Characterization of Prismaticin-14, a novel matrix protein from the prismatic layer of the Japanese pearl oyster (*Pinctada fucata*)

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The mollusc shell is a hard tissue consisting of calcium carbonate and organic matrices. The organic matrices are believed to play important roles in shell formation. In the present study, we extracted and purified a novel matrix protein, named Prismaticin-14, from the acid-insoluble fraction of the prismatic layer of the shell of the Japanese pearl oyster (*Pinctada fucata*), and determined its whole amino acid sequence by a combination of amino acid sequence analysis and MS analysis of the intact protein and its enzymic digests. Prismaticin-14 consisted of 105 amino acid residues, including PIYR repeats, a Gly/Tyr-rich region and N- and C-terminal Asp-rich regions. Prismaticin-14 showed inhibitory activity on calcium carbonate precipitation and calcium-binding activity *in vitro*. The scanning electron microscopy images revealed that Prismaticin-14 affected the crystallization of calcium

carbonate *in vitro*. A cDNA encoding Prismaticin-14 was cloned and its expression was analysed. The amino acid sequence deduced from the nucleotide sequence of Prismaticin-14 cDNA was identical with that determined by peptide sequencing. Northern-blot analysis showed that a Prismaticin-14 mRNA was expressed only at the mantle edge. *In situ* hybridization demonstrated that a Prismaticin-14 mRNA was expressed strongly in the inner side of the outer fold of the mantle. These results suggest that Prismaticin-14 is a framework protein that plays an important role in the regulation of calcification of the prismatic layer of the shell.

Key words: biomineralization, calcification, matrix protein, mollusc shell, pearl oyster, prismatic layer.

INTRODUCTION

Biomaterials are hard tissues that consist mainly of inorganic compounds. They are observed in many organisms and their functions include maintenance of the body structure, protection from enemies, magnetic and gravity sensing and storage of minerals [1]. They also contain a small amount of organic matrices that are believed to play important roles in their formation. Many organic matrices have been identified from various biomaterials, e.g. bone, teeth, coccoliths, otoliths, exoskeleton of crustaceans, seashells, eggshells and bacterial magnetite [2–6]. Many of these matrices are acidic compounds and are supposed to be responsible for mineralization. These acidic matrices are supposed to interact specifically with crystal surfaces to induce orientated nucleation, to intercalate into the crystal lattice itself, to determine the mineral phase or to function as the framework of biomaterials [7].

The shell of the pearl oyster consists of two mineralized layers, nacreous and prismatic layers. Both the layers are composed of calcium carbonate and an organic matrix. Previous works on mollusc shells showed that the major components of organic matrices are Asp-rich calcium-binding proteins [7,8]. In the course of shell formation, the periostracum, which is not mineralized and covers the external surface of the shell, is formed first; subsequently, the prismatic layer is formed on the periostracum. Finally, the nacreous layer is formed on the prismatic layer [9]. The shell makes contact with the mantle, which supplies the

two layers with inorganic ions and organic matrices through the extrapallial fluid.

The nacreous layer is made of aragonite. The aragonite crystal compartment in the nacreous layer is sandwiched between sheets of organic matrices. Many proteins, such as nacrein [10], MSI60 [11], lustrin A [12], N16 [13], Mucoperlin [14] and Perluslin [14], have been identified from the nacreous layer. Nacrein has carbonic anhydrase activity and may supply the shell with carbonate ions. MSI60 is rich in alanine residues and may constitute the framework of the nacreous layer. Lustrin A has eight tandem repeats of a Gly/Cys-rich and a Pro-rich region, and is homologous with the organic matrices from bone and eggshell. Thus various matrix proteins from the nacreous layer have been extensively characterized.

In contrast, little is known about the matrix proteins of the prismatic layer. The prismatic layer is composed of columnar calcite surrounded by organic matrices. Only a matrix protein MSI31 has been characterized from the prismatic layer [11]. MSI31 has a Gly-rich region at the N-terminus and acidic repeats of ESEED-X at the C-terminus. MSI31 is probably a framework protein in the prismatic layer. However, the function of this protein *in vivo* is not known.

As a first step towards clarifying the mechanism of shell formation, we tried to identify the organic matrices associated with calcification. In the present study, we report the isolation and determination of the complete amino acid sequence of a novel

Abbreviations used: CAP-1, calcification-associated peptide; CBB, Coomassie Brilliant Blue; DIG, digoxigenin; DTT, dithiothreitol; GAMP, gastrolith matrix protein; ORF, open reading frame; RACE, rapid amplification of cDNA ends; RT, reverse transcriptase; SEM, scanning electron microscopy; TFA, trifluoroacetic acid.

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matrix protein, named Prismalin-14, from the prismatic layer of the Japanese pearl oyster (*Pinctada fucata*). Functional and expression analyses of Prismalin-14 were also performed.

