suda, Tatsuo, 1997). Usually osteoclasts are controlled by binding OPG with receptor activator of NF-κB ligand (RANKL), which is a cytokine promoting osteoclast precursor cells (Boyle, William J, 2003). When RANKL is activated after releasing OPG, osteoclasts make acidic conditions by Cathepsin K and degrade bone matrix (Nakagawa, Nobuaki, 1998). To maintenance of homeostasis with calcium levels in blood, bone remodeling is balanced in aspects of hormones. In case of low calcium levels in blood, PTH is secreted for activation of osteoclasts, while calcitonin and estrogen inhibit osteoclast differentiation (Rodan, Gideon A 1981; Carter, Percy H, 2006).

The balance between bone formation and bone resorption will be leaned with age, especially in the menopausal woman, heredity, steroid, smoking, obesity, etc. Estrogen is important to inhibit bone resorption through both accelerating apoptosis of osteoclasts by increasing level of transforming

growth factor β (TGF-β) , which restrain osteoclastogenesis, and inactivation of osteoclast by induction of OPG (Hughes, Davis E, 1996; Hofbauer, Lorenz C, 1999). This hormones are derived from reproductive cells and adipocytes , however, the hormone levels in post-menopausal woman is rapidly fallen (Cooke,P.S., 2004). This change cause loss of bone mass and density. Moreover, estrogen are supplied by adipocyte for maintaining homeostasis of hormone, and obesity is accelerated by promotion of adipocyte differentiation (Zhao, Lan-Juan, 2007 ; Syed, Farhan A, 2008). These complex symptoms lead to bone-related disease such as osteoporosis. Osteoporosis is occured without distinction of gender. As time goes by, the ratio of calcium to phosphate could be changed due to regulation of both osteoblasts and osteoclasts (Francis & Marion.D, 1970; Raynaud.S, 2002). Also the activity of vitamin D is declined by reducing metabolic capability, and the absorption rate of calcium in small intestine is decreased, too. For homeostasis, secretion of PTH, which promote bone resorption, will be increased and results in typical osteoporosis (Holick,M.F., 2004).

As mentioned above, Imbalance between bone formation and resorption results in development of a variety of bone-decreasing disorders including hypercalcemia, rheumatoid arthritis, tumor metastasis into bone, periodontitis and Paget's disease as well as osteoporosis (Rodan & Martin, 2000). Currently, a number of therapeutic drugs for osteoporosis such as Bisphosphonate, which induce osteoclasts apoptosis by inhibition of their enzyme, are available (Marx, Robert E, 2005). Unfortunately, however, the long-term use of anti-osteoporotic medications is associated with serious side

effects (Yu et al., 2013). Since osteoblasts play a crucial role in bone formation, activation of mesenchymal stem cells (MSCs) to an osteoblast lineage is an important step in the process of new bone formation. Therefore, the development of osteogenic agents without side effects is urgently needed.

Many natural substances including flavonoid, sulphated polysaccharide, and collagen-derived dipeptide have been developed to treat bone-related diseases (Cho, Jung, Kim, Choi, & Kim, 2009; Kimira et al., 2014; Yoon et al., 2011). These natural products have shown beneficial effects on osteoblast differentiation and bone regeneration through activation of bone morphogenetic proteins (BMPs) and transcriptional factors including runt-related gene 2 (Runx2) and osterix, which regulate the expression of the osteoblast-related genes encoding alkaline phosphatase (ALP), type I collagen and osteocalcin. However, few studies have examined marine protein-derived food factors that have a beneficial effect on bone health. Ark shell (*Scapharca subcrenata*) belongs to the phylum Mollusca, class Bivalvia, suborder Taxodonta and family Arcidae and is important shellfish which is widely cultured in Korea. In 2013, the total output of ark shell was approximately 5,000 M/T. Previous works reported that shellfish protein-derived peptides have shown versatile bioactivities such as antioxidant, antihypertensive, and anticancer (Harnedy & FitzGerald, 2012; Park, Ahn, & Je, 2014). Therefore, the aim of this study was to produce and purify ark shell protein-derived bioactive peptides with beneficial effects on bone health and to investigate them for promoting osteoblast differentiation on mesenchymal stem cells (MSCs) as shown in Fig. 1.

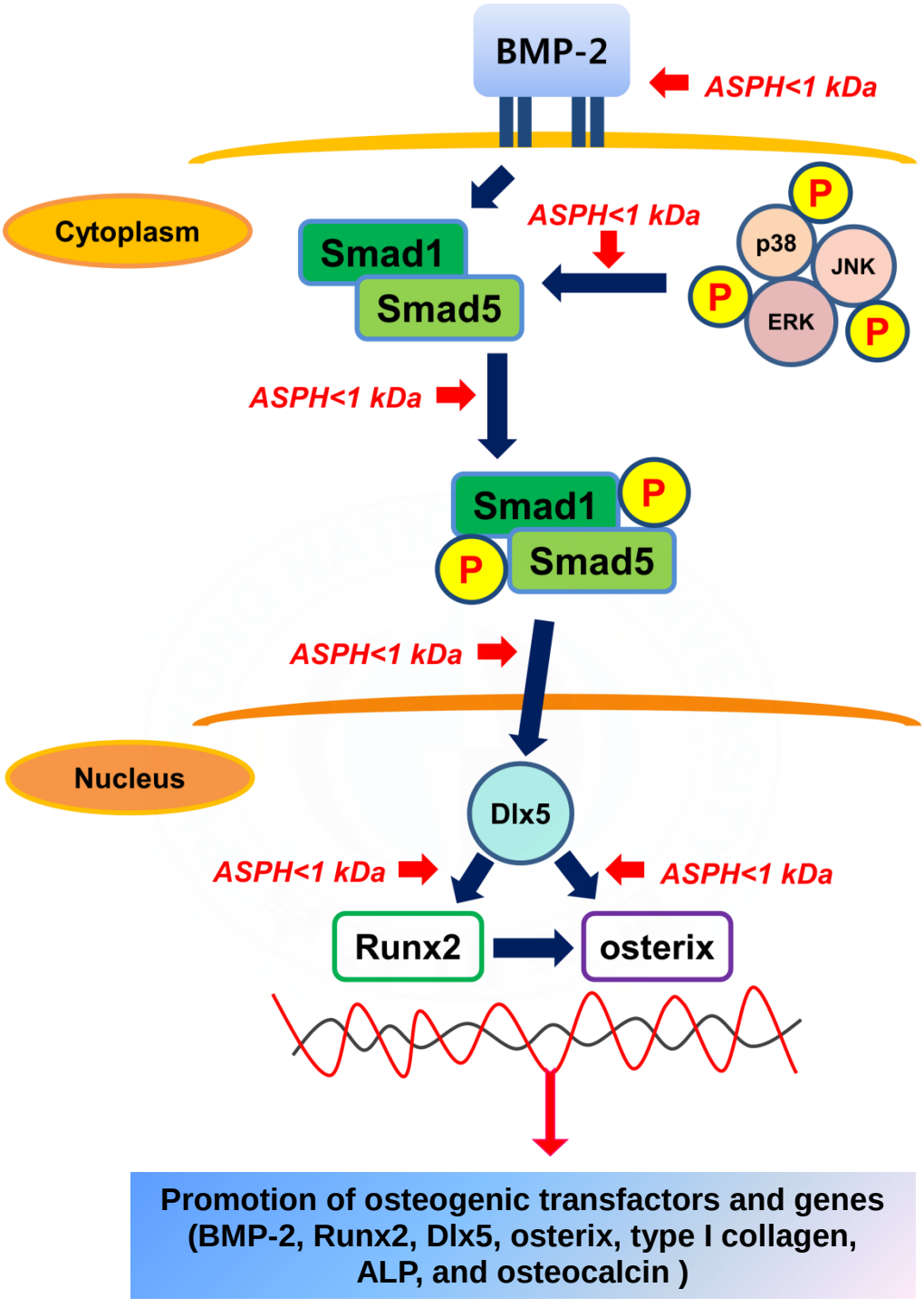


Fig. 1 Schematic diagram for osteoblastic differentiation by ASPH < 1 kDa

1. Materials and methods

1-1. Materials

Ark shell was purchased from local fish market (Suncheon, Korea) and protein content of ark shell was 68.24% (dry basis) by Kjeldahl method. Cell culture media and materials were purchased from Gibco-BRL (Gaithersburg, MD, USA). 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). All other chemicals were purchased from Sigma-Aldrich (St. Louis, MO, USA).

1-2. Preparation and membrane fractionation of ark shell protein hydrolysates (ASPH)

Before digestion, ark shell was washed using tap water and boiled to inactivate internal enzymes for 10 min. Then ark shell was freeze-dried and pulverized. ASPH were prepared by gastrointestinal digestion with pepsin at enzyme/substrate (E/S) ratios of 1:100, 1:500, and 1:1000 for 120 min, and then the reaction was terminated by boiling for 10 min to inactivate pepsin. After measuring ALP activity, new digestions using the selected E/S ratio were conducted to determine the optimal digestion time for 30, 60, 120, 240, and 360 min followed by measuring ALP activity. ASPH by enzymatic reaction of selecting E/S ratio and hydrolysis time were further fractionated based on its molecular weight using molecular weight cut-off (MWCO) membrane of 10, 3,

and 1 kDa using Quixstand benchtop system (GE Healthcare, Bucking hamshire, UK). The resulted permeates were designated as 3-10 kDa, 1-3 kDa and <1 kDa peptide fractions. All fractions were freeze-dried and stored at -20°C until needed for further analysis.

1-3. Purification of low molecular weight peptide fraction (ASPH<1 kDa)

Among the ASPH separated by MWCO membrane, the peptide showing the most stimulative ALP activity was dissolved in distilled water and fractionated by ion-exchange chromatography on a SP-Sephadex C-25 column (GE Healthcare, Buckinghamshire, UK) equilibrated with 50 mM sodium acetate buffer and eluted with a linear gradient of NaCl concentrations from 0 to 1.0 M in the same buffer at a flow rate of 1.3 ml/min (Zhao, Yuanhui, 2009). After desalination and freeze-dried, the fraction exhibiting the highest ALP activity was separated by reversed-phase high-performance liquid chromatography (RP-HPLC) on a YMC pack Pro C18 column (30 x 10mm I.D.) using a linear gradient of acetonitrile (0-60% in 30 min) containing 0.1% trifluoroacetic acid (TFA) at a flow rate of 2.5ml/min, and appeared at 280nm (Zhang, Junhui, 2010). Among the purified peptides, the fracton showing the most stimulative activity was conducted on the column to HPLC controlling a linear gradient of acetonitrile (0-30% in 20 min) containing 0.1% trifluoroacetic acid (TFA) at a flow rate of 1ml/min. The fraction indicating the most highest ALP activity was measured on the same column with a linear gradient of acetonitrile (0-30% in 20 min) containing 0.1% trifluoroacetic acid (TFA) at a flow rate of 1ml/min (Zhang, Yufeng, 2012)

1-4. Cell culture

Murine mesenchymal stem cells (D1 cell, CRL-12424) were purchased from the American Type Culture Collection (ATCC, Manassas, VA, USA). The cells were maintained in Dulbecco's low glucose modified Eagle's medium (DMEM) contained 10% fetal bovine serum (FBS) and 1% penicillin /streptomycin in a 5% CO_2 incubator at 37°C. All experiments were started when the cells reached 80% confluence and used for the assays at passage 2 to 6 (Zhao, Hong-Bin, 2014). For Osteogenic differentiation, culture media were changed at 2 days to ODM (DMEM supplemented with 50 μg/mL ascorbic acid, 10 mM β-glycerolphosphate, and 10^{-7} M dexamethasone) (Juffroy, Olivier 2009; George A, 2000). Cells were harvested for gene expression determination after 7 days culture.

1-5. ALP activity assay

Cells were seeded into 96 well-plates at a density of 5×10^3 cells/well and treated with various concentrations of ASPH and its membrane fractions for 6 days. After treatments, the cells were washed two times with PBS and lysed with 25 mM sodium carbonate buffer (pH 10) containing 0.1% triton X-100. After centrifugation at 13000 rpm for 15 min at 4°C, 100 μL of supernatant was incubated with 100 μL of an enzyme assay buffer (25 mM carbonate buffer containing 1.5 mM $MgCl_2$, 3.8 mM p-nitrophenyl phosphate (p-NPP)) at 37°C for 90 min (Karadeniz, 2014; Nguyen, Minh Hong Thi, 2014). The

absorbance was measured at 405 nm using a microplate reader and calculated following equation in which A and A_0 were relative absorbance with and without the sample, respectively.

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

1-6. ALP staining

Cells were seeded into 12well-plates and treated with diverse concentrations of ASPH for 7 days. After treatment, the cells were washed two times with PBS and fixed with 10% formalin for 5 min at RT, and carefully aspirate the formalin and wash the cells with PBS. Then add enough BCIP/NBT substrate solution to cover the cellular monolayer for 15 min at incubator (Juffroy, Olivier, 2009). Images of stained cells were collected with a microscope.

