Cellular properties of the fermented microalgae *Pavlova lutheri* and its isolated active peptide in osteoblastic differentiation of MG-63 cells

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Abstract. Fermented microalgae *Pavlova lutheri* (*P. lutheri*), the product of *Hansenula polymorpha* fermentation, exhibited an increase in alkaline phosphatase (ALP) activity in MG-63 osteoblastic cells when compared to that of non-fermented *P. lutheri*. Fractionation of the fermented *P. lutheri* resulted in identification of the active peptide [peptide of *P. lutheri* fermentation (PPLF)] with the sequence of EPQWFL. PPLF significantly increased ALP release from MG-63 cells and mineralization in a dose-dependent manner. In addition, the intracellular levels of ALP and osteocalcin (OCN) proteins were augmented by PPLF treatment. To identify the molecular mechanism underlying the effect of PPLF on osteoblastic differentiation, the phosphorylation levels of the mitogen-activated protein kinases, p38, extracellular signal-regulated kinases 1/2 and Jun, and nuclear factor (NF)-κB were determined following PPLF treatment and the differences in expression were analyzed using p38 and NF-κB selective inhibitors. These results concluded that PPLF from fermented *P. lutheri* induced osteoblastic differentiation by increasing ALP and OCN release in MG-63 cells via the p38/p65 signaling pathway, indicating that PPLF supplement may be effective for therapeutic application in the field of bone health.

Introduction

For a long time, microbial fermentation was applied in milk, soybean, fruit and grain as source of enzymes as a means to provide palatability, nutritional value, preservative and medicinal properties (1). Protein fermentation that mainly occurs through hydrolysis by digestive, microbial, and plant proteolytic enzymes can release the bioactive peptides corresponding to cryptic sequences from native proteins (2-4). Also, the degree of proteolysis depends on the microbial strain used for fermentation and is reported to be directly related to the biological activity of the released peptides (5).

Marine microalgae have been of interest as a biofuel source with their large biomass in the marine environment and recently reported in various studies in the field of pharmaceuticals and nutraceuticals as unconventional protein source (6). We have previously suggested that the fermented of *Pavlova lutheri* (*P. lutheri*; microalgae) by *Hansenula polymorpha* or *Candida rugopelliculosa* as protein source has potential antioxidant activity via reduction of hydroxyl radical or the increment of antioxidant related enzymes at protein levels in hydroxyl radical-induced oxidative stress (7).

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Bone structure is maintained by upholding a balance between bone formation by osteoblasts and bone resorption by osteoclasts (8-10). Osteoblasts originating from mesenchymal stem cells (MSCs) can translate mechanical signals into biological responses to regulate bone remodelling in intact bone in the process of bone repair (11). The mechanical signal in the cells involves the sequential activation (via phosphorylation cascade) of various intracellular signalling molecules, including MAPKs and phosphoinositide 3-kinase (PI3k)/Akt, resulting in the activation of transcription factors such as activator protein-1 (AP-1) and nuclear factor (NF)-κB and subsequent modulation of the expression of genes that regulate osteoblast maturation and mineralisation (12-17). The cellular responses in osteoblasts have influence on the interaction between various systemic factors, such as alkaline phosphatase (ALP), bone morphogenetic proteins (BMPs), osteocalcin (OCN), collagen type I (Col I) that are generally considered stimulants for osteoblast adhesion and differentiation (9,18).

In the present study, the effect of the *P. lutheri* fermentation on osteoblastic differentiation in MG-63 osteoblastic cells was compared with the non-ferment. The active peptide [peptide of *P. lutheri* fermentation (PPLF)] responsible for the effect was subsequently purified and the mechanism by which PPLF is involved in the regulation of osteoblastic differentiation of MG-63 cells was explored.

