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Osteoblastic differentiation effects of a novel peptide from biodiesel by-products of microalgae *Nannochloropsis oculata* on MG-63 and D1 cells

Graduate School of Chosun University

College of Natural Sciences

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해양 미세조류 *Nannochloropsis oculata* 바이오디젤 부산물로부터 분리된 신규 펩타이드의 MG-63 및 D1 조골세포 분화 효능

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LIST OF ABBREVIATIONS

NOP	*Nannochloropsis oculata* peptide
ALP	Alkaline phosphatase
BMP	Bone morphogenic protein
cDNA	complementary Deoxyribonucleic acid
Da	Dalton
DMEM	Dulbecco's modified Eagle's medium
DMSO	Dimethyl sulphoxide
ERK	Extracellular-signal-regulated kinases
FBS	Fetal bovine serum
GAPDH	Glyceraldehyde-3-phosphate dehydrogenase
HPLC	High performance liquid chromatography
IL	Interleukin
JNK	c-Jun N-terminal kinases
MAPK	Mitogen-activated protein kinases
mRNA	Messenger ribonucleic acid
MSCs	Mesenchymal stem cells
MTT	3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide
NF-κB	Nuclear factor kappa-light-chain-enhancer of activated B cells
RT-PCR	Reverse-transcription polymerase chain reaction
R-Smad	Receptor-regulated Smads
TGF-β	Transforming growth factor-β
TNF	Tumor necrosis factor

초 록

해양 미세조류 *Nannochloropsis oculata* 바이오디젤 부산물로부터 분리된 신규 펩타이드의 MG-63 및 D1 조골세포 분화 효능

응구엔 띠 홍 민

지도교수: 정 원 교

해양생명과학과

조선대학교 대학원

조골세포는 체내 골 형성 역할을 담당하며 골 조직 파괴 및 흡수를 담당하는 파골세포와 함께 골 대사기전의 항상성을 유지하는 주요 골 조직 관련 세포이다. 조골세포와 파골세포 생리작용의 불균형은 골다공증 등과 같은 주요 골 질환을 유발하게 된다. 지금까지 많은 연구들이 골 다공증과 같은 주요 골 질환을 치료하기 위해 골 미네랄 형성 역할을 담당하는 조골세포의 분화 및 활성을 증가시키고자 수행되었다. 본 연구에서는 최근 바이오디젤 원으로써 연구되고 있는 해양 미세조류 중, *Nannochloropsis oculata* 의 바이오 디젤 생산공정의 부산물을 고부가가치 천연 의약학 소재로 활용하고자 다양한 생리활성을 검토하였으며, 그 결과 미세조류 부산물 효소 가수분해물이 조골세포 분화 및 활성을 증가시키는 것을 확인하였다. 액체 크로마토그래피를 통해 가수분해물들로부터 단일 활성물질, MPDW (분자량 601 Da) 펩타이드를 분리할 수 있었으며 질량분석을 통해 서열을 규명하였다. 이 펩타이드는 조골세포 MG-63 및 조골세포 분화 줄기세포 D1 수준의 실험을 통해 ALP, osteocalcin 및 골 미

네랄화를 농도의존적으로 유도하는 것을 확인하였으며, 분자세포 수준의 작용기전 실험을 통해 MAPK 및 Smad pathway 분자들의 인산화를 유도함을 알 수 있었다. 이러한 결과는 해양 미세조류 *Nannochloropsis oculata* 펩타이드가 골 질환에 유의한 천연 건강기능성 식품 및 의약학 소재로써의 가능성을 제시할 수 있다.

I. INTRODUCTION

Osteoporosis is a systemic skeletal disease characterised by low bone mineral density and micro-architectural deterioration of bone tissue, consequently resulting in increases in bone fragility and susceptibility to fracture. According to the World Health Organisation (WHO) definition, osteoporosis affects 30% of post-menopausal women, a proportion that rises to 70% in women aged over 80 years (Fazzalari, 2008). Several therapies for treating osteoporosis were provided in Table 1 and Table 2 (Gallagher and Sai, 2010). In addition, some drugs routinely used for treatment of multiple diseases have detrimental effects on either skeleton or induce osteoporosis disease such as in women treated with aromatase inhibitors for breast cancer, in men receiving anti-androgen therapy for prostate cancer, in postmenopausal women treated with high doses of thyroxine, and in men and women treated with thiazolinediones for type 2 diabetes mellitus. Bone loss with fractures also occurs in patients treated with drugs targeting the immune system, such as calcineurin inhibitors, antiretroviral drugs, selective inhibitors of serotonin reuptake, anticonvulsants, loop diuretics, heparin, oral anticoagulants, and proton pump inhibitors (Gherardo et al., 2010).

Osteoporosis, that bone resorption is greater than bone formation, has becoming an important public health problem leading to an increased risk of developing spontaneous and traumatic fractures. This disease can be treated via osteoclast and/or osteoblast action (Khajuria et al., 2011). Naturally, bone undergoes continuous destruction, called resorption, carried out by osteoclasts, and formation by osteoblasts. In the adult skeleton, the two processes are in balance, maintaining a constant, homeostatically controlled amount of bone. The imbalance between the two processes resulted in significant bone loss, mimicking an osteoporosis phenotype (Rodan, 1998). Therefore, drugs that would act via promoting bone formation and/or inhibiting bone resorption could be a tool for desirable therapy.

Table 1. Osteoporosis treatment: established therapies

Agents	Clinical trials	Type
Bisphosphonates	FDA approved for both prevention and treatment	Anti-resorptive
Estrogen	FDA approved only for the prevention of post menopausal osteoporosis	Anti-resorptive
Selective estrogen receptor modulators (SERM)- Raloxifene	FDA approved for the prevention and treatment of post menopausal osteoporosis	Anti-resorptive
Calcitonin	FDA approved for the treatment of post menopausal osteoporosis in women > 5 years since menopausal and who can not take other medications	Anti-resorptive
PTH	FDA approved for treatment of osteoporosis	Anabolic
Strontium	Approved in Europe but not in US	Both anabolic and anti-resorptive
RANKL antibody - Denosumab	Approved in Europe but not in US	Anti-resorptive

Table 2. Osteoporosis treatment: novel therapies

Agents	Clinical trials	Type
Cathepsin K inhibitors (balicatib and odanacatib)	Phase 1 studies with balicatib terminated due to serious adverse events like morphea, phase 2 studies of odanacatib ongoing	Anti-resorptive
Glucagon like protide (GLP-2)	Phase 1	Anti-resorptive
Osteoprotegerin (OPG)	Phase 1	Anti-resorptive
$A_{\gamma}\beta_3$ intergrin antagonists (L-000845704)	Phase 1 and 2	Anti-resorptive
Sclerostin neutralizing antibodies (AMG 167)	Phase 1	Anabolic
rhBMP	Phase 2	Anabolic
Activin antibody (ACE-011)	Phase 1	Anabolic

A. Osteoblasts and mesenchymal stem cells

Osteoblasts are mono-nucleated cells that are responsible for bone formation. Osteoblasts and osteoclasts are the bone cells that are present and active in mature bone, interacting in bone remodeling units and their balance activities remain bone homeostasis. As bones grow, osteoblasts lay down an organic matrix that is then mineralized by deposition of calcium and phosphate in the form of hydroxyapatite which is essential for hardness and strength of bones. If the process is not properly regulated, the result can be too little mineral or too much, either of which can compromise bone health. Osteoclasts are cells responsible for bone resorption that play a crucial role in modeling bones as they grow. The disturbances in the relationship between these cell types are found in many disease states (Rodan and Martin, 2000; Cho et al., 2009; Ryu et al., 2009). Osteoblasts are probably derived from pluripotent mesenchymal stem cells that can differentiate into bone, cartilage, and fat cells (Fig. 1). Osteoblasts no longer divide and are cuboidal cells found on the bone surface actively laying down new bone. When these cells become surrounded by bone, they become stellate osteocytes. If, instead, osteoblasts stop synthesizing matrix and remain on the bone surface, they flatten out as bone lining cells. Not all preosteoblasts and osteoblasts mature; a variable number die by apoptosis (Bringhurst et al., 2011).

The activity of osteoblasts can vary during bone formation. Some cells are tall and closely packed and produce a large amount of matrix in a small area; others are flatter and produce matrix at a slower rate over a larger area. Nevertheless, all differentiated osteoblasts share certain features. They are connected by gap junctions and contain a dense network of rough endoplasmic reticulum and a large Golgi complex. They secrete collagen and noncollagen proteins in an oriented fashion and produce more type I collagen and alkaline phosphatase than other mesenchymal cells. Some products, such as osteocalcin, are produced almost uniquely by osteoblasts; consequently changes in serum levels of osteocalcin reflect changes in osteoblast activity. Mature osteoblasts have a finite capacity to produce matrix, and bone formation is sustained by the arrival of new populations of cells at the bone surface. The number and the function of osteoblasts are determined by hormones and local signals. The ultimate fate of a mature osteoblast is death by apoptosis. However, an osteoblast may become embedded in the matrix as an osteocyte, or may be converted to flattened lining cells, which cover a large percentage of the surface of bone with a thin cytoplasmic layer (Lorenzo et al., 2011).

Mesenchymal stem cells, or MSCs, are multipotent stem cells that can differentiate into cells of the mesodermal lineage, such as adipocytes, osteocytes and chondrocytes, as well as cells of other embryonic lineages (Fig. 2). (Uccelli et al., 2008). Mesenchymal stem cell (MSCs) derived from bone marrow have been used to repair skeletal bone and hard tissues. Because of their multilineage potential and plasticity, MSCs can commit to form various non-hematopoietic tissues, such as, bone, cartilage, tendons and ligaments.

Since the first study by Friedenstein and colleagues (Friedenstein et al, 1966), the field of MSC investigation has gained increasing attention and popularity, particularly in the past decade. Using 'mesenchymal stem cell' as a key word in a PubMed search, we retrieved 304 papers in 2002, 3776 in 2010, and 3889 in 2011. The amounts of publication on MSCs were increased by years make MSC-based cell therapy a hotly pursued subject of investigation in regenerative medicine and could be a therapeutic for treating several disease states (Table 3) (Uccelli et al., 2008). Initial studies focused on MSC characterization, tissue origin, and the basic biology of MSC growth and differentiation regulation. These studies led to the realization that MSCs can be easily isolated from various tissue sources, readily expanded in culture, and appropriately differentiated under suitable stimulation. These characteristics make MSCs an ideal candidate cell type for tissue engineering efforts aiming to regenerate replacement tissues for diseased structures. Further studies discovered that the regenerative effects of MSCs do not merely rely on their ability to structurally contribute to tissue repair. MSCs possess potent immunomodulatory and anti-inflammatory effects, and through either direct cell-cell interaction or secretion of various factors, MSCs can exert a tremendous effect on local tissue repair through modulating local environment and activation of endogenous progenitor cells (Chen and Tuan, 2008).