1-7. Alizarin red S staining

The level of mineralization was determined by Alizarin Red S staining using 12 well-plates after 21 days treatment (Ovchinnikov, Dmitry, 2009). Briefly, The cells were rinsed with PBS, fixed with 10% formalin for 5 min at RT, and then stained with 2% Alizarin Red S Solution (pH 4.2) for 15 min at Incubator. The cells were washed four times with distilled water. Images of calcium deposits were collected with a microscope. For quantification of mineralization, the cells were destained for 15 min with 10% of cetylpyridium chloride in 10 mM sodium phosphate buffer pH 7.0 (Wu, Jyun-Yi, 2012). Then the extracted

stain was measured at 562 nm using a microplate reader. The level of mineralization was calculated following equation in which A and A_0 were relative absorbance with and without the sample, respectively.

$$\text{Mineralization level (\%)} = (A - A_0) / A_0 \times 100$$

1-8. BMP-2, type I collagen, and osteocalcin assay

The levels of BMP-2 (BMP-2 Quantikine ELISA Kit, R&D systems Inc., MN, USA), type I collagen (SIRCOL Collagen Assay Kit, Biocolor, UK), and osteocalcin were determined using the culture media according to the manufacture's instructions.

1-9. Western blot analysis

Cell lysates were prepared using RIPA lysis buffer (Sigma Chemical Co.) containing protease and phosphatase inhibitor (Roche Applied Science, IN, USA). Protein concentration was quantified using the BCA protein assay kit (Thermo Scientific, MA, USA). Proteins were separated using 10% SDS-PAGE and transferred onto nitrocellulose membrane. After 1 h blocking using 5% skim milk or BSA in TBST (20 mM Tris-HCl, 150 mM NaCl, 0.1% Tween-20) at room temparature, the membrane was incubated with indicated primary antibodies in TBST containing 1% BSA (overnight 4°C). After washing with TBS-T, the membrane was incubated with horseradish peroxidase-conjugated secondary antibody in TBST containing 1% BSA at room temperature for 2 h.

The blot was visualized by the enhanced chemiluminescence western blotting detection kit (Pierce Biotechnology, IL, USA). The bands were imaged on Davinch-Chemi^{TM} imaging system (Core Bio, Seoul, Korea). The basal levels of the proteins were normalized by analyze the level of β-actin.

1-10. Identification of osteogenic peptides

Molecular weight and amino acid sequence of osteoblastogenic peptides was investigated by using Hybrid Quadrupole-TOF LC/MS/MS mass spectrometer (Bruker Daltonics, 255748 Germany) coupled with ESI source, the data were acquired with a scan range of 50-2000 (m/z). The peptides was separately infused into the electrospray source, and molecular mass was determined by double charged state in the mass spectrum. Following MS analysis, the peptides was automatically selected for fragmentation, and sequence information was obtained by tandem MS analysis and *De novo* sequencing was performed by *De-novo* sequencing program (ABSciex Instruments, CA, USA).

1-11. Statistics

All results are expressed as the mean ± standard deviation of three determinations. Differences between means of each group were assessed 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.

2. Results and Discussion

2-1. Production of ASPHs by enzymatic hydrolysis for osteoblasto-genesis

There are several degradation methods for acquisition of bioactive peptides from ark shell products : radiolytic, thermal, and enzymatic decomposition. The peptides produced by radioactive degradation are possible to damage the human body and also thermal hydrolysis is difficult to set an appropriate condition to raise production efficiency and maintain biological activity (Alvira, Petal, 2010; Mandels, Mary, 1974). However, enzymatic hydrolysis is a biodegradable, harmless method and further has low-energy requirement in the decomposition reaction (Grethlein, Hans E, 1985). Thus, this method is available as the best strategy against all of the above problems.

Enzyme reaction rates are affected by several factors such as pH, temperature, the concentration of substrates, and time for enzymatic reactions (Migneault, Isabelle, 2004; Mateo, Cesar, 2007; Eigen, Manfred, 2006). Each enzyme has suitable pH, temperature conditions in their reaction (Kernohan, JC, 1965; Cano, M Pilar, 1997; Sizer, Irwin W, 2006). Increasing the substrate concentration and reaction time accelerates the production rate including that the enzyme activity will be stopped in case of reaching a saturation point (Teipel, John, 1969; Liao, Fei , 2003). In this study, the enzymatic hydrolysis was used by pepsin, which is a digestive enzyme secreted in stomach, degradable from proteins to peptides, and It is usually activated in acidic conditions, about 37℃ (Dunn, Ben M, 2002; Fruton,

Joseph S, 2014). Reaction conditions to produce bioactive peptides, which can induce osteoblastic cell differentiation in MSCs, were controlled by the ratio of enzyme /substrate and reaction time maintaining constant pH, temperature. Firstly, three E/S ratios of 1:100, 1:500, and 1:1000 with hydrolysis time of 120 min were employed and ALP activities of the resulted bioactive peptides were displayed in Fig. 2A. ASPHs differently affected on ALP activities in MSCs and ASPH produced by E/S ratio of 1:500 exhibited the most ALP activity with 202% at 400 μg/mL compared to the blank group (without treatment). Cytotoxicities of the resulted ASPHs on MSCs were determined using the MTT assay and all ASPHs showed no cytotoxicity against MSCs in Fig. 2B. So as to determine optimal hydrolysis time at E/S ratio of 1:500, enzymatic hydrolysis was conducted by different reation time (0.5, 1, 2, 4, and 6 h). As shown in Fig. 2C, ALP activities were increased up to 120 min thereafter these increments were decreased. Thus, the E/S ratio of 1:500 with hydrolysis time of 120 min was selected and the resulted ASPH was further separated into 3-10 kDa, 1-3 kDa, and <1 kDa peptide fractions using a MWCO membrane. The peptide fractions were evaluated their stimulation effects on ALP in MSCs. As depicted in Fig. 2D, all peptide fractions stimulated ALP activities, and moreover low molecular weight peptide fraction (ASPH<1 kDa) showed the most stimulation activity compared to other peptide fractions in a dose-dependent manner.

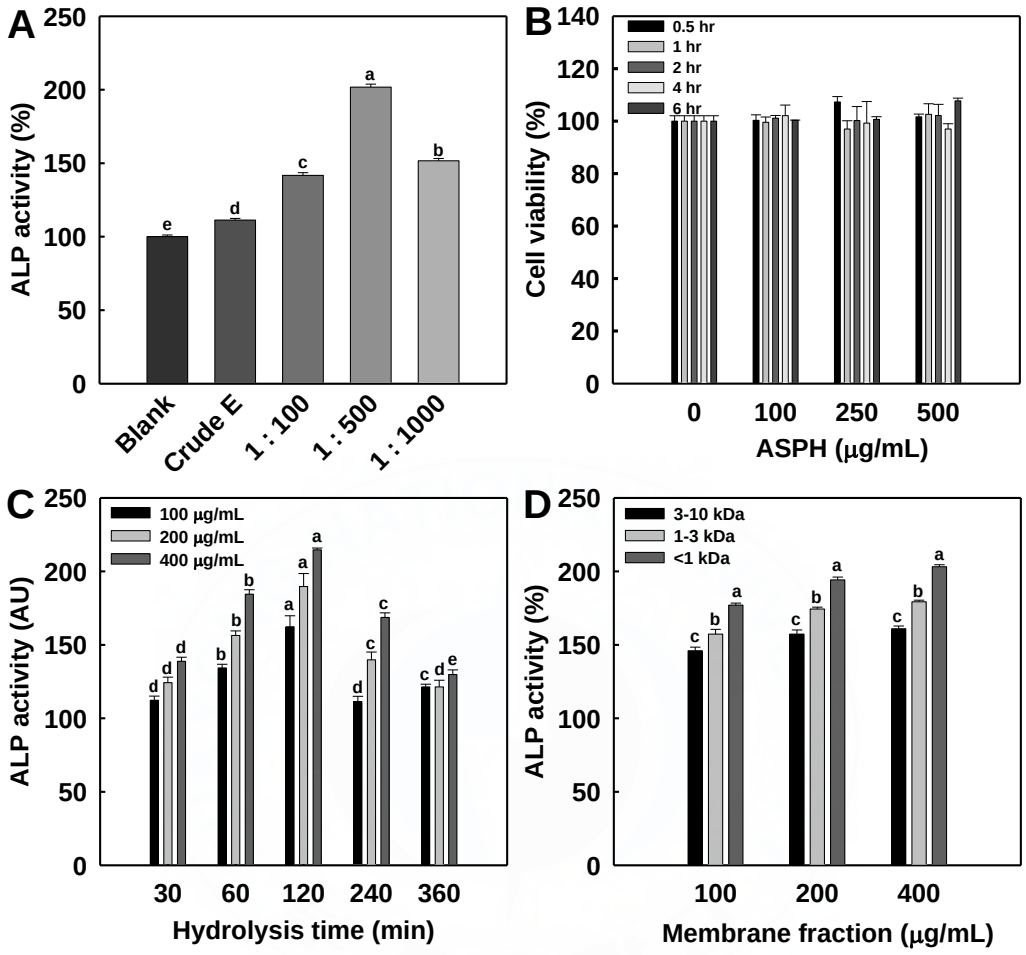


Fig. 2 (A) Effects of ASPHs prepared from different E/S ratio on ALP acticity, (B) Cell cytotoxicity of ASPHs at different time and concentrations, (C) Effects of the E/S ratio (1:500) on ALP activity at dissimilar time, (D) Effects on ALP of the peptides separated by using a MWCO membrane. Data were represented as mean ± S.D. of three independent experiments. Bars with different letters are significantly different ($p<0.05$).

2-2. Effects of ASPH < 1 kDa on osteoblastic cell differentiation by ALP staining and Alizarin red S staining

ALP expressed in the early stage of osteogensis is a representative marker of osteoblastic cell differentiation (Orimo, Hideo, 2010). Its activity generally influences the differentiation from stem cells to osteoblasts. In initial steps, ALP acitivty affect on the expression of various osteogenic transcription factors, genes, and secretion of hormones (Rawadi, Georges, 2003 ; Golub, Ellis E, 2007). During second mature osteoblast differentiation about 2-3 weeks, ALP promotes calcification known as calcium deposition in bone matrix, and osteoid, organic tissue, and becomes stiffened by deposit of calcium salts (Lalles,J.P., 2010; Meng, Shiyun, 2011). This process is called bone mineralization. In many other studies, ALP activity is measured by using following reactions that p-NPP is dephosphorylated in the presence of ALP as enzyme, and emits yellow light at 405 nm. The mineralization is investigated by Alizarin Red, 1,2-dihydroxyanthraquinone which bind to calcium ion, staining (Gregory, Carl A, 2004).

In order to demonstrate the effect of ASPH < 1 kDa on ALP activity for osteoblast differentiation, MSCs treated with ASPH < 1 kDa (400μg/mL) for 7 days were investigated by measuring ALP staining. As shown in Fig. 3A, the results indicated that MSCs in the presence of ASPH < 1 kDa (400 μg/mL) for 7 days significantly visualized more expression of ALP compared to the control group. This evident difference revealed that targeting peptide fractions enhanced and accelerated osteoblast differentiation and could be inferred about modulating BMP signaling, Wnt signaling pathway known as a typical

mechanism of bone formation. In addition, The peptide fractions enhanced latterly mature osteoblast differentiation through promoting calcium deposition. Its effect is determined using Alizarin red S staining on MSCs treated with or without ASPH < 1 kDa (400µg/mL) in the presence of ODM for 21 days (Fig. 3B). The illustrated image of mineralization in MSCs after incubation with ASPH substantially increased the intensity of calcium deposit compared to control group (without treatment). Mineralized amount after treatment with ODM in the presence of ASPH < 1 kDa was increased 338% in comparison with the untreated ODM-only control group (Fig. 3C).

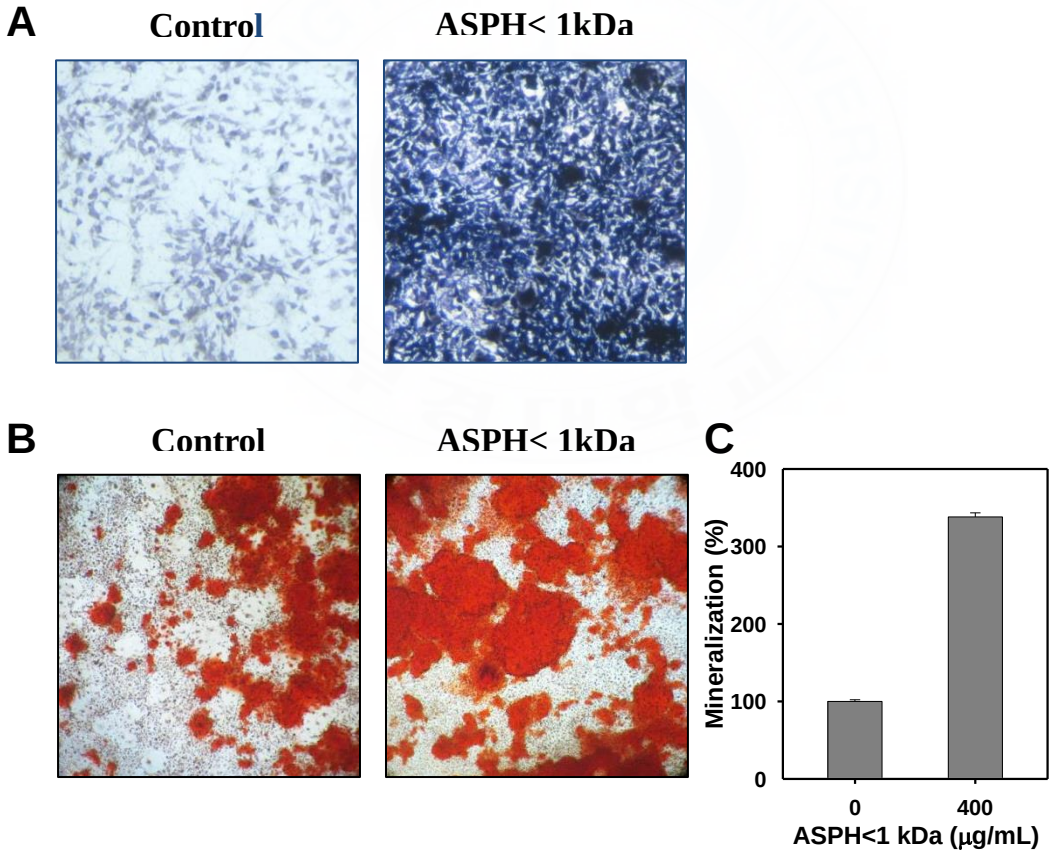


Fig. 3 (A) Effect of ASPH < 1 kDa on ALP activity visualized by ALP staining, (B) Effect of ASPH < 1 kDa on mineralization obtained by Alizarin red S staining. (C) Quantification of mineralization extracted from cetylpyridinium chloride solution. Data were represented as mean ± S.D. of three independent experiments. Bars with different letters are significantly different ($p<0.05$).