Materials and methods

General. In the previous study, we fermented microalgae *Pavlova lutheri* by *H. polymorpha* as below (7). The cellulose-degraded microalga *P. lutheri* was autoclaved at 121˚C for 30 min in a buffer (pH 7) solution at a ratio of 1:15 (w/v). Subsequently, *H. polymorpha* was inoculated with the autoclaved solution at a concentration of 1% (v/v) and incubated at 37˚C for 12 days. Fermented microalgae was lyophilized and stored at 80˚C until used. Human osteoblastic (MG-63) cells were obtained from American Type Culture Collection (Manassas, VA, USA). Dulbecco's Modified Eagle's Medium (DMEM), Trypsin-ethylenediaminetetraacetic acid (EDTA), penicillin/streptomycin, fetal bovine serum (FBS) and other materials required for culturing cells were purchased from Gibco BRL, Life Technologies (Grand Island, NY, USA). The p38 inhibitor (SB203580), NF-κB inhibitor [pyrrolidine dithiocarbamate (PDTC)] and 3-(4,5-dimethyl-2-yl)-2,5-diphenyltetrazolium bromide (MTT) reagent were purchased from Sigma-Aldrich (St. Louis, MO, USA). All other chemicals and reagents used in this study were of analytical grade.

Cell culture and viability assay. Human osteoblastic MG-63 cells were cultured in DMEM containing 5% FBS in a humidified atmosphere with 5% CO_2 at 37˚C. Cells were sub-cultured at 3-day intervals using trypsin-EDTA and were seeded in 96-well plates at a density of $5x10^3$ cells per well. 24 h post plating, media was replaced with DMEM without serum and cells were subjected to sample treatment for another 3 days. Subsequently, 100 μl of 1 mg/ml MTT reagent was added to each well, and incubated for 3 h. The supernatant was removed and formazan was dissolved in DMSO and its formation was observed by monitoring the signal at 540 nm using a microplate reader.

ALP activity assay. Cells were seeded into 12-well plates at a density of $5x10^5$ cells per well for 24 h and then treated with samples in DMEM media without serum for 3 days. Cells were then washed three times with PBS, and were lysed with a lysis buffer containing 20 mM Tris-HCl (pH 7.5), 150 mM NaCl and 1% Triton X-100. Briefly, the lysate was mixed with p-NPP substrate solution which consists of 1.5 mM $MgCl_2$ and 15 mM p-nitrophenyl phosphate (p-NPP). The activity of phosphatases to catalyze the hydrolysis of p-NPP to p-nitrophenol was evaluated by measuring the absorbance at 405 nm (19). ALP activity was normalized according to the cellular total protein content of cell lysate determined by BCA protein assay (Sigma-Aldrich).

Preparation of fermented microalgae peptide. The lyophilized fermented microalgae was dissolved in distilled water and passed through disposable Silica-based bonded-phase cartridges (Sep-Pak Vac C_{18} 20 cc/5 g; Waters, Milford, MA, USA). The cartridges were activated with 100% methanol in 0.1% trifluoroacetic acid (TFA) and subsequently the cleaned product was eluted with 10% methanol in 0.1% TFA. The eluent was lyophilized then further purified as described below.

Purification procedures. The cleaned eluent from previous step was loaded onto HiPrep 16/10 DEAE ion exchange column equilibrated with 20 mM sodium acetate buffer (pH 4.0) and eluted with a linear gradient of NaCl (0-2 M) at a flow rate of 1.0 ml/min by 280 nm absorbance using FPLC (Amersham Bioscience Co., Uppsala, Sweden). Separated fractions were pooled and desalted by an electrodialyzer (Micro Acilyzer model G3; Asahi Chemical Industry Co., Tokyo, Japan) with a 100 Da molecular mass cutoff membrane (Asahi Chemical Industry Co.). The desalted fractions were lyophilized and investigated for their ALP activity. The fraction which had the highest ALP activity was further purified on a Primesphere 10 C_{18} (20x250 mm) column at reversed-phase HPLC (Dionex Corp., Sunnyvale, CA, USA) with a linear gradient of acetonitrile (0-30% in 30 min) containing 0.1% TFA at a flow rate 1.0 ml/min by 215 nm absorbance. Elution peaks were collected and tested for ALP activity. The highest active fraction was re-applied to a Acclaim 120 C_{18} analytical column with 15% acetonitrile (20% v/v, in 15 min) containing 0.1% TFA at flow rate of 1 ml/min. The purified peptide was subjected to amino acid sequence analysis.