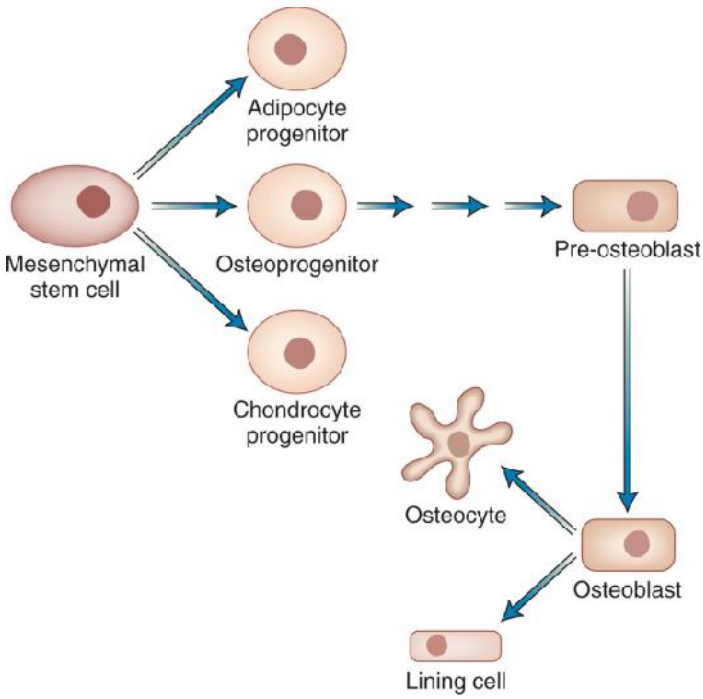


Fig. 1. Osteoblast lineage. All precursors of osteoblasts can proliferate; osteoblasts are transformed to osteocytes and lining cells without further proliferation. Some data suggest that lining cells might revert to osteoblast function after parathyroid hormone stimulation. At each stage in the lineage, apoptotic cell death is probably an alternative fate (Bringhurst et al., 2011).

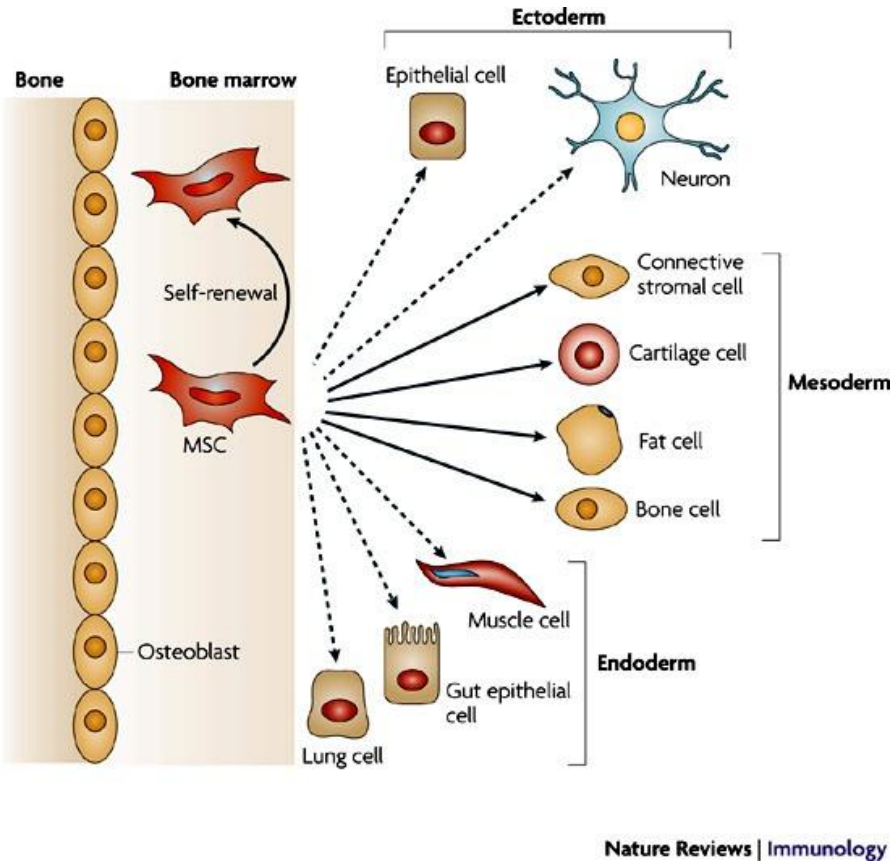


Fig. 2. The multipotentiality of MSCs. This figure shows the ability of mesenchymal stem cells (MSCs) in the bone-marrow cavity to self-renew (curved arrow) and to differentiate (straight, solid arrows) towards the mesodermal lineage. The reported ability to transdifferentiate into cells of other lineages (ectoderm and endoderm) is shown by dashed arrows, as transdifferentiation is controversial *in vivo* (Uccelli et al., 2008).

Table 3. Biological effects of MSCs in preclinical models of disease. CNS, central nervous system; EAE, experimental autoimmune encephalomyelitis; HSC, haematopoietic stem cell; IGF1, insulin-like growth factor 1; IL, interleukin; MSC, mesenchymal stem cell; SFRP2, secreted frizzled-related protein 2.

Disease	Species	Target organ	Mechanism of MSC effects
Co-transplantation with human HSCs	Sheep	Haematopoietic organs	Support engraftment and increased haematopoiesis
Myocardial infarction	Mouse	Heart	Generation of new myocytes and vascular structures
Skin-graft rejection	Monkey	Skin	Inhibition of T cells
Stroke	Rat	CNS	Release of trophic factors and induction of neurogenesis
Melanoma	Mouse	Skin	Inhibition of tumour-specific T cells by $CD8^+$ T cells
Acute renal failure	Rat	Kidney	Inhibition of pro-inflammatory cytokine production and induction of anti-apoptotic and trophic factors
EAE	Mouse	CNS	Inhibition of myelin-specific T cells and induction of peripheral tolerance
Diabetes	Mouse	Pancreas and renal glomeruli	Induction of local progenitor cells and inhibition of macrophage infiltration

EAE	Mouse	CNS	Inhibition of production of myelin-specific antibodies and encephalitogenic T cells; decreased axonal loss
Rheumatoid arthritis	Mouse	Joint	Inhibition of T cells and of production of pro-inflammatory cytokines; induction of regulatory T cells
Retinal degeneration	Rat	Eye	Decreased retinal degeneration through anti-apoptotic and trophic molecules
Acute lung injury	Mouse	Lung	Inhibition of production of pro-inflammatory cytokines
Acute lung injury	Mouse	Lung	Inhibition of production of pro-inflammatory cytokines and increased production of IL-10
Acute renal failure	Mouse	Kidney	Tubular-cell regeneration through IGF1 secretion
Myocardial infarction	Rat	Heart	Anti-apoptotic and mitogenic effect by the WNT-related molecule SFRP2
Hepatic failure	Rat	Liver	Inhibition of leukocyte invasion through the release of cytokines and chemokines
Diabetes	Mouse	Pancreas	Induction of local progenitors and inhibition of β-cell-specific T cells

B. Osteoblast differentiation and function

Process of osteoblast differentiation is necessary for bone strength and remodeling. This can be subdivided in three subsequent stages including proliferation stage, extracellular matrix synthesis and maturation stage, and mineralization stage (Zamurovic et al., 2004). The phenotypic markers of each stage are distinctively expressed. Active osteoblasts have high expression of alkaline phosphatase (ALP), collagen type I, early markers of osteoblast differentiation, while osteocalcin appears late, concomitantly with mineralization. Briefly, four steps of osteoblast differentiation were simplified in Figure 3.

Osteoblast differentiation process is under central (hormonal and neuronal), and local control (various growth factors and cytokines bound to extracellular matrix ECM). Many factors have an effect on osteoblast differentiation process and the ultimate cell maturation is the result of their synchronized action. It relies on complex signaling pathways to achieve proper rates of growth and differentiation. These signaling pathways include the action of several hormones, including parathyroid hormone (PTH), vitamin D, growth hormone, steroids, and calcitonin, as well as several cytokines.

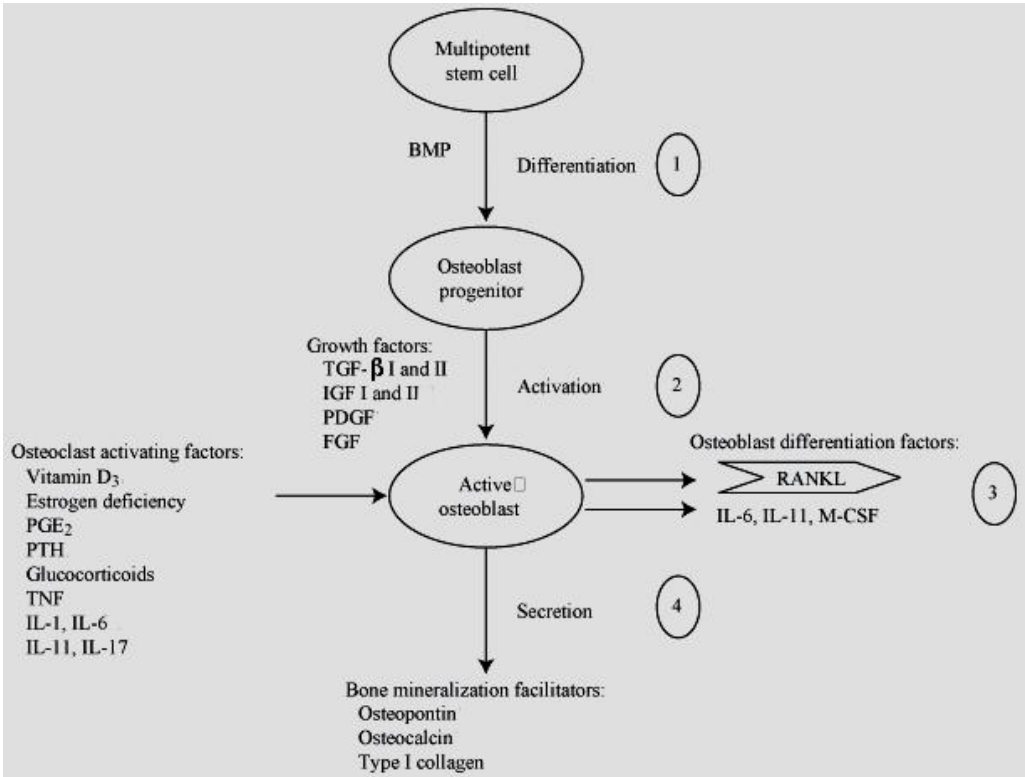


Fig. 3. Osteoblast differentiation and function. Step 1: multipotent stem cells undergo differentiation by bone morphogenic protein (BMP) to osteoblast progenitors. Step 2: growth factors activate osteoblast progenitor cells to active osteoblasts. Step 3: osteoclast-activating factors stimulate active osteoblasts to produce osteoclast differentiation factors. Step 4: active osteoblasts secrete bone mineralization facilitators (Qaqish and Sims, 2004). TGF-β: transforming growth factor-β; IGF: insulin-like growth factor; PDGF: platelet-derived growth factor; FGF: fibroblast growth factor; PGE_2: prostaglandin E2; PTH: parathyroid hormone; TNF: tumor necrosis factor; IL: interleukin; RANK: receptor activator of nuclear factor-κ B; RANKL: RANK-ligand; M-CSF: macrophage colony-stimulating factor.

C. MAPK and Smad pathway

Osteoblast differentiation is regulated by various signaling pathways such as BMP-Smads (Nöth et al., 2003; Cao and Chen, 2005; Wan and Cao, 2005), mitogen-activated protein kinases (MAPKs) (Gallea et al., 2001; Xiao et al., 2002; Zhang, & Liu, 2002; Hu et al., 2003; Ge et al., 2007), and nuclear factor kappa-light-chain-enhancer of activated B cells (NF-κB) (Novack, 2011).