2-3. ASPH < 1 kDa promotes BMP-2 expression

BMP-2 is a low molecular weight glycoprotein as TGF-β superfamily and main osteogenic biomarkers on osteoblast differentiation (Riley, Edward H, 1996). In bone development, BMP-2 initiates bone formation through activating bone forming pathway. In the presence of BMP-2, the last formation process, which is replacement from cartilage to solid bone, is performed and proliferation and maturation of chondrocytes is controlled (Reddi, A Hari, 1992 ; Chen,G., 2012). In this study, BMP-2 concentration was measured in cultured media using ELISA kit. As shown in Fig. 4, BMP-2 concentration was significantly (p<0.05) increased in a dose-dependent manner after treatment with ODM in the presence of ASPH<1 kDa (0-400 μg/mL) for 7 days, suggesting that enhancing osteoblast differentiation on MSCs may attribute to stimulation of BMP-2 expression by ASPH<1 kDa.

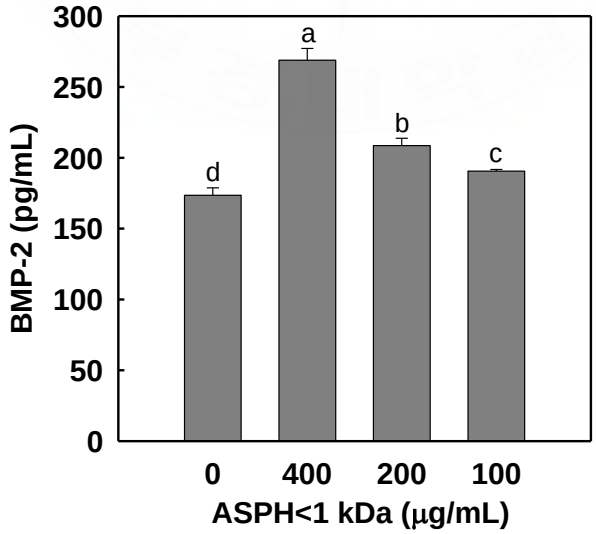


Fig. 4 Investigation of BMP-2 in MSCs treated with ASPH < 1 kDa for 7 days. The expression of BMP-2 in the media was determined using ELISA kits. Data were represented as mean ± S.D. of three independent experiments. Bars with different letters are significantly different (p<0.05).

In other studies, Metformin, a biguanide compound used to treat people with type 2 diabetes, enhanced osteoblast differentiation and mineralization through stimulating BMP-2 expression as well as AMP-activated protein kinase (AMPK) signaling pathway (Kanazawa, Ippei, 2008). Pitavastatin for the treatment on hyperlipidemia also accelerated BMP-2 and osteocalcin expressions, suggesting that it is available as osteogenic agent against osteoporosis (Ohnaka, Keizo, 2001). Unfortunately, these materials still have some serious side effects in reason that they were made up of organic synthesis. But the bioactive peptides as natural substances made from ark shell have hardly side effects. Thus, these peptides can be offered as a prospective possibility of an alternative medicine.

2-4. Effects of ASPH < 1 kDa on BMP-2 signaling pathway

The pathways for BMP-2 signal transduction are typically divided into two classes: canonical BMP-2 signaling as the Smad-dependent pathways and non-canonical BMP-2 signaling as the Smad-independent pathways (Retting-,K.N. 2009). When it comes to the canonical pathways, BMP-2 initiates the process by being combined with BMP receptors and the activated receptors cause phosphorylation of R-Smads (Nohe,A., 2002), thereafter the complex bound to Co-Smad to protect gene information formed translocates to the nucleus to stimulate various transcriptional factors (Dlx5, Runx2, and osterix) and also target genes such as type I collagen, alkaline phosphatase, osteocalcin. To verify BMP-2 signaling mechanisms of ASPH < 1 kDa on

osteoblastic cell differentiation, MSCs were cultured with ODM in the presence of ASPH < 1 kDa (400 μg/mL) for 7 days, and then the cell lysates were prepared for western blot analysis. As described in Fig. 5, Western blot analysis revealed that ASPH < 1 kDa stimulated BMP-2/4 expression and downstream BMPs signaling pathway. The expression of p-Smad1/5 was up-regulated by treatment with ASPH < 1 kDa, while total forms of Smad1/5/8 were not changed. The upregulation of Smads phosphorylation in the presence of ASPH< 1 kDa may significantly contribute to BMP-mediated osteoblastic cell differentiation. the expressions of Runx2, Dlx5, and osterix proteins were also measured by treatment with ASPH < 1 kDa (Fig. 5). All of the osteoblastogenic factors exhibited high expression in MSCs treated with ODM in the presence of ASPH < 1 kDa (400 μg/mL), suggesting that ASPH < 1 kDa enhances bone formation through modulating BMPs signaling pathway.

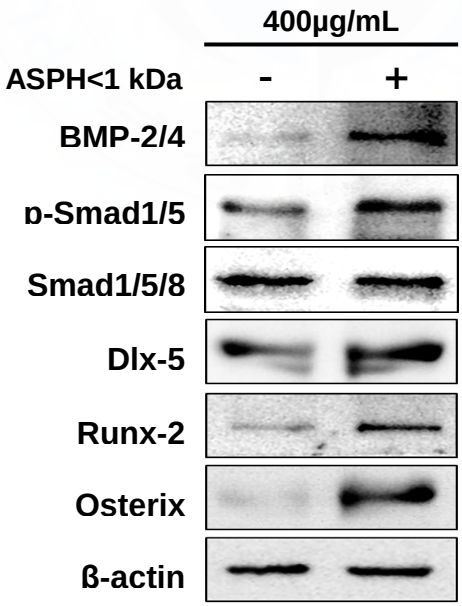


Fig. 5 Effects of ASPH < 1 kDa on the expression of osteogenic biomarkers (BMP-2/4, p-Smad1/5, Smad1/5/8, Dlx5, Runx-2, and osterix), and the results were analyzed by western blotting.

2-5. ASPH<1 kDa stimulates type I collagen and osteocalcin expressions

Osteogenic transcription factors stimulate osteoblastic target genes such as type I collagen and osteocalcin. Type I collagen is produced at an early stage of osteoblast differentiation and osteocalcin is formed at a terminal step of osteoblastogenesis. Their expressions were measured using ELISA kits. As shown in Fig. 6, type I collagen and osteocalcin in cultured media were significantly (p<0.05) elevated after treatment with ODM in the presence of ASPH < 1 kDa (400 μg/mL) for 7 days, indicating that ASPH<1 kDa enhanced type I collagen and osteocalcin protein productions in dose-dependent manner. Type I collagen is a source to compose bone matrix structures, and the above results suggested that ASPH < 1 kDa increases the ratio of calcium deposition, mineralization. Osteocalcin is secreted by osteoblast and regulate metabolic activity and bone building process (Pittas, Anastassios G, 2009). It is also related in bone mineralization and calcium ion homeostasis (Ducy, P, 2011). Osteocalcin acted as a hormone in the body leads to release more insulin and adipocytes to secrete adiponectin, which increases sensibility to insulin (Clemens, Thomas L, 2011 ; Rochefort, Gaël Y, 2011). Thus, high expressions of type I collagen and osteocalcin after treatment with ASPH < 1 kDa induce beneficial effects on bone formation and metabolic process.

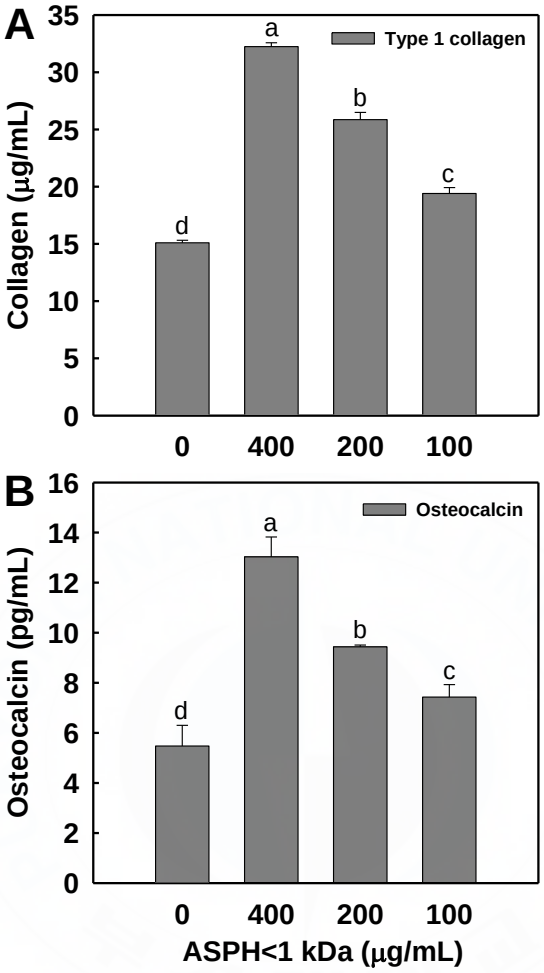


Fig. 6 Determination of type I collagen and osteocalcin in MSCs. MSCs were treated with ASPH < 1 kDa for 7 days. Collagen and osteocalcin in the media were measured using ELISA kits. Data were represented as mean ± S.D. of three independent experiments. Bars with different letters are significantly different ($p<0.05$).

2-6. Effects of ASPH < 1 kDa on MAPK signaling pathway

The above results demosnstrated that ASPH < 1 kDa promoted osteoblast differentiation through stimulating BMPs signaling pathway. MAPKs are also activated along with a diversity of osteoblastic markers (Xiao, Guozhi, 2002). MAPKs play an essential role in complicated cellular programs such as prolifieration, differentiation, development, transformation, and apoptosis (Zhang, Wei & Liu. Hui Tu, 2002). In this research, it was assessed whether ASPH < 1 kDa influences non-canonical MAPK signaling pathway, testing phosphorylation of MAPKs such as ERK, JNK, and p38 known as involvement in cell differentiation (Ding, Qingming, 2001). The MSCs were cultured with ODM in the presence of ASPH < 1 kDa (400 μg/mL) for 7 days, and the cell lysates were prepared for western blot analysis. As shown in Fig. 7, phosphorylation levels of ERK, JNK, and p38 in MSCs were significantly increased by treatment with ASPH<1 kDa, but not changed the expressions of total forms of ERK, JNK, and p38, indicating that ASPH<1 kDa is capable of participating in osteoblast differentiation through activation of MAPK signaling pathway.

In previously study, it has been reported that MAPKs could modulate the levels of Smads by phosphorylation (Javelaud, Delphine, 2005). Especially, ERK has been known as the linker of phosphorylation of Smads. However, the roles of MAPKs on osteoblastic cell differentiation are disputable. MAPKs indirectly affect Smad signaling by controlling Smad7 expression (Iwai,T., 2008). The induction of Smad7 leads to a negative feedback through

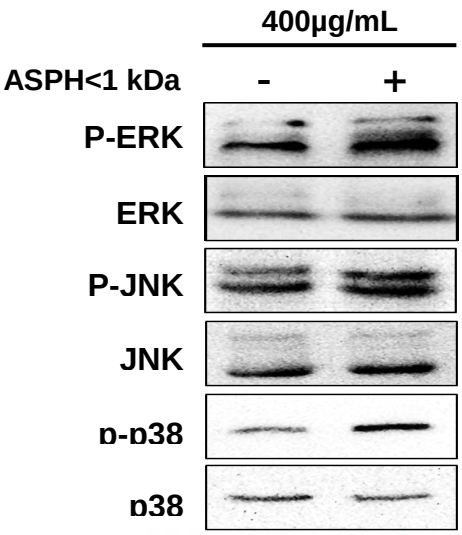


Fig. 7 Effects of ASPH < 1 kDa on the expressions of MAPKs in MSCs. the results were analyzed by western blotting.

inhibiting the expression of TGF-β, which promotes the apoptosis of osteoclast (Yan,X., 2009). In contrast, p38 modulates the sumoylation of Smad4 by specific proteins, contributing to an increment of Smad4 (Xu, Xun, 2008). Smad4 is the only common Smad for both BMP pathway and TGF-β signaling. Reportedly, suppressing the expression of Smad4 occurs various developmental defects (Wang, Rui-Hong, 2005). Partial deletion of Smad4 on osteoblast differentiation results in decreased osteoblasts and bone formation rates, while enhancing the expression of Smad4 is a good way to up-regulate bone formation (Chen,G., 2012). It is suggested that ASPH < 1 kDa has a synergetic effect on the expression of phosphorylated R-Smads by up-regulation of both BMPs and phosphorylated MAPKs. The stained picture clearly showing the difference also support this synergetic strategy on osteoblastic cell differentiation.