Amino acid sequence of the purified peptide. The accurate molecular mass and amino acid sequence of the purified peptide was determined by quadrupole time-of-flight mass spectroscopy (Q-TOF MS; Micromass UK Ltd., Altrincham, UK) coupled to electrospray ionization (ESI) source. The molecular weight of the purified peptide dissolved in methanol/water (1:1, vol/vol) was detected by a doubly charged $(M+2H)^{+2}$ state in the mass spectrum and the amino acid sequence was identified by tandem MS analysis. Spray voltage was 4,000 V and nitrogen was maintained at 40 psi for nebulization. Mass spectra were acquired over the range from 50 to 1,500 m/z.

Mineralization assay. The levels of mineralization were determined in the 24-well plates using Alizarin Red staining
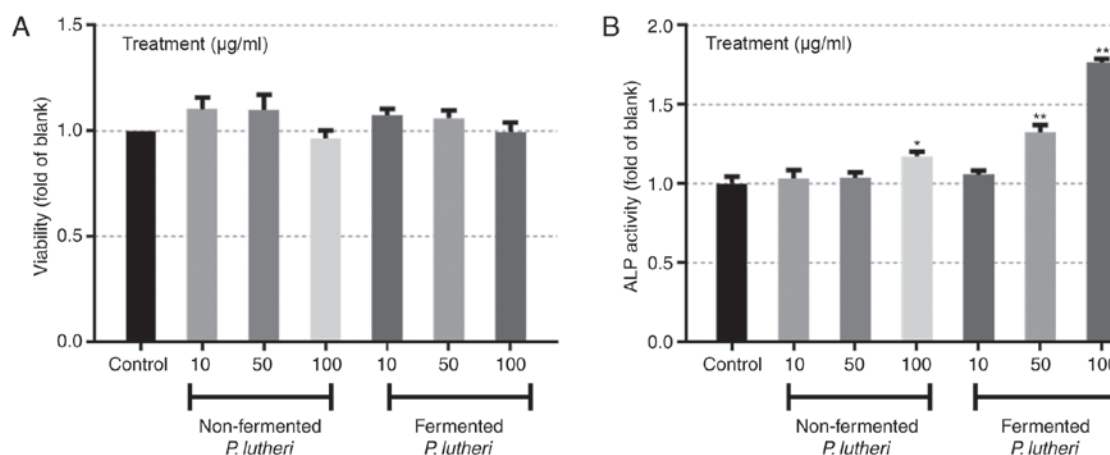
Figure 1. Effects of the non-fermented and fermented *P. lutheri* on (A) cell viability and (B) ALP activity in MG-63 cells. Cells were cultured with different concentrations of either fermented or non-fermented *P. lutheri* (10, 50 and 100 µg/ml) for 3 days and analyzed by MTT and ALP assays. Results were normalized to the control groups, which were incubated in Dulbecco's modified Eagle's media without treatment. *P<0.05 and **P<0.01 vs. control. *P. lutheri, Pavlova lutheri*; ALP, alkaline phosphatase; MTT, 3-(4,5-dimethyl-2-yl)-2,5-diphenyltetrazolium bromide.

(Sigma-Aldrich) after 7 days treatment with the peptide. Briefly, cells were fixed with 70% (v/v) ethanol for 1 h and then stained with 40 mM Alizarin Red S (pH 4.2) for 15 min at room temperature. After removing Alizarin Red S solution by aspiration, cells were incubated in PBS for 15 min at room temperature on an orbital rotator. Cells were then rinsed once with fresh PBS and subsequently distained for 15 min with 10% cetylpyridinium chloride in 10 mM sodium phosphate (pH 7.0). The extracted stain was then transferred to a 96-well plate and the absorbance at 562 nm was measured using a microplate reader (Tecan Austria GmbH, Grödje, Austria).

