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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 powered 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.

Keywords: Bone marrow cells; MC3T3-E1 osteoblast; Mineralization nodules; Mother of pearl; Nacrein; *Pinctada fucata*

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 laeviagata* 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 HCO_3^- 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*-glycosyation sites. They are considered to influence $CaCO_3$ 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 $CaCO_3$ 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 $CaCO_3$ 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 $ZnSO_4$. 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^{SQ}, 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 $CaCO_3$ 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.

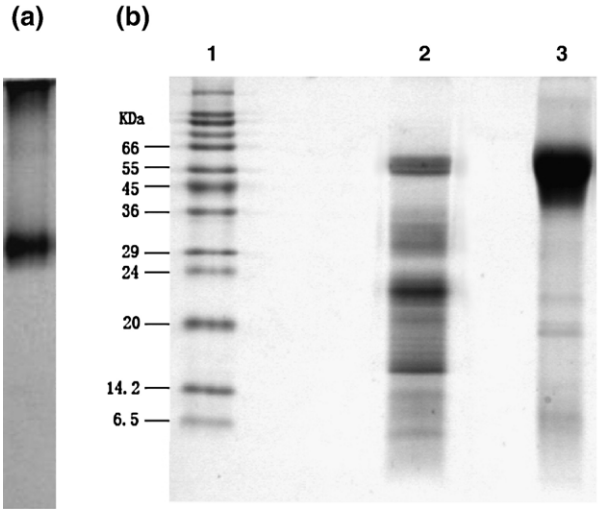

Fig. 1. Electrophoresis analyses of the protein complex P60 extracted from *Pinctada fucata*. (a) Native-PAGE analysis. (b) SDS-PAGE analysis. 1, molecular mass standards (Sigma M4038); 2, P60 treated with β-mercaptoethanol and SDS; 3, P60 treated with SDS only.

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 μg/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 μg/ml streptomycin. The cells were plated at a concentration of 2×10^6 cells/cm^2. The cells grew at 37 °C and 5% CO_2 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 μg/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.

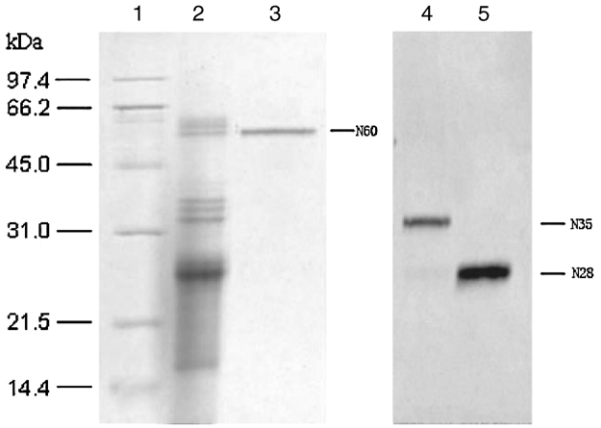

Fig. 2. SDS-PAGE analysis of the isolated N60, N35 and N28 proteins. 1, molecular mass standards; 2, P60; 3, N60; 4, N35; 5, N28.

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,

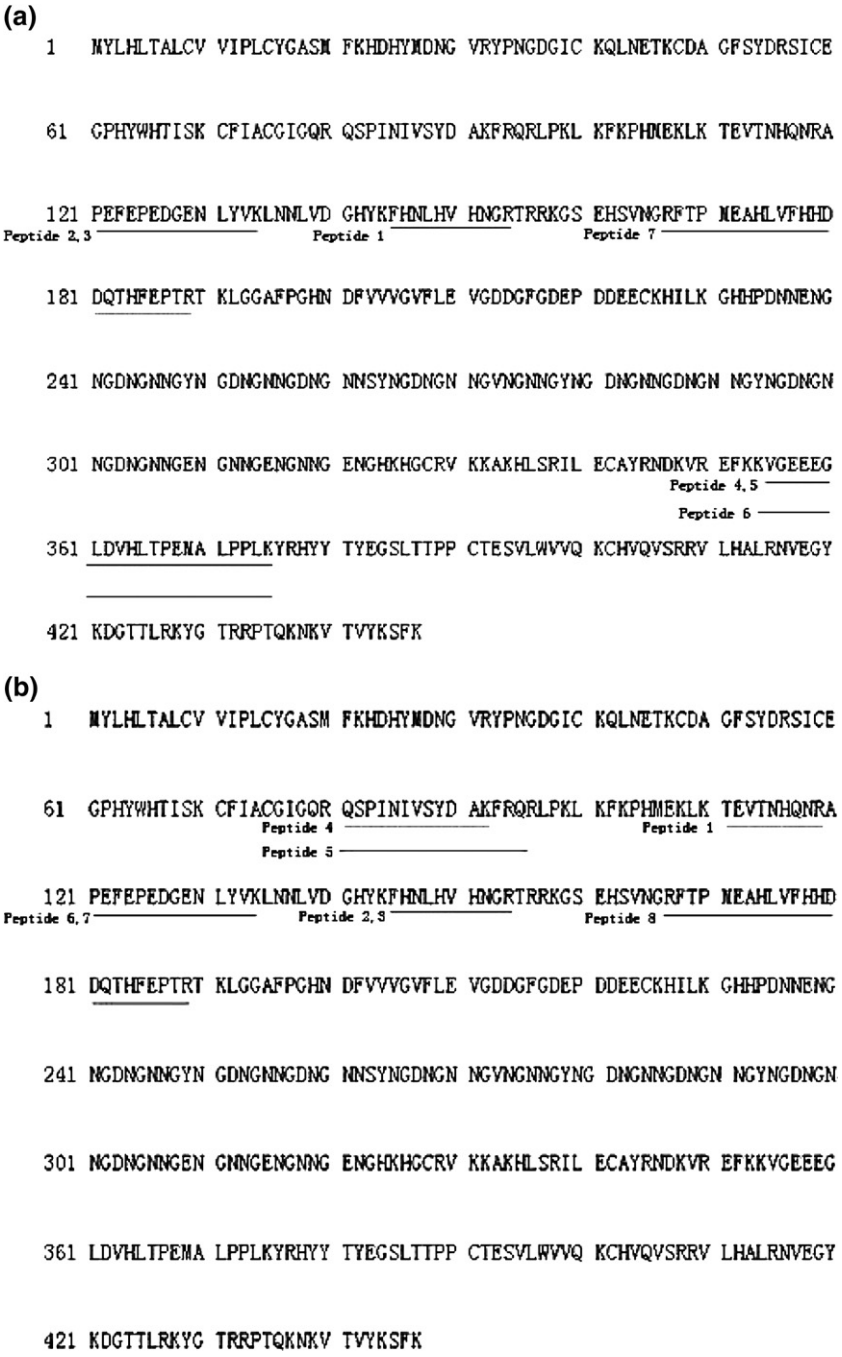

Fig. 3. Alignment of peptide sequences of N35 and N28 proteins with the sequence of nacrein. The amino acid sequence of nacrein (GenBank BAA11940) is shown in capital letters. Peptides of N35 and N28 proteins identified by MALDI-TOF MS analyses are underlined. (a) Seven peptides of N35 are identical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