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ISOLATION OF ORGANIC MATRIX NACREOUS PROTEINS FROM *HALIOTIS DIVERSICOLOR* AND THEIR EFFECT ON *IN VITRO* OSTEOINDUCTION

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ABSTRACT

The organic matrix proteins of molluscan nacre are known to engage biocompatible and osteoinductive properties. In this study, we aimed to extract *Haliotis diversicolor* nacreous proteins and assayed their effect on pre-osteoblastic cell differentiation. We found that acid extracted nacreous proteins (AEP) consisted primarily of a major protein band of 25 kDa and three other minor proteins. AEP enhanced alkaline phosphatase (ALP) activity of MC3T3-E1 cells both in time- and concentration-dependent manners. Transcriptional up-regulation of osteogenic markers, including collagen type I (COL-I), osteopontin (OPN) and osteocalcin (OCN), was also apparent in days 7 and 14 upon AEP treatments. At the translational level, higher protein expression of COL-I was evident in AEP treated cells, and the protein was presumably laid down as extracellular matrix. Further *de novo* sequencing of an AEP major protein revealed a match with the abalone mantle protein sometsuke. Conclusively, we demonstrated that *H. diversicolor* AEP contains a factor, potentially the mantle protein sometsuke, which may impart in the osteoblastic cell differentiation.

Key words: *Haliotis diversicolor*, nacre, bone, cell differentiation, osteogenesis.

INTRODUCTION

Bone is a mineralized connective tissue containing osteocytes, extracellular matrix and deposited minerals. In order to maintain bone integrity, a fine balance in bone formation and remodelling is required (Freemont, 1993; Hill, 1998; Katagiri & Takahashi, 2002; Proff & Romer, 2009; Raggatt & Patridge, 2010). Nevertheless, the activation of bone formation by osteoblasts has been viewed as the primary regimen to improve bone mass and bone architecture during bone repair (Horton, 2003; Caetano-Lopes et al., 2007; Lee et al., 2008; Suomi et al., 2008). Therefore, much research has been focused on controlling osteoblast differentiation using naturally extracted biomaterials to replace many known chemical stimulants, such as ascorbic acid and β -glycerophosphate (Rousseau et al., 2003, 2008). Osteoblast differentiation is under the control of central (hormonal and neuronal) and local (growth factors and cytokines) signalling (Komori et

al., 1997; Komori, 2000; Canalis et al., 2003; Zamurovic et al., 2004; Caetano-Lopes et al., 2007; Grabowski, 2009). In particular, the TGF- β superfamily plays many crucial roles in cell proliferation, differentiation, apoptosis, adhesion, migration and extracellular matrix protein production. The most potent local factors that belong to the TGF- β superfamily, bone morphogenetic proteins (BMPs), are also known to regulate osteoblast differentiation. BMPs bind to type I and II BMP receptors, which are transmembrane serine/threonine kinases and further phosphorylate Smad 1, 5 and 8. As a consequence, the binding complex translocates into the nucleus together with Smad 4 and regulates transcription of the targeted genes (Yamaguchi et al., 2000; Canalis et al., 2003; Chen et al., 2004; Kahai et al., 2004; Zamurovic et al., 2004; Suomi et al., 2008; Proff & Romer, 2009).

Recently, many experimental studies in osteoblast differentiation have relied on *in vitro* testing of the cultured cells, with one well-established

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in vitro model being a mouse calvarial cell line, MC3T3-E1. In response to the known inducers, these cells demonstrate an increase in alkaline phosphatase activity and up-regulation of collagenous and non-collagenous gene transcription and translation (Yamaguchi et al., 2000; Almeida et al., 2001; Kahai et al., 2004; Zamurovic et al., 2004; Carinci et al., 2005).

Nacre, or mother-of-pearl, is a natural biomaterial consisting of organic matrix proteins embedded in aragonite tablets. The organic matrix has been classified into soluble and insoluble matrices depending on their ability to solubilise in water (Marin & Luquet, 2004; Rahman et al., 2006). The soluble matrix controls aragonite crystal carbonate formation and deposition (Feng et al., 2000; Thompson et al., 2000; Zhang et al., 2006; Gries et al., 2011). In molluscs, bio-mineral complexes are essential for exoskeleton formation, the composition of which is comparable to that of vertebrates where the biominerals are crucial materials of endoskeleton (Wilt et al., 2003; Omelon et al., 2009; Lefebvre & Bhattaram, 2010). The significance of biomaterials is not only fundamental for skeletal biosynthesis, but their stimulatory and self-repairing capacity is also recognized as one of their bioactive properties. In the past decade, strong evidence from numerous shell studies has indicated that the soluble fraction of organic matrix extracts contain crucial factors to induce osteogenesis, both *in vitro* and *in vivo* (Lopez et al., 1992; Atlan et al., 1997, 1999; Lamghari et al., 1999; Almeida et al., 2001; Moutahir-Belqasmi et al., 2001; Rousseau et al., 2008). It is believed that diffusible substances from a piece of nacre inserted into a site of bone fracture can stimulate bone repair without showing any inflammatory side effect, providing excellent evidence for *in vivo* osteogenic activity of nacre (Atlan et al., 1999; Lamghari et al., 1999). Apart from the osteogenic property, some small extracted nacreous proteins inhibit cathepsin K, a protease that is secreted by osteoclasts during bone resorption (Duplat et al., 2007a, b).