EXPERIMENTAL

Animals and collection of shells

The Japanese pearl oyster *P. fucata* was cultured at the Omura Bay (Nagasaki Prefecture, Japan) and only the shells were sent to our laboratory in Tokyo and stored at room temperature (10–20 °C) for protein analyses.

RNA samples for cDNA cloning were prepared from live Japanese pearl oysters, which were supplied by the Fisheries Research Division, Mie Prefectural Science and Technology Promotion Center, Japan.

Extraction and purification of the organic matrix

Shells (50 g) were decalcified in 1 litre of 1 M acetic acid for 1 week at 4 °C, and the water-insoluble material originating from the prismatic layer was collected. This insoluble material was washed with distilled water and treated with 50 ml of 1 % SDS/10 mM DTT (dithiothreitol)/50 mM Tris/HCl (pH 8.0) at 100 °C for 10 min. The extract from the nacreous layer was also obtained in a similar manner. The extract from the prismatic layer was passed through a Sep-Pak Plus C₁₈ environmental cartridge (Waters, Milford, MA, U.S.A.), which was eluted with 80 % (v/v) acetonitrile/0.05 % TFA (trifluoroacetic acid). The eluate was concentrated, and the concentrate was subjected to reverse-phase HPLC on a PEGASIL-300 C4P column (4.6 mm × 250 mm; Senshu Kagaku, Tokyo, Japan). Elution was performed first with 0.05 % TFA for 5 min and then with a gradient of 0–60 % acetonitrile/0.05 % TFA for 30 min at a flow rate of 1 ml/min. Protein elution was monitored by measuring the absorbance at 225 nm. Fractions were collected manually. The amount of protein was estimated by the Bradford method using BSA (Nacalai Tesque, Kyoto, Japan) as a standard [15].

SDS/PAGE

The crude extract and the protein purified by reverse-phase HPLC were subjected to SDS/PAGE (15 % gel) under reduced conditions. After electrophoresis, the gel was stained with CBB (Coomassie Brilliant Blue; Nacalai Tesque) or Stains-all (Sigma, St. Louis, MO, U.S.A.).

Enzymic digestion

Prismalin-14 (5 µg) was dissolved in 300 µl of 2 M urea/1 % ammonium bicarbonate. To each solution, 1 µg of trypsin (Sigma), thermolysin (Sigma) or pepsin (Sigma) was added. Each mixture was incubated for 18 h at 37 °C, and 200 µl of 1 M HCl was added to stop the digestion. Each digest was separated by reverse-phase HPLC with a Capcell Pak C₁₈ column (2.0 mm × 250 mm; Shiseido, Tokyo, Japan). Elution was performed first with 0.05 % TFA for 5 min and then with a gradient of 0–40 % acetonitrile/0.05 % TFA for 40 min at a flow rate of 0.2 ml/min. Protein elution was monitored by measuring the absorbance at 225 nm.

Prismalin-14 (5 µg) was dissolved in 300 µl of 10 mM DTT/1 mM EDTA/50 mM sodium phosphate buffer (pH 7.0). To this solution, 0.5 µg of pyroglutamate aminopeptidase (Takara, Kyoto, Japan) was added. The resulting mixture was incubated for 18 h at 37 °C and 200 µl of 1 M HCl was added to stop the digestion.

MS analysis

Mass spectra were measured on a matrix-assisted laser-desorption/ionization–time-of-flight mass spectrometer (Voyager-DE STR; Applied Biosystems, Foster City, CA, U.S.A.) with cinapic acid, α -cyano-4-hydroxycinnamic acid or dehydrobenzoic acid as the matrix.

Amino acid sequencing

The amino acid sequences of the enzymic fragment peptides were analysed on a protein sequencer (model 476A, 492HT or 491cLC, Applied Biosystems).

Inhibition of calcium carbonate precipitation

Effect of Prismalin-14 on calcium carbonate precipitation from its supersaturated solution was examined by the method described previously [16]. In brief, formation of calcium carbonate precipitates was monitored by recording the turbidity of a solution containing 100 µl of 20 mM NaHCO₃ (pH 8.7) and 10 µl of sample solution after the addition of 100 µl of 20 mM CaCl₂ to the solution. Changes in the turbidity of the solutions were measured every 1 min for 5 min by the absorbance at 570 nm using a spectrophotometer.

Calcium-binding assay

Calcium-binding activity of Prismalin-14 was characterized using the ⁴⁵Ca²⁺ overlay analysis [17]. Prismalin-14 blotted on to PVDF membranes was incubated in a 10 mM imidazole/HCl buffer (pH 6.8) containing ⁴⁵Ca²⁺ (1 µM, 20 kBq/ml) in the absence or presence of Ca²⁺ (100 mM) for 10 min and subsequently washed with distilled water for 5 min. The ⁴⁵Ca²⁺ bound to protein bands was detected on an FLA 2000 computerized densitometer scanner (Fuji Film, Tokyo, Japan).

