N40, a Novel Nonacidic Matrix Protein from Pearl Oyster Nacre, Facilitates Nucleation of Aragonite in Vitro

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A novel nonacidic matrix protein from pearl oyster nacre has been purified by cation-exchange chromatography. It was designated N40 for the nacreous protein of approximately 40 kDa. On the basis of the extraction method (with Tris-buffered Milli-Q water) and amino acid compositions (Gly- and Ala-rich), N40 was inferred to be a conventional "insoluble matrix protein". Crystallization experiments showed that N40 could facilitate the nucleation of aragonite drastically. So far, among the macromolecules that have been purified from the shell, N40 is an exclusive protein that can nucleate aragonite by itself, without the need for adsorption to a substrate. Thus, the present study has proposed the possibility that the nonacidic shell protein (maybe a conventional "insoluble framework protein") can also directly participate in aragonite nucleation and even act as a nucleation site. It is a valuable supplement to the classic biomineralization theory, in which the soluble acidic proteins of the shell are generally believed to function as a nucleation site.

Introduction

The molluscan shell is a composite of $CaCO_3$ crystals, matrix proteins, and other biopolymers, and together they exhibit exceptional hardness and strength, more so than the pure mineral of $CaCO_3$.[1] Although they account for less than 2% of the shell weight, the organic matrix proteins are thought to direct the nucleation, polymorphism, orientation, and morphology of growing $CaCO_3$ crystals, and they contribute in this way to the extraordinary properties of the shell.[2] The shells of the genus *Pinctada* are composed of two polymorphs of $CaCO_3$: calcite in the prismatic layer and aragonite in the nacreous layer (nacre). During shell formation, the prismatic layer is first deposited, and then the nacre is added as the shell grows in thickness.[1] Therefore, the *Pinctada* shell is a suitable model for investigating the polymorphic transition in the formation of the shell.

So far, several matrix proteins have been identified in the molluscan shell, and their distributions and functions in controlling $CaCO_3$ crystal formation have also been extensively investigated.[3−14] As for *Pinctada fucata*, heretofore, only two nacreous proteins[3,4] have been characterized from the nacre. This is due to the difficulty of purification, resulting from the unusual cross-link and aggregation of the shell protein. In addition, prismalin-14[5] was purified from the prismatic layer of *P. fucata*, and very recently, two tyrosinase proteins were localized in the same layer.[6]

Generally, the molluscan shell proteins are thought to be acidic, and this view has been supported by many works.[7−9] Therefore, among the various methods of protein purification, anion-exchange chromatography has become a common choice to isolate the shell matrix proteins.[3,10−12] We noted, however, that a few nonacidic matrix proteins were also identified in recent studies.[13−16] However, the method of cation-exchange chromatography has never been used to isolate these proteins from molluscan shells in previous studies, so in this study, we have attempted to purify certain novel nonacidic proteins from the pearl oyster shell using cation-exchange resin.

In general, after purification of the shell proteins, in vitro $CaCO_3$ crystallization and precipitation experiments are used to investigate the functions of matrix proteins on crystal nucleation, growth, and polymorphic transitions.[5,17] Through these approaches, the aragonite-associated proteins were shown to have the ability to regulate the growth of calcite[18−20] or nucleate aragonite.[21−23] For example, N16,[4] a nacre protein from *P. fucata*, can facilitate the formation of aragonite crystals when it is fixed on the substrate EISM membrane. Also, macromolecules extracted from the aragonitic layers of mollusks can induce aragonite formation when they are adsorbed to a substrate of β-chitin and silk fibroin.[21]

In this study, we have purified a novel nonacidic matrix protein using cation-exchange chromatography from the pearl oyster nacre. The protein was designated N40 because it was a nacreous protein of approximately 40 kDa, and a partial characterization was performed. The in vitro crystallization experiments showed that N40 could facilitate the nucleation of aragonite crystals drastically.

Materials and Methods

Abbreviations. DTT (dithiothreitol), PMSF (phenylmethanesulfonyl fluoride), SDS−PAGE (sodium dodecyl sulfate−polyacrylamide gel electrophoresis), MALDI-TOF MS (matrix-assisted laser desorption/ionization time-of-flight mass spectrometry), SEM (scanning electron microscopy), FTIR (Fourier transform infrared spectroscopy), EDTA (ethylenediaminetetraacetic acid), EISM (EDTA-insoluble matrixes), CBB (Coomassie Brilliant Blue), BSA (bovine serum albumin).