Western blot analysis. Cells were treated with p38 inhibitor (SB203580, 20 µM) or NF-κB inhibitor (PDTC, 10 µM) for 1 h prior to treatment with the peptide (in the presence of inhibitor) for 72 h. Cells were then harvested in lysis buffer (RIPA; Sigma-Aldrich). Cleared total cell lysates (20 µg) were separated by 10% SDS-polyacrylamide gel electrophoresis and transferred onto a polyvinylidene fluoride membrane (Amersham Pharmacia Biotech, Amersham, UK). Membrane was blocked with 5% skim milk and probed with the primary antibody (diluted 1:1,000) followed by incubation with a secondary antibody conjugated with horseradish peroxidase (diluted 1:5,000) at room temperature. The proteins of interest were detected using a chemiluminescent ECL assay kit (Amersham Pharmacia Biotech) according to the manufacturer's instructions. Primary and secondary antibodies including ALP (sc-373737), OCN (sc-365797), β-actin (sc-47778), p-p38 (sc-7973), p-pERK (sc-7383), p-Jun (sc-822), p-p65 (sc-166748) and anti-mouse IgG-HRP (7076) purchased from Santa Cruz Biotechnology Inc. (Santa Cruz, CA, USA) and Cell Signaling Technology, Inc. (Danvers, MA, USA). 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).

Statistical analysis. All experiments were repeated at least three times and expressed as the means ± SD. Differences between the means of individual groups were assessed by two-way ANOVA with Tukey's multiple comparisons test

using the statistical software package GraphPad Prism 6 software (San Diego, CA, USA).

Results

Comparison of the ALP activities of fermented and non-fermented P. lutheri. We previously investigated the antioxidant activities of the fermented *P. lutheri* microalgae by *H. polymorpha* or *C. rugopelliculosa* and suggested the potential of fermented *P. lutheri* as protein source possessing antioxidant activity (7). In this study, we investigated the difference between the effect of fermented and non-fermented *P. lutheri* on osteoblastic differentiation in MG-63 cells. The lyophilized *P. lutheri* was treated with cellulose (1% dry powder weight of the raw substance) to break its cell wall and was subsequently fermented by inoculation with *H. polymorpha* for 12 days (7). Different concentrations (10, 50, 100 µg/ml) of fermented and non-fermented *P. lutheri* (with degraded cell-wall) were added to the cells for 3 days followed by measuring their toxicity and ALP activity. The cells treated with either fermented or non-fermented *P. lutheri* did not show any significant difference in viability compared to control which is non-treatment group (Fig. 1A), however a significant increase in the ALP activity of the cells were observed when treated with 100 µg/ml non-fermented *P. lutheri* with P<0.05 and 50 and 100 µg/ml fermented *P. lutheri* with P<0.01 (Fig. 1B).

We then further traced the purification profile of peptide from fermented *P. lutheri* and characterized the effect on osteoblastic differentiation in MG-63 cells.

