

Characterization and *in vitro* mineralization function of a soluble protein complex P60 from the nacre of *Pinctada fucata*[☆]

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Abstract

A soluble protein complex P60 from the powdered nacre of *Pinctada fucata* was extracted and partially characterized. The biological activity of the P60 on pre-osteoblast cell line MC3T3-E1 and bone marrow stroma cells (MSCs) was investigated. The P60 protein from the decalcified powdered nacre was solubilized with acetic acid and then purified by liquid chromatography. The P60 protein was a protein complex composed of several subunits with disulfide bridges. The known protein nacrein, and its two derivatives, N28 and N35, were included in the P60 protein complex. The most abundant amino acids in the P60 that account for 68.3% of the total residues are glycine (32.1%), aspartic acid (17.4%), alanine (13.6%), and glutamic acid (5.2%). The *in vitro* study of the crystallization showed that this protein complex could control the formation and size of calcium carbonate. The assay of biological activity of the P60 protein complex on the pre-osteoblast cell line MC3T3-E1 and MSCs suggested that the P60 could stimulate the formation of mineralized nodules.

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1. Introduction

The organic matrix of nacre is generally classified as two components: water-soluble matrix and water-insoluble matrix. Since Crenshaw (1972) first identified a water-soluble matrix containing a Ca-binding glycoprotein, a number of nacre matrix proteins have been isolated. Most of these proteins control the formation of nacre. Perlucin and perlustrin are two proteins extracted from *Haliotis laevis* nacre. While perlucin belongs to a heterogeneous group of proteins (Mann et al., 2000), perlustrin is a minor component which has certain

similarity to part of the much larger collagen-like protein lustrin A from *Haliotis rufescens* nacre (Shen et al., 1997; Weiss et al., 2000). Further research indicated that perlustrin consisted of a single insulin-like growth factor binding protein N-terminal domain (Weiss et al., 2001). Nacrein and N66 have carbonic anhydrase domains participating in calcium carbonate crystal formation by catalyzing the HCO₃⁻ formation (Miyamoto et al., 1996; Kono et al., 2000). Some of these nacre matrix proteins have particular amino acid repeat domains. Nacrein has a Gly–Xaa–Asn (Xaa=Asp, Asn, or Glu) repeat domain which possibly binds calcium (Miyamoto et al., 1996). N14 and N16 proteins have an Asn–Gly repeat domain which not only has the capacity for Ca-binding but also serves for adsorption of the protein to other matrix components or aragonite crystals (Samata et al., 1999), so it can induce platy aragonite layers which are highly similar to the nacreous layer (Kono et al., 2000). MSI7 has a Gly-rich domain which is proposed to be critical for Ca⁺ binding (Zhang et al., 2003). MSI60 and MSI31 are rich in Gly–Ala and Gly–Glu repeat domains respectively.

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Both of them have neither carbonic anhydrase functional domains nor *N*-glycosylation sites. They are considered to influence CaCO₃ formation by selectively binding soluble aspartate-rich matrix glycosylation and carbonic anhydrase (Sudo et al., 1997).

During nacre formation, the organic matrix proteins can control the mineralization of aragnite (Kalpana and Dinesh, 2006). In vitro crystallization experiments indicated that the matrix proteins can control the size, quantity, or structure of CaCO₃ crystal (Shen et al., 1997; Samata et al., 1999; Kono et al., 2000; Weiss et al., 2000). When it is implanted in animal bones as a biomaterial, evidence showed the organic matrix proteins play a primary role in controlling the formation of interfacial structure and biocompatibility with bone as well as the stability of biogenic tissues (Kim et al., 2002). Bioactivity research on the matrix proteins extracted from *Pinctada maxima* suggested that matrix proteins had osteo-inductive effects on mammal osteoblastic precursor cells by promoting cell proliferation, increasing alkaline phosphatase activity, increasing Bcl-2 expression, and bone nodules formation (Lamghari et al., 1999; Almeida et al., 2000; Moutahir-Belqasmi et al., 2001; Mouries et al., 2002; Rousseau et al., 2003).