MAPKs families play an important role in complex cellular programs like proliferation, differentiation, development, transformation, and apoptosis (Zhang et al., 2002). MAPK signaling also has a critical role in osteoblast differentiation (Fig. 4). During the bone di□erentiation of osteogenic cells, the MAPK signaling cascade is activated along with the expression of various osteomarkers (Xiao et al., 2002). Various osteogenic stimuli, including BMP2, are known to induce the expression of osteomarkers such as alkaline phosphatase (ALP), osteocalcin along with the activation of MAPK signaling molecules (Schindeler and Little, 2006).

Smads play important roles in osteoblast di□erentiation (Fig. 4 and 5). The BMP-specific R-Smads, Smad1, 5, and 8, transiently and directly interact with activated BMPR-Is and become phos phorylated at SSXS motifs at their C termini. Smad1/ 5/8 then form heteromeric complexes with Co-Smad Smad4 and translocate into the nucleus where they regulate transcription of various target genes. The Smad pathway is a well-characterized BMP signaling pathway. However, BMPs also initiate non-Smad interacellular signaling pathways (Fig. 4). Several lines of evidence suggested that BMPs activate the MAPK family of signaling molecules, i.e., ERK1/2, p38, and stress-activated protein kinase/Jun N-terminal kinase (Wan and Cao, 2005)

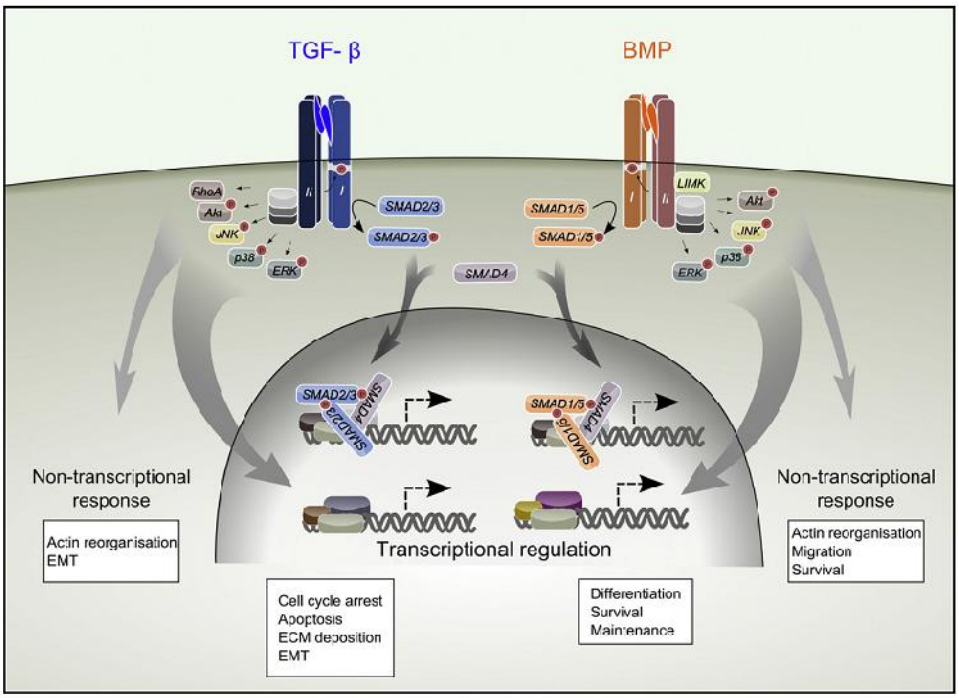


Fig. 4. TGF-β and BMP signalling elicit diverse cellular responses through Smad and non-Smad pathways. Binding of TGF-β or BMP to cognate receptors induces the formation of active receptor signalling complexes in which the type I receptor is phosphorylated and thus activated by the type II receptor. Subsequently, R-Smad proteins are phosphorylated by the type I receptor, associate with SMAD4, and accumulate in the nucleus. By binding to DNA, SMADs regulate gene expression in concert with transcriptional co-factors, co-activators, or co-repressors. Non-SMAD pathways originate at the receptor complex. Their activation in most cases involves proteins that interact with one or both receptor types, as well as additional upstream factors (both indicated in grey) that mediate the activation of non-Smad signalling targets which in turn initiate transcriptional or non-transcriptional responses. P, phosphorylation; EMT, epithelial–mesenchymal transition; ECM, extracellular matrix (Horbelt et al., 2012).

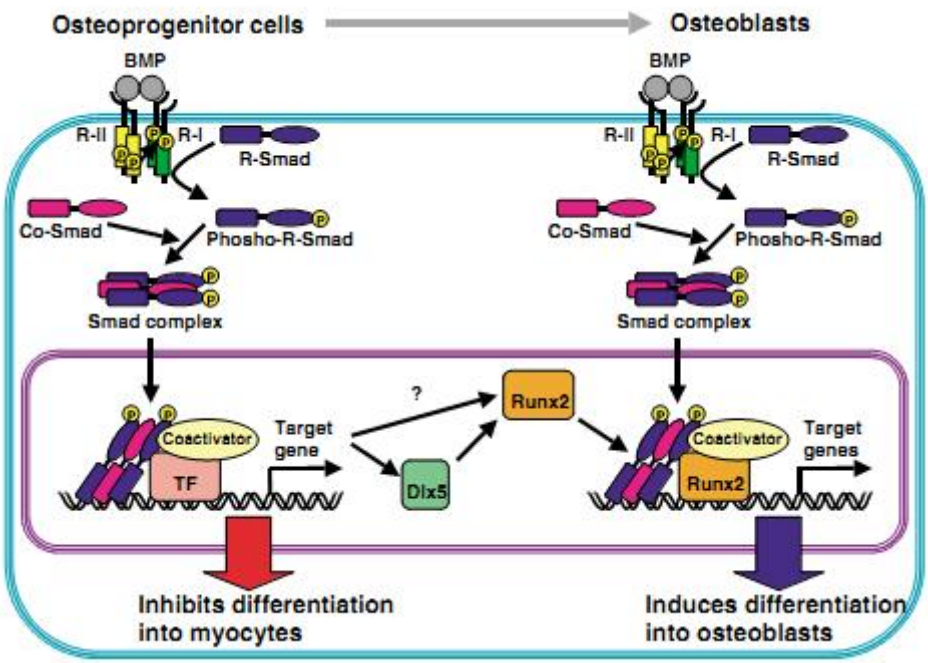


Fig. 5. Induction of osteoblastic differentiation by BMPs (Miyazono et al., 2004)

D. Bone remodeling

Bone remodeling (or bone metabolism) is a lifelong process where mature bone tissue is removed from the skeleton (a process called bone resorption) and new bone tissue is formed (a process called ossification or new bone formation). Bone remodeling follows a time sequence that lasts about 6 months. There are four stages: (1) activation of osteoclast precursors that mature into multinuclear osteoclasts under the direction of cytokines and hormones, (2) resorption of bone by osteoclasts causing a resorption cavity – a process that lasts about 3 weeks, (3) reversal of the resorption signal and (4) formation of new bone that fills up the resorption cavity with new bone and lasts several months (Fig. 6) (Gallagher and Sai, 2010).

No single factor has been proven to link the two processes, bone formation and bone resorption in bone remodeling. Existing evidence suggests that multiple factors probably are involved in the maintenance of bone homeostasis. Growth factors found in bone, such as IGFs or TGFbs, were proposed to be released during resorption and initiate local bone formation. Factors deposited on the bone surface by osteoclasts at the end of the resorption phase were proposed to initiate the bone formation that follows. Humoral factors, such as parathyroid hormone and prostaglandin E, that stimulate both bone resorption and bone formation, could increase the two processes in tandem. The action of these factors and other hormones and cytokines on osteoclasts was proposed to be mediated by osteoblast-lineage cells, which possess the cognant receptors, intimately linking osteo-blast– osteoclast interaction to bone turnover (Rodan, 1998).

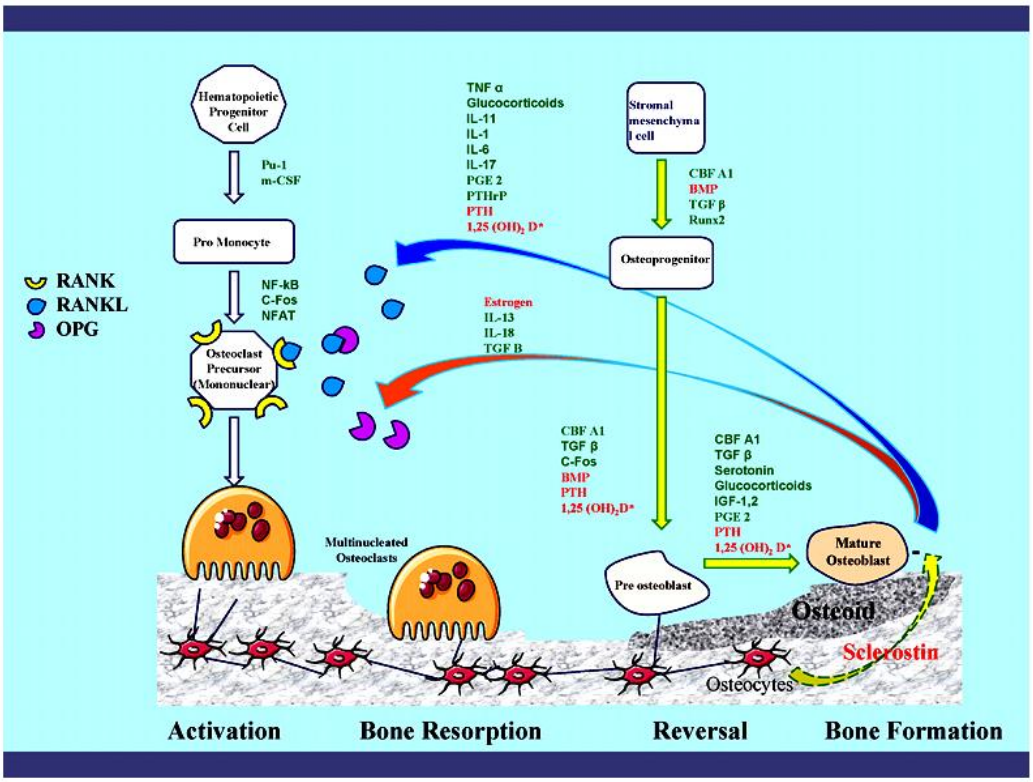


Fig. 6. Bone Remodeling – showing the various stages and the factors involved. Also shown is the development of osteoblasts and osteoclasts from precursors. The factors in red are currently being used and/or under active investigation in clinical studies, others in green are potential targets based on animal and *in vitro* studies. RANKL (receptor activator of nuclear factor kappa B ligand), OPG (osteoprotegerin), TNF-α (tumor necrosis factor alpha), IL (interleukin), PGE 2 (prostaglandin E 2), PTHrP (parathyroid hormone related peptide), PTH (parathyroid hormone), 1,25(OH) 2 D 3 (1,25-dihydroxyvitamin D 3), CBF A1 (core binding factor alpha 1), BMP (bone morphogenic protein), TGF-β (transforming growth factor beta), IGF (insulin like growth factor), m-CSF (monocyte colony stimulating factor), NFAT (nuclear factor of activated T-cells). *The function of Vitamin D3 in bone is complex and is dependent on serum calcium. If serum calcium is low, vitamin D increases bone resorption and if its high/normal, vitamin D promotes bone formation (Gallagher and Sai, 2010).