Extraction of the Total Nacre Proteins. Adult specimens of *P. fucata* were purchased from the Guofa Pearl Farm, Guangxi Province,

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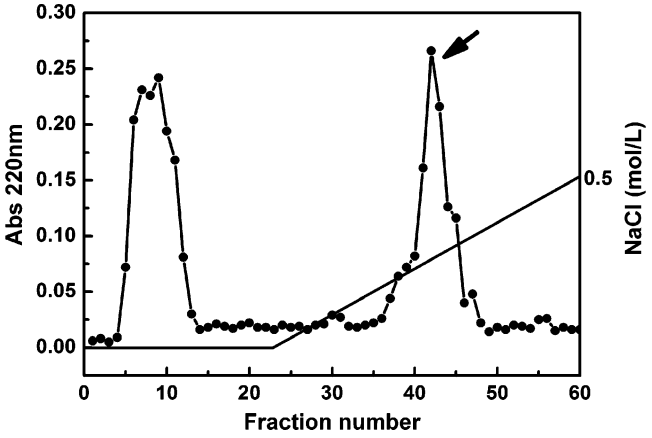

Figure 1. Chromatography of the N40 protein on a CM-Sepharose fast flow column. The starting buffer was 25 mM sodium phosphate buffer (pH 7.0). A linear gradient of 0−0.5 M NaCl was applied to the starting buffer. The arrow shows the N40 fraction.

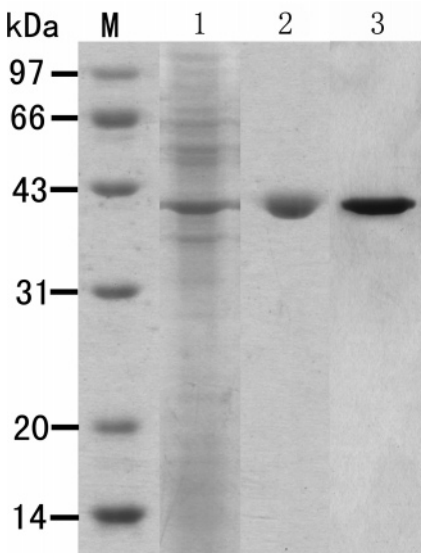

Figure 2. Gel electrophoresis of N40 purification: M, molecular mass markers; lane 1, total nacre proteins stained by CBB; lane 2, purified N40 stained by CBB; lane 3, purified N40 stained by silver. The molecular mass (kDa) is shown on the left of the gel.

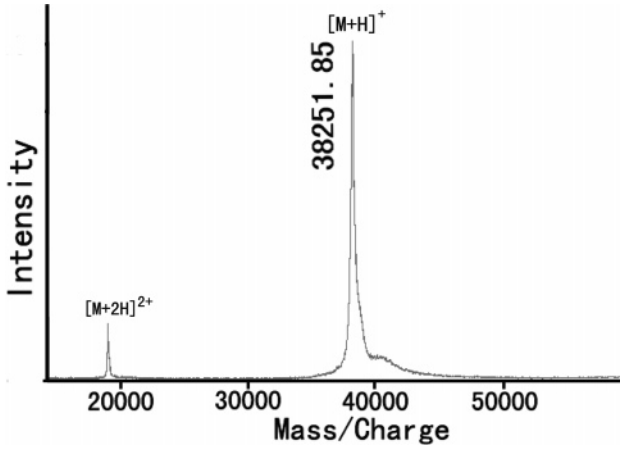

Figure 3. MALDI-TOF mass spectrum of purified N40.

China. After being cleaned, the shells were immersed in 5% NaOH for 24 h and subsequently rinsed in distilled water to remove the organic substance that adheres to the inner surface of the nacre. After air-drying, the prismatic layer was removed mechanically, and the nacre was examined under a converted microscope (Leica, DMIRB) to ensure that the inner organic substance and the prismatic layer had been removed completely. Then the nacre was powdered (100 g, particle size <100 μm) and suspended in 300 mL of extracting solution (20 mM Tris−HCl buffer, pH 7.5/1 mM DTT/0.2 mM PMSF/0.1% NaN_3) for 24 h at room temperature with continuous stirring. The supernatant was collected by centrifugation at 13000 rpm for 30 min at 4 °C, and then it was concentrated to a volume of 20 mL by ultrafiltration (Millipore ultrafiltration tube, cutoff of 5 kDa). The concentrated solution was subjected to ammonium sulfate fractionation. The precipitate (by 90% saturation) was dissolved in 4 mL of Milli-Q water, and then it was extensively dialyzed against Milli-Q water and lyophilized.