Fractionation profile of fermented P. lutheri and the effect on ALP activity. The lyophilized fermented *P. lutheri* was dissolved in distilled water and passed through disposable Sep-Pak Vac C$_{18}$ cartridges to salt out. The cleaned product was then concentrated and further separated using a weak anion exchange chromatographic method on a FPLC at 280 nm. The monitored peaks were desalted and analyzed for their ALP activity (data not shown). The selected fraction with highest ALP activity was split to four portions through reversed-phase chromatographic method on a HPLC at 215 nm (Fig. 2A). Each portion was
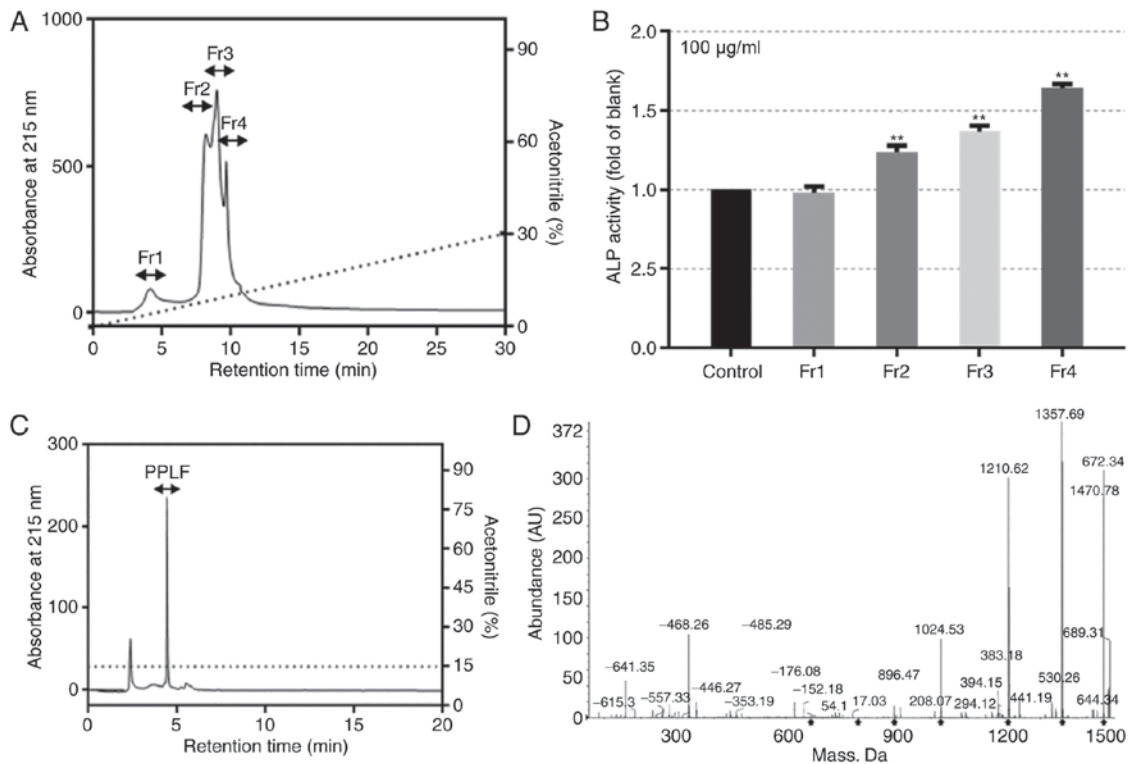
Figure 2. Fractionation of fermented *Pavlova lutheri* using chromatographic methods and isolation of an active peptide. (A) Fractions from the reversed-phase separation of the fermented product were incubated at a 100 µg/ml concentration with cells for 3 days and their (B) ALP activity was measured. ALP activity was normalized to the control group. **P<0.01 vs. control. Fraction 4 (Fr. 4) was selected based on its ALP activity, and (C) was reanalyzed and (D) identified as Glu-Pro-Gln-Trp-Phe-Leu (MW 908.9 Da) by electrospray ionization/mass spectroscopy. ALP, alkaline phosphatase; PPLF, peptide of *Pavlova lutheri* fermentation.
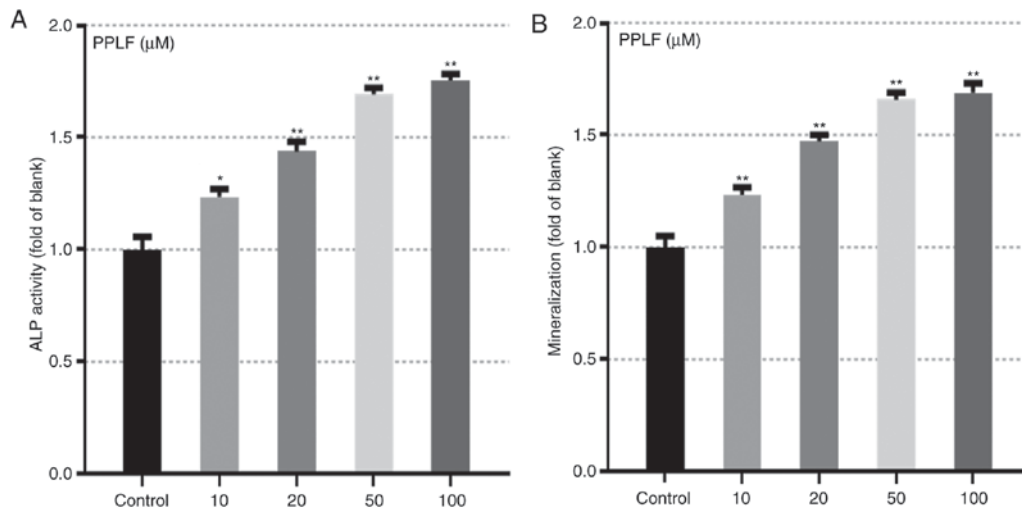

Figure 3. Effects of the purified peptide (PPLF) on (A) ALP activity and (B) mineralization in MG-63 cells. Cells were treated with different concentrations of PPLF (10, 20, 50 and 100 µM) and measured for their ALP activity at day 3 and mineralization at day 7. The results were normalized to the control groups. *P<0.05 and **P<0.01 vs. control. PPLF, peptide of *Pavlova lutheri* fermentation; ALP, alkaline phosphatase.

measured for its ALP activity at 100 µg/ml and Fraction 4 (Fr.4) showed the highest activity with of 1.642 fold compared to the control (P<0.01; Fig. 2B). The peptide PPLF was purified from the additional analysis using an Acclaim 120 C_{18} analytical column and was determined as Glu-Pro-Gln-Trp-Phe-Leu (MW 908.9 Da) by ESI/MS spectroscopy (Fig. 2C and D).