Observation of calcium carbonate morphology with SEM (scanning electron microscopy)

A solution of CaCl₂ was added to two wells of a 96-well plate to a final concentration of 20 mM. Next, Prismalin-14 was added to one well to a final concentration of 2 µM, whereas distilled water was added to the other well as a control. Each total volume was 200 µl. This plate was placed in a closed desiccator that also contained solid (NH₄)₂CO₃ crystals. Sublimation of (NH₄)₂CO₃ at 20 °C for 48 h yielded a saturated calcium carbonate solution. After incubation, the resulting crystals in the solution were taken up, air-dried and analysed on a scanning electron microscope (Hitachi S-4000, Tokyo, Japan).

RT (reverse transcriptase)–PCR

Total RNA was prepared from the mantle using ISOGEN (Nippongene, Tokyo, Japan) according to the manufacturer's instructions. First-strand cDNA was synthesized with 1 µg of total RNA using a SMARTTM RACE cDNA Amplification kit (Clontech, Palo Alto, CA, U.S.A.) according to the manufacturer's instructions. For RT–PCR, four degenerate oligonucleotide primers, F, R, NF and NR, were designed based on the amino acid sequences.

A cDNA fragment of Prismalin-14 was amplified by two rounds of PCR. In the first PCR, the first-strand cDNA was used as a template, and the amplification was primed by the set of primers F/R (F, AACGGITAYTTYGGNTAYTT, and R, TCICCGAANC-CRTARTANCC). In the second PCR, the first PCR product was

used as a template, and the amplification was primed by the set of nested primers NF/NR (NF, GGITACTTYGGNTAYTTY-CC, and NR, CCGAAICRTARTANCCRTA). The following programme was used for PCR amplification: 30 cycles of 30 s at 94 °C (3 min 30 s only for the first cycle), 30 s at 55 °C and 1 min at 72 °C (3 min only for the last cycle).

5'- and 3'-RACE (rapid amplification of cDNA ends)

Two specific primers, 5R and 5RN, were prepared based on the nucleotide sequence of the Prismalin-14 cDNA fragment amplified by RT-PCR. A cDNA fragment encoding the 5'-region of Prismalin-14 was amplified by two rounds of PCR. In the first PCR, the first-strand cDNA was used as a template, and the amplification was primed by the set of primers UPM (universal primer mix)/5R (UPM: a mixture of CTAATACGACTCACTATA-GGGCAAGCAGTGGTAACAACGCAGAGT and CTAATACG-ACTCACTATAGGC; and 5R: TATAATCCTAGTCCCTCCGTA-ACCACCGT). In the second PCR, the first PCR product was used as a template, and the amplification was primed by the set of primers NUP (nested universal primer)/5RN (NUP: AAGC-AGTGGTAACAACGCAGAGT; and 5RN: TCCTAGTCCTCC-GTAACCACCGTTAAAT). The following programme was used for PCR amplification: 5 cycles of 5 s at 94 °C and 3 min at 72 °C; 5 cycles of 5 s at 94 °C, 10 s at 70 °C and 3 min at 72 °C; and 25 cycles of 5 s at 94 °C, 10 s at 68 °C and 3 min at 72 °C.

Two specific primers, 3R and 3RN, were prepared based on the nucleotide sequence of the Prismalin-14 cDNA fragment amplified by 5'-RACE. A cDNA fragment encoding the 3'-region of Prismalin-14 was amplified by two rounds of PCR. In the first PCR, the first-strand cDNA was used as a template, and the amplification was primed by the set of primers 3R/RTG (3R: AAAGAAATACTTAACTGGTGCTA; and RTG: AACTGGAA-GAATTCGCGGCCG). In the second PCR, the first PCR product was used as a template, and the amplification was primed by a set of 3RN/RTG-N (3RN: TTAAGTGGTGCTACATTTCCATT; and RTG-N: TGGAAGAATTCGCGGCCGCAG). The following programme was used for PCR amplification: 30 cycles of 30 s at 94 °C (3 min 30 s only for the first cycle), 30 s at 55 °C and 1 min at 72 °C (3 min only for the last cycle).

Confirmation of the nucleotide sequence of Prismalin-14 cDNA

The nucleotide sequence of Prismalin-14 cDNA obtained by RACE over the coding region was confirmed by PCR amplification using the specific primers 3R/Prismalin-14-R (3R: AAA-GAAATACTTAACTGGTGCTA; and Prismalin-14-R: CATG-AGCAGCCCCGGGTCCAAA).

Nucleotide sequence analysis

All PCR products were ligated into a pCR 2.1 vector (Invitrogen, Carlsbad, CA, U.S.A.) using a TA Cloning kit (Invitrogen) according to the manufacturer's instructions. Both strands of the inserted DNA were sequenced on a Long-Read Tower™ DNA sequencer (Amersham Biosciences, Little Chalfont, Bucks., U.K.) using a Thermo Sequenase Cy5 and Cy5.5 Dye Terminator Cycle Sequencing kit (Amersham Biosciences).