Table 1. Amino Acid Analysis of N40

amino acid	concn, mol %	amino acid	concn, mol %	amino acid	concn, mol %
Asx	6.45	Ser	8.33	Leu	6.27
Glx	3.17	Cys	6.87	Tyr	1.84
Gly	10.90	Val	9.88	Phe	6.28
Ala	15.73	Met	1.76	Lys	6.60
Thr	9.50	Ile	1.87	Arg	4.55

N40 Protein Purification. The total nacre proteins were loaded at room temperature onto a CM-Sepharose fast flow column (Amersham, 1.5 × 8 cm) which had been preequilibrated with 25 mM sodium phosphate buffer, pH 7.0. The column was washed with the same buffer until the absorbance at 220 nm reached the baseline, and then it was eluted with a linear gradient of 0−0.5 M NaCl in the same buffer at a flow rate of 0.15 mL/min. Fractions of 0.45 mL of eluate were continuously collected and subsequently analyzed by SDS−PAGE. The N40-containing fractions were pooled and extensively dialyzed against Milli-Q water at 4 °C. After concentration by ultrafiltration (Millipore, cutoff 5 kDa), N40 was freeze-dried. The protein yields were measured by a BCA assay kit (Pierce).

MALDI-TOF MS and Amino Acid Analysis. Purified N40 was resuspended in Milli-Q water, and small aliquots were taken for MALDI-TOF MS measurements and amino acid analysis. The protein was analyzed in an α-cyano-4-hydroxycinnamic acid matrix. Mass spectra were measured on a Voyager BioSpectrometry workstation (PerSeptive Biosystems). The sample spots were irradiated using a 6 MW N_2 laser (Laser Science, Inc.) with a 337 nm wavelength. N40 protein ions were accelerated with a 20 kV accelerating voltage.

In preparation for amino acid analysis, lyophilized N40 was hydrolyzed under vacuum in 6 N HCl at 110 °C for 24 h. Samples and amino acid standards (Sigma) were analyzed on a Beckman System 6300 automated amino acid analyzer, which was equipped with a 25 cm column and quantified by ninhydrin reaction. The amino acid compositions, expressed as a mole percentage, represent the average of three independent determinations.

In Vitro Crystallization Experiment. The calcitic crystallization solution was prepared according to the method of Xu et al.[24] by purging a stirred aqueous suspension of $CaCO_3$ with CO_2 for 6 h. Then excess solid $CaCO_3$ was removed by filtration (0.2 μm), and subsequently, the filtrate was purged with CO_2 for another hour. Crystallization experiments were carried out by adding proteins to the freshly prepared crystallization solution on a cover glass at 20 °C. After 24 h, the crystallization solution was removed, and the crystals were stored in a desiccator for characterization. The experiments were repeated several times.

Crystal Characterization. For morphological observation and identification of the induced crystals, SEM and Raman and FTIR spectroscopy were used. The scanning electron micrographs were obtained using a SIRION 200 scanning electron microscope (FEI). After the crystallization experiments, the cover glass was coated with gold before imaging. Raman spectra of the crystals were recorded with a Renishaw RM2000 spectrometer. The samples were positioned on a cover glass and placed under a microscope (50×) that can focus the