Marine microalgae, or phytoplankton, are mostly represented in ocean populations: the best known are the diatoms (Bacillariophyta), the dinoflagellates (Dinophyta), the green algae (Chlorophyta) and the blue-green algae (Cyanophyta). The unicellular marine microalgae were considered to be an abounding resource for carotenoids, lipids, and polysaccharides, and were widely investigated in the fields of food supplements and bio-fuel production (Liau et al, 2010). While terrestrial plants in temperate climates can achieve a photoconversion efficiency of only below 1 %, microalgae can convert up to 5% of the solar energy into chemical energy (Rösch et al, 2011). Microalgae are efficient in converting solar energy into metabolites, such as lipids, proteins, carbohydrates, pigments and vitamins. Moreover, microalgae can provide various types of renewable energy sources, such as methane, biohydrogen, and biodiesel (Borges et al, 2011). Thus, they can apply in food, feed, natural compounds, or aquacultures field.

Nanochloropsis oculata is microalgae that mostly been known from the marine environment but also occur in fresh and brackish water. *Nannochloropsis* is able to build up a high concentration of a range of pigments such as astaxanthin, zeaxanthin and canthaxanthin, and has been shown to be suitable for algal biofuel production due to its ease of growth and high oil content (28.7% of dry weight), mainly unsaturated fatty acids and a significant percentage of palmitic acid. It also contains enough unsaturated fatty acid, linolenic acid and polyunsaturated acid (>4 double bonds) for a quality biodiesel. Morever, *N. oculata* has a high rate of protein as well as carbohydrate (Table 4). Thus, the use of *Nannochloropsis* has been recognized for human diets and biomass, and the beneficial effect of reducing blood pressure in hypertensive rats have demonstrates by feeding experiments (Rebolloso-Fuentes et al., 2001).

Many studies have been reported that marine bioactive peptides can be used as functional foods, nutraceuticals, or pharmaceuticals due to their therapeutic potential in the treatment or prevention of various diseases. Peptides- derived from marine microalgae have potential activity on anti-oxidant, antihypertensive, anticancer (Kim and Kang, 2011). *N. oculata* possess a numerous of protein which break down to peptide, amino acid, or protein fractions by protease hydrolysis. We would like to check whether biodiesel by-products of *N. oculata* after applying to enzymatic hydrolysis can induce osteoblastic cell differentiation.

Table 4. Biomass, proximate composition and time period at harvest of culturing *Nannochloropsis oculata* outdoor under shade and under laboratory condition (Banerjee et al., 2011).

Parameter	*Nannochloropsis oculata*	
	Outdoor	Laboratory
Biomass (g/l dw)	2.11 ± 1.70	1.6 ± 0.20
Protein (% dw)	32.82 ± 3.11	34.11 ± 0.70
Lipid (% dw)	13.02 ± 0.91	12.51 ± 1.01
Carbohydrate (% dw)	26.13 ± 2.70	19.81 ± 2.02
Time period (days) (scaling up from 250 ml to 1l)	16	20

II. MATERIALS AND METHODS

A. Materials

Human osteosarcoma (MG-63) was obtained from American Tissue Culture Collection (ATCC). Murine mesenchymal stem cells (D1) were purchased from ATCC (Manassas, VA). Dulbecco's Modified Eagle's Medium (DMEM) and fetal bovine serum (FBS) was obtained from Gibco-BRL, Life Technologies (Grand Island, NY, USA). All chemicals required for purification, including hydrolysate enzymes, were obtained from Sigma Chemical Co., USA and Novo Co., Denmark. Alizarin red-S, para-nitrophenyl phosphate (p-NPP), cetylpyridinium chloride monohydrate, dexamethasone, and 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) were purchased from Sigma (St. Louis, MO, USA). RT-PCR reagents were purchased from Promega (Madison, WI, USA). Primary and secondary antibodies used for Western blot analysis were purchased from Santa Cruz Biotechnology Inc. (Santa Cruz, CA, USA) and Amersham Pharmacia Biosciences (Piscataway, NJ, USA), respectively. Other chemicals and reagents used were of analytical grade.

B. Preparation of enzymatic hydrolysates

To extract peptide induced osteoblast differentiation from biodiesel by-products of marine microalgae, enzymatic hydrolysis was performed using various commercial enzymes Alcalse, Flavourzyme, Neutrase, PTN, and Protamex (Novo Co., Denmark) with each optimal condition (Table 5). At enzyme/substrate ratio of 1/100 (w/w), 1% substrate and enzyme were mixed. The mixture was incubated for 8 h at each optimal temperature with stirring and then heated in a boiling water bath for 10 min to inactivate the enzyme. Degree of hydrolysis was determined by measuring the nitrogen content soluble in 10% (v/v) trichlroacetic acid and lyophilized hydrolysates were stored under -80 ˚C until used.

Table 5. Hydrolysis conditions of *Nanochloropsis oculata* by different enzymes

Commodity	Name / Purpose	pH	Temperature (˚C)	Form	Max. pH / Max. ˚C
Alcalase 2.4L	Protease Upgrade protein hydrolysis and the growth of yeast; hydrolyse protein of animal and plant origin	6.5-8.5	60	Liquid	7.0 60
Flavourzyme 1000L	Aminopeptidase High exopeptidase activity, hydrolyse protein of animal and plant origin	5.5-6.5	45-55	Liquid	6.0 50
Neutrase 0.8L	Protease Hydolyse protein of animal and plant origin	5.5-7.5	45-55	Liquid	6.0 50
PTN 6.OS	Trypsin Upgrade fermentation, protein hydrolysis, and growth of yeast	7.0-8.0	45-50	Powder	7.0 50
Protamex	Protease (subtilisin) Hydolyse protein of animal and plant origin	5.5-7.5	35-60	Powder	6.0 50

C. Purification of osteoblast activating peptide

1. Recyling preparative HPLC (high-performance liquid chromatography)

Osteoblast activating peptide was purified from enzymatic hydrolysates using recycling preparative HPLC on a Jaigel W253 column. The lyophizied protein (20 mg/ml) was loaded onto the column equilibrated with 20 mM phosphate buffer (pH 6.8), and eluted at a flow rate of 3 ml/min. Each fraction was monitored at 215 nm, and dried using a lyophilizer under - 80 ˚C. Osteoblast differentiation activity was also investigated, and a highest osteoblast activating fraction was determined to purify by chromatography as the next step.

2. High-performance liquid chromatography (HPLC)

The fraction exhibiting osteoblast differentiation activity was further purified using reversed-phase high-performance liquid chromatography on a YMC pack pro C18 column with a linear gradient with a linear gradient of acetonitrite (0 – 60% in 60 min) at a flow rate of 1.0 ml/min. Elution peaks was detected at 215 nm. Potent peaks were collected, lyophized, and then evaluated osteoblast differentiation activity. The finally purified peptide was analyzed amino acid sequence.

D. Determination of amino acid sequence

Accurate molecular mass and amino acid sequence of the purified peptide were determined with a Q-TOF mass spectrometer (Micromass, Altrincham, UK) coupled with an electrospray ionization (ESI) source. The purified peptide was injected into the electrospray source following dissolution in methanol/water (1:1, v/v), and its molecular mass was determined by a doubly charged $(M+2H)^{2+}$ state in the mass spectrum. Following molecular mass determination, the peptide was automatically selected for fragmentation and its sequence information was obtained by tandem mass spectroscopy (MS) analysis.

E. Culture of cells and viability determination

D1 cells were maintained and MG-63 cells were cultured in Dulbecco's Modified Eagle's Medium (DMEM) supplemented with 10% fetal bovine serum (FBS) and antibiotics (100 IU/ml of penicillin G and 100 μg/ml of streptomycin) at 5% CO_2 and 37 ˚C humidified atmosphere.Cells were seeded at 1×10^4 cells/cm^2 and experiments were performed after cells had reached about 80% confluence. To induce osteogenic differentiation, culture mediawere changed at 3 days to ODM (DMEM supplemented with 50 μg/ml ascorbic acid, 10^{-8} M dexamethasone, and 10 mM β-glycerolphosphate. After culture for another 3 days, one group was cultured only for ODM, while another groupwas cultured for ODM plus the purified peptide. Cells werethen analyzed 24 or 48 h later.

Cell viability was measured by using MTT as an indicator of cell viability. The principle of this determining based on mitochondrial-dependent reduction to formazan. The cells were grown in 96 well-plates and then treated with various concentrations of the enzyme hydrolysates. After 24 h incubation, cells were rewashed and 100 μl of MTT (1 mg/ml) was added and incubated for 4 h. Finally, 150 μl dimethyl sulphoxide (DMSO) was added to solubilize the formazan salt formed and amount of formazan salt was determined by measuring the OD at 540 nm using a microplate reader (PowerWave XS model; BioTek Instruments, Inc., Winooski, VT, USA).

F. Alkaline phosphatase (ALP) activity

For estimation of ALP activity, osteosarcoma cells and murine mesenchymal stem cells were grown to confluence in 24-well plates. The medium was replaced by DMEM or ODM respectively supplemented with the enzyme hydrolysates or the purified peptide, and the cells were incubated for 48 h. After the incubation, the cells were rinsed with PBS buffer, homogenized in 25 mM carbonate buffer (pH 10.3) containing 0.1% Triton X-100. Next the cellular activity was measured by incubating for 1 h at 37 ^oC in 250 mM carbonate buffer containing 1.5 mM $MgCl_2$ and 15 mM p-NPP. In the presence of ALP, p-NPP is transformed to para-nitrophenol and inorganic phosphate. The ALP activity of the samples was determined from the absorbance at 405 nm using a spectrophotometer.

G. Mineralization

The level of mineralization was determined by alizarin red-S staining using 6-well plates. Briefly,cell cultures were washed twice with PBS, fixed in ice-cold 70% (v/v) ethanol for 1 h, and then air-dried. The ethanol fixed cells and matrix were stained with 40 mM alizarin red-S (pH 4.2) for 1 h and extensively washed with water. The cells were subsequently destained for 15 min with 10% of cetylpyridium chloride in 10 mM of sodium phosphate buffer (pH 7.0). The optical density was measured at 562 nm using UV microplate reader (Tecan Austria GmbH, Grödig, Austria).

H. RT-PCR analysis

RNA was isolated with TRIzol reagent. Aliquots (2 μg) of total RNA were reverse transcribed to cDNA using AMV reverse transcriptase. The oligonucleotides used for PCR were showed in Table 6. PCR products were detected by 1.2% agarose gel electrophoresis and photographed.

I. Western blot analysis

Western blotting was performed according to standard procedures. Different antibodies (Santa Cruz Biotechnology Inc.) were used to detect the respective proteins with a chemiluminescent ECL assay kit (Amersham Pharmacia Biosciences), according to the manufacturer's instructions. Western blots were visualized using an LAS3000® luminescent image analyzer and protein expression was quantified by MULTI GAUGE V3.0 software (Fujifilm Life Science, Tokyo, Japan).

J. Statistical analyses

All data are presented as means ± S.D. The mean values were calculated based on the data taken from at least three independent experiments conducted on separate days using freshly prepared reagents. Statistical analyses were performed using Student's t-test.