Northern-blot analysis

Total RNAs were prepared separately from the mantle edge, the dorsal mantle, gill, muscle, stomach and gonad of *P. fucata*. The samples of total RNA were subjected to electrophoresis on a 1 % agarose gel in 40 mM 3-(*N*-morpholino)-propanesulphonic

acid (pH 7.0), containing 18 % (v/v) formamide and blotted on to a Hybond N⁺ nylon membrane (Amersham Biosciences). These RNA samples were probed with the Prismalin-14 cDNA fragment corresponding to nucleotides 18–498. Labelling of the cDNA probe, hybridization, washing and detection were performed using the Alkphos Direct Labeling and Detection System with CDP-*Star* (Amersham Biosciences) according to the manufacturer's instructions. Chemiluminescence of the blot was detected on a lumino image analyser, LAS-1000 Plus (Fuji Film).

In situ hybridization

In situ hybridization of Prismalin-14 mRNA was performed on paraffin sections. Sense and antisense DIG (digoxigenin)-labelled RNA probes were transcribed *in vitro* from the cDNA encoding Prismalin-14 using a DIG RNA Labelling kit (Roche, Basel, Switzerland).

The mantle tissues were fixed overnight in 10 mM phosphate buffer (pH 7.4) containing 4 % (w/v) paraformaldehyde and then dehydrated in ethanol and embedded in paraffin. Cross-sections (8 µm thick) were de-paraffinized in ethanol and rehydrated before prehybridization in 50 % formamide/5 × SSC/5 × Denhardt's/baker's yeast RNA (250 µg/ml)/salmon sperm DNA (500 µg/ml) and hybridization with DIG-labelled sense or antisense RNA probes (0.5 µg/ml). Hybridization was performed overnight at 60 °C. After hybridization, the tissue sections were washed with 0.2 × SSC at 60 °C for 2 h and incubated with anti-DIG-alkaline phosphatase, Fab fragments (Roche) dissolved in 0.1 M Tris/HCl (pH 7.5)/0.15 M NaCl for 18 h at 4 °C. The reaction with alkaline phosphatase was carried out by incubation with a Nitro Blue Tetrazolium/5-bromo-4-chloroindol-3-yl phosphate stock solution (Roche) dissolved in 0.1 M Tris/HCl (pH 7.5)/0.1 M NaCl/0.05 M MgCl₂ for 4 h. The sections were washed in TE buffer (10 mM Tris/HCl, pH 7.5 and 1 mM EDTA) to stop the reaction, then dehydrated in ethanol, cleared in xylene and mounted with Bioleat (Okensoji, Tokyo, Japan).

RESULTS

Isolation of a protein from the prismatic layer of *P. fucata*

Organic matrices were extracted separately from the prismatic and nacreous insoluble materials of the decalcified shell of the Japanese pearl oyster *P. fucata* using the SDS/DTT solution. Both extracts were analysed by SDS/PAGE. Three major bands were detected after CBB staining in the extract of the prismatic layer but not in that of the nacreous layer (Figure 1A). Of the three bands, only the 14 kDa protein stained blue with Stains-all, suggesting that this protein was negatively charged (Figure 1B). To purify this 14 kDa protein, the extract was subjected to reverse-phase HPLC, and the eluate was divided into seven fractions (Figure 2). The shaded fraction contained the 14 kDa protein, and yielded a single band on SDS/PAGE. Since this 14 kDa protein was specific to the prismatic layer (Figure 1A), we designated it Prismalin-14. Estimation of the amount of protein by the Bradford method showed that the yield of Prismalin-14 was approx. 4.5 µg/g of dry shell.

Determination of the amino acid sequence of Prismalin-14

The mass spectrum of Prismalin-14 showed a singly protonated ion peak at *m/z* 11 890.8. N-terminal amino acid sequence analysis of Prismalin-14 by Edman degradation did not yield any sequence, suggesting that the N-terminus was blocked. Hence, digestion of

Figure 1 SDS/PAGE of extracts from insoluble matrices in the prismatic and nacreous layers

Extracts from the insoluble matrices in the prismatic and nacreous layers were separated by SDS/PAGE and visualized by staining with CBB (A) or Stains-all (B). Molecular-mass standards with masses in kDa are indicated on the left. Lane 1, extract from the prismatic layer; lane 2, extract from the nacreous layer.

Figure 2 Purification of Prismaticin-14 by reverse-phase HPLC

Prismaticin-14 was purified by reverse-phase HPLC with a PEGASIL-300 C4P column (4.6 mm × 250 nm). Elution was performed with 0–60% acetonitrile in 0.05% TFA at a flow rate of 1 ml/min; ---, concentration of acetonitrile. Prismaticin-14 was recovered from the shaded peak.