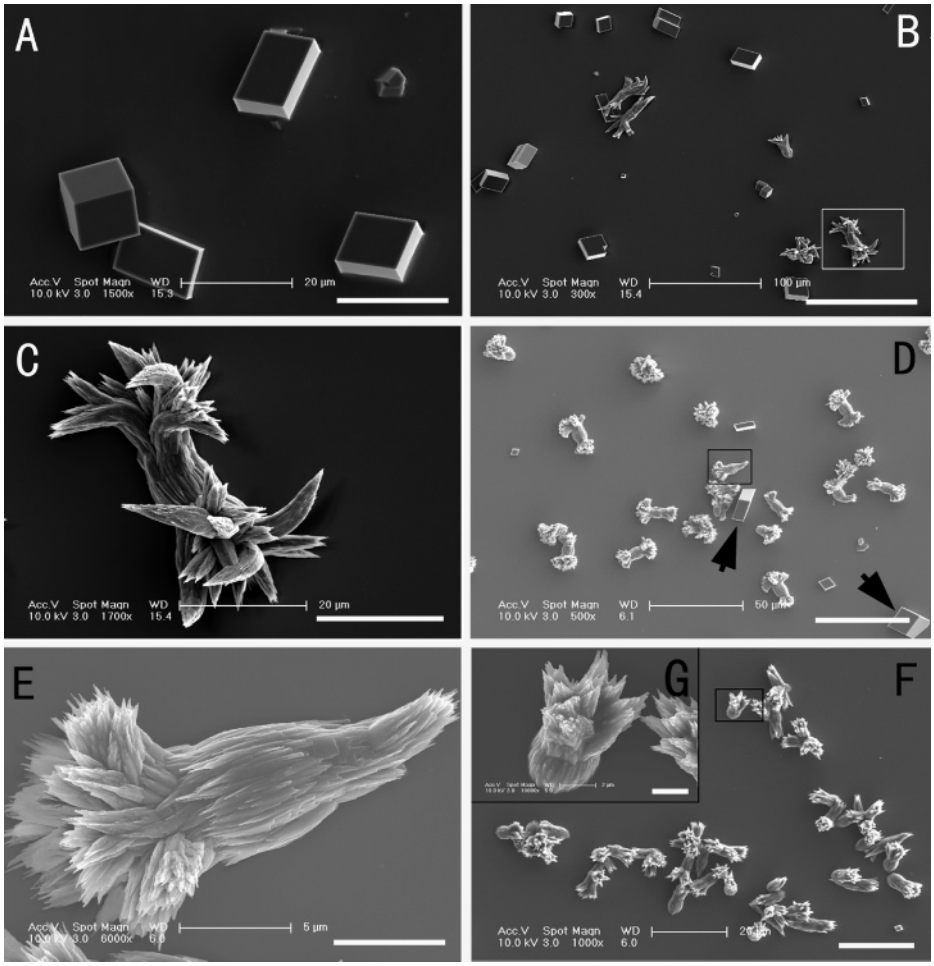

Figure 4. SEM images of in vitro crystallization experiments in the presence of N40. Crystals were grown in the presence of 30 μg/mL BSA (A), 10 μg/mL N40 (B), 20 μg/mL N40 (D), and 30 μg/mL N40 (F). The black arrows in (D) indicate that the calcite crystals remained in the solution. (C), (E), and (G) are enlarged images of the boxed part in (B), (D), and (F), respectively. Scale bars: A, C, F, 20 μm; B, 100 μm; D, 50 μm; E, 5 μm; G, 2 μm.

laser beam on the sample while collecting the backscattered light. The Raman spectra were obtained with an excitation wavelength of 514 nm, which was provided by an argon laser limited to 4.6 mW power. The spectra were recorded from 100 to 1500 cm^{-1}. For FTIR investigations, the crystals were collected from the cover glass, and KBr pellets were produced with a 1% sample and analyzed on a Perkin-Elmer model 1600 FTIR spectrometer. All the spectra were recorded at 4 cm^{-1} resolution with 64 scans with a strong Norton−Beer apodization.

Results

N40 Protein Purification and Partial Characterization. The total nacre proteins were loaded onto a CM-Sepharose fast flow column, and the N40-containing fractions were pooled (Figure 1). The purified N40 was detected by SDS−PAGE with CBB R-250 and silver staining. As shown in Figure 2 (lanes 2 and 3), only one band at about 40 kDa was visualized on the gels. The subsequent identification by MALDI-TOF MS showed that the exact mass of the protein (N40) was 38.3 kDa (Figure 3). The result of total amino acid analysis is presented in Table 1. The results show that the percentage of acidic residues (Asx and Glx) and basic residues (Lys and Arg) were 9.6 and 11.2 mol %, respectively. We also noted that N40 is rich in Gly and Ala (26.6 mol %).

Aragonite Grows in the Presence of N40. To investigate the influence of N40 on the crystal growth, in vitro crystallization experiments were performed, and the precipitated crystals were examined. As shown in Figure 4, aragonite crystals were induced in the presence of N40, while the crystals of the control experiments conducted without protein (data not shown), or in the presence of BSA (Figure 4A), were all the typical rhombohedra of calcite. In the presence of N40, at a concentration of 10 μg/mL, some aragonites (about 30%) occurred together with calcite (Figure 4B). When a higher concentration (20 μg/mL) of N40 was present, the aragonite crystals became dominant (about 80%) in the crystallization solution (Figure 4D). The N40 protein exhibited its maximal influence on the crystal polymorph at a concentration of 30 μg/mL, when there was no calcite observed in the crystallization solution, and the induced crystals were shown to be all aragonite (Figure 4F).