In this study, we postulated that an osteoblast differentiation factor may be present in an acid-extracted nacreous protein (AEP) from abalone shell that plays a role in inducing a downstream cascade of gene regulation involved in osteoblast differentiation. Using the MC3T3-E1 cell line as an *in vitro* model, we found that AEP provoked an increased alkaline phosphatase activity and transcriptionally up-regulated many osteoblast markers, and enhanced secretion of

a ground-matrix protein for mineral deposition. The major protein isolated from AEP was a sometsuke-like protein, which may be responsible for induction of osteogenesis. This study fundamentally enhances our understanding of an invaluable biomaterial with high potential for future therapeutic applications.

MATERIALS AND METHODS

Extractions of Nacreous Proteins and Sodium Dodecyl Sulphate Polyacrylamide Gel Electrophoresis (SDS-PAGE)

Abalone (*Haliotis diversicolor* Reeve, 1846) shells were kindly provided by the Phuket Abalone Farm, Phuket, Thailand. The outer surface of the shells were cleaned by thorough brushing with metal brush or sandpaper followed by extensive washing with ultrapure water. Shells were either fragmented into small pieces (3–10 mm) or ground into fine powder. Two demineralization methods were used for extraction: (1) 10% acetic acid and (2) 100 mM EDTA. The fragments or powder were continuously stirred with demineralizing agents for 24 h at 4°C and were subjected to centrifugation (4,000 × g, 4°C, 20 min) to get rid of remaining debris. Thereafter, the supernatants were collected and dialysed with a 12-kDa cut-off membrane (Spectrum Laboratories, Breda, The Netherlands). The retentate was pooled and partially purified using two-step MicroCon centrifugal devices (Millipore, Billerica, Maryland, USA) with 50 and 100 kDa cut-off. This method allowed us to collect three sub-fractions: (I) < 50 kDa; (II) 50–100 kDa, and (III) > 100 kDa. Protein concentration was determined using a Bradford protein assay (Bradford reagent; Sigma-Aldrich, St. Louis, Missouri, USA) and measured at 595 nm.

For protein profiling, SDS-PAGE was performed according to methods described by Laemmli (1970). Approximately 10–15 µg of the fractionated proteins were loaded into the well and separated by 12.5% SDS-PAGE under reducing condition. Gels were either stained with Coomassie Brilliant Blue G-250 or subjected to silver staining (Mortz et al., 2001) using Proteo-Silver Silver staining kit (Sigma-Aldrich).

Cell Culture and Treatment

An MC3T3-E1 cell line (passage 16; ATCC, CRL-2593, USA) was initially propagated in an alpha minimum essential medium (α-MEM;

Gibco, Grand Island, New York, USA) supplemented with 2 mM L-glutamine, 10% fetal calf serum and 100 µg/ml penicillin/streptomycin. Treatment conditions on cultured cells followed the methods described by Lao et al. (2007). Culture cells were propagated until approaching confluence. They were subsequently subcultured and plated into 4-well plates at a density of 2,500 cells/well in the humidified atmosphere at 5% CO₂ with the culture medium changed every three days. Testing substances were added every two days within a 14-day period according to these following conditions: (1) culture cells maintained in a Dulbecco's modified Eagle's medium (DMEM; Invitrogen, Carlsbad, CA, USA) as "sham control", (2) cells treated with osteoinductive medium containing 50 µg/ml ascorbic acid in 10 mM β-glycerophosphate (Sigma; St.Louis, Missouri, USA) as "positive control" (Rousseau et al., 2003) and (3) cells treated with 50 and 100 µg/ml acid extracted proteins (AEP) which were resuspended in DMEM medium. After 14 days, the cells in each individual culture well were harvested to monitor their osteogenic activities at both the transcriptional and translational levels as described below.

Measurement of Alkaline Phosphatase (ALP) Activity

ALP activity was determined on the cultured cells at days 1, 7 and 14 post-AEP treatments. Cultured cells were mechanically detached from the cultured wells and washed with ice cold PBS. The ALP activity was determined with the cell lysates based on formation of *p*-nitrophenol (PNP) using the *p*-nitrophenylphosphate kit (Spinreact, St. Esteved'enBas, Girona, Spain) following manufacturer's instructions.

The optical density of PNP was measured at 405 nm using a microplate reader. The specific ALP activity was expressed as µmole PNP/min/mg protein calculated from independently triplicate experiments. Statistical analysis between each group was computed by a paired Student's *t*-test and a *P* value < 0.05 was considered statistically significant.