Prismaticin-14 with pyroglutamate aminopeptidase was tried. The mass of the resulting product after digestion decreased to 11 784.2. The difference in mass values and the substrate specificity of the enzyme indicated that the N-terminal amino acid residue of Prismaticin-14 was pyroglutamate. N-terminal amino acid sequence

Figure 3 Amino acid sequence analysis of Prismaticin-14

Peptides used for sequencing are indicated as follows: T1–T4, tryptic peptides; Th1, thermolysin peptide; P1–P3, pepsin peptides; Nt, the N-terminal sequence after digestion with pyroglutamate aminopeptidase. < Q indicates a pyroglutamate residue.

Table 1 Amino acid sequence analysis and MS analysis of trypsin, thermolysin and pepsin digests

	Amino acid sequence	<i>m/z</i> (<i>M</i> + <i>H</i>) ⁺	Calculated molecular mass (<i>M</i> + <i>H</i>)
Trypsin			
T1	—*	744.0	N.C.†
T2	GGDDDDNGFFGGDDDDNGYFGYFPR	2505.9	2505.4
T3	—	3230.8	N.C.
T4	FSYPIYRPIYRPIIYYPQIIRPFYGYGY	8679.3	N.C.
Thermolysin			
Th1	LYGGYGGFGGYGYRP	1584.9	1584.7
Pepsin			
P1	YGFDDDDGGFDD	1443.2	1443.4
P2	YGYGGFNGGYGGLGL	1452.7	1452.5
P3	GGYGYRPFPSYGYNPFPSYGY	2216.7	2216.3

* The amino acid sequence was not identified.
† The molecular mass was not calculated.

analysis of the digested product unambiguously identified 34 amino acid residues (Figure 3).

To determine the amino acid sequence of the remaining part of Prismaticin-14, it was digested with some enzymes. The tryptic digest was separated by reverse-phase HPLC to afford four major fragments, named T1–T4, which were subjected to MS analysis and amino acid sequence analysis (Table 1). Their mass values suggested that T3 consisted of T1 and T2, and T3 and T4 constitute the whole molecule of Prismaticin-14. The mass value of only T2 agreed well with that calculated from the amino acid sequence. Neither T1 nor T3 showed any sequence, suggesting that they were N-terminal fragments. Since the amino acid sequence analysis of T4 identified only 36 amino acid residues, some 40 residues were yet to be determined.

Thermolysin and pepsin digestions and subsequent separations by reverse-phase HPLC afforded one and four major fragments respectively (Table 1), which were then subjected to MS analysis and amino acid sequence analysis. MS data of these fragments agreed well with the mass values calculated from the amino acid sequences.

By combining all the sequences of the enzymic digests, the complete amino acid sequence of Prismaticin-14 could be

Figure 4 Inhibitory activity of Prismalin-14 on calcium carbonate precipitation

Changes in the turbidity of the assayed solutions are shown. ○, 10 mM Tris/HCl (pH 7.5) alone; ●, 0.2 μM Prismalin-14; △, 0.4 μM Prismalin-14; and ▲, 2.0 μM Prismalin-14.

determined (Figure 3). The observed mass value of Prismalin-14 [11890.8, ($M + H$)⁺] was almost identical with its calculated value [11888.5, ($M + H$)⁺]. Prismalin-14 is composed of 105 amino acid residues and contains only 11 kinds of amino acids. There are many hydrophobic residues in the central part and many hydrophilic residues in both the terminal parts. It has pyroglutamate at the N-terminus, four tandem PIYR repeats from Pro³² to Arg⁴⁸, a Gly/Tyr-rich region from Tyr⁵¹ to Gly⁹⁷ and two Asp-rich regions in the N- and the C-terminal sections.

Inhibitory activity of Prismalin-14 on calcium carbonate precipitation and calcium-binding activity

The inhibitory activity of Prismalin-14 on the precipitation of calcium carbonate from its supersaturated solution was examined by the method of Inoue et al. [16]. The precipitation of calcium carbonate was monitored using a spectrophotometer. Prismalin-14 inhibited calcium carbonate precipitation in a dose-dependent manner and completely inhibited it at a concentration of 2.0 μM (Figure 4).

In view of the inhibitory activity of Prismalin-14 on calcium carbonate precipitation, we tested its ability to bind calcium. The crude extract from the prismatic layer and purified Prismalin-14 were subjected to SDS/PAGE, followed by blotting on to a PVDF membrane; the membrane was then subjected to ⁴⁵Ca²⁺ overlay analysis (Figure 5A). In the lane loaded with the crude extract, a major band was detected at approx. 14 kDa, whereas a minor band was observed in the high-molecular-mass region. The purified Prismalin-14 bound ⁴⁵Ca²⁺ (Figure 5A). In the unlabelled inhibition experiments, no signal was detected (Figure 5B). These results indicated that Prismalin-14 had an ability to bind calcium ion specifically and was a major component of the calcium-binding proteins in the crude extract.