Recently, it has been reported that there was a linker, TGF-β activated kinase1 (TAK1), between BMPs and MAPKs for osteogenesis and bone homeostasis (Gazit, Dan, 2009; Guo, Fen, 2013). TAK1 and TAK1 binding protein (TAB1) are the up-stream proteins of p38 (Greenblatt, 2010 & Shim, 2009). p38 accelerates the induction of Runx2 by promoting its co-activator CREB binding protein (CBP) required to regulate osteoblastogenic process. Thus, the relationship between TAK1 and p38 is important for bone formation. Surprisingly, deficiency of TAK1, however, seems to affect not only stimulation of p38 but also the expression of Smad1/5/8. Thus, the activation of MAPKs in MSCs treated with ODM in the presence of ASPH<1 kDa (400μg/mL) may attribute to TAK1 stimulation.

2-7. Purification and identification of ASPH < 1 kDa

Ion-exchange chromatography separates proteins based on a molecular charge (Jungbauer, Alois, 2009). These proteins are applied to oppositely charged gel matrix. They are bound by electrostatic interactions. The attached proteins are eluted in order of the least to the most strongly bound molecules by increasing the ionic strength (Harinarayan, C, 2006). Each fraction is gathered and analyzed respectively. Meanwhile, HPLC is a highly elaborate analytical method of column chromatography. Normally, RP-HPLC is the most commonly used. In this case, the column size is same, however the silica is altered to make it non-polar by attaching long hydrocarbon chains on its surface with either 8 or 18 carbon atoms (Tanaka, Takashi, 2007).

The most stimulative peptide fraction determined by ALP activity assay was purified by ion-exchange chromatography on a SP-Sephadex C-25 column and RP-HPLC on a YMC pack Pro C18 column (30 x 10mm I.D.). Firstly, ASPH < 1 kDa was separated by ion fractionation using a linear gradient of NaCl concentrations from 0 to 1.0 M, and ALP activities of the fractions were investigated. As shown in Fig. 8, the peptide fractions differently affected ALP activity in MSCs after treatment with ODM in the presence of them (200μg/mL) for 7 days. F5 separated in 0.1 M NaCl condition exhibited the most ALP activity with 210.6% compared to the control group (without treatment). Thereafter, F5 was fractionated on the HPLC column using a linear gradient of acetonitrile (0-60% in 30 min) containing 0.1% TFA at a flow rate of 2.5ml/min (Fig. 9). Effects of the collected fractions appeared by measuring ALP activity on osteoblast differentiation in MSCs cultured with ODM and respective fractions of F5 (100 μg/mL) for 7 day. As shown in Fig. 10, F5-D fraction showed the most ALP activity with 222.75 % compared to the control group. F5-D fraction was also separated on the same column using a linear gradient of acetonitrile (0-30% in 20 min) containing 0.1% TFA at a flow rate of 1.0ml/min in shown as Fig. 11, and ALP activities of the assorted fractions were measured. As depicted in Fig. 12, F5-D-1 fraction displayed the most ALP activity with 294.7% compared to the control group. F5-D-1 fraction was separated on the HPLC column using a linear gradient of acetonitrile (0-30% in 20 min) containing 0.1% TFA at a flow rate of 1.0ml/min in described as Fig. 13, and ALP activities of the separated fractions were determined. F5-D-1-a fraction exhibited the most ALP activity with 249.40 % compared to the control

group (Fig. 14). F5-D-1-a fraction was purified by on the HPLC column using a linear gradient of acetonitrile (10-20% in 20 min) containing 0.1% TFA at a flow rate of 0.9ml/min, and the most stimulative peak was finally obtained in shown as Fig. 15. Since the peak wasn't made up of the exclusively single peptide, the peak was separated by LC-MS analysis. Two major ions (m/z 640.3 and 480.2 Da) were selected, and their primary sequences were identified using Hybrid Quadrupole-TOF LC/MS/MS mass spectrometer. A mass spectrum of ion m/z 640.3 was depicted in Fig. 16A, and an MS/MS spectrum of a singly charged ion with m/z 640.3 Da was shown in Fig, 16B. The sequencing was determined to be Ala-Try-Leu-Asn-His (AWLNH) by y-type fragmentation causing the cleavage C-terminal in peptide amide bonds. As illustrated in Fig. 17A, a mass spectrum of ion m/z 480.2 also appeared, and an MS/MS spectrum of a singly charged ion with m/z 480.2 Da was exhibited in Fig. 17B. The sequencing was identified to be Pro-His-Asp-Leu (PHDL) by b-type fragmentation related to the cleavage N-terminal in peptide amide bonds. In this study, for the first time, the amino acid sequences purified from ASPHs demonstrated that they have effects on encouraging osteoblast differentiation. In order to obviously investigate their osteoblasto-genic activity, both AWLNH and PHDL were synthesized as a respectively high purity, which was measured on a Shiseido capcell pak C18 column (4.6 x 50mm) using a gradient of acetonitrile (3-60% in 20 min) containing 0.1% TFA at a flow rate of 1.0ml/min (Fig. 18).

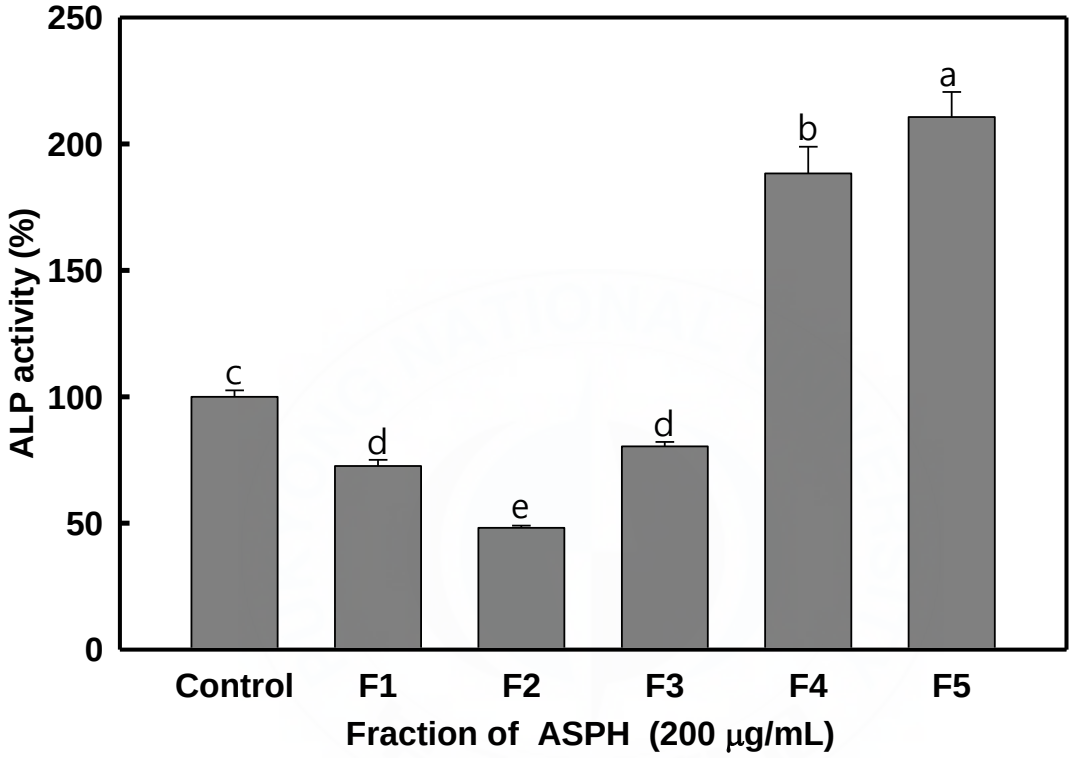


Fig. 8 Effects of the separated fractions from SP-Sephadex C-25 were measured by ALP activity (F1: non-absorption, F2: 0~0.2 M NaCl, F3: 0.2~0.4 M NaCl, F4: 0.4~0.6 M NaCl, F5: 0.6~1.0 M NaCl fractions). Data were represented as mean ± S.D. of three independent experiments. Bars with different letters are significantly different ($p<0.05$).

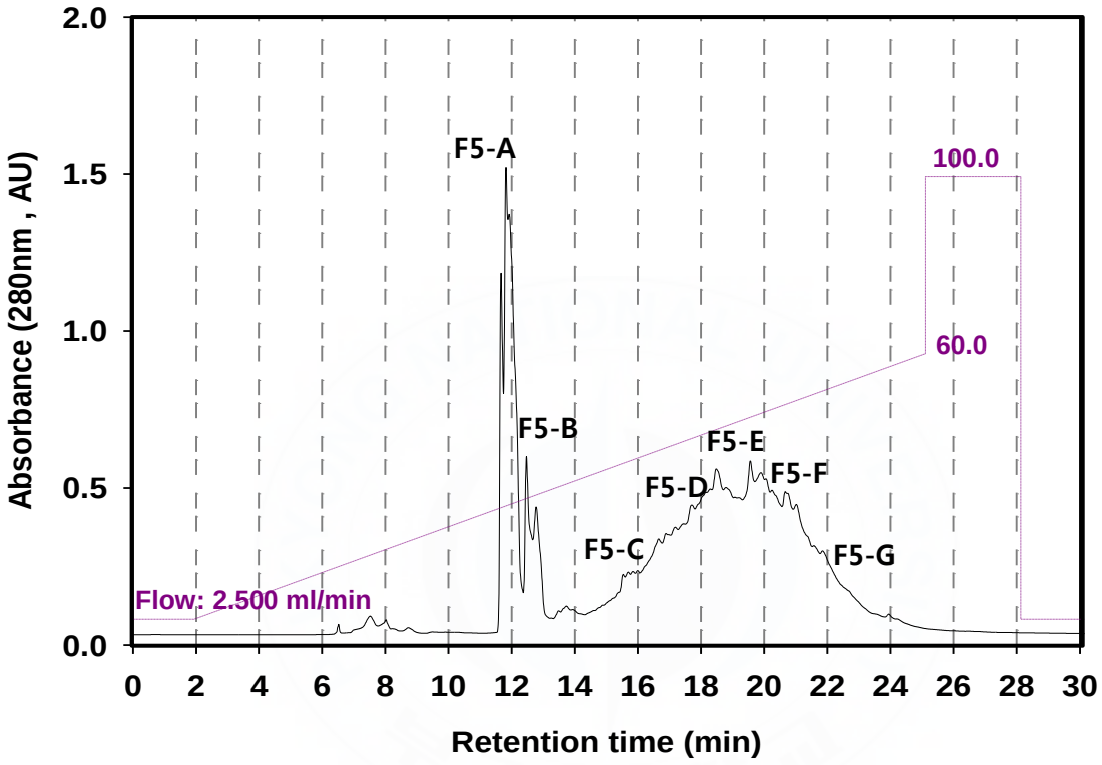


Fig. 9 Chromatogram of F5 on HPLC. The fractions were collected by every 2 min at a linear gradient of acetonitrile (0-60% in 30 min) containing 0.1% TFA at a flow rate of 2.5ml/min.

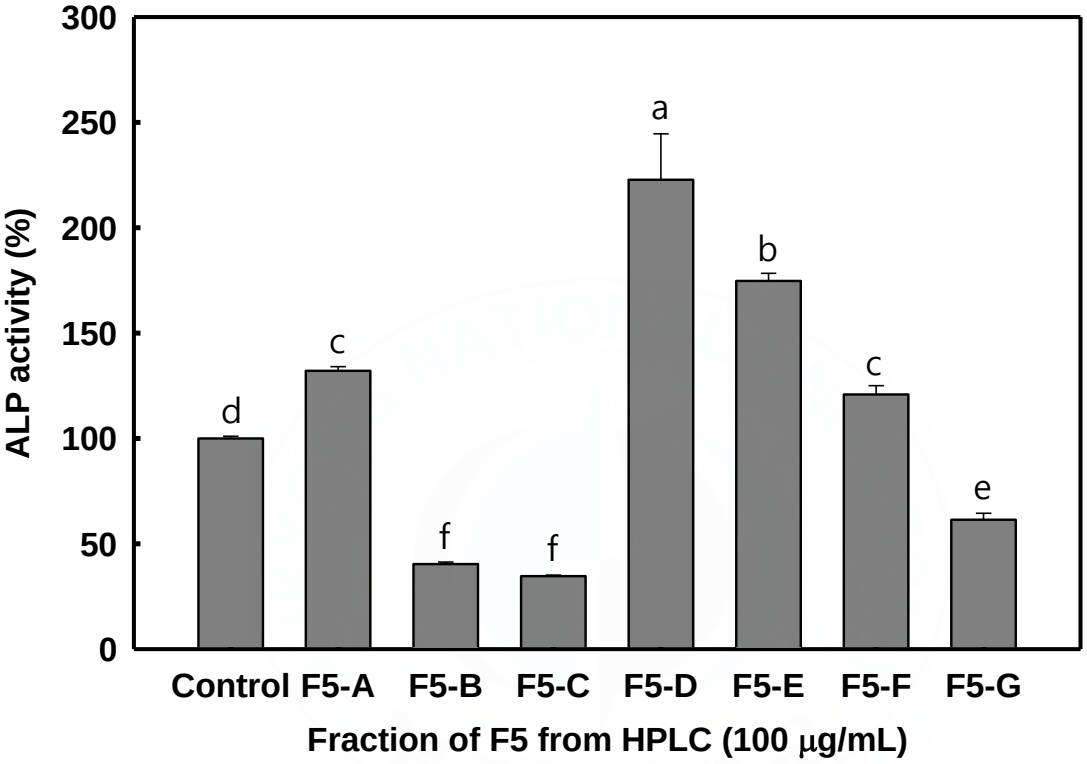


Fig. 10 Effects of the separated fractions from HPLC were measured by ALP activity. Data were represented as mean ± S.D. of three independent experiments. Bars with different letters are significantly different ($p<0.05$).