In this study, we have extracted a protein complex P60 by acetic acid and purified it by liquid chromatography from *Pinctada fucata*. The protein showed one band in native-polyacrylamide gel electrophoresis (native-PAGE) but revealed several major bands in SDS-polyacrylamide gel electrophoresis (SDS-PAGE), which suggested the subunits of P60 were linked by disulfide bridges. N-terminal sequencing and MALDI-TOF MS analysis revealed that the known protein nacrein, and two new proteins N28 and N35, which are homologous to nacrein, are the components of the protein complex P60. Similar to other *P. fucata* proteins reported, P60 has a high ratio of glycine, aspartic acid, alanine and glutamic acid. In vitro study in crystallization suggested that P60 affected CaCO₃ formation in size and link mode. To certain extent, P60 could also promote the mineralized nodules formation in MC3T3-E1 cells and MSCs cells.

2. Materials and methods

2.1. Extraction of the soluble protein

Raw nacre, which is free of other calcite-rich shell elements of *P. fucata*, was crushed to a fine powder (0.75 μm). The powder (20 g) was suspended in 200 ml of 33% acetic acid at 4 °C for 3 h with continuous stirring. The suspension was then centrifuged for 20 min at 14,000 rpm. After filtration, the supernatant was loaded onto a Sephadex G-15 column (5 × 15 cm) previously equilibrated with 25 mM Tris-HCl (pH 7.2), and eluted with the same buffer. The fractions were detected by an UV detector (280 nm) and a conductivity detector. The first peak detected by UV detector, which was separated from the salt peak detected by conductivity detector, was collected and lyophilized. The lyophilized fraction was dissolved and loaded onto a Sephadex G-15 column (5 × 15 cm) again. The fraction eluted with 25 mM PB (pH7.2) was used for

biological activity assay. The content of protein was analyzed by Bradford procedure with bovine serum albumin as standard. This protein was named P60.

2.2. Chromatography analysis

P60 was fractionated by a series of chromatography procedures: size-exclusion chromatography using a Sephadex G-75 (1 × 85 cm) column, ion-exchange chromatography using a HiTrap CM FF column (Amersham), a HiTrap DEAE FF column (Amersham) and HiLoad Q Sepharose FF column (Amersham), and hydrophobic interaction chromatography using a HiLoad Phenyl Sepharose High Performance column (Amersham).

2.3. Native PAGE and SDS-PAGE analysis

Non-denatured PAGE was performed in a Laemmli buffer system (Sambrook et al., 1989) in the absence of SDS at 4 °C. SDS-PAGE was conducted using the buffer system of Laemmli (Sambrook et al., 1989). Gels were stained with Coomassie brilliant blue R-250.

2.4. N-terminal sequencing and MALDI-TOF MS analysis

The protein complex P60 was separated by SDS-PAGE. The gel was stained with ZnSO₄. Three bands corresponding to about 60-, 35- and 28-kDa protein (named N60, N35 and N28) was excised and electroeluted. The electroeluted protein samples were dialyzed against 50 mM Tris-HCl (pH 8.0) and subjected to SDS-PAGE. N60 protein was blotted to PVDF membrane (Immobilon-P^{SO}, Cat. no. ISEQ10100, Millipore, Bedford, MA, USA) and sequenced at its N-terminus using the Edman degradation procedure by Wei-Qun Shen (Perking University). N28 and N35 proteins were excised from SDS-PAGE gel and subjected to MALDI-TOF MS analysis.

2.5. Amino acid analysis

Analysis was performed in Instrumental Analysis and Research Center of South China Agricultural University. P60 was hydrolyzed in 6 M HCl at 110 °C for 22 h. Hydrolysate was concentrated under vacuum. Amino acids were separated by HITACHI L-8800 amino acid analyzer using a HITACHI 855-3506 ion-exchange column.

2.6. Crystallization

P60 solution was dialyzed against 3 mM Tris-HCl (pH8.2) at 4 °C. The dialyzed fraction protein concentration was determined using the CBB method and diluted into 0.1 mg/ml.

Supersaturated CaCO₃ solutions were prepared according to Wiess et al. (2000). Sample solution 50 μl of 0.1 mg/ml P60 in Tris (pH8.2), was added to 150 μl of the supersaturated solution. A buffer solution without protein was used as the control. Each of the solutions was incubated at 4 °C for 1 week. A JEOL JSM-6330F Field Emission Scanning Electron Microscope was used for morphological observation of the induced crystals.