Morphology of the crystals induced by Prismalin-14 *in vitro*

To investigate the effect of Prismalin-14 on the morphology of calcium carbonate crystals, the crystals precipitated *in vitro* were examined by SEM. Crystals in the control experiment were completely hexahedral, which was a typical morphology of calcite (Figure 6A). In the presence of Prismalin-14, the overall morphology of the crystals was as a hexahedron as in the control experiment, but the surface became rough and steps were formed (Figures 6B and 6C).

Figure 5 Calcium-binding analysis of the extract from insoluble matrices in the prismatic layer and purified Prismalin-14

(A) Calcium-binding assays were performed in a buffer containing ⁴⁵Ca²⁺ (1 μM, 20 kBq/ml). (B) Unlabelled inhibition experiments were performed in the same buffer containing ⁴⁵Ca²⁺ (1 μM, 20 kBq/ml) and Ca²⁺ (100 mM). Lane 1, extract from insoluble matrices in the prismatic layer; lane 2, purified Prismalin-14.

Molecular cloning of a cDNA encoding Prismalin-14

To elucidate the structure of the precursor of Prismalin-14, a cDNA encoding Prismalin-14 was cloned. First, a cDNA fragment encoding Prismalin-14 was amplified by RT-PCR using degenerate oligonucleotide primers designed based on the amino acid sequence of Prismalin-14. Next, the 5'- and 3'-regions of the cDNA were amplified by 5'- and 3'-RACE respectively using specific primers, which were synthesized based on the nucleotide sequences obtained by RT-PCR and 5'-RACE respectively. Finally, to confirm the whole nucleotide sequence of the cDNA, the cDNA fragment covering the ORF (open reading frame) was amplified using specific primers corresponding to the 5'-UTR (5'-untranslated region) and 3'-UTR.

The nucleotide and deduced amino acid sequences of the cDNA encoding Prismalin-14 are shown in Figure 7. The cDNA was of 569 bp, comprising a 5'-UTR (64 bp), an ORF (363 bp), a stop codon (TAA) and a 3'-UTR (139 bp). The ORF encoded a precursor of Prismalin-14 comprising a signal peptide and Prismalin-14. The 16-amino-acid peptide from Met⁻¹⁶ to Ala⁻¹ included a high proportion of hydrophobic amino acid residues and, therefore, it was probably a signal peptide. The amino acid sequence from Gln¹ to Asp¹⁰⁵ was completely identical with that of Prismalin-14 determined previously by peptide sequencing.

Tissue-specific gene expression of Prismalin-14

To examine the tissue-specific gene expression of Prismalin-14, Northern-blot analysis was performed. Samples of total RNA were prepared separately from the mantle edge, the dorsal mantle, gill, muscle, stomach and gonad of *P. fucata*. A transcript of approx. 0.6 kb, whose size agreed well with that of the Prismalin-14 cDNA, was clearly detected only in the total RNA from the mantle edge (Figure 8).

Figure 6 Morphological changes in calcium carbonate crystals induced by Prismalin-14

SEM images of a calcium carbonate crystal precipitated *in vitro* from a supersaturated calcium carbonate solution (A) without Prismalin-14 as a control and (B) with Prismalin-14. (C) Enlarged image of the boxed part in (B). Scale bar: (A) 15 μm , (B) 100 μm and (C) 15 μm .

Prismalin-14 mRNA expression at the mantle edge

To determine the more precise expression site of Prismalin-14 mRNA in the mantle tissue of *P. fucata*, *in situ* hybridization was performed with paraffin sections (Figure 9). The hybridization signals for Prismalin-14 mRNA detected in the inner epithelial cells of the outer fold were strong signals and those detected in a part of the inner epithelial cells of the inner fold were weak ones. No hybridization signal was detected at the

dorsal mantle or in the middle fold. Hybridization with a control sense probe gave no significant signals.

DISCUSSION

In the present study, we have isolated and characterized a novel, prismatic layer-specific protein named Prismalin-14. Since Prismalin-14 is an acid-insoluble, detergent-soluble macromolecule, it may bind rather tightly to other insoluble macromolecules, such as chitin, and therefore serves as a framework constituent controlling nucleation of calcium carbonate crystals or crystalline polymorphism.

Prismalin-14 is the second macromolecule so far characterized from the prismatic layer of *P. fucata*. Prismalin-14 consists of 105 amino acid residues with an N-terminus blocked by a pyroglutamate residue (Figure 3). It has a peculiar amino acid composition comprising only 11 kinds of amino acids with high proportions of glycine (27.6%) and tyrosine (20.0%) residues. According to a previous report [18], high contents of glycine (23.0%) and tyrosine (12.3%) were also found in the crude matrix proteins from the prismatic layer of *P. fucata*. These findings suggest the possibility that the other proteins having a similar amino acid composition also exist, although such proteins have not been isolated so far. Prismalin-14 is an acidic protein with a pI value of 3.98, as determined by staining of SDS/polyacrylamide gels with Stains-all (Figure 1). Acidic amino acid residues are located near both the N- and C-termini. This acidic nature of Prismalin-14 is similar to that of many other macromolecules isolated and characterized from various biominerals and is supposed to be essential for direct interactions with calcium ions and/or the surface of calcium carbonate crystals [19,20]. Indeed, Prismalin-14 showed an ability to bind calcium ions (Figure 5) and showed inhibitory activity on calcium carbonate crystallization in the *in vitro* assay (Figure 3).