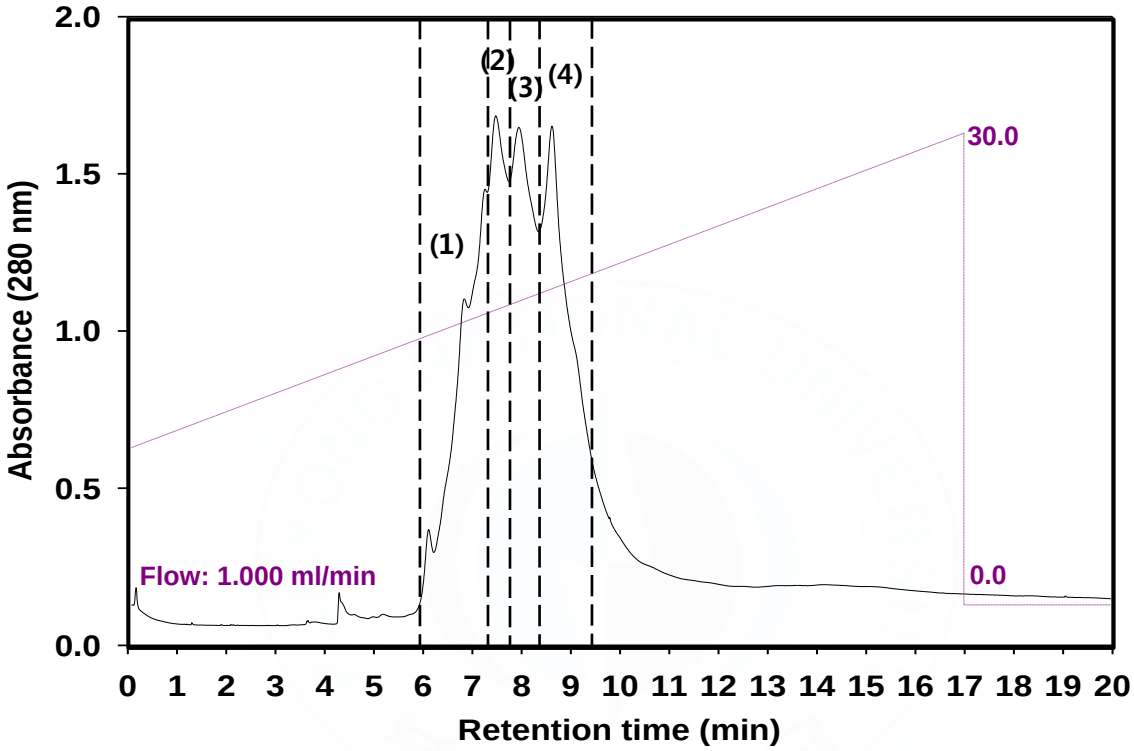


Fig. 11 Chromatogram of F5-D on HPLC. The fractions were collected at a linear gradient of acetonitrile (0-30% in 20 min) containing 0.1% TFA at a flow rate of 1.0ml/min.

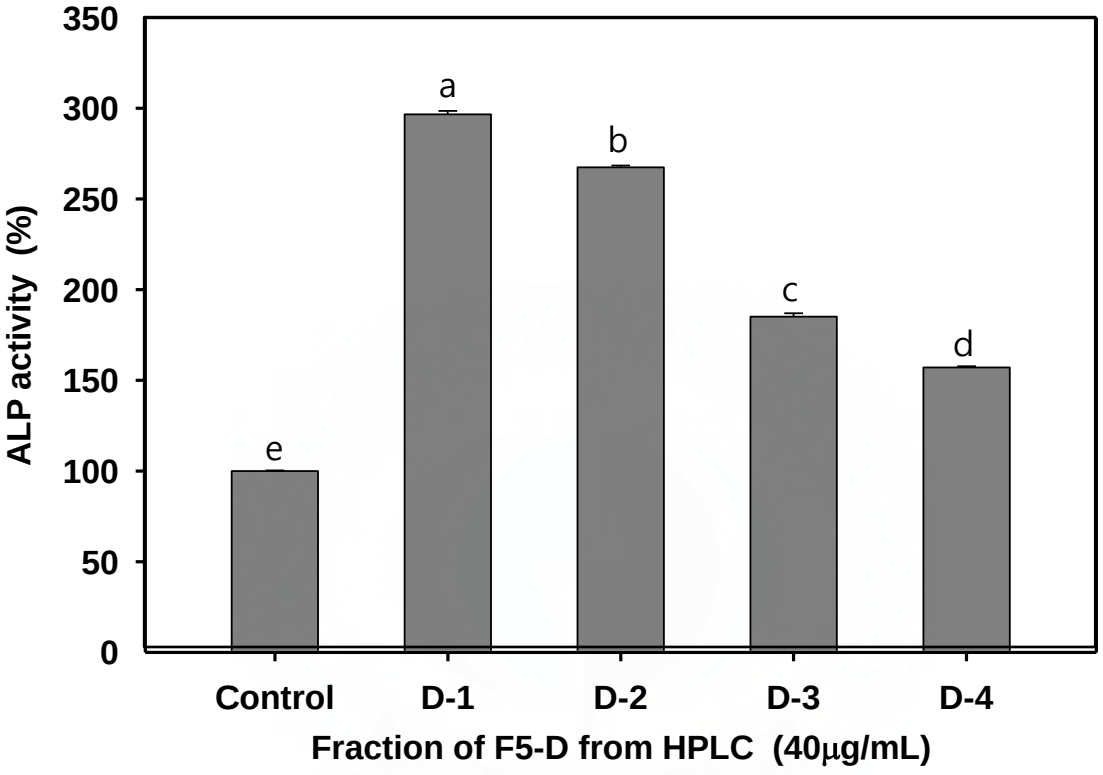


Fig. 12 Effects of the separated fractions from HPLC were measured by ALP activity. Data were represented as mean ± S.D. of three independent experiments. Bars with different letters are significantly different ($p<0.05$).

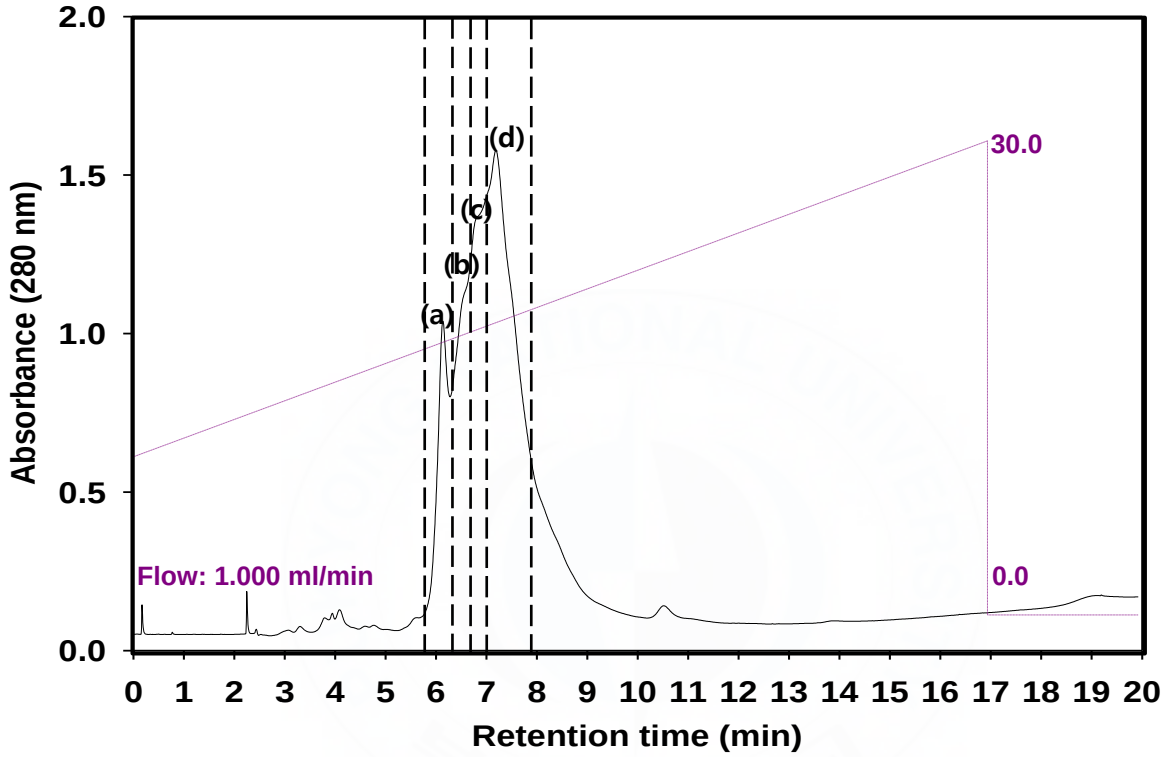


Fig. 13 Chromatogram of F5-D-1 on HPLC. The fractions were collected at a linear gradient of acetonitrile (0-30% in 20 min) containing 0.1% TFA at a flow rate of 1.0ml/min.

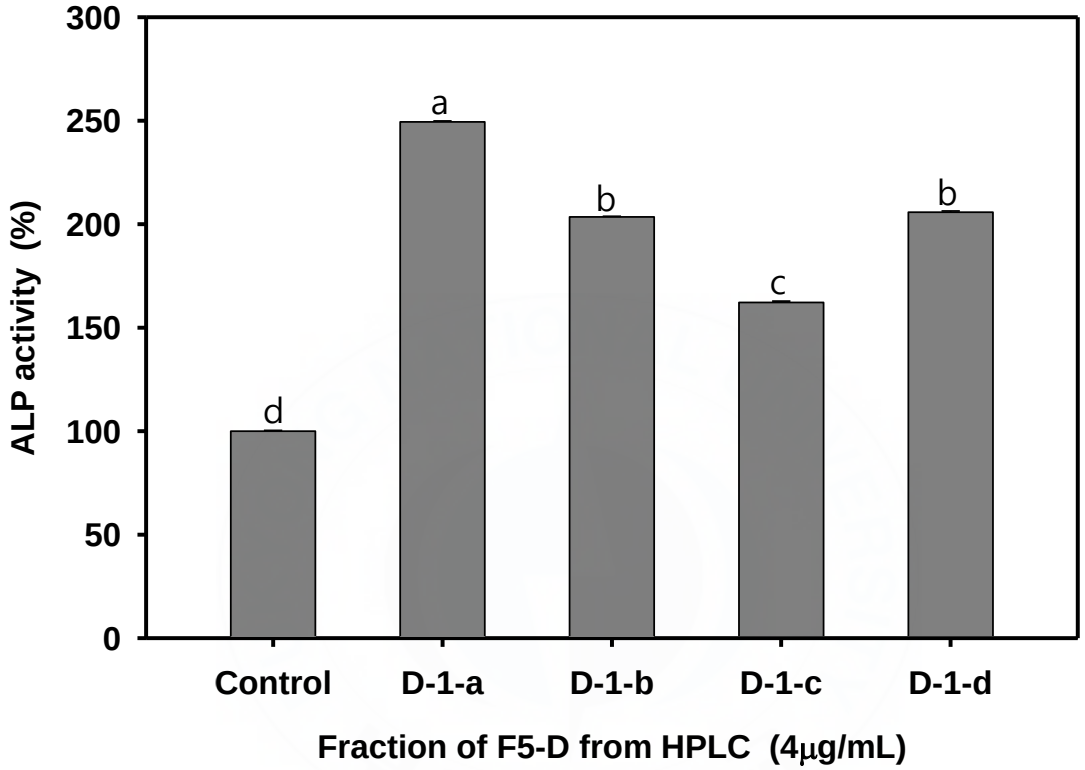


Fig. 14 Effects of the separated fractions from HPLC were measured by ALP activity. Data were represented as mean ± S.D. of three independent experiments. Bars with different letters are significantly different ($p<0.05$).