Analysis of Osteogenic Transcripts using Reverse Transcriptase Polymerase Chain Reaction (RT-PCR)

MC3T3-E1 cells cultures were trypsinized on days 1, 7 and 14 for total RNA isolation using Trizol reagent (Invitrogen, Carlsbad, California, USA). First-strand cDNA was synthesized with 1 µg of total RNA using a SuperScript™ III Reverse Transcriptase (Invitrogen) and oligo-dT according to the manufacturer's protocol. Forward and reverse primers of mouse osteogenic markers including collagen type I (COL-I), osteopontin (OPN), osteocalcin (OCN) and their expected product size were listed in Table 1. GAPDH mRNA was used as an internal control. For PCR amplification, the PCR conditions included a cycle at 94°C (5 min), 30 cycles at 94°C (30 sec each), 2 cycles at T_m (see Table 1) for 30 sec and at 72°C (1 min), and a final step at 72°C (10 min). Densitometric analysis of RT-PCR products in the same, pixel area was performed using ImageJ software (NIH, Bethesda, Maryland, USA) and the data were expressed as mean ± S.D. calculated from triplicate experiments. Statistical analysis of the expression levels within different days of treatments and among the treatment groups in each treatment period was performed using ANOVA. Statistical difference was considered at the *P* value ≤ 0.5.

TABLE 1. Primers for osteogenic markers used in PCR and their expected product size.

Primer	Sequence (5'-3')	T _m (°C)	Product size (bp)
Collagen type IF	ACTGGTACATCAGCCCGAAC	62.4	476
Collagen type IR	GGTGGAGGGAGTTTACACGA	62.4	
Osteopontin F	CGATGATGATGACGATGGAG	60.4	434
Osteopontin R	TCTCCTGGCTCTCTTTGGAA	56.3	
Osteocalcin F	TATGTGTCCTCCGGTTCAT	60.4	455
Osteocalcin R	GCCCTCTGCAGGTCATAGAG	64.5	
GAPDH F	AACTTTGGCATTGTGGAAGG	58.4	472
GAPDH R	CCCTGTTGCTGTAGCCGTAT	62.4	

Immunofluorescence and Confocal Laser Scanning Microscopy

MC3T3-E1 cells were cultured as described above with round coverslips placed on the bottom of the wells. During cell treatments with different testing agents, cells in each well were washed with PBS at days 7 and 14. They were then fixed with 4% paraformaldehyde in phosphate buffer overnight and rinsed with PBS three times. Fixed cells were further permeabilized with cold methanol for 30 sec and transferred to PBS. The following steps were carried out in a moist chamber at room temperature. Cells were blocked with 5% normal goat serum (NGS) and incubated (90 min) with rabbit anti-collagen type I (Merck KGaA, Darmstadt, Germany) at 1:1000 dilution in PBS containing 2% NGS. After rinsing in PBS several times, cells were further incubated (60 min) with Alexa 488 conjugated goat anti-rabbit IgG (Molecular Probes, Eugene, Oregon, USA) at a dilution of 1:500. Cell nuclei were counter-stained with TO-PRO-3 (Molecular Probes) at 1:1000 dilution and mounted with glycerol/PBS (1:1, v/v). The fluorescent images were acquired by a FV1000 confocal laser scanning microscope (Olympus, Tokyo, Japan) using argon (excitation wavelength = 488 nm) and krypton (excitation wavelength = 633 nm) lasers and a Kalman's line-by-line scanning mode.

Reverse Phase High Performance Liquid Chromatography (RP-HPLC) and Mass Spectrometry (MS/MS)

The partially purified AEP was resuspended with 0.1% TFA and subjected to RP-HPLC (Discovery BIO Wide Pore C5 column; 15 cm x 2.1 mm) using a two-step linear gradient (0–5% CH₃CN containing 0.1% TFA in 5 min, then 5–60% CH₃CN containing 0.1% TFA in 90 min at a flow rate of 0.2 ml/min). Selected fractions were pooled, lyophilized, and repurified using different gradient conditions (0–39% CH₃CN containing 0.1% TFA in 10 min, then 39–50% CH₃CN containing 0.1% TFA in 60 min at the flow rate of 0.2 ml/min). Proteins were detected at 210 nm. Selected fractions were collected, lyophilized and subjected to in-solution tryptic peptide digestion for overnight. The peptides were desalted, concentrated in 0.1% formic acid and 2% acetonitrile and further analysed by one-dimensional (1D) LC MS/MS and N-terminal sequencing at the Australian Proteome Analysis Facility (NSW, Australia).

RESULTS

Profiles of Partially Purified Nacreous Proteins

We compared protein yields among four nacreous protein extraction methods from *Haliotis diversicolor* shells which were based on physical (powder and small chips) and chemical demineralization (acetic and EDTA) methods. As shown in Table 2 shells ground into powder and extracted with acetic acid (AEP) appeared to give the highest yield of protein of ~37 mg/kg dry weight powder. Therefore, this extraction method was selected for further purification and used for experiments in this study. Our preliminary data of the partial purification using two-step cut-off (50 and 100 kDa) centrifugation