Prismalin-14 has a Gly/Tyr-rich region from Tyr⁵¹ to Gly⁹⁷. The amino acid sequence of this region is similar to that of keratin [21,22] produced by vertebrates to some extent, and Prismalin-14 shares common functions with keratin as an extracellular matrix protein, although the functions of other parts of Prismalin-14 are completely different. This region may also form the structural motif termed as the glycine loop. The glycine loop may contribute to the elasticity and flexibility of the molecular conformation [23,24]. The prediction of the secondary structure of Prismalin-14 suggested that the region of the PIYR repeat from Pro³² to Arg⁴⁸ has a β -strand conformation. Many matrix proteins identified so far have characteristic repeating sequences consisting of amino acid residues ranging from 3 to 98 residues, but the PIYR repeat is unique among them. Although the significance of the repeated sequence has not been clarified, it may be related to the regular arrangement of atoms in inorganic crystals or to the repeated structure of polymers such as chitin. As described above, Prismalin-14 may bind to chitin in the prismatic layer, but it does not have a known chitin-binding consensus sequence. Therefore Prismalin-14 may have a novel chitin-binding motif. Prismalin-14 has no sequence similarity to MSI31, which is another protein characterized from the prismatic layer of *P. fucata*.

Prismalin-14 inhibited calcification in a dose-dependent manner (Figure 4). Least inhibition was observed at 0.2 μM and maximum inhibition was attained at 2.0 μM . This activity is weaker than those of CAP-1 (calcification-associated peptide) isolated from crayfish exoskeleton and of GAMP (gastrolith matrix protein) from crayfish gastrolith by one or two orders of magnitude with the same assay system. The strength of activity may be related to the number and distribution of acidic amino acid

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1  ACGCGGGACACAGTCCCAAGAAATACTTAACTGGTGCTACATTTCATTTCATCAGCAACAACAAATGCGATCTCTGCTAGTCTCTCTTGC
-16                                     M R S L L V L L A

91  CTTAGTCTGCTTGTGCCAGTGCACAGTACTTCTCCGTGGTGGGACGATGACAACGGTTTCTTCGGAGGAGATGACGACAACGGCTACTT
-7  L A A C A S A Q Y F F R G G D D D N G F F G G D D D N G Y F
    -1 1

181 CGGCTATTTCGCCGTTTCTCTATCCCATTTACCGTCCAAATCTACCGCCCAATTACTATCTCAATCATAAGACCATTCTACGGCTA
24  G Y F P R F S Y P I Y R P I Y R P I Y Y P Q I I R P F Y G Y

271 TGGGTATGGCGGATTTAACGGTGGTTACGGAGGACTAGGATTATATGAGGATATGGTGGGTTTCGGAGGCTATGGGTACAGGCCATTCTC
54  G Y G G F N G G Y G G L G L Y G G Y G G F G G Y G Y R P F S

361 TTACGGATACAATCCATTCTCTTATGGATACTACGGATTGGTGACGATGATGGCGGATTGGATGATTAAGTTGACGGTACTTTCTAGCG
84  Y G Y N P F S Y G Y Y G F G D D D G G F D D *

451 GAATCCAGCGAATCCATGTCATTGTTTTTGGACCGGGCTGCTCATGTTGTTACCATGTCCTTTGTTTCGTTCTTTGTTGTTGTTGA

541 TTTCATCACAAAATATACTATTATATTT (A)n

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Figure 7 Nucleotide and deduced amino acid sequences of a cDNA for the Prismalin-14 precursor of the Japanese pearl oyster *P. fucata*

The deduced amino acid sequence is shown below their respective codons. The nucleotides and amino acids are numbered on the left. The putative signal peptide is shown underlined; ---, the putative polyadenylation signal (AATATA).