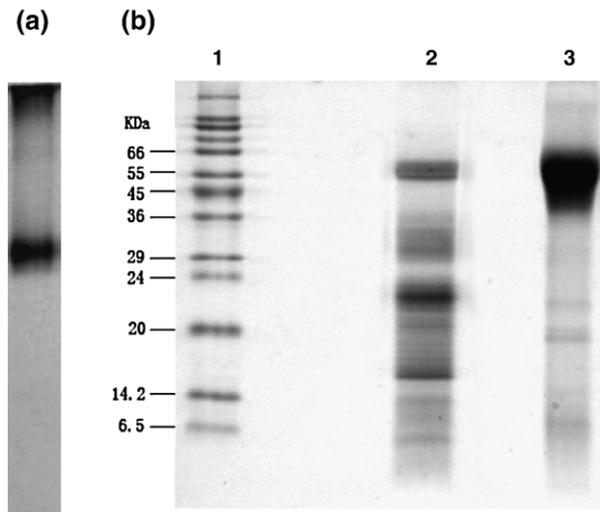


Fig. 1. Electrophoresis analyses of the protein complex P60 extracted from *Pinctada fucata*. (a) Native-PAGE analysis. (b) SDS-PAGE analysis. 1, molecular mass standards (Sigma M4038); 2, P60 treated with β -mercaptoethanol and SDS; 3, P60 treated with SDS only.

2.7. Cell culture conditions

MC3T3-E1 pre-osteoblast cells, obtained from Chinese Academy of Medical Sciences and Peking Union Medical College cell bank, were cultured in complete Dullbecco's Modified Eagle Medium (DEME, high glucose; Gibco) containing 10% fetal calf serum (Hyclone), 100 U/ml penicillin and 100 μ g/ml streptomycin. MC3T3-E1 cells were plated out in 12-well dishes at a concentration of 5×10^4 cell/well for 24 h.

MSCs was obtained from the femur and tibia of young SD rats aged 30–40 days by inserting a 21-gauge needle into the shaft of the bone and flushing it with complete Dullbecco's Modified Eagle Medium (DEME, low glucose; Gibco) containing 10% fetal calf serum (Hyclone), 100 U/ml penicillin and 100 μ g/ml streptomycin. The cells were plated at a concentration of 2×10^6 cells/cm². The cells grew at 37 °C and 5% CO₂ and was washed with phosphate-buffered saline (PBS) after 48 h. The medium was replaced every two days. The non-adherent cells were removed by washing with PBS and medium changes. The primary MSCs were passaged when the cell density reached 80–90% confluence. The second passage MSCs were used for experiments. MSCs were plated out in 12-well dishes at a concentration of 5×10^4 cell/well for 24 h.

2.8. Biological assay

MC3T3-E1 cells and MSCs cells were treated for 8 days with the following: (1) BSA solution (0.1 mg/ml); (2) P60 solution (0.1 mg/ml); (3) Dex(Dexamethasone, 10^{-8} M), GP(β -glycerophosphate, 10 mM), AA(ascorbic acid, 50 μ g/ml).

The cells were washed with PBS and then were fixed with neutral formalin and stained by the von Kossa method. Freshly prepared 1% silver nitrate was added to plates which then were incubated in ultraviolet radiation for 15 min. The plates were

rinsed with distilled water, then incubated in 5% sodium thiosulfate for 2 min. After rinsing with distilled water 1% neutral red was added, and then rinsed with distilled water again. The cells were photographed using a Nikon camera and an inverted light microscope.

3. Results

3.1. Extraction of the soluble nacre protein

In the nacre, proteins are strongly bound to the mineral. In our protocol, very fine nacre power was dissolved in acetic acid solution. By centrifugation, the matrix was separated into two parts: the soluble and the insoluble fractions. The soluble fraction was desalted on a Sephadex G-15 column. The major protein peak was collected and lyophilized. After a second desalting, the protein was dissolved in PBS at a concentration of about 1 mg/ml for further analysis.

3.2. Characterization of the protein complex P60

To further purify the soluble nacre protein obtained above, a series of chromatography procedures were employed, including size-exclusion chromatography (Sephadex G-75), ion-exchange chromatography (HiTrap CM FF, HiTrap DEAE FF and HiLoad Q Sepharose FF), and hydrophobic interaction chromatography (HiLoad Phenyl Sepharose High Performance). All elution profiles from these columns, however, revealed only one major protein peak. This major protein peak showed one major band on native-PAGE gel (Fig. 1a). SDS-PAGE of unreduced protein sample (without β -mercaptoethanol) also yielded a major band of approximately 60 kDa (Fig. 1b, line 3) whereas the reduced (with β -mercaptoethanol) protein yielded several bands (Fig. 1b, line 2). Accordingly this soluble nacre protein obtained in this study was designated P60. The result shown in Fig. 1 suggests that P60 is a protein complex consisting of several subunits linked through interchain disulphide bridges.