Table 6. Sequence of gene specific primers

Target	Sequence	
ALP	Forward	5'-CCACGTCTTCACATTTGGTG-3'
	Reverse	5'-AGACTGCGCCTAGTAGTTGT- 3'
Osteocalcin	Forward	5'-ATGAGAGCCCTCACACTCCTC-3'
	Reverse	5'-GCCGTAGAAGCGCCGATAGGC-3'
Type I collagen	Forward	5'- CCAGATTGAGACCCTCCTCA -3'
	Reverse	5'- ATGCAATGCTGTTCTTGCAG -3'
GAPDH	Forward	5'-TTCACCACCACCATGGAGAAGGC-3'
	Reverse	5'- GGCATGGACTGTGGTCATGA-3'

III. RESULTS

A. Preparation of protein hydrolysates from biodiesel by-products of microalgae *Nannochloropsis oculata* and cell viability properties

Biodiesel by-product (BD) protein was hydrolyzed by alcalse, flavourzyme, neutrase, PTN, and protamex (abbreviation as BD-A, BD-F, BD-N, BD-PT, and BD-Pr, respectively) for the extraction of osteoblast activating peptides (Table 5). To investigate the effect of the hydrolysates on viability of human osteoblastic (MG-63) and murine mesenchymal stem (D1) cells, we used the MTT assay. Treatment of either cell line with hydrolysates (10–1000 μg/ml) for 24 h did not affect the cell viability (Fig. 7), so hydrolysate concentrations of 20–500 μg/ml were used to examine their effects on osteoblastic and mesenchymal stem cells differentiation.

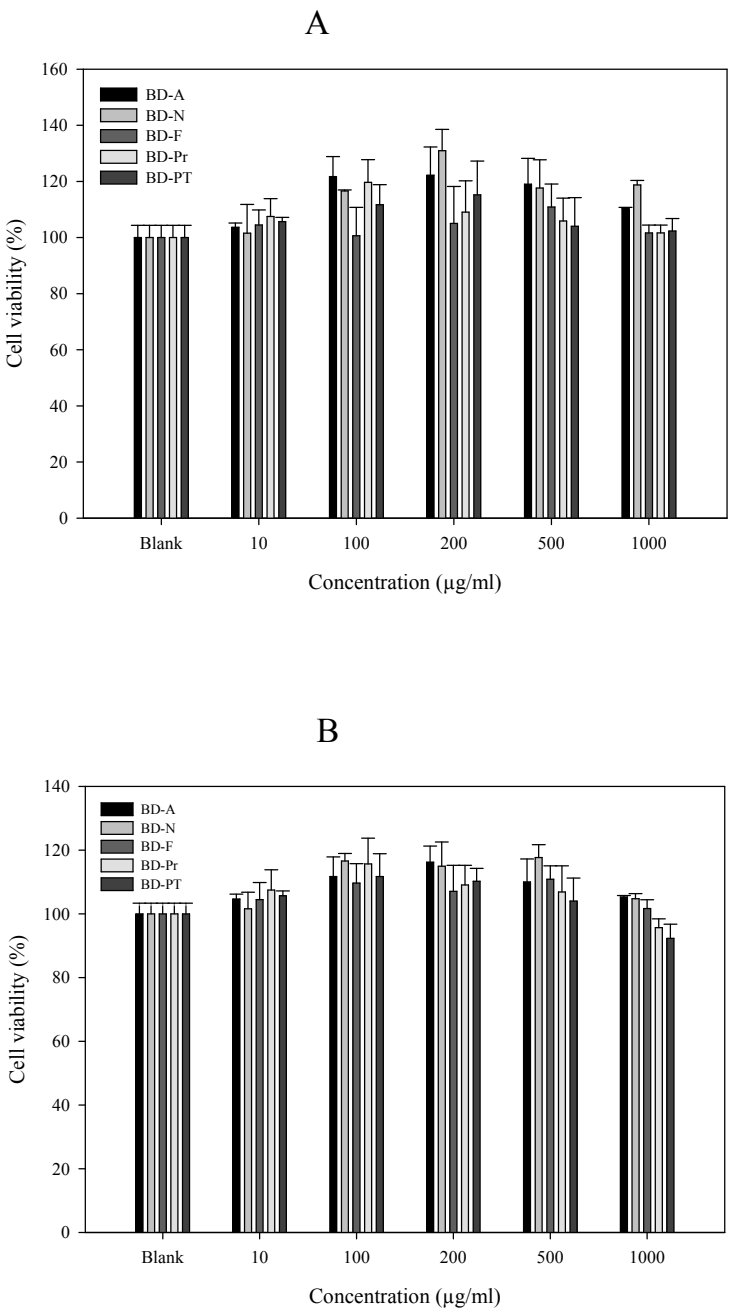


Fig. 7. Effects of the different hydrolysates on cell viability of MG-63 (A) and D1 (B) cells. Viable cells were detected by MTT assay. Each value is the average of triplicate cultures, and each bar indicates means ± S.D.

B. Effect of hydrolysates on alkaline phosphatase (ALP) activity in MG-63 and D1 cells

Alkaline phosphatase (ALP) activity initiates at early stages of osteoblast differentiation and continues to increase until the mineralization phase, when it is decreased (Stein et al., 1990; Beck et al., 1998; Candeliere et al., 1999). ALP activity in the specific cell line will be increased in the presence of bioactive substance that has positive effects on the osteoblastic differentiation process. Thus, the effect of hydrolysates on the process was studied by determining ALP activity in MG-63 and D1 cells. As shown in Figure 8, BD-A has expression highest ALP activity compared with those of other hydrolysates in both of human osteosarcoma and murine mesenchymal stem cells. In addition, alkaline phosphatase activity was increased in dose-dependent manner in the presence of alcalase hydrolysate of biodiesel by-product (BD-A) in MG-63 cells, but it has reached peak at concentration 20 μg/ml in D1 cells. Therefore, BD-A hydrolysate was selected for further study.

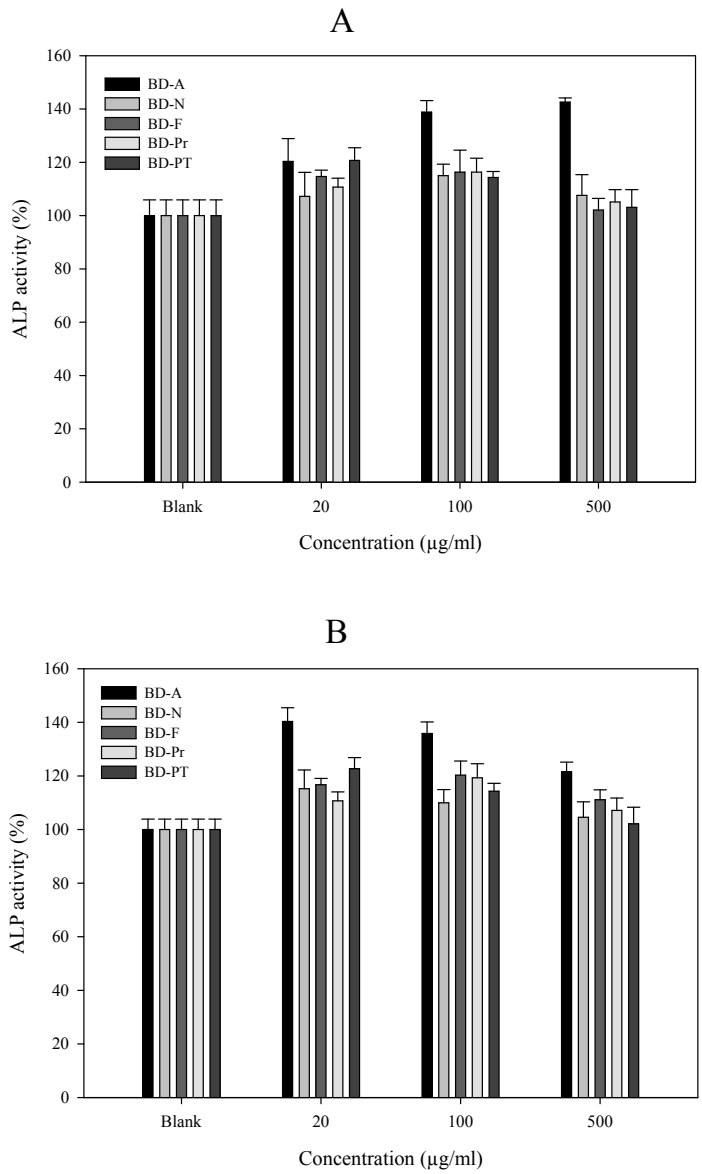


Fig. 8. Effects of hydrolysates on alkaline phosphatase (ALP) activity in MG-63 (A) and D1 (B) cells. The cells were treated with various concentrations of those hydrolysates (20 – 500 μg/ml) for 48 h. Alkaline phosphatase activity was assessed by the conversion of p-nitrophenyl phosphate in 0.1 M $NaHCO_3 – Na_2CO_3$ buffer, pH 10, containing 1.5 mM $MgCl_2$ and 0.1% Triton. Each value is the average of triplicate cultures, and each bar indicates means ± S.D.

C. Purification profiles of peptides from biodiesel by-products of microalgae, *Nannochloropsis oculata*

The lyophilized BD-A protein was dissolved in 20 mM phosphate buffer (pH 6.8), and loaded onto a jaigel W253 column. Elution peaks were monitored at 215 nm (Fig. 9). Each fraction was pooled, lyophilized, and its ALP activity was measured. Fraction II exhibited the highest osteoblast differentiation to increase ALP activity in both of MG-63 and D1 cells compared with other fractions (Fig. 10). The lyophilized active fraction II was further separated by RP-HPLC on YMC pack pro C18 column with a linear gradient of acetonitrile (0–60%), and a clear fraction was collected (Fig. 11). Finally, one peptide having potent activities potencies was purified. The amino acid sequence of *Nannochloropsis oculata* peptide (NOP) was determined to be MPDW (MW 601 Da). The NOP determined by ESI/MS spectroscopy was in excellent agreement with the theoretical mass calculated from the sequence (Fig. 12).

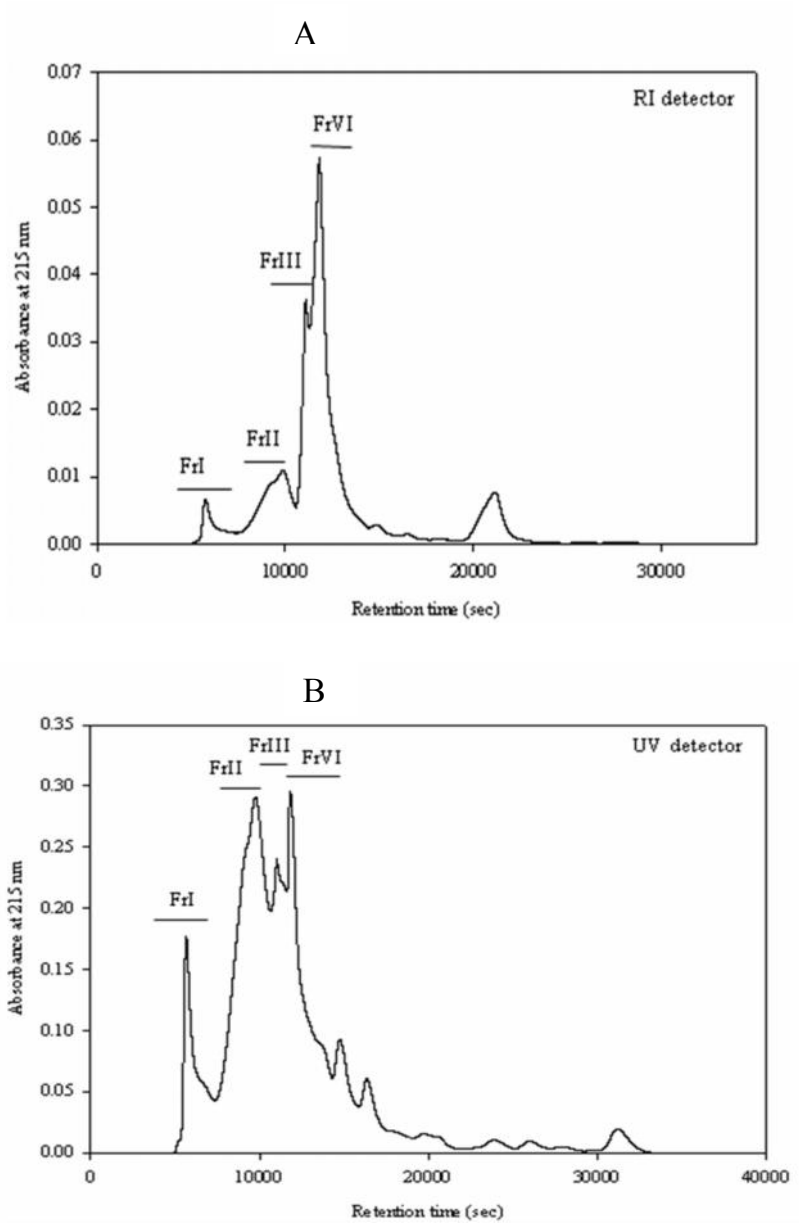


Fig. 9. Separation of activity peptide from alcalase hydrolysates of biodiesel by-products of microlagae *Nannochloropsis oculata* by recycling preparative HPLC, Jaigel W253 column with refractive index (RI) detector (A), and UV detector (B). Elutions was performed at 2.5 ml/min of flow rate.