Figure 8 Tissue-specific gene expression of Prismalin-14 by Northern-blot analysis

Samples of total RNA (10 µg), prepared from the mantle edge (lane 1), the dorsal mantle (lane 2), gill (lane 3), muscle (lane 4), stomach (lane 5) and gonad (lane 6), were subjected to Northern-blot analysis. The blot was probed with alkaline phosphatase-labelled Prismalin-14 cDNA and visualized with a lumina image analyser. The lower panel shows rRNA stained with ethidium bromide before blotting.

residues in each protein. Prismalin-14 has two (Asp)₃ sequences in the N-terminal part and one (Asp)₃ sequence in the C-terminal part, whereas CAP-1 has a cluster of acidic residues, Ser(PO₄)-Ser-Glu-(Asp)₆, at the C-terminus and two sets of triple acidic residues each in the N-terminal and central parts [16]. In the N-terminal part of GAMP, there are 17 tandem repeats of a ten-amino-acid sequence containing the acidic amino acid residues, QAAQEQAQEG [25]. For the three compounds, the order of inhibitory activity is GAMP > CAP-1 > Prismalin-14. However, the intensity of activity *in vitro* does not necessarily reflect the importance of the activity in the tissue *in vivo*. The inhibitory activity may indicate that Prismalin-14 binds to the surface of the fine crystals first formed in the supersaturated solution of calcium carbonate to stop further crystal growth. In fact, the SEM examination of the calcium carbonate crystals precipitated *in vitro* from the supersaturated calcium carbonate solution using

Figure 9 Detection of Prismalin-14 mRNA in the mantle of *P. fucata* by *in situ* hybridization

Hybridization signals with an anti-sense probe were observed in the inner side of the outer fold and at the central part of the inner side of the inner fold. Hybridization signals are indicated by arrowheads. The inset shows a control section stained with a sense probe. The signal in the middle fold was non-specific. OF, outer fold; MF, middle fold; IF, inner fold. Scale bar, 0.5 mm.

Prismalin-14 as a control showed changes in the morphology of the crystal when compared with the control (Figure 6). The surface of crystals became rough and many steps were formed. This phenomenon shows that Prismalin-14 probably binds steps and kink sites and inhibits the layer-by-layer growth of calcium carbonate crystals. Prismalin-14 is a major calcium-binding protein in the prismatic layer as indicated by incubation with ⁴⁵Ca²⁺ (Figure 5). This result also indicates that Prismalin-14 has an effect on calcification.

Since the mantle is responsible for shell formation and is supposed to be the site of synthesis of Prismalin-14, we prepared mRNA from the mantle of *P. fucata* and, using this mRNA preparation, cloned a cDNA encoding Prismalin-14 (Figure 7). The ORF encoded a precursor protein consisting of a signal peptide and Prismalin-14. The existence of a signal peptide indicated that Prismalin-14 is secreted from the mantle epithelial cells. The deduced amino acid sequence of Prismalin-14 was completely identical with that determined by peptide sequencing. The

Figure 10 Schematic representation of the possible functions of each part of Prismalin-14

The N- and C-terminal Asp-rich region of Prismalin-14 may bind Ca^{2+} , the PIYR repeat may form a β -sheet and the Gly/Tyr-rich region may possess a random structure that gives flexibility to Prismalin-14.

N-terminal residue was encoded as a glycine residue, which is probably converted into a pyroglutamate residue posttranslationally, thereby being resistant to degradation. The specific distribution of Prismalin-14 in the prismatic layer was demonstrated by SDS/PAGE (Figure 1), and Prismalin-14 mRNA was detected only in the tissue from the mantle edge by Northern-blot analysis (Figure 8). This is a reasonable observation, given that the dorsal mantle facing the nacreous layer is supposed to be responsible for the formation of the nacreous layer and the mantle edge near the prismatic layer is responsible for the formation of the prismatic layer. Thus the epithelia of the mantle can be divided into at least two parts spatially and functionally. For example, MSI60 is synthesized at the ventral part, whereas MSI31 is synthesized at the edge [11]. Moreover, *in situ* hybridization indicated that Prismalin-14 was synthesized at two sites: inner-side cells of the outer fold and inner-side cells of the inner fold (Figure 9). These two sites are separated physically by the periostracum, which is believed to be formed by acid glycoproteins secreted from the inner side of the outer fold [26,27], from the cells at or near the junction between the outer and middle folds [9,28]. Therefore Prismalin-14 synthesized in the inner-side epithelial cells of the outer fold may be secreted into the extrapallial fluid and incorporated into the prismatic layer. The results of *prismalin-14* expression show that the inner fold may have functions other than controlling the osmotic pressure of the haemolymph [26], since Prismalin-14 is also synthesized in the inner side cells of the inner fold.

On the basis of the structural and functional characteristics of Prismalin-14, we propose a molecular model for this protein as shown in Figure 10. This molecule has four functional parts.

The Asp-rich regions at the N- and C-terminal parts may bind calcium ions to regulate calcification or they may interact with the surface of calcium carbonate crystals to modulate crystal growth. The PIYR repeat region may form a β -sheet structure to contribute to the construction of the framework. The Gly/Tyr-rich region may form a glycine-loop structure to give the molecule flexibility. In addition, this molecule is associated with chitin at an unknown region. The *in vivo* function of Prismalin-14 is still not clear. A more detailed analysis is required for understanding the mechanism of calcification of the prismatic layer of the shell of *P. fucata*.

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