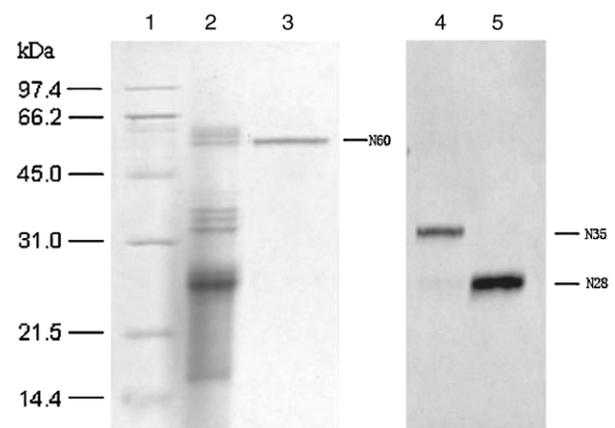


Fig. 2. SDS-PAGE analysis of the isolated N60, N35 and N28 proteins. 1, molecular mass standards; 2, P60; 3, N60; 4, N35; 5, N28.

3.3. Components of the protein complex P60

To characterize the components of the protein complex P60, three bands corresponding to about 60-, 35- and 28-kDa protein (named N60, N35 and N28) was excised from SDS-PAGE gel (Fig. 2). Sequencing revealed that the N60 has exactly the same N-terminal sequence (ASMFK HDHYM DDG) as nacrein purified from the same species *P. fucata* (Miyamoto et al., 1996), suggesting N60 is the known protein nacrein. Other two proteins, N35 and N28, were subjected to MALDI-TOF MS

analysis. Seven peptides from N35 (Fig. 3a) and eight peptides from N28 (Fig. 3b) were found to be identical with the corresponding sequences of nacrein. This result suggests that both N35 and N28 are derived from nacrein.

3.4. Amino acid analysis

Amino acid analysis of the soluble protein complex P60 was illustrated in Table 1. Four types of amino acids represented 68.3% of the total residues: 32.1% glycine, 17.4% aspartic acid,