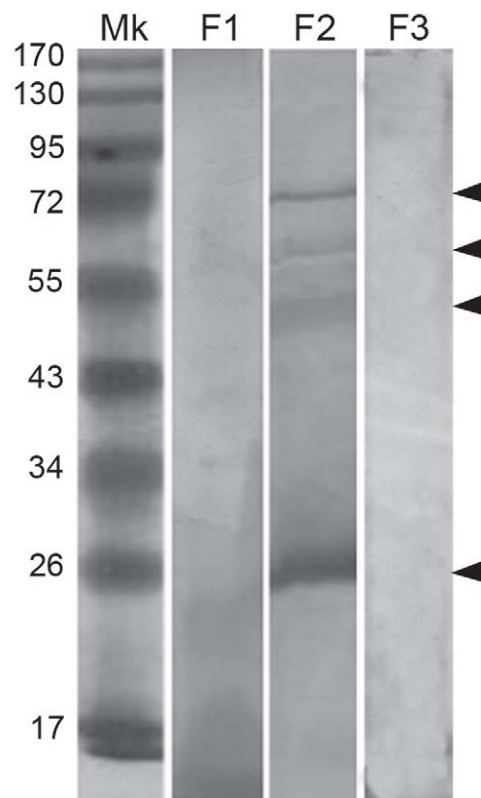


FIG. 1. SDS-PAGE and silver stain profiles of the partially purified *H. diversicolor* acid nacre protein extracts (AEP) by two-step filtration centrifugation. The three fractions shown are F1: < 50 kDa cut-off, F2: 50–100 kDa and F3: > 100 kDa. Arrowheads indicate major proteins and their molecular masses. Mk: standard marker.

TABLE 2. Relative nacreous protein concentration and protein ratio in dry weight by different physical and demineralization methods.

Extraction method	Relative protein concentration (mg/ml)	Protein ratio in dry weight (mg/kg)
Powder + Acetic acid	0.56	37
Fragments + Acetic acid	0.51	34
Powder + EDTA	0.46	31
Fragments + EDTA	0.43	29

indicated that the majority of nacreous proteins were found in fraction 2 (Fig. 1, lane F2). Therefore, we chose to conduct experiments exclusively with F2 in this study. The profile of AEP in F2 as revealed by silver staining showed a prominent protein band at 25 kDa and additional faint bands at 52, 60 and 72 kDa of much lower intensity (Fig. 1). Coomassie Blue staining of these partially purified AEP revealed only one major band at a 25 kDa (data not shown), indicating that the 25 kDa band is of the major protein in this extraction methodology.

AEP Enhanced Alkaline Phosphatase (ALP) Activity

To evaluate the effect of AEP on the pre-osteoblast MC3T3-E1 cells, we compared enhancement of ALP activity, one of the well-known markers for osteoblast differentiation. Generally, ALP activity gradually increased in a time- and concentration-dependent manner when treated with osteoinductive medium (positive control) and AEP, compared with the control groups (Fig. 2). At day 1, ALP activity

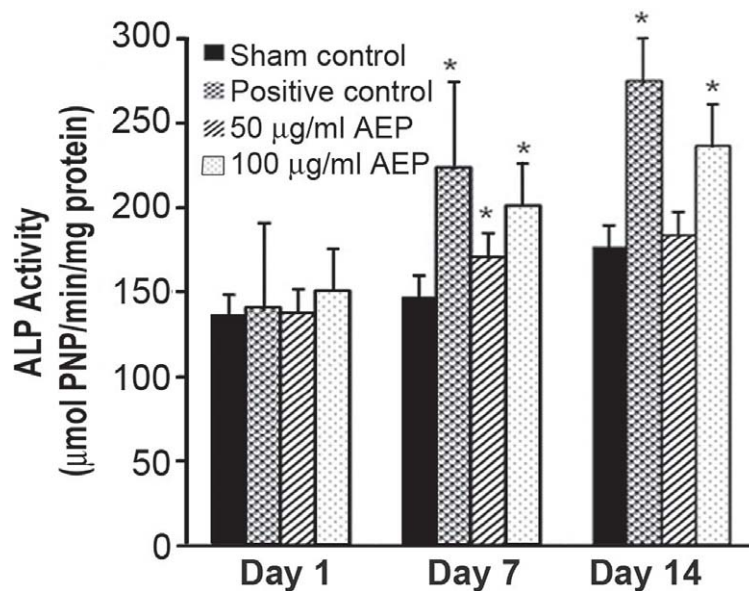


FIG. 2. Time-course and concentration-dependent effect of AEP on alkaline phosphatase activity in MC3T3-E1 cells. Cells were treated with DMEM (sham control), 10mM β -mercaptoethanol and 50 $\mu\text{g/ml}$ ascorbic acid (positive control), 50 and 100 $\mu\text{g/ml}$ AEP. Results are expressed as mean \pm S.D calculated from triplicate experiments. *indicates significant difference ($P < 0.05$) to control treatment within analysis day.