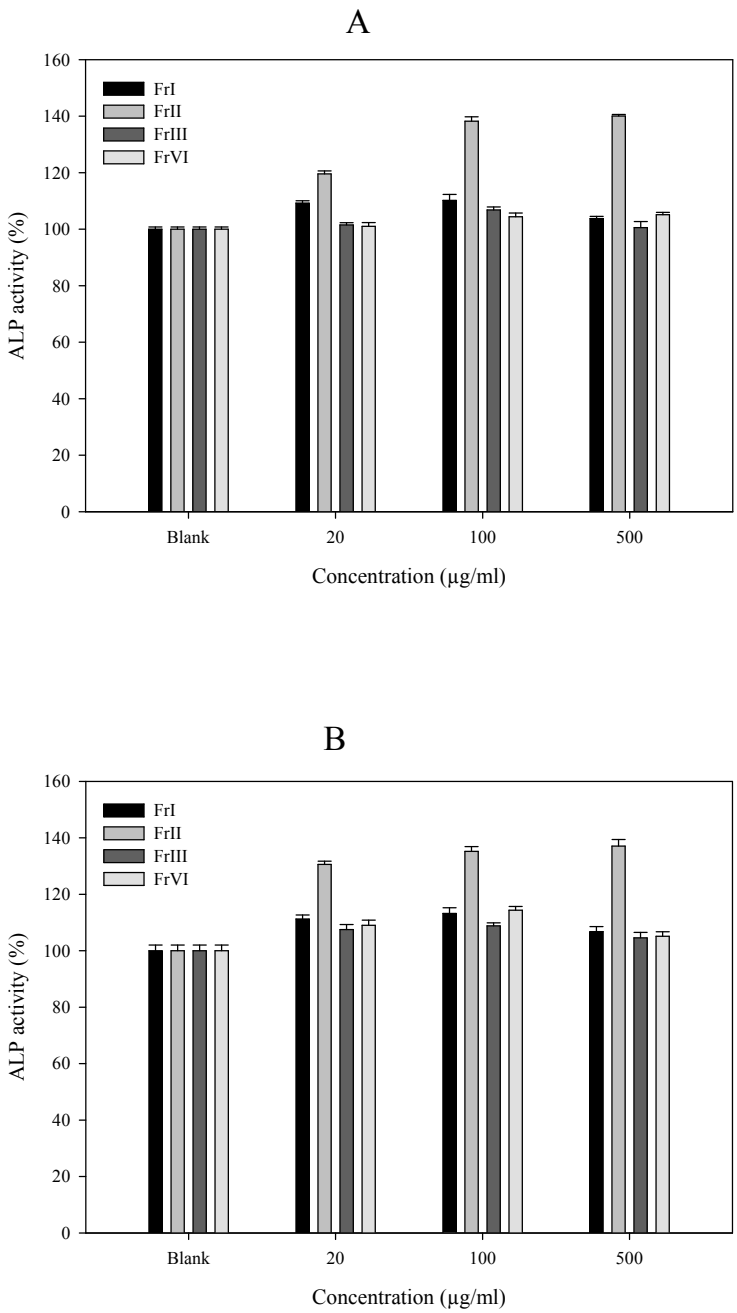


Fig. 10. Effect of separated fractions on ALP activity in MG-63 (A) and D1 (B) cells. Each value is the average of triplicate cultures, and each bar indicates means ± S.D.

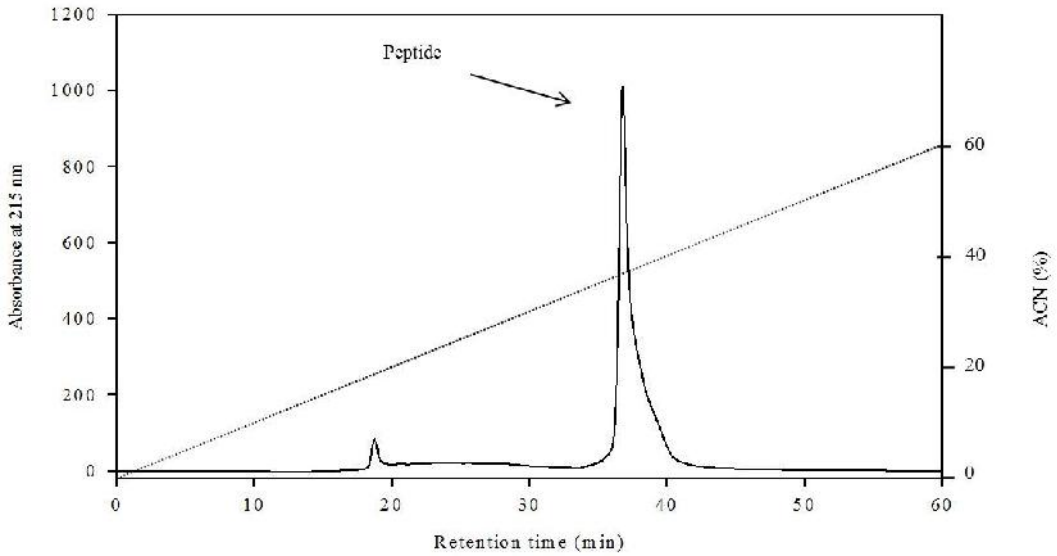


Fig. 11. Reversed-phase HPLC pattern on YMC pack pro C18 column of active fraction from Recycling preparative HPLC fraction FrII. HPLC operation was carried out with 60% acetonitrite as mobile phase at 1 ml/min of a flow rate using UV detector at 215 nm

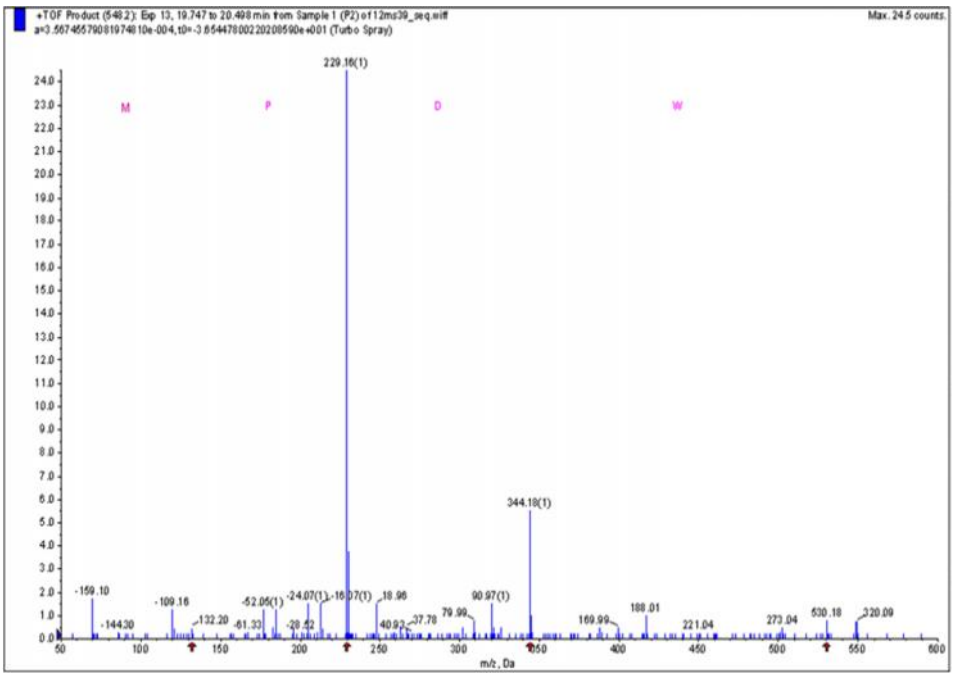
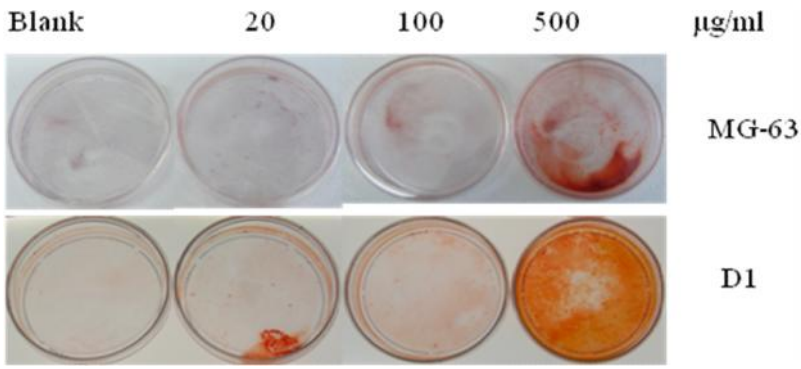


Fig. 12. Identification of molecular mass and amino acid sequence of peptide. MS/MS experiments were performed on a Q-TOF tandem mass spectrometer (Micromass Co., Manchester, UK) equipped with a nano-ESI source. Sequencing of active peptide was acquired over the *m/z* range 50-2,500 and sequenced by using the PepSeq de novo sequencing algorithm.

D. Effect of NOP on mineralization in MG-63 and D1 cell

ALP known as a marker for early stage of osteoblast differentiation, bone mineralization has to be known as terminal stage. Bone mineral is formed by small imperfect hydroxyapatite crystals, which contain carbonate, magnesium, sodium, and potassium. Mineralization occurs by two distinct mechanisms. The initial mineralization of calcified cartilage and woven bone probably occurs by means of matrix vesicles. These membrane-bound bodies are released from chondrocytes and osteoblasts, contain alkaline phosphatase, and can form a nidus for crystallization. Mineralization requires calcium, phosphate, and alkaline phosphatase. The alkaline phosphatase probably functions to hydrolyze local inhibitors of mineralization, such as pyrophosphate. The mineralization process is impaired in vitamin D deficiency, with its low calcium-phosphate product and hypophosphatasia, a disorder of mutated alkaline phosphatase genes (Lorenzo et al., 2011).