PPLF peptide prompted the markers for differentiation. The effect of PPLF on ALP activity and mineralization which

are main makers for osteoblastic differentiation (8,18) was assessed in MG-63 cells. Cells treated with PPLF showed significantly increased levels of ALP release (Fig. 3A) and mineralization (P<0.05 and P<0.01) (Fig. 3B). In order to further assess the effects of PPLF, we analyzed protein levels of two of the known early markers of differentiation ALP and OCN in cells treated with PPLF (10, 20, 50 µM) for 3 days. Both protein levels showed to be up-regulated by PPLF treatment compared to the non-treated cells (P<0.05; Fig. 4). These
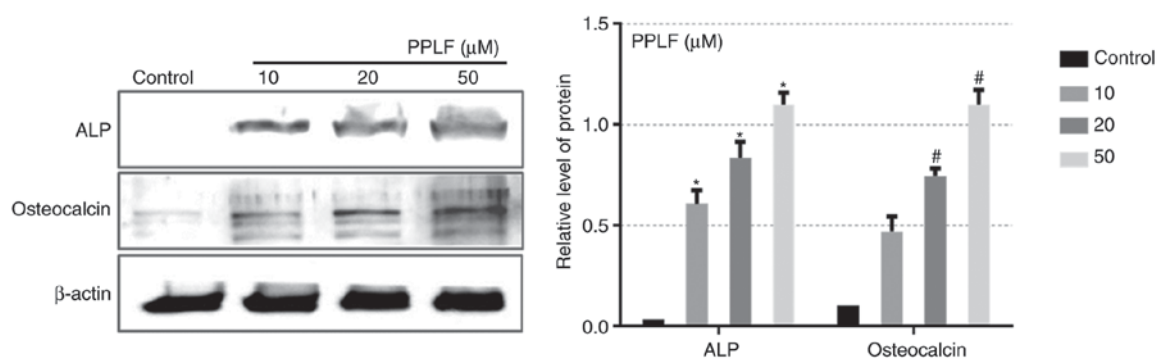
Figure 4. Effects of PPLF on the protein levels of ALP and osteocalcin in MG-63 cells. Cells were incubated with PPLF (10, 20 and 50 μM) for 3 days, then the protein levels of ALP and osteocalcin were assessed by western blotting. The results were then normalized to β-actin. *P<0.05 vs. ALP control; #P<0.05 vs. osteocalcin control. PPLF, peptide of *Pavlova lutheri* fermentation; ALP, alkaline phosphatase.
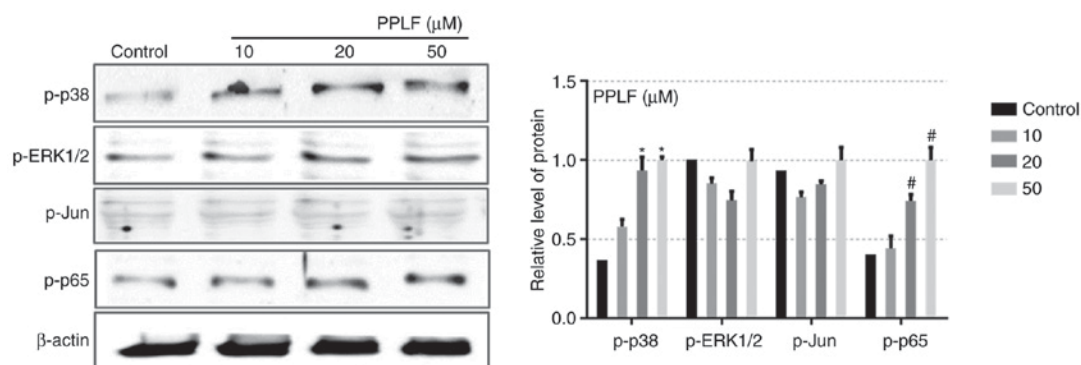