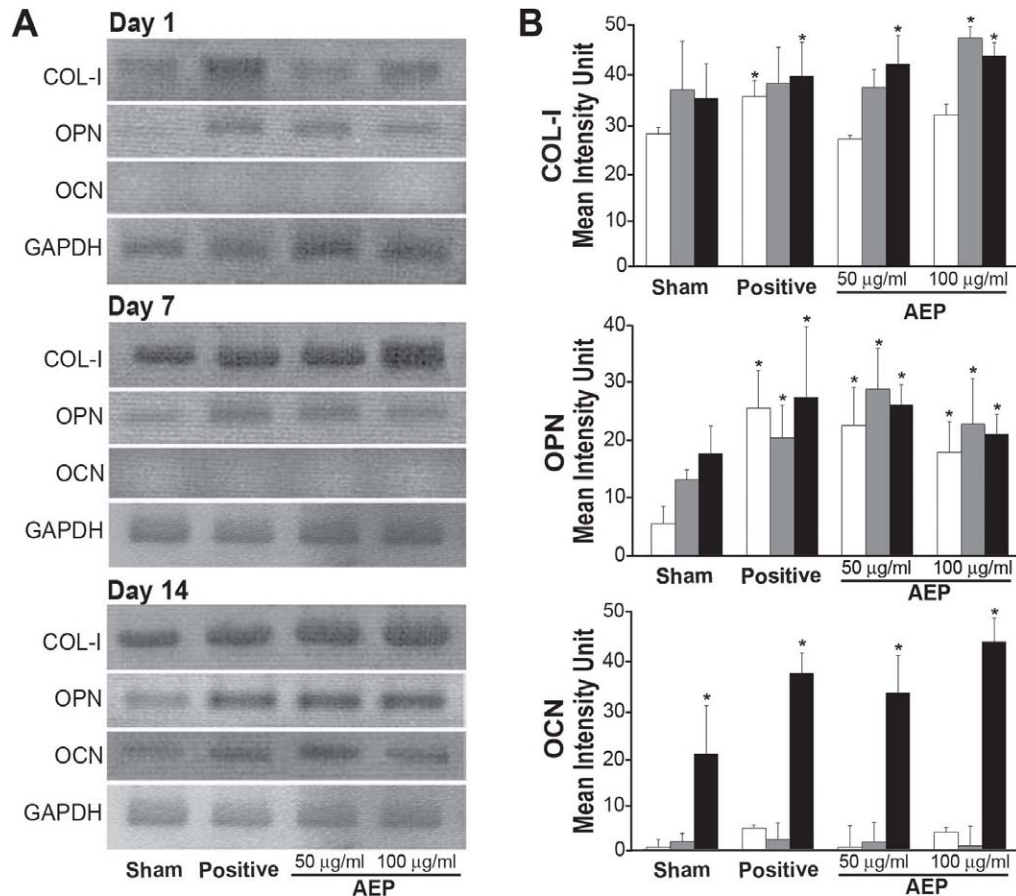


FIG. 3. Effect of AEP on up-regulation of osteoblastic gene markers in MC3T3-E1 cells in day 1, 7 and 14 culture. A: Semi-quantitative RT-PCR was used to amplify gene expression products including COL-I (collagen type I), OPN (osteopontin) and OCN (osteocalcin) in sham control (lane 1), positive control (lane 2), 50 and 100 µg/ml AEP treatments (lanes 3, 4); B: The band intensities of PCR products in different days (day 1: empty bars, day 7: gray bars, day 14: solid bars) of the triplicate experiments were quantified by densitometric analysis. GAPDH is used as internal control.

was relatively comparable in all treatment and control groups. However, the activity was significantly ($P < 0.01$) enhanced in day 7 and was even more pronounced in day 14, particularly in 100 µg/ml AEP and positive control groups. The highest ALP activity in 100 µg/ml AEP-treated cells at day 14 was 236.02 ± 0.33 U/L/min/mg protein, which was slightly less than positive control (275.07 ± 0.24 U/L/min/mg protein) but significantly greater ($P < 0.05$) than 50 µg/ml AEP (183.46 ± 0.33 U/L/min/mg protein) or the sham control (176.85 ± 0.13 U/L/min/mg protein).

Up-regulation of Osteogenic Markers' Transcripts following AEP Treatment

In order to investigate the effect of AEP on osteoblast differentiation, RT-PCR and densitometric quantitation of the amplified products including COL-I, OPN, and OCN was performed (Fig. 3). Following seven days of MC3T3-E1 cell cultivation, COL-I gene expression was up-regulated in all groups studied. In particular, the COL-I band density in 100 µg/ml AEP-treated cells (50.14 arbitrary density units) was apparently higher than the other groups and