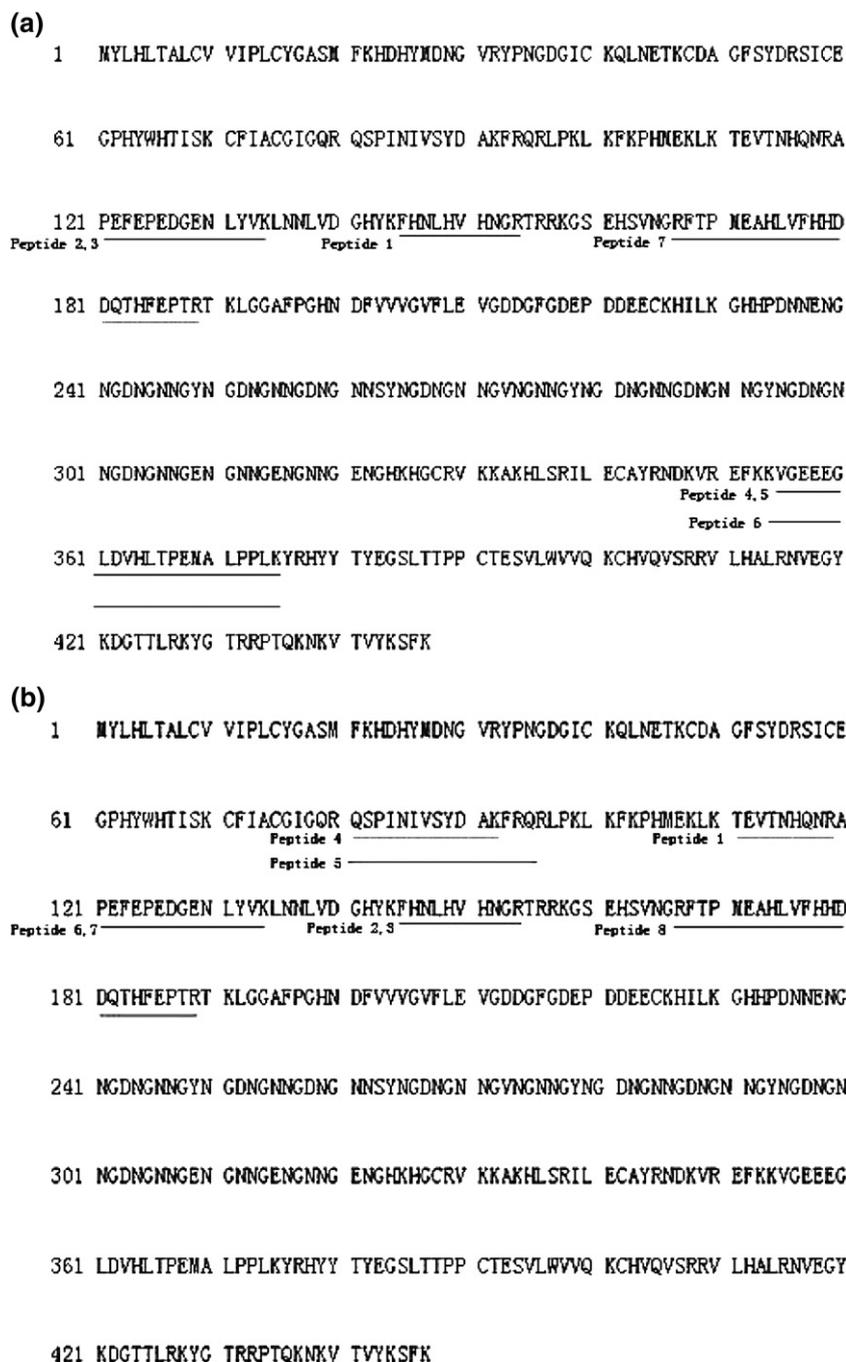


Fig. 3. Alignment of peptide sequences of N35 and N28 proteins with the sequence of nacrein. The amino acid sequence of nacrein (GenBank BAA11940) is shown in capital letters. Peptides of N35 and N28 proteins identified by MALDI-TOF MS analyses are underlined. (a) Seven peptides of N35 are identical with the corresponding sequence of nacrein. (b) Eight peptides of N28 are identical with the corresponding sequence of nacrein.

Table 1
Amino acid content of the protein complex P60 extracted from *Pinctada fucata*

| Amino acid | Content (mol%) |
|------------|----------------|
| Asx | 17.39 |
| Thr | 2.15 |
| Ser | 3.75 |
| Glx | 5.21 |
| Gly | 32.07 |
| Ala | 13.58 |
| Cys | 0.42 |
| Val | 2.75 |
| Met | 1.31 |
| Ile | 1.33 |
| Leu | 4.13 |
| Tyr | 2.41 |
| Phe | 2.06 |
| Lys | 3.21 |
| His | 1.75 |
| Arg | 3.37 |
| Pro | 2.96 |
| Hypro | 0.14 |
| Total | 100.00 |

13.6% alanine, 5.2% glutamic acid. Glycine was the most abundant residue which consisted one third of the amino acid content. Potentially acidic amino acid (Asx+Glx) represented

22.6%, basic amino acid (Lys+Arg+His) accounted for 8.3%. The content of collagen-associated amino acids was low: 2.96% Pro, 0.14% Hypro.

3.5. Crystallization

The crystallization experiment was carried out with the protein complex P60 solution. The buffer solution without proteins was used as control. In the crystallization solution with protein, most of the crystals grew smaller (Fig. 4b) than that in control (Fig. 4a), and they stuck to one another like a “brick and wall” structure (Fig. 4b) or like chains (Fig. 4c). Needle-like crystals (Fig. 4d) were also found when protein was present.

3.6. Biological assay

Both MC3T3-E1 cells (Fig. 5b) and MSCs cells (Fig. 5c) cultured with protein complex P60 were induced to mineralization after 8 days. Under an inverted light microscope, the centers of mineralized nodules were glistening and were surrounded by cells like irradiation forms (Fig. 5a). The effect was remarkable in MC3T3-E1 cells. By the von Kossa method, the mineralized nodules were stained to black. MSCs cells and MC3T3-E1 cell cultured with Dex+GP+AA little formed