Figure 5. Phosphorylation levels of the three major types of mitogen-associated protein kinases, p38, ERK1/2 and Jun, and NF-κB (p65) following PPLF treatment in MG-63 cells. Cells were treated with PPLF (10, 20 and 50 μM) for 3 days and the levels of p-p38, p-ERK1/2, p-Jun and p-p65 were assessed by western blotting. The results were normalized to β-actin. *P<0.05 vs. p-p38 control; #P<0.05 vs. p-p65 control. PPLF, peptide of *Pavlova lutheri* fermentation; ALP, alkaline phosphatase; ERK, extracellular signal-regulated kinase; NF, nuclear factor; p-, phosphorylated.
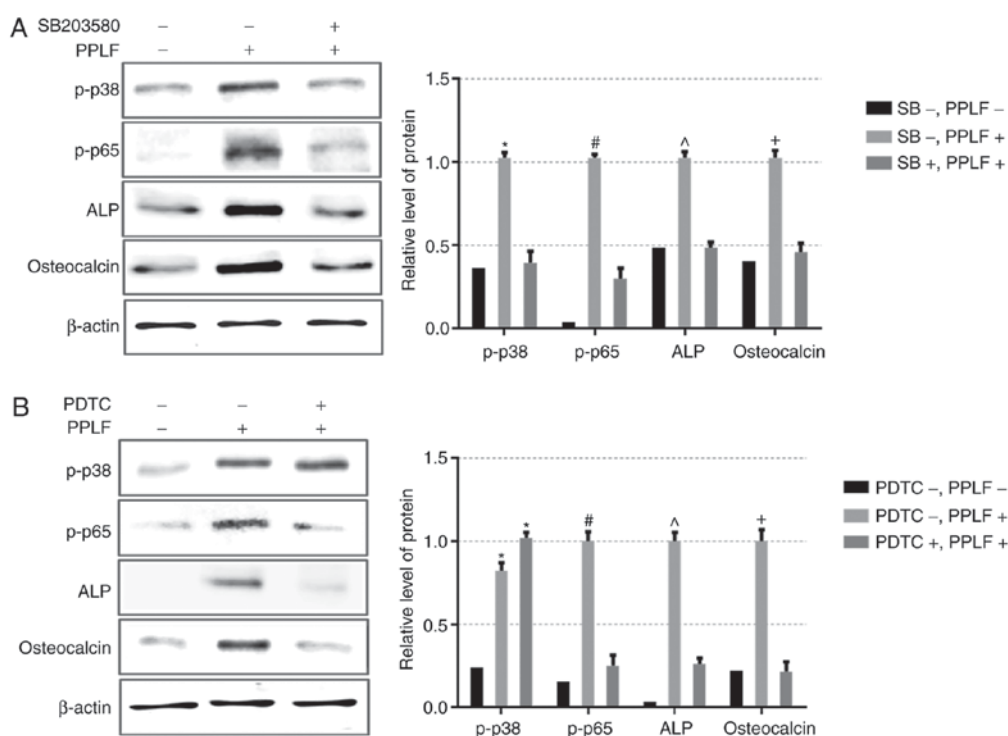

Figure 6. Effect of the selective inhibitors of p38, (A) SB203580, and p65, (B) PDTC, on osteoblastic differentiation. MG-63 cells were treated with PPLF (50 μM) and assessed for the levels of p-p38 and p-p65, ALP and osteocalcin with SB203580 (20 μM) or PDTC (10 μM). The results were normalized to β-actin. *P<0.05 vs. p-p38 control (PPLF-); #P<0.05 vs. p-p65 control. (PPLF-); ^P<0.05 vs. ALP control (PPLF-); +P<0.05 vs. osteocalcin control (PPLF-). PPLF, peptide of *Pavlova lutheri* fermentation; ALP, alkaline phosphatase; PDTC, pyrrolidine dithiocarbamate; p-, phosphorylated.

results suggest that PPLF induces the expression of the early markers of osteoblastic differentiation in MG-63 cells.