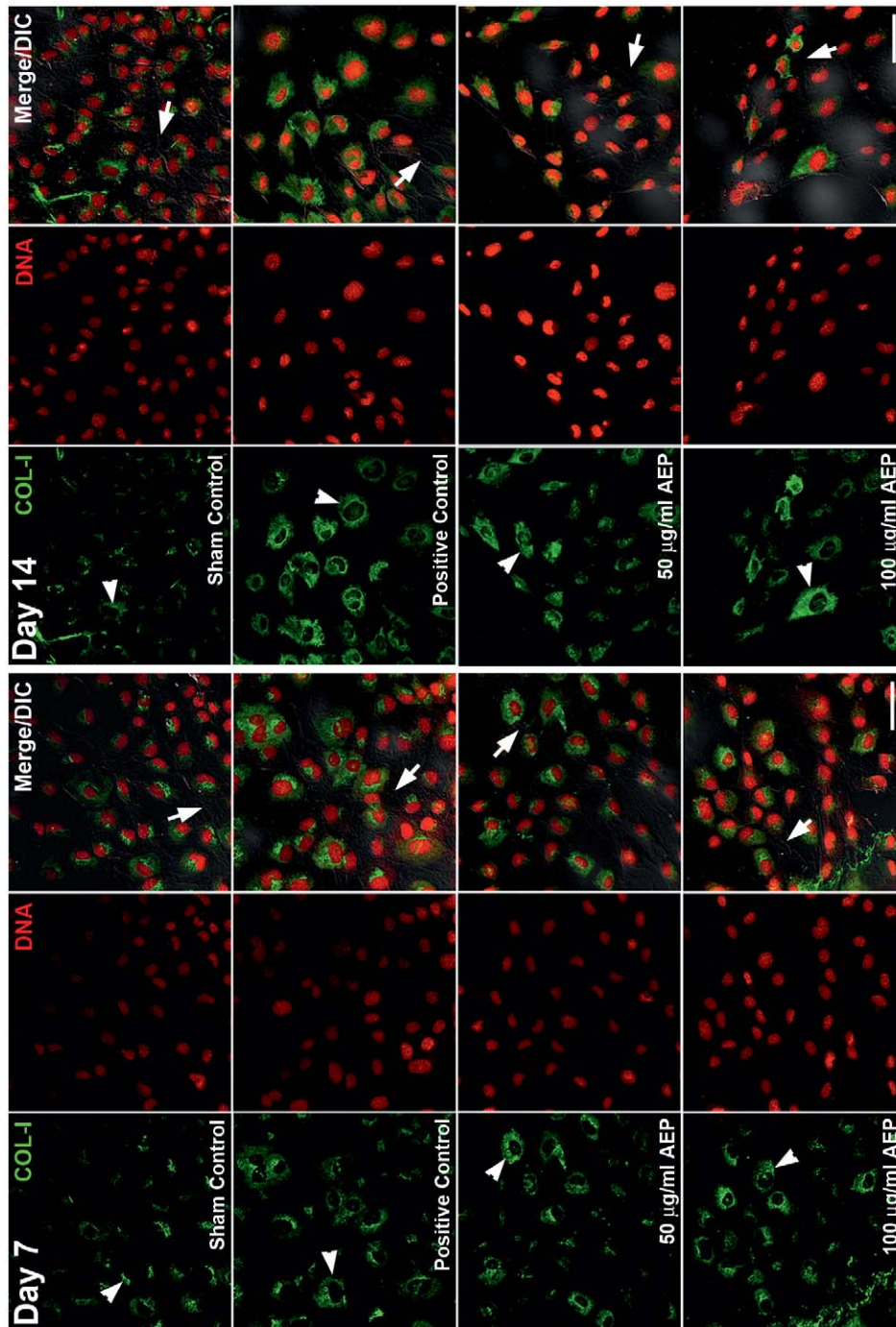


FIG. 4. Immunofluorescence and confocal microscopy of collagen type I (COL-I) expression in MC3T3-E1 cells treated with AEP at days 7 and 14. In each panel, green fluorescence represents COL-I staining (left column), red fluorescence represents DNA staining by TO-PRO 3 (middle column), and the right column is merged fluorescent micrographs with DIC. Note the crescent-like staining pattern in sham control but spreading throughout cytoplasm in AEP-treated cells (arrowheads). Arrows indicate the fibrillar structure of the extracellular matrix.

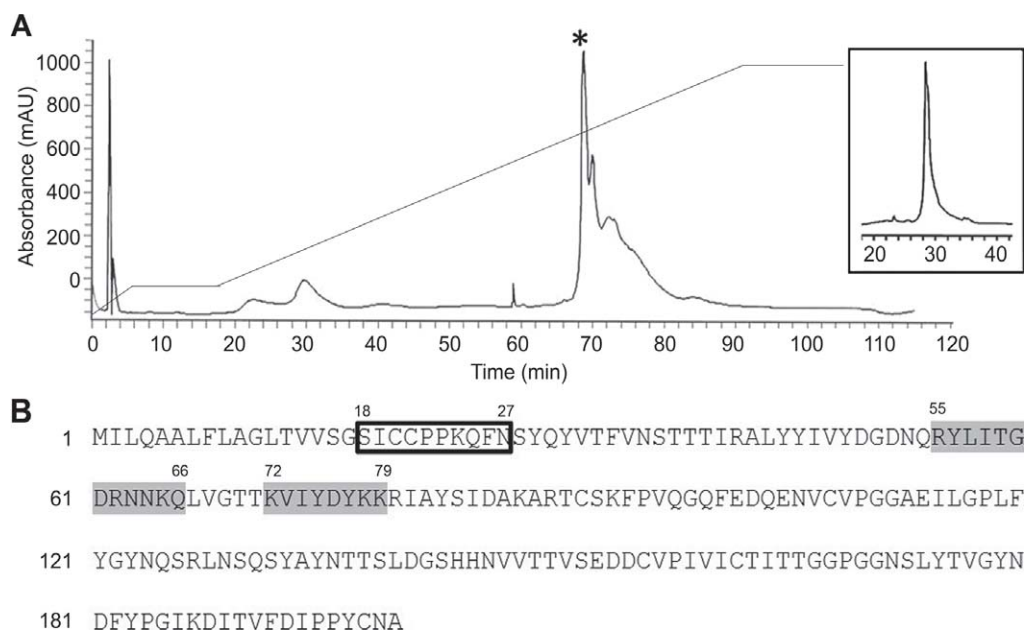


FIG. 5. Purification and sequencing of AEP 25 kDa protein from *H. diversicolor*. A: Representative chromatogram of primary RP-HPLC purification with the major 25 kDa peak indicated by an asterisk which was re-purified (inset) for sequencing; B: Sometsuke protein sequence of *H. asinina* showing the sites matching the *H. diversicolor* AEP 25 kDa protein as determined by 1D MS/MS spectra (shaded in grey) and N-terminal sequencing (boxed).