In the present study, bone mineralization, process by which organic tissue becomes hardened by the physiologic deposit of calcium salts, was quantification by optical density measurement of alizarin red-S extracted from stained culture cells in the presence of NOP. Figure 13 illustrated that both of MG-63 and D1 cells showed clearly red color depends on increased concentrations (20-500 μg/ml) of NOP.



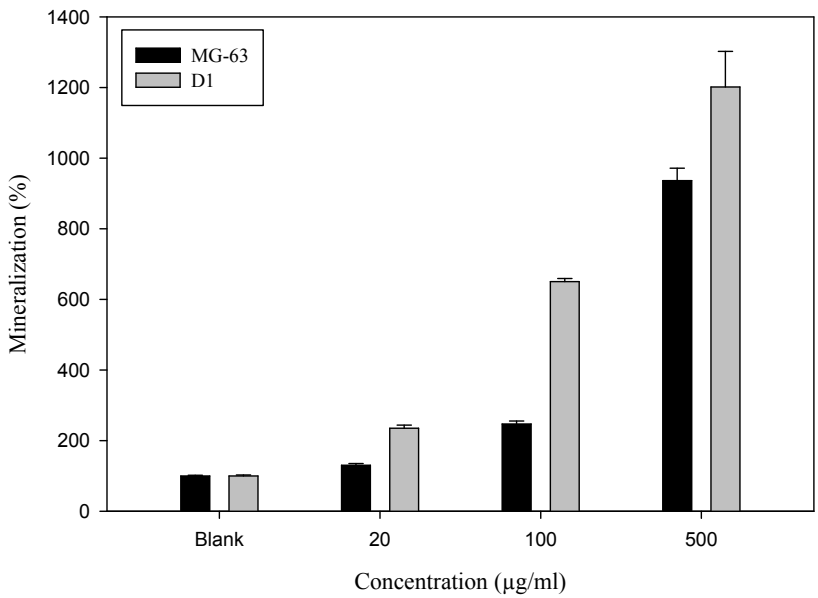


Fig. 13. Effects of NOP on mineralization of MG-63 and D1 cells. The degree of mineralization was assayed by Alizarin Red S staining. Each value is the average of triplicate cultures, and each bar indicates means ± S.D.

E. Effect of NOP on expression of several specific genes in MG-63 and D1 cell

Some of the most frequently used markers of osteoblast differentiation process are alkaline phosphatase (ALP), collagen type I, and osteocalcin. Type I collagen is the most abundant protein of the bone matrix, and it is a rigid, rodlike, insoluble molecule composed of two α1 chains and one α2 chain. Collagenase plays a critical function in bone remodeling. The synthesis of collagenase by osteoblasts is regulated by hormones and by cytokines present in the bone microenvironment acting by transcriptional and posttranscriptional mechanisms (Lorenzo et al., 2011). Osteocalcin and alkaline phosphatase (ALP) have to be known as terminal and early marker of osteoblast differentiation process, respectively. Thus, we examined gene expression of these markers in MG-63 and D1 cells. The result shown that NOP resulted in increase of mRNA expression of all these genes in both of MG-63 and D1 cells (Fig. 14).

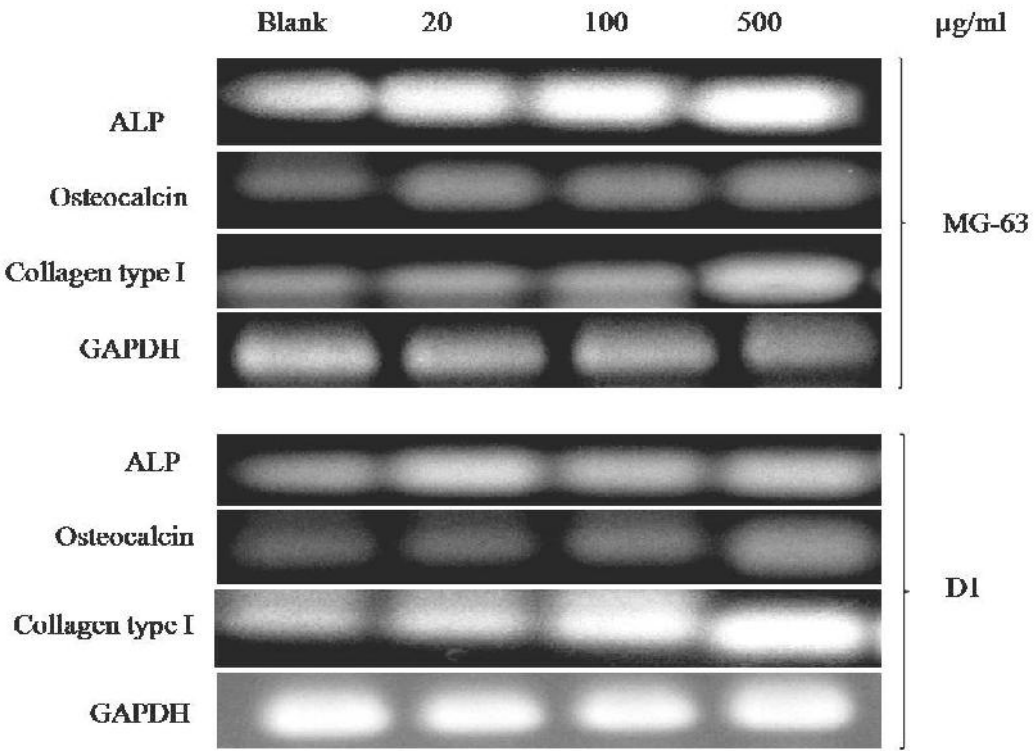


Fig. 14. Effects of NOP on mRNA expression of several specific markers in osteoblast differentiation process in MG-63 (A) and D1 (B) cells. Cells were treated with different concentrations (20-500 μg/ml) of peptide. Total RNA was collected and specific ALP, osteocalcin, and collagen type I primers used to polymerize the reverse transcribed cDNA and visualized by ethidium bromide staining followed by electrophoresis separation. GAPDH mRNA expression levels were used to confirm the equal amounts of RNA used for cDNA synthesis.

F. Effect of NOP on MAPK and Smad pathway

The common mechanism of osteoblast activating is seemly mediated by serine-threonine kinases of the mitogen-activated protein kinase (MAPK) and Smad family. Binding of BMPs to preformed heteromeric receptor complexes, two major types of membrane-bound serine/threonine kinase receptors subsequently known as the type-I and type-II receptors, results in activation of MAPKs and Smad pathway. Therefore, investigation the effects of NOP on expression of Smad1/5/8 and three different MAPKs such as ERK, JNK, p38 was performed. The result showed that phosphorylation of ERK, JNK, and p38 expression are significantly increased in the presence of peptide in both MG-63 and D1 cells (Fig. 15). The expression of the phosphorylation of these kinases was increased in dose-dependent manner in MG-63 cells (Fig. 15A). However, they were reached peak at concentration 20 μg/ml and then decreased but still higher than blank (untreated) in D1 cells (Fig. 15B). In addition, the presence NOP was resulted in significantly increase the expression of phosphorylation of Smad1/5/8 in both of MG-63 and D1 cells (Fig. 16). Hence, it could be suggested that the activation of osteoblastic differentiation was resulted from MAPK and Smad mediated up-regulation.

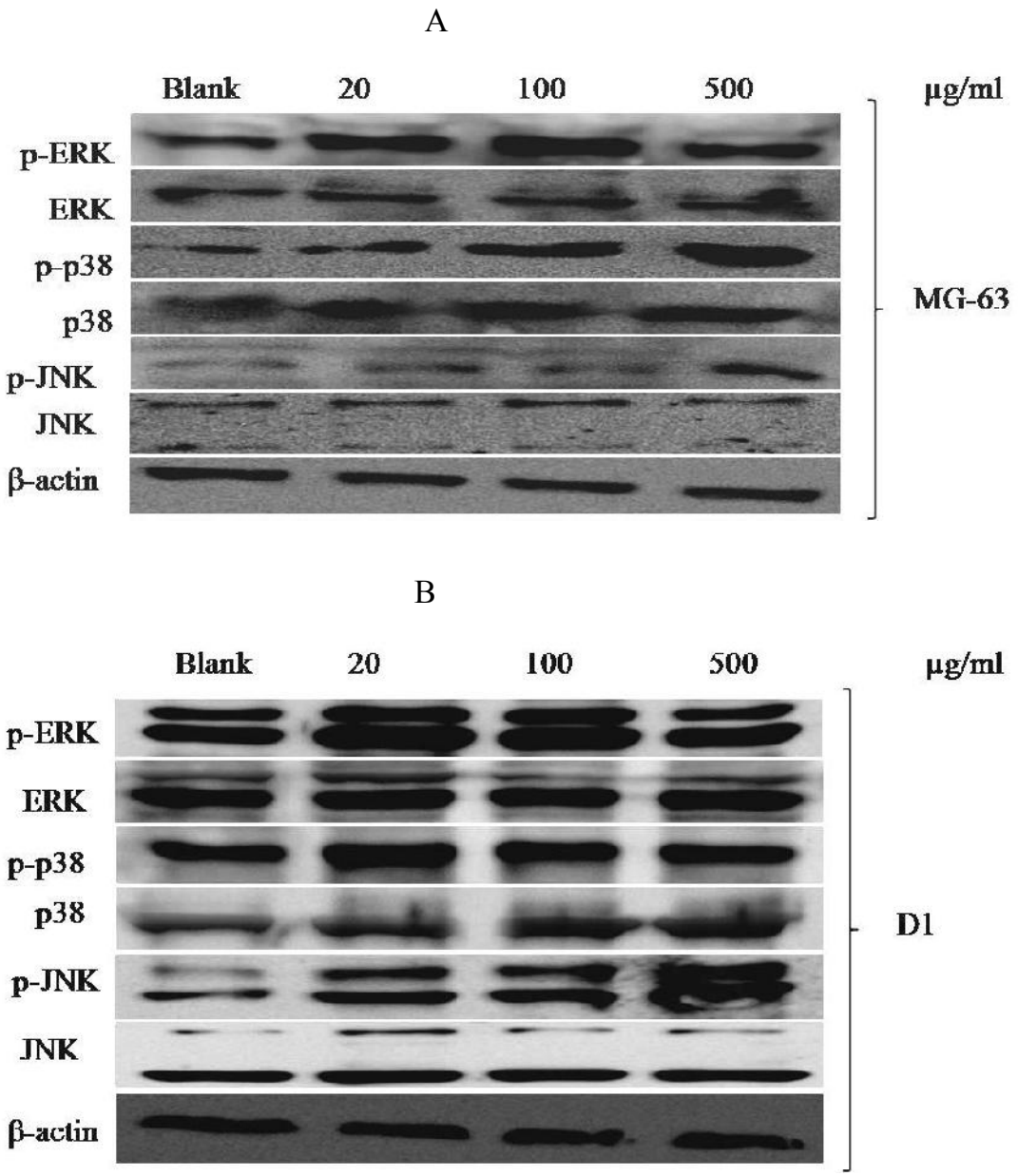


Fig. 15. Effects of NOP on MAPK pathway in MG-63 (A) and D1 (B) cells. Equal amounts of protein in the cell lysates were electrophorased and levels of p-ERK, ERK, p-JNK, JNK, p-p38, and p38 protein expressions were determined using specific antibodies for them. β-actin was used as control.