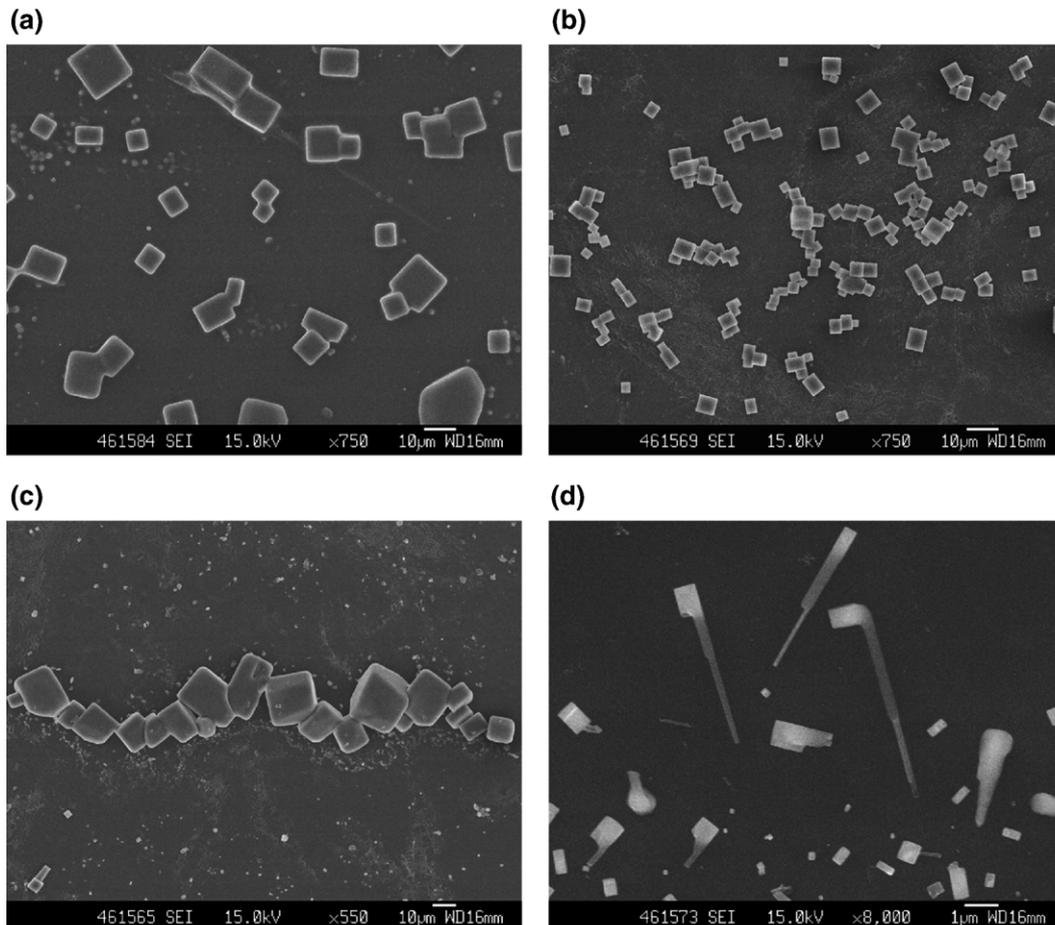


Fig. 4. Scanning electron microscope images of crystals grown in supersaturated CaCO_3 solutions: (a) Crystals formed in Tris-HCl control buffer; (b–d) Crystals formed in the presence of P60.