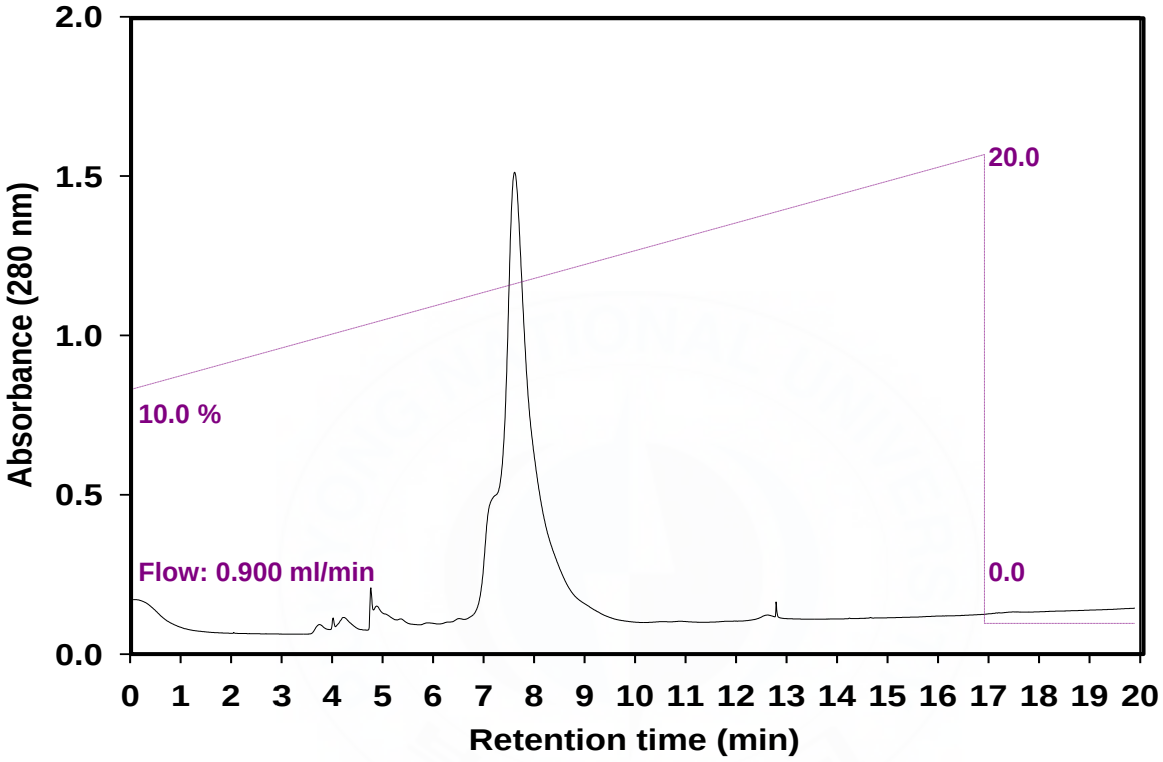


Fig. 15 Chromatogram of F5-D-1-a on HPLC. The fraction was collected at a linear gradient of acetonitrile (0-30% in 20 min) containing 0.1% TFA at a flow rate of 1.0ml/min.

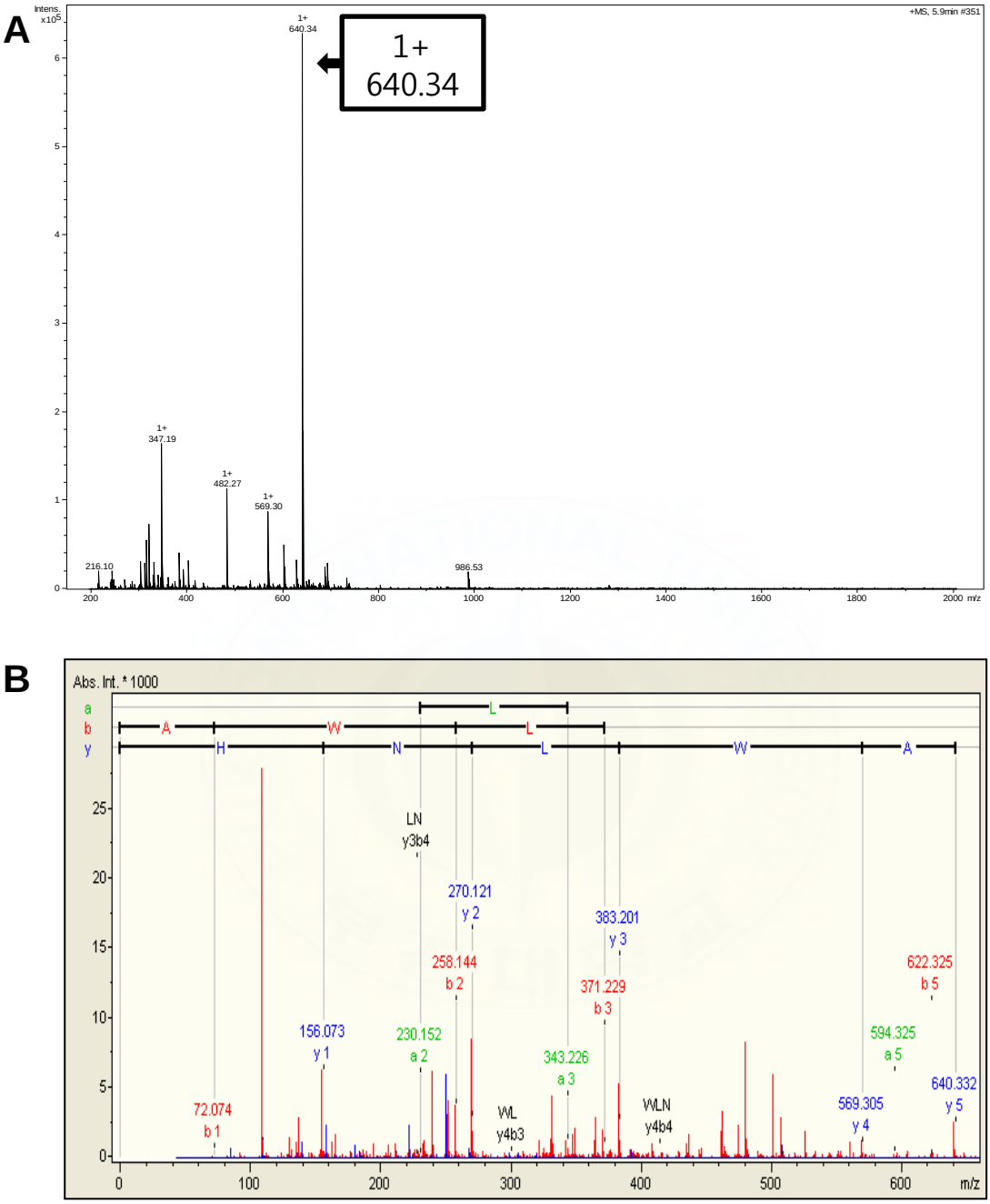


Fig. 16 Identification of the purified peptide: (A) mass spectrum of ion m/z 640.3, (B) MS/MS spectrum of a single charged ion with m/z 640.3 Da. Sequencing was determined over the m/z range of 200-2000 or 50-2000, and sequenced by using De-novo sequencing program. Data were represented as mean ± S.D. of three independent experiments.

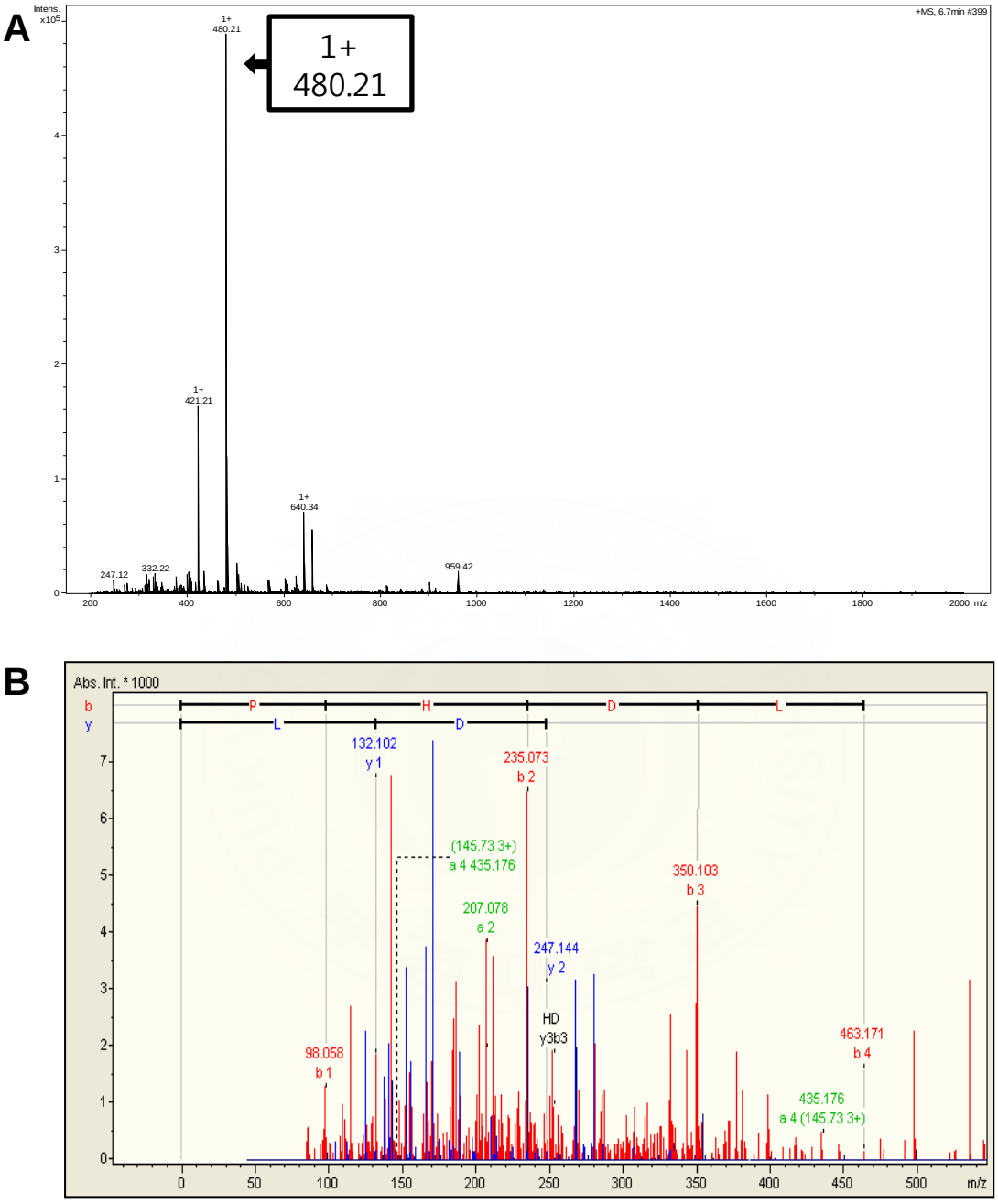
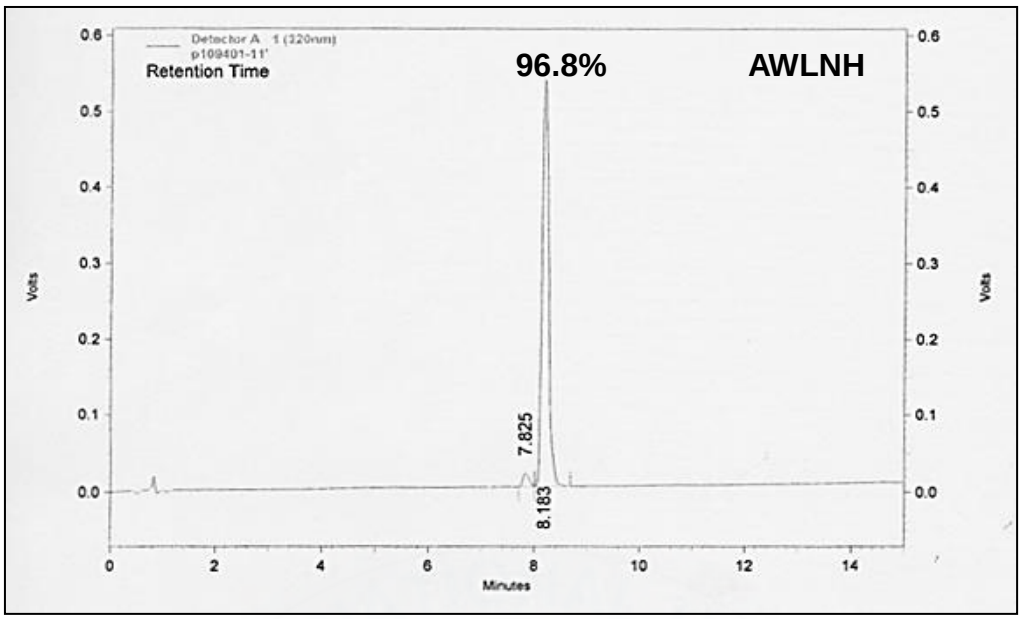


Fig. 17 Identification of the purified peptide: (A) mass spectrum of ion m/z 480.2, (B) MS/MS spectrum of a single charged ion with m/z 480.2 Da. Sequencing was determined over the m/z range of 200-2000 or 50-2000, and sequenced by using De-novo sequencing program. Data were represented as mean ± S.D. of three independent experiments.