kept relatively constant towards day 14 (52.63 units). This similar trend of increased COL-I gene expression was also observed in the 50 $\mu\text{g/ml}$ AEP-treated and positive control groups. The expression of OPN, a non-collagenous marker for osteoblast differentiation, was only at the detectable level on day 1 of the sham control (8.47 units). However, upon AEP treatment even at day 1, the OPN transcriptional level was greatly enhanced by ~ 3 folds in both 50 and 100 $\mu\text{g/ml}$ AEP-treated cells. At day 7, the increase in band intensity was relatively low compared with day 14, where the band intensity peaked up to 77.9 units in both 50 and 100 $\mu\text{g/ml}$ AEP treatments. Positive control cells constantly displayed high OPN transcription in MC3T3-E1 cells over the three period of culture. OCN transcription, another specific and late marker for osteoblast differentiation, was undetectable at day 1 or day 7. However, its transcriptional level was greatly enhanced at day 14 in all groups with band intensity of ~ 21 – 26 units that were ~ 1.4 – 1.8 fold higher than that detected in the sham control.

Enhancement of COL-I Protein in Pre-osteoblasts Upon AEP Treatments

To test whether AEP treatments also stimulated COL-I expression at the protein level, MC3T3-E1 cells were subjected to anti-COL I immunohistochemistry and their staining distribution compared at days 7 and 14. As shown in Figure 4, cultured cells showed an intense crescent-like staining pattern (arrowheads) over one side of the nuclear region which was similar in both days 7 and 14 (left column in each panel). Upon treatment with AEP (both 50 and 100 $\mu\text{g/ml}$; third and fourth rows), anti-COL I staining spread throughout the cytoplasm of most cells in addition to the crescent-like pattern which was comparable to the pattern observed in the positive control (second row). The lay-down of ground matrix between the cellular spaces as seen by differential interference contrast (DIC) microscopy (second and fourth rows) was quite apparent, particularly positive control and 100 $\mu\text{g/ml}$ AEP treatment (arrows).

Identification of a Major Protein Sequence in AEP Extract

The AEP extract used in this study was further purified by reversed-phase HPLC and characterized by 1D LC-MS/MS and *N*-terminal sequencing. Initially, three protein peaks were obtained: a major peak at 65–80 min, and two minor peaks at 20–34 and 58–60 min (Fig. 5A). The major peak was re-purified to provide a single peak (Fig. 5A, inset) and further subjected to sequence analysis. Following 1D LC-MS/MS, the MASCOT search engine was used for protein identification by comparing the mass spectra of the fragmented peptides (2+ up to 4+ charged) with the proteins present in the NCBI database. Two peptides matched with *Haliotis asinina* Linnaeus, 1758, sometsuke (GenBank accession number DW986219) (Fig. 5B). The peptide sequences matched at positions 55–66 (12 amino acids) and 72–79 (eight amino acids). In addition, *N*-terminal sequencing confirmed that the 10 amino acids at position 18–27 were identical to the sometsuke protein sequence. This AEP sequence analysis thus suggests that sometsuke is a major part of the organic matrix in *H. diversicolor* nacreous layer that may induce osteoblast differentiation.

DISCUSSION

In our study, we used mechanical- and chemical-based methods to isolate proteins from the *H. diversicolor* nacreous layer and showed that AEP extraction was the most effective method in nacreous protein isolation while it still maintained the biological function of the extracted proteins in stimulating osteoblast differentiation. This is consistent with studies on *Pinctada maxima* (Jameson, 1901), where osteogenic activity exists within the soluble organic matrix of nacre (Lamghari et al., 1999; Almeida et al., 2001; Moutahir-Belqasmi et al., 2001; Mouriès et al., 2002; Rousseau et al., 2003, 2008). At a first step towards isolating the osteogenic differentiation factors, we showed that 10% acetic acid gave the highest extracted protein concentration (0.56 mg/ml) equivalent to 37 mg dry nacreous protein/kg nacre weight. This AEP extract showed a prominent protein band at 25 kDa, which was at a lower molecular mass than the cut-off molecular mass of the centrifugal device used for purification. One

explanation for this phenomenon is that this nacreous protein may be in a dimeric or multimeric form bound together by disulfide bridges or by electrostatic interaction as also reported for other nacreous proteins (Marin et al., 2005; Kong et al., 2009), which are subsequently disrupted by reducing or high salt conditions. This finding is consistent with the results reported by Bédouet et al. (2007), indicating the presence of low molecular weight molecules in the shell matrix. Interestingly, further purification of this AEP protein followed by partial peptide sequencing indicated that our AEP corresponded to the abalone mantle protein, sometsuke, a ~20 kDa protein expressed in the anterior zone of the outer fold of abalone mantle (Jackson et al., 2006). Its expression correlates with nacre and prism soluble matrix in *H. asinina*. Physiologically, it has been suggested that sometsuke possesses the ability to organize the shell matrix framework and has calcium binding properties (Marie et al., 2010). Besides, sometsuke has also been known to engage its function in shell pigmentation patterning in *H. asinina*, as shown by Jackson et al. (2006).