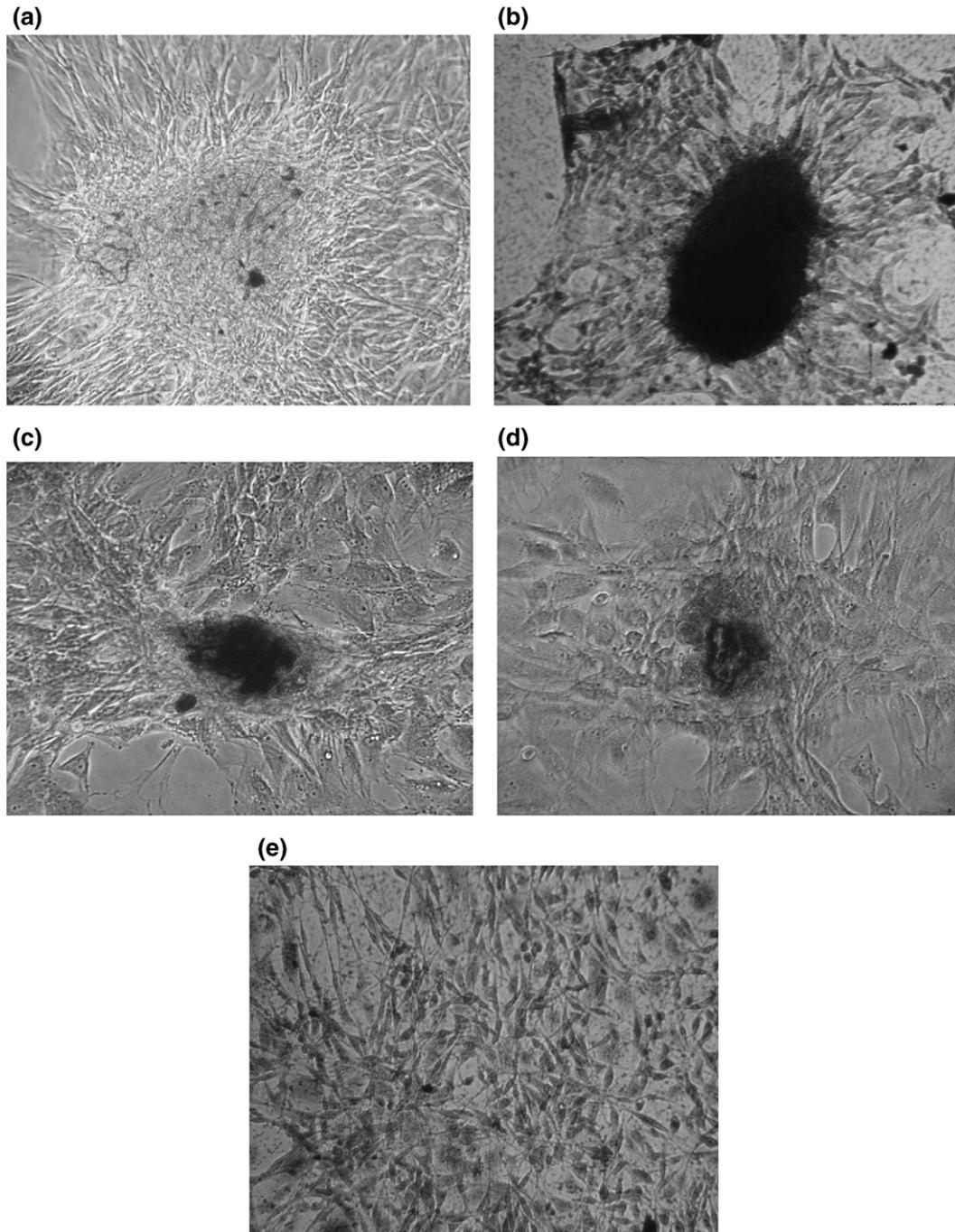


Fig. 5. Light micrographs of mineralized nodules of control and protein complex P60 treated MC3T3-E1 cells and MSCs cells: (a–c) Mineralized nodules formed in the presence of protein complex P60. (b) and (c) Von Kossa stain was used to visualize mineralized nodules; (d) Cell treated with Dex+GP+AA; (e) Cell treated with BSA solution.

mineralized nodules as illustrated (Fig. 5d). There is no mineralized nodule found in the cells cultured with control (Fig. 5e). The result seems to suggest that the protein complex P60 could accelerate the differentiation of both MC3T3-E1 cell and MSCs cells.

4. Discussion

This study reports the characterization and biological activity of protein complex P60 from nacre of *P. fucata*. We observed the mineralization functions of this protein complex during

calcium carbonate growth and its biological effect on mammal animal cells.

In this study, we removed the periostracum and primatic layer of the shell by a mechanical method using a grinding wheel, then ground it into a very fine powder in liquid nitrogen. In nacre, the organic matrix was bound firmly with CaCO_3 . Decalcification is a usual method to free such proteins. Acetic acid, EDTA, and others, are generally used in this process. In this study, we obtained free proteins from the calcium carbonate by acetic acid at a low temperature within a short time.

Table 2
Contents of four amino acids in P60, nacrein, N16, MSI60, MSI31 and MSI7 (mol%)

| | P60 | nacrein | N16 | MSI60 | MSI31 | MSI7 |
|-----|------|---------|------|-------|-------|------|
| Gly | 32.1 | 12.8 | 13.9 | 38.0 | 25.1 | 35.5 |
| Ala | 13.6 | 2.6 | 1.9 | 27.1 | 6.0 | 2.6 |
| Asx | 17.4 | 20.0 | 18.5 | 7.8 | 7.0 | 3.9 |
| Glx | 5.2 | 8.6 | 9.3 | 1.0 | 10.8 | 1.3 |

Asx: sum of Asp and Asn; Glx: sum of Glu and Gln.