A



B

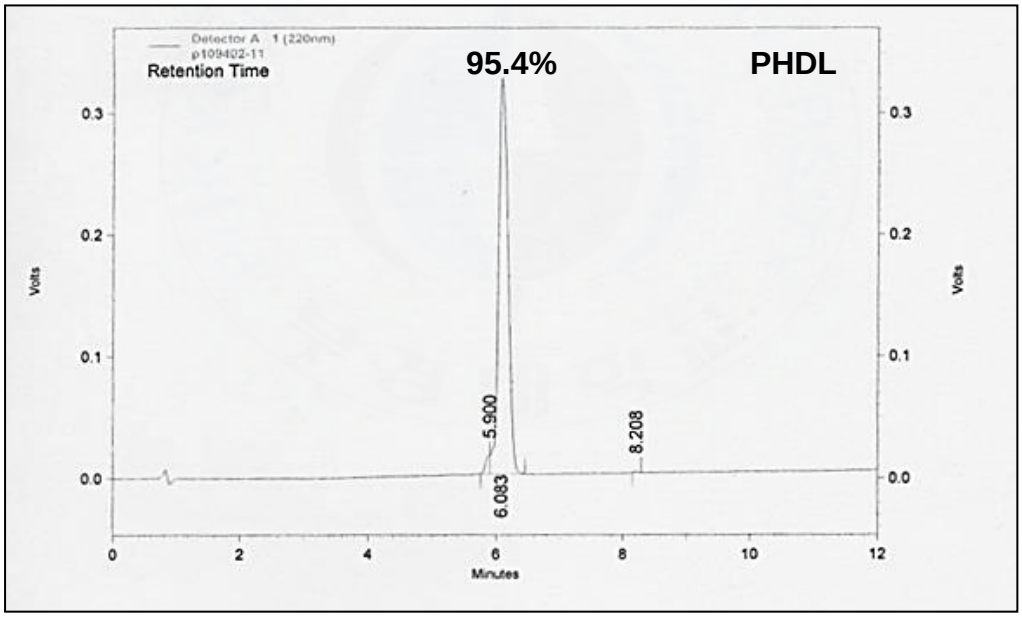


Fig. 18 (A) Chromatogram of AWLNH on HPLC, (B) Chromatogram of PHDL on HPLC. The fraction was collected at a gradient of acetonitrile (3-60% in 20 min) containing 0.1% TFA at a flow rate of 1.0ml/min, and detected at 220nm.

2-8. Effects of AWLNH and PHDL on ALP activity

To clarify osteoblastogenic ability of AWLNH and PHDL alike, a simple method was used by measuring ALP activity in MSCs. MSCs were cultured with ODM in the presence of either AWLNH or PHDL (0-8 μM) for 7 days. As depicted in Fig. 19A, ALP activity by treatment with AWLNH (8 μM) in MSCs was increased 257.78% compared with the untreated only-ODM control group. All MSCs treated with PHDL for 7 days also exhibited stimulative ALP activity, and moreover the cells after treatment with a 8 μM concentration for 7 days showed the most ALP activity with 256.55% compared to the blank group (without treatment) as described in Fig. 19B. The results suggest that both AWLNH and PHDL can accelerate osteoblast differentiation since ALP is representative osteogenic biomarker, and 4 μM concentrations of AWLNH and PHDL were selected to assess the following experiments in details.

2-9. Mineralization of AWLNH and PHDL by Alizarin red S staining

Calcium deposits in extracellular matrix determine whether the latter stages of osteoblast differentiation normally proceed in MSCs. To verify both AWLNH and PHDL increase calcium accumulation, MSCs were incubated with ODM in the presence of either AWLNH or PHDL (4 μM) for 21 days, and examined by Alizarin red S staining. As illustrated in Fig. 20A, the mineralization image in MSCs treated with AWLNH or PHDL for 21 days showed considerably increased calcium deposits compared with the only-ODM control group. Quantitative data revealed calcium in MSCs cultured with AWLNH/PHDL was more accumulated 289.4 % or 259.2% than the untreated group (Fig. 20B).

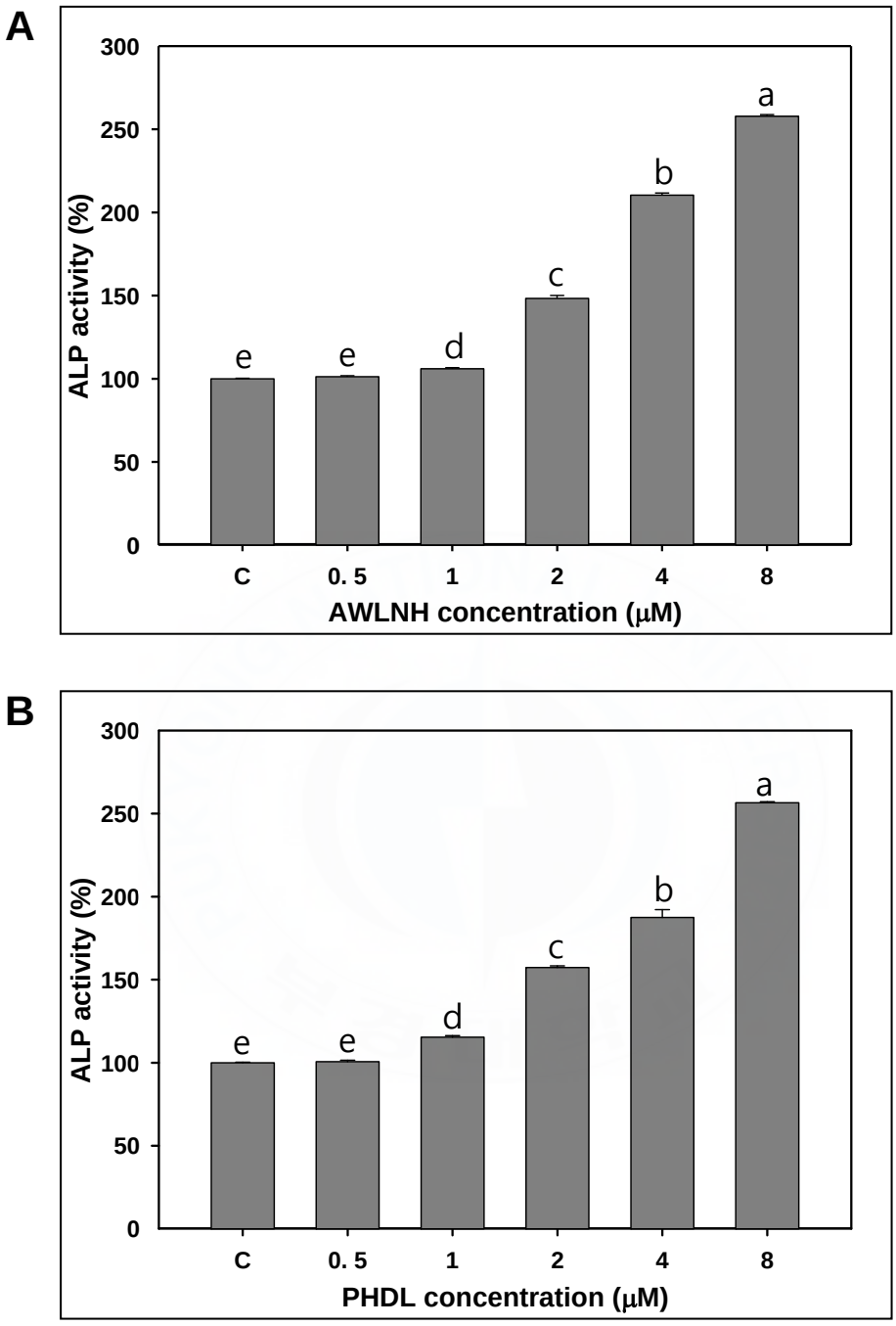


Fig. 19 ALP activities of (A) AWLNH and (B) PHDL. MSCs were cultured with ODM in the presence of AWLNH/PHDL (0-8 μM) for 7days, and Data were represented as mean ± S.D. of three independent experiments. Bars with different letters are significantly different ($p<0.05$).

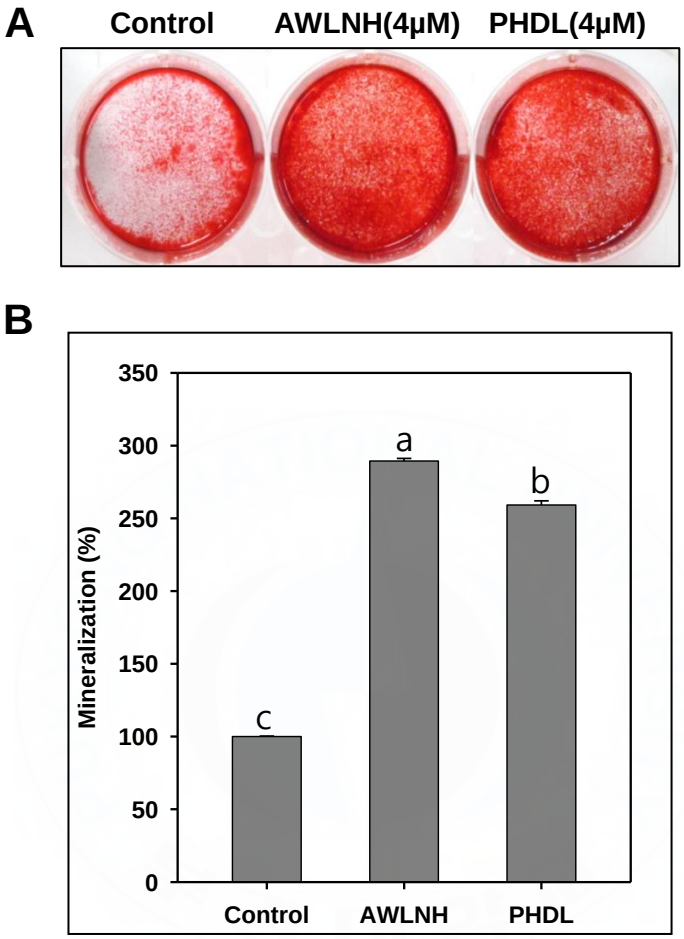


Fig. 20 Mineralization of AWLNH and PHDL. (A) MSCs were cultured with ODM in the presence of AWLNH/PHDL (4 μM) for 21days, and calcium deposits were determined by Alizarin red S staining, (B) quantification of mineralization was measured by extracts with 10% of cetylpyridium chloride at 562 nm. Data were represented as mean ± S.D. of three independent experiments. Bars with different letters are significantly different ($p<0.05$).

2-10. Effects of AWLNH and PHDL on BMP-2 signaling pathway

To investigate underlying mechanisms of AWLNH and PHDL on osteoblast differentiation, MSCs were incubated with ODM in the presence of AWLNH or PHDL (4 μM) for 7 days. Then the cell lysates were obtained for western blot analysis. As depicted in Fig. 21, the results showed AWLNH and PHDL substantially affect BMP-2/4 expression though their concentrations were extremely low (4 μM). Analysis for downstream BMP signaling pathway was also made, and the expressions of p-Smad1/5, Dlx-5, Runx2, osterix, collagen proteins were up-regulated by treatment with AWLNH or PHDL. These expressions are attributed to up-regulation of BMP-2/4 levels by treatment with AWLNH or PHDL. Moreover, the phosphorylation levels of p38, ERK, and JNK were significantly increased in MSCs added to AWLNH or PHDL (4 μM). But the expressions of total forms of MAPKs weren't changed, indicating AWLNH and PHDL is able to modulate osteoblast differentiation through activation of MAPK signaling pathway.

2-11. BMP signaling modulation of AWLNH and PHDL in the presence of noggin

Noggin proteins needed to cartilage formation are known as BMP inhibitors blocking molecular interaction between BMP proteins and receptors (Groppe, Jay, 2002). They obstruct BMP binding to the cell surface receptors by binding to BMP proteins. Treatment with noggin on osteoblast differentiation showed decreasing bone formation, causing osteopenia, and indicated that noggin has an effect on enhancing osteoclast formation (Devlin, RD, 2003). In other-

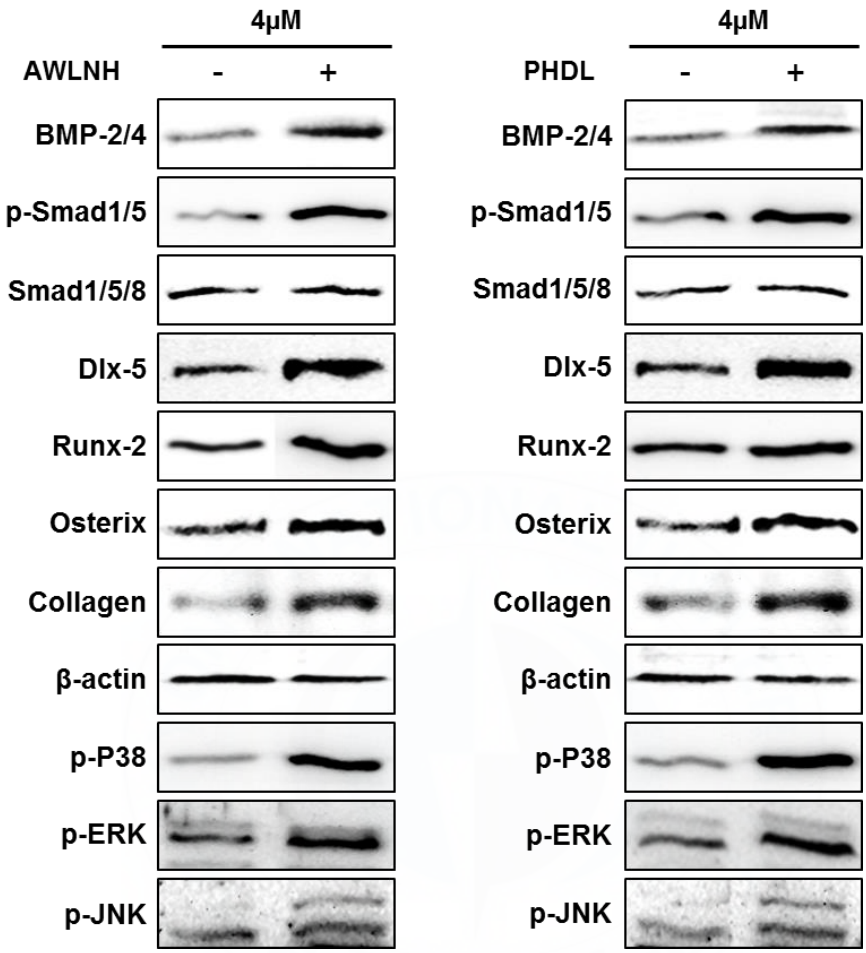


Fig. 21 Effects of AWLNH and PHDL on the expressions of osteogenic biomarkers (BMP-2/4, p-Smad1/5, Dlx5, Runx-2, osterix, and collagen) and MAPKs in MSCs. The results were subjected to Western blot analysis.

studies, osteogenic effects of *Herba Epimedii* flavonoids on BMP signaling were demonstrated by using noggin proteins, and the results revealed significant inhibition of various osteogenic transcription factors and genes (Zhang, Jin-fang, 2010). Similarly, to investigate whether the identified peptide directly stimulates BMP proteins and modulate downstream BMP signaling pathway, MSCs were incubated with noggin proteins (100ng/mL) for 2h prior to treatment of AWLNH or PHDL (4 μM) for 7 days. Then protein extracts were subjected to Western blot analysis. As illustrated in Fig. 22, AWLNH and PHDL enhance the expressions of BMP-2/4 proteins than BMP blocking group by noggin proteins, and also considerably increase p-Smad1/5, Runx2 levels. However, the phosphorylation levels of p38, ERK, and JNK remained constant whether or not noggin proteins were treated in MSCs. MAPKs is important in osteoblast differentiation, but the roles of p38, ERK, and JNK in osteoblastic cell differentiation are disputable. It has been reported that the activation of p38 is required for the expression of ALP in primary calvarial osteoblasts but inhibition of ERK had no significant effect on cell differentiation (M.Wan, X. Cao, 2005). In contrast, Kim *et al.,* demonstrated that osteoblast differentiation induced by fucoidan in human mesenchymal stem cells is required for activation of ERK and JNK but not p38 (B. S. Kim *et al*, 2015). It has been also demonstrated that the activation of ERK, which is modulated by Smads, up-regulates fibronectin and osteopontin expression (A. Nohe *et al*, 2004). These discrepant roles of MAPKs in osteoblast differentiation may be attributed to the different cell types.