In fact, the proposed function of the sometsuke protein in calcium binding has drawn attention to the biomineralization function of shell organic matrix, the knowledge of which has gradually accumulated in other *Haliotis* species. Perlustrin, a 9 kDa nacre soluble organic matrix protein extracted from *Haliotis laevigata* Donovan, 1808, contains an insulin-like growth factor binding protein (IGF-BP) domain that enables IGF-binding, known to be crucial for cell interactions (Weiss et al., 2000). Other smaller nacreous proteins of *H. laevigata* include perlucin, an 18 kDa nacre soluble organic matrix protein that has a C-type lectin domain which facilitates calcium carbonate precipitation (Weiss et al., 2000). In *Haliotis rufescens* Swainson, 1822, lustrin A, a nacre insoluble protein of 142 kDa has been reported to adhere nacre tablets, assuming a function similar to that of muscle protein titin, an excitation-contraction coupling protein (Shen et al., 1997). The last two identified abalone aragonite proteins, AP7 and AP24, are moderately acidic and considerably novel, since they show no homology with any known proteins. While AP7 contains four cysteine residues involved in its conformation through disulfide bridging, AP24 contains an *N*-glycosylated domain which is believed to participate in calcium

binding (Michenfelder et al., 2003). Despite the fact that these abalone nacreous proteins play significant roles in biomineralization, most of them are related to organization of the nacreous layer within the shell. Unlike the studies reported in some oyster and abalone species, additional biomedical roles in osteoinduction both *in vitro* and *in vivo* have also been experimentally validated, making them potentially valuable pharmaceutical agents.

During *in vitro* osteogenesis, the three check points are generally considered including: (1) incremental ALP activity as an initial indicator of osteoblast differentiation (Hessle et al., 2002), (2) changes in osteoblast-specific gene markers throughout the cell differentiation process (Spreafico et al., 2006; Sila-asna et al., 2007) and (3) collagenous protein synthesis and its lay-down to become extracellular matrix (Li et al., 2011; Reffitt et al., 2003). In our study, we found that AEP at 100 µg/ml strongly increases ALP activity comparable to that of a positive control known to stimulate ALP activity (Son et al., 2007). This finding agrees well with the study in *Pinctada fucata* Röding, 1798, in which the mantle extract PFMG3 at 10 µg/ml considerably stimulates ALP activity following a 48 h incubation with MC3T3-E1 cells (Wang et al., 2011). In addition, water-soluble organic material (WSM, 1300 µg/ml) increases ALP activity in MRC5 cells when incubated for three days, and rat bone marrow stromal cells (135 µg/ml) when treated for seven days (Mouries et al., 2002). Notably, the concentration of AEP used in our study (at the microgram level) was in the same range as that used by other studies, suggesting a comparable physiological potency of our extracting compounds with other existing compounds. Inversely, Rousseau et al. (2003) found that WSM inhibited ALP activity, which may have occurred because of differences in the concentrations of WSM used to treat cells. In fact, we found that AEP at 200 µg/ml was rather toxic to MC3T3-E1 cells (data not shown).

We also found that AEP exerted up-regulation of gene markers (COL-I, OPN and OCN) related to osteoblast differentiation. Most notably, both 50 and 100 µg/ml AEP stimulated a relatively higher level of expression of COL-I and OPN after one day of culture. This appears to be a quicker response of MC3T3-E1 cells to AEP when compared with those treated with 200 µg/ml of ethanol soluble matrix (ESM)

extracted from oyster nacre (Rousseau et al., 2008). Concurrently, ESM enhances OPN transcription more than RP-HPLC fractions and the cells reach their maximum transcription level on day 21 (Rousseau et al., 2008). In the late stage of osteoblast differentiation concomitant with mineralization, osteocalcin is expressed (Caetano-Lopes et al., 2007). Our results revealed that OCN mRNA was present at day 14 but not at day 1 or 7. In contrast, the expression of OCN mRNA was reduced when cultivated with ESM and its HPLC fractions (Rousseau et al., 2008). Finally, we found that pre-osteoblasts were induced to translate higher levels of collagen type I by day 7 up to day 14 of culture, consistent with the positive control. This collagen is presumably laid down extracellularly by mature osteoblasts to become the most predominant component in the ground substances of the bone matrix (Caetano-Lopes et al., 2007).

In conclusion, our study has shown that AEP from *Haliotis diversicolor* increased ALP activity, up-regulated all osteoblastic differentiation markers studied, and stimulated collagen ground substance lay-down *in vitro*. We believe that the organic extracts from nacreous layer of *H. diversicolor* constitute the signalling molecules, one of which could be a 25-kDa sometsuke-like protein, which play a role in promoting osteoblast differentiation and osteogenesis presumably through its calcium binding property. While the other function of sumetsuke protein in shell coloration patterning is also of particular interest in this species, however, it still requires further extensive investigations to address this question.

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