Protein complex P60 showed only one major peak when fractionated by various liquid chromatography procedures, such as size exclusion chromatography, ion exchange chromatography and hydrophobic chromatography. P60 showed a major band on native PAGE and unreduced SDS-PAGE. Several subunits of P60 were revealed on reduced SDS-PAGE. These results indicate P60 is a protein complex consisting of several subunits linked by disulphide bridges. This oligomeric structure might be used to protect the P60 from degradation during the synthesis of nacre, as suggested by Bedouet et al. (2001). Several other protein complexes have also been isolated from *Macrocallista nimbosa* (Marsh, 1986), *P. maxima* (Bedouet et al., 2001) and *P. fucata* (Matsushiro et al., 2003).

By N-terminal sequencing and MALDI-TOF MS analysis, the known nacre protein nacrein and its two derivatives, N28 and N35, were identified as three components of the P60. It's likely that N28 and N35 derived from the posttranslational processing of nacrein. Based on the amino acid contents (Table 2), P60 had similar characteristics with several characterized proteins from *P. fucata*, i.e. they all contains a high content of four amino acids, glycine, aspartic acid, alanine and glutamic acid. These four amino acids could form the characteristic repeat sequences, which had been identified in many known nacre proteins and may be responsible for nacreous formation controlled by proteins. These characteristic repeat sequences include Gly–Xaa–Asn repeat sequence in nacrein (Miyamoto et al., 1996; Miyamoto et al., 2005), Asn–Gly repeat sequence in N16 (Samata et al., 1999), Gly–Ala repeat sequence in MSI60 and Gly–Glu repeat sequence in MSI30 (Sudo et al., 1997), and Gly-rich repeat sequence in MSI7 (Zhang et al., 2003). All these repeat sequences were believed to regulate the crystallization process of calcium carbonate. P60 had a high ratio of glycine (32.1%), aspartic acid (17.4%), alanine (13.6%), and glutamic acid (5.2%), suggesting a high probability for forming some repeat sequences in the protein complex P60, like Gly–Xaa–Asn (Xaa=Asp, Asn, or Glu), Asn–Gly, Gly–Ala or Gly–Glu.

At present, nacre organic materials, especially soluble protein, are considered to play an important role during the formation of nacre. In vitro, crystallization indicated that the soluble proteins can enhance or suppress calcium carbonate formation (Miyamoto et al., 1996; Kono et al., 2000; Weiss et al., 2000; Zhang et al., 2004). Under some special conditions, they can control and orient the calcium carbonate form (Samata et al., 1999). P60 play different functions from them: first, contacting crystal as a bridge to form the crystal chains or stacks. The protein complex P60 solution may act like cement to

“stick” the crystals together. Secondly, to a certain extent, P60 may inhibit big crystal formation. When the crystal is small, the stacks are well formed. Thirdly, it may induce aragonite formation which is a needle-like structure. Since the protein has a similar amino acid components to the proteins reported, we hypothesize that if the protein subunits have similar functions as the reported proteins, the protein complex P60 may show a composite effect of inhibiting, inducing or promoting crystal growth.

As reported in research on *P. maxima*, the soluble proteins could affect ALP activity and Bcl-2 expression of osteoblast precursor cells (Moutahir-Belqasmi et al., 2001; Mouries et al., 2002), which were the key factors in cell differentiating into mature osteoblasts, and forming bone nodules. For water soluble proteins of *P. maxima*, MC3T3-E1 cells showed that ALP activity was blocked and had an earlier mineralization within 6 days (Rousseau et al., 2003), but MSCs cells showed that ALP activity was enhanced and had a relative late mineralization for 14 days (Mouries et al., 2001). In our mineralization study of MC3T3-E1 cells and MSCs cells with P60, mineralized nodules in both the two cell had occurred in 8 days. The mineralization phenomena was weak in MSCs and strong in MC3T3-E1 cells. Differentiation of MC3T3-E1 cells into mature osteoblasts requires incubation in the presence of both GP and AA for at least 21 days, by which time mineralization has occurred (Matsumoto et al., 1991), and dexamethasone can caused a significant increase in the number of mineralized nodules in enzymatically released rat calvaria cells (Bellows et al., 1986). In our study cells cultured with Dex, GP and AA showed little mineralization nodules on day 8. This result seemed to suggest that protein complex P60 solution accelerated the differentiation of osteoblast precursor cells to an earlier mineralization.

Our findings indicated that soluble protein complex P60 of nacre from *P. fucata* contains the known protein nacrein and its two derivatives. P60 shares some similar physical and chemical characters with other known nacre proteins. Furthermore, P60 was showed to have bio-mineralization function in vitro. The further studies of P60 structure and its role in calcium carbonate growth and enhancement of the bone nodules formation in osteoblast precursor cells, are underway.

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