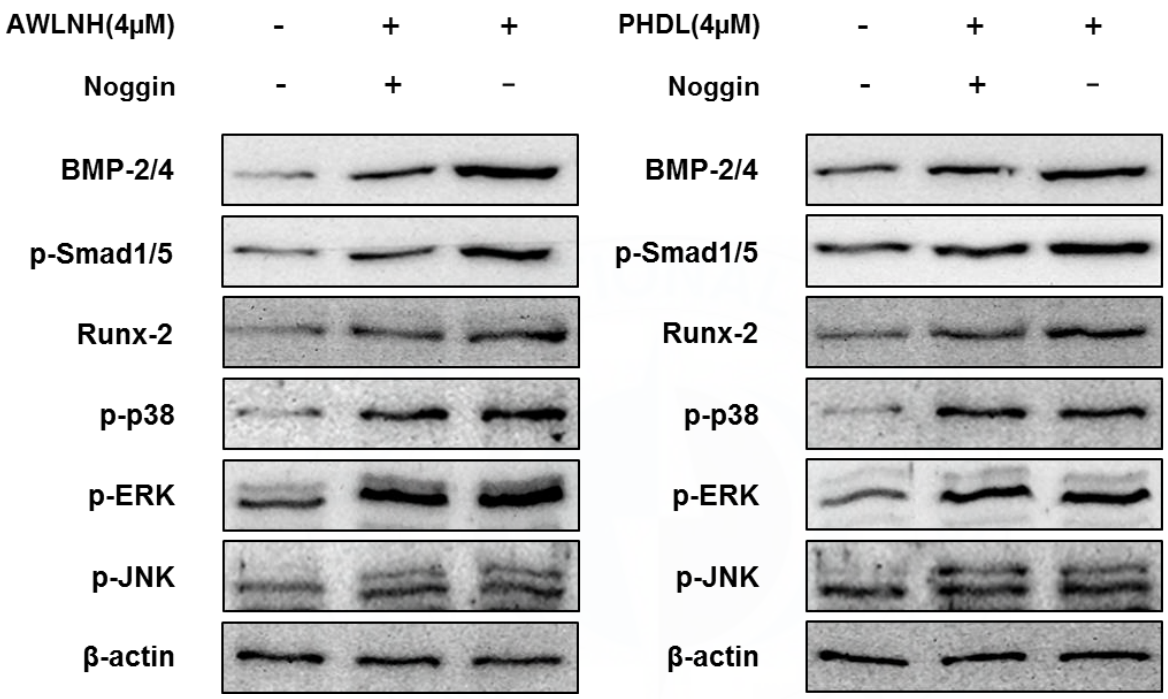


Fig. 22 Effects of noggin proteins on the expressions of BMP-2/4, p-Smad1/5, Runx2, and MAPKs in MSCs treated with AWLNH and PHDL. The results were subjected to Western blot analysis.

2-12. Effects of MAPK inhibitors on osteoblast differentiation in the presence of AWLNH and PHDL

In the present study, to examine whether MAPKs interact with BMP proteins at upstream positions, MSCs were pretreated with SB203580 (10 μM), PD98059 (20 μM) or SP600125 (10 μM) for 2h and incubated with ODM in the presence of AWLNH or PHDL (4 μM) for 7 days, and the cells were subjected to ALP staining analysis. As displayed in Fig. 23, the ERK inhibitor (PD98059) and JNK inhibitor (SP600125) prevented the effects of AWLNH and PHDL on osteoblast differentiation, but the p30 inhibitor (SB203580) had no effect on osteogenic process. In addition, the experiments to determine if the ERK and JNK inhibitors directly affect the expression of BMP-2/4 proteins and downstream BMP signaling pathways in MSCs were performed. The cells were cultured with the respective MAPK inhibitors for 2h prior to treatment of AWLNH or PHDL (4 μM), and incubated for 7 days. The cell lysates were obtained for Western blot analysis. As shown in Fig. 24, the ERK and JNK inhibitors significantly decreased the expression of BMP-2/4 and also depressed p-Smad1/5 and Runx2 levels in MSCs treated with AWLNH or PHDL. But the p38 inhibitor didn’t work in the presence of AWLNH or PHDL. Therefore, the results demonstrated that AWLNH/PHDL-induced osteoblast differentiation was regulated by ERK and JNK signaling pathways. To assess how the ERK and JNK inhibitors can work against the effects of AWLNH and PHDL on osteoblast differentiation, further studies are required.

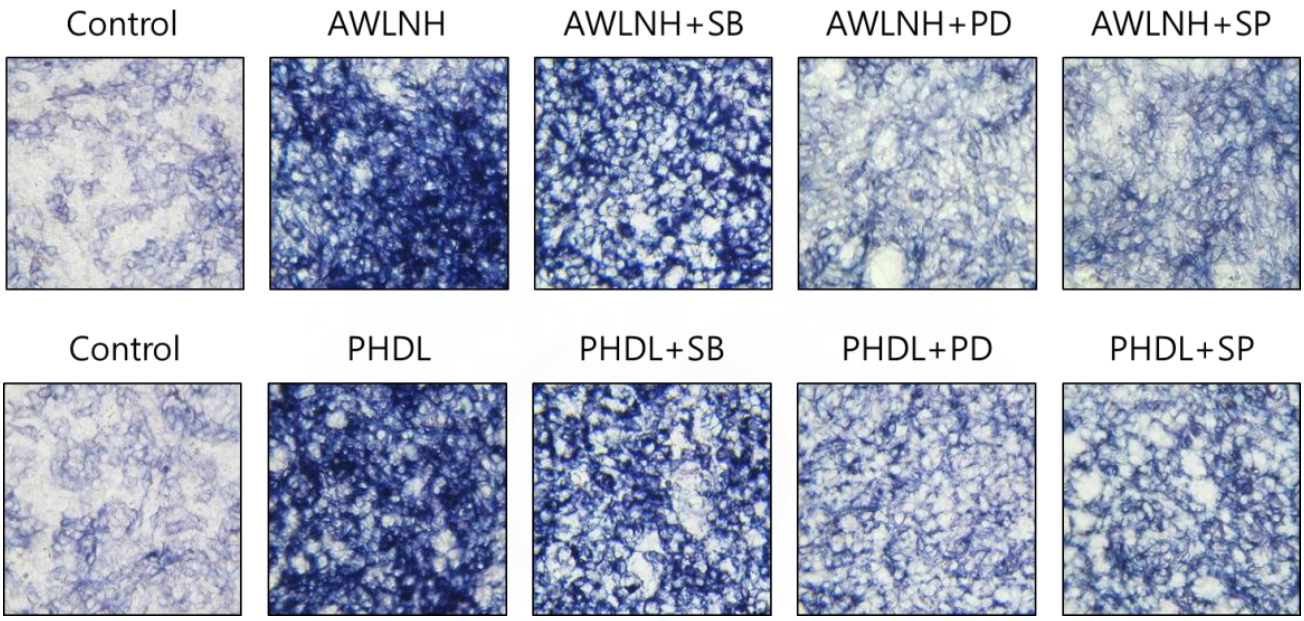


Fig. 23 Effects of MAPK inhibitors on AWLNH/PHDL induced osteoblast differentiation. The results were visualized by ALP staining analysis.

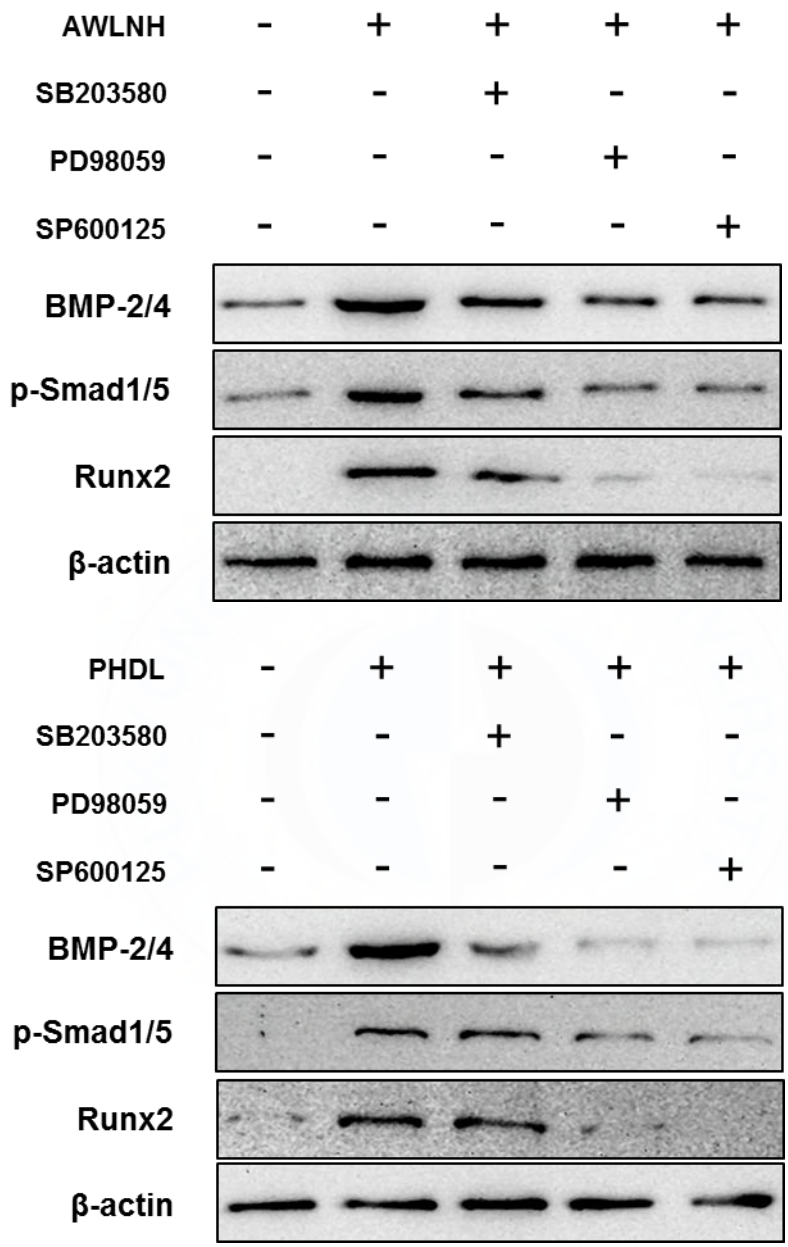


Fig. 24 Effects of MAPK inhibitors on the expressions of BMP-2/4, p-Smad1/5, and Runx2 proteins in the presence of AWLNH or PHDL (4 μM), the results were subjected to Western blot analysis.

3. Conclusion

In conclusion, it was demonstrated about the effects of marine bioactive materials, ark shell protein hydrolysates on osteoblast differentiation. The aim of this research was to purify ark shell protein-derived bioactive peptides with profitable effect on bone health and to investigate the underlying mechanisms on osteoblast differentiation through modulating BMP signaling known as mainly osteogenic pathway in MSCs. ALP activity assay was used as the purification guideline determining the most bioactive peptide on osteoblast differentiation in MSCs. The purified and identified peptides (AWLNH and PHDL) promoted overall osteoblast differentiation in mouse MSCs. AWLNH and PHDL up-regulated ALP activity, mineralization, and the expressions of both canonical BMPs signaling and non-canonical MAPKs signaling pathways on osteoblast differentiation. Since the findings, effects of the identified peptides on osteoblast differentiation, is reported for the first time, these observations suggest that these peptides could be promising pharmacological agents for osteoporosis, otherwise may be useful as an ingredient in functional foods, which can promote bone health.

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