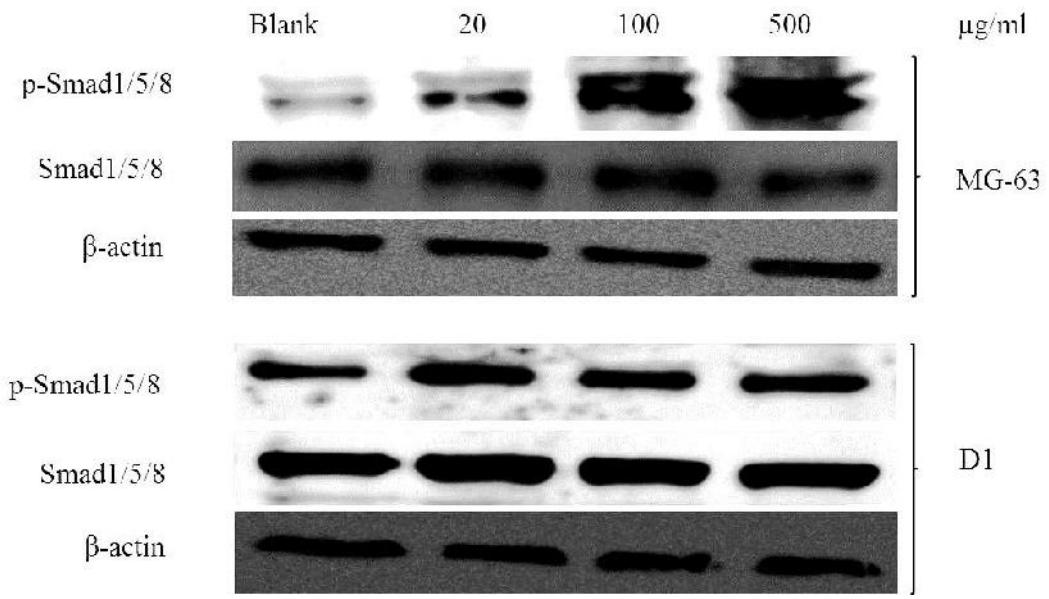


Fig. 16. Effects of NOP on Smad pathway in MG-63 and D1 cells. Equal amounts of protein in the cell lysates were electrophorased ans levels of p-Smad1/5/8, and Smad1/5/8 protein expressions were determined using specific antibodies for Smad1/5/8, and Smad1/5/8, respectively. β-actin was used as control.

IV. DISCUSSION

Protein can be broken down to their constituent amino acids by a variety of methods. Enzymatic hydrolysis is widely utilized to meliorate their functional and nutritional properties (Calderón et al., 2000; Moure et al., 2005). Bioactive peptides can be obtained from organisms proteins by enzymatic hydrolysis of proteins (Je et al., 2005). In our study, biodiesel by-products of microalgae *Nannochloropsis oculata* were hydrolyzed with various enzymes (alcalse, flavourzyme, neutrase, PTN, and protamex) to extract osteoblast activating peptide. Among these enzyme hydrolysates, alcalase hydrolysate expressed the highest ALP activity and further used for purification. Alcalase has been used in the past for the production of anti-oxidant peptide (Park et al., 2006) and anti-hypertension peptide (Li et al., 2006; Lee et al., 2009). Alcalase have endo-peptidease characteristics. Bioactive peptides produced by Alcalase are resistant to digestive enzymes such as pepsin, trypsin and α-chymotrypsin, which would allow for absorption of peptides contained in these sort of hydrolysates. Moreover, Alcalase produce shorter peptide sequences as well as terminal amino acid sequences responsible for various bioactivities including Angiotensin I converting enzyme (ACE) inhibition (Saito et al., 1994; Lee et al., 2009).

Alcalse hydrolysate was subjected to purification using consecutive HPLC. Amino acid sequence was identified using MS/MS and shown to be Met – Pro – Asp – Tryp (601 Da). Methionine, one of the two sulfur-containing amino acids, has a non-polar thioether group in its side chain. Proline has an aliphatic side chain with a distinctive cyclic structure. The secondary amino group of proline residues is held in a rigid conformation that reduces the structural flexibility of polypeptide regions containing proline. Proline is known to be prevent digestion of enzymes and may pass from the capillary into the circulation of blood in the sequence of short peptides (Pan et al., 2005; Korhonen and Pihlanto, 2006). Aspartic acid is an acidic amino acid which has a second carboxyl group. Tryptophan, with its aromatic side chains, is relatively nonpolar (hydrophobic). It can participate in hydrophobic interactions. Tryptophan is significantly more polar than phenylalanine because of the nitrogen of the tryptophan indole ring. The diverse biological activities of functional peptides depend on structural properties and molecular size of precursor proteins, amino acid compositions, or free amino acids (Elias et al., 2008). The sequence of purified peptide is a short peptide (oligopeptide) and has three per fourth hydrophobic amino acids.

Recent research has indicated that high levels of hydrophobic and aromatic amino acids may aid in healing after multiple trauma (Brennan et al., 1986). Oligopeptides are frequently synthesized, and their biological activity is assessed. Short peptides are likely to be extremely dynamic in solution. They were modeled on the cytoplasmic regions of transmembrane proteins and were identified from an evolutionary sequence analysis of specificity-determining and conserved residues (Parthasarathi et al., 2006).

Although marine organisms are enormous promising resource of bioactive substances and natural products, there are limited reports on them. Marine organisms represent a valuable source of new compounds. In this study, we found out marine the bioactive peptide from microalgae *Nannochloropsis oculata* that induces osteoblast differentiation. The discovery of the bioregulatory role of different endogenous peptides in the organism as well as the understanding of the molecular mechanisms of action of some new bioactive peptides obtained from natural sources on specific cellular targets, contributed to consider peptides also as promising lead drug candidates (Aneiros and Garateix, 2004). Functional peptides, can be induced from enzymatic hydrolysis of various proteins, may act as potential physiological modulators of metabolism during intestinal digestion of nutrients (Lee at al., 2009). These peptides exhibited various bioactivities such as antioxidative (Rajapakse et al., 2005), antihypertensive (Byun and Kim, 2001) and antimicrobial (Kim et al., 2001a,b). Bioactive peptides from sponges, ascidians, mollusks, sea anemones and seaweeds with their pharmacological properties and obtainment methods are reviewed by Aneiros and Garateix (Aneiros and Garateix, 2004). Thus marine organisms contain potential of natural products for industry or pharmacy and a great number of them are undiscover.

V. CONCLUSION

In our study, we sought to investigate the effects of purified peptide derived from microalgae *Nannochloropsis oculata* on osteoblast differentiation in human osteoblast (MG-63) and murine mesenchymal stem (D1) cells. NOP was purified and characterized by consecutive HPLC purification system and tandem mass analysis for the amino acid sequence. It showed that peptides purified from biodiesel by-products of marine algae, has to stimulated osteoblast differentiation at various stages in human osteoblastic and murine mesenchymal stem cells. The presence of NOP significantly induced osteoblastic cell differentiation in both of early and terminal stage by increase the levels of alkaline phosphatase, osteocalcin and bone mineralization in MG-63 and D1 cells. Furthermore, NOP increased phosphorylation of MAP kinases (p-ERK, p-JNK, p-p38) and Smads (p-Smad1/5/8) expression. It was simplified our study in Figure 17. From the results, the present study provides the proofs on the positive effects of NOP for the osteoblastic cell differentiation. To evaluate positively for its application in bone health supplement, its bioavailability will be exmanined by furthure *in vivo* studies.

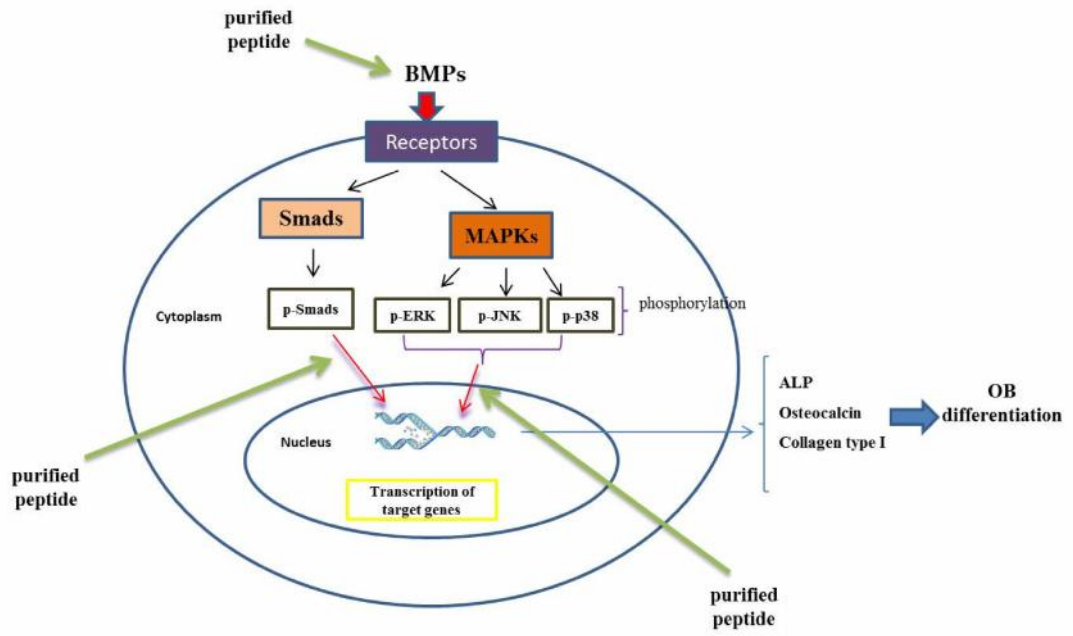


Fig. 17. Sumary of the effects of NOP on osteoblastic differentiation via BMPs-Smads and MAPKs pathway.

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논문제목	**한글:** 해양 미세조류 *Nannochloropsis oculata* 바이오디젤 산물로부터 분리된 신규 펩타이드의 MG-63 및 D1 조골세포분화 효능 **영문:**Osteoblastic differentiation effects of a novel peptide from biodiesel by-products of microalgae *Nannochloropsis oculata* on MG-63 and D1 cells				

본인이 저작한 위의 저작물에 대하여 다음과 같은 조건 아래 -조선대학교가 저작물을 이용할 수 있도록 허락하고 동의합니다.

- 다 음 -

1. 저작물의 DB 구축 및 인터넷을 포함한 정보통신망에의 공개를 위한 저작물의 복제, 기억장치에의 저장, 전송 등을 허락함

2. 위의 목적을 위하여 필요한 범위 내에서의 편집·형식상의 변경을 허락함.
다만, 저작물의 내용변경은 금지함.

3. 배포·전송된 저작물의 영리적 목적을 위한 복제, 저장, 전송 등은 금지함.

4. 저작물에 대한 이용기간은 5 년으로 하고, 기간종료 3 개월 이내에 별도의 의사표시가 없을 경우에는 저작물의 이용기간을 계속 연장함.

5. 해당 저작물의 저작권을 타인에게 양도하거나 또는 출판을 허락을 하였을 경우에는 1 개월 이내에 대학에 이를 통보함.

6. 조선대학교는 저작물의 이용허락 이후 해당 저작물로 인하여 발생하는 타인에 의한 권리 침해에 대하여 일체의 법적 책임을 지지 않음

7. 소속대학의 협정기관에 저작물의 제공 및 인터넷 등 정보통신망을 이용한 저작물의 전송·출력을 허락함.

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동의 여부: 동의 (O) 조건부 동의( ) 반대 ( )

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