PPLF induces ALP and OCN expression via p38/p65 pathway. To determine the mechanism of the PPLF effect on differentiation in MG-63 cells, the phosphorylation levels of MAPK pathway (p38, ERK1/2, Jun) and NF-κB (p65) which are known to control ALP and OCN expression and are involved in osteoblastic differentiation were examined (8,10,18). As shown in Fig. 5, p-p38 and p-p65 exhibited a significant increment by PPLF at 20 and 50 μM (P<0.05), however p-ERK1/2 and p-Jun did not show any change with PPLF treatment. The result suggests that PPLF treatment activates p38/p65 signaling pathway. To examine whether the p38/p65 phosphorylation by PPLF treatment was connected to the expression of ALP and OCN, we assessed the expression of ALP and OCN in the cells treated PPLF (50 μM) when pre-treated with p38 inhibitor SB203580 (20 μM) or NF-κB inhibitor PDTC (10 μM). Pre-treatment with SB203580 inhibited both p38 and p65 phosphorylation levels and resulted in a decrease in the expression of ALP and OCN (Fig. 6A). We also observed the same result on ALP and OCN expression by PDTC treatment, however p38 phosphorylation showed no-significant change compared to PPLF treatment suggesting that p38 is upstream of p65 (Fig. 6B). These data suggest that treatment with PPLF induces ALP and OCN expression via p38/p65 signaling pathway in MG-63 cells.

Discussion

Microbial fermentation by proteolytic action can produce small molecule peptides from the parent proteins with various activities. These peptides have been reported to have role as part of dietary proteins in controlling and influencing health (3,20). In the present study, we have shown that the fermented of microalgae *P. lutheri* (by *H. polymorpha*) causes a significant elevation in differentiation at a concentration of 50 and 100 μg/ml in human osteoblastic MG-63 cells compared to the cells treated with non-fermented *P. lutheri* (Fig. 1B). Following this, we fractionalized the fermented *P. lutheri* using chromatography separation and measured the ALP activity of each separated fraction to purify the active responsible peptide (Fig. 2). Through ESI/MS spectroscopy, the purified peptide (PPLF) was sequenced as Glu-Pro-Gln-Trp-Phe-Leu (MW 908.9) which has rich-hydrophobic amino acids including one acidic residue (Glu), four basic residues (Pro, Trp, Phe, Leu), and one aromatic residue (Trp). High levels of hydrophobic and aromatic amino acids have been shown to rejuvenate cellular activity, cell differentiation and metabolism (21).

Subsequently, we showed that PPLF significantly promoted ALP release and mineralization concentration-dependently and further observed an increase in the protein levels of ALP and OCN by PPLF treatment (Figs. 3 and 4). ALP, OCN and mineralization are specific markers of differentiation that are known to be involved in differentiation of mature osteoblasts and induced by p38 MAPK and p65 (a subunit of NF-κB) (22). p38/p65 are widely conserved family of serine threonine protein kinases implicated in several cellular programs such as cell proliferation, calcification, and apoptosis (23). A number of studies have reported that p38 and p65 pathways are required

in osteoblast differentiation and act downstream of BMP receptors and play important roles in osteoblast differentiation and bone remodeling (16,24,25). Indeed, p38 pathway has been found to contribute to bone formation by phosphorylating the p65 subunit of NF-κB transcription complex and thus increasing NF-κB transcriptional activity (23). In this study, we showed that PPLF increased the activation of p38 and p65 whereas did not have any effect on the ERK1/2 and Jun phosphorylation (Fig. 5). Furthermore, we demonstrated that selective inhibitors of p38 and p65 significantly inhibited the expression of differentiation specific markers (ALP and OCN) indicating that PPLF induces osteoblastic differentiation in MG-63 cells by triggering activation of p38/p65 signaling pathway (Fig. 6).

Our data shows that fermented *P. lutheri* promotes differentiation of MG-63 cells and the peptide PPLF which was purified from the fermented *P. lutheri* is responsible for the increased differentiation in MG-63 cells through p38/p65 activation. Our work shows the potential of the fermented *P. lutheri* as an inducer of osteoblast differentiation and further suggests PPLF peptide as a likely candidate for the treatment of bone loss and promotion of bone health.

Acknowledgements

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