Polymorph Determination. To ascertain the polymorph of the induced crystals in the crystallization experiments, the Raman spectrum was used to characterize the induced crystals. As shown in Figure 5, the aragonites induced in the presence of N40 were validated (upper panel), while the crystals grown in the control experiments were shown to be calcite (lower panel). Because Raman spectra can only identify the single crystals on the cover glass, it is difficult to reveal the characteristic of whole crystals in the crystallization solution, so we collected the crystals grown on the cover glass and characterized them by FTIR. The crystals induced by N40 at a concentration of 30 μg/mL demonstrated the typical peaks of aragonite (Figure 6A) in the FTIR spectra, indicating that the

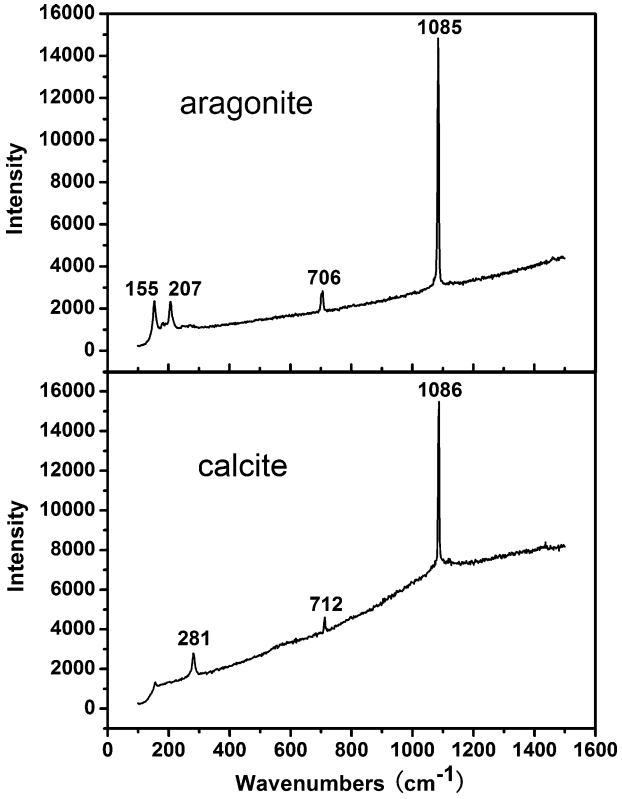

Figure 5. Raman spectra of the crystals induced in a crystallization solution. The aragonite (induced in the presence of N40) and calcite (control experiments) were validated with the characteristic peaks of calcite at 281, 712, and 1086 cm^{-1} and of aragonite at 207, 706, and 1085 cm^{-1}.

crystals induced at this concentration were all aragonite, while the crystals in the control crystallization solution demonstrated the characteristic peaks of calcite in the spectra (Figure 6C). In the presence of N40 at a concentration of 10 μg/mL, the characteristic peaks of calcite (Figure 6B, indicated by circles) and aragonite (Figure 6B, indicated by stars) were observed to occur simultaneously.

Discussion

The matrix proteins are thought to play crucial roles in molluscan shell formation. Some matrix proteins have even been found to have enzymatic functions in biomaterial formation.[3] However, only a few shell matrix proteins have been characterized so far. One reason is that most matrix proteins are believed to bind tightly with $CaCO_3$ crystals in the shell. Thus, studying them is very difficult due to the fact that only low quantities of these proteins can be recovered from the shell matrix. Generally, the shell must be decalcified in a calcium-chelating agent such as EDTA or a weak acid before the matrix protein is isolated. In a recent study,[25] a new nondecalcified method, in which the matrix protein is extracted with ultrapure water, was proposed. The new method was considered a preferred choice since it seemed milder than the conventional demineralization method, which has a possibility of alterating macromolecule activity, so in the present study, the technique of extraction with Milli-Q water (Tris-buffered), not EDTA or acid,[3,26] was followed. Actually, we noted that the mixture of the isolation solution and the nacre powder in this study was basic and the pH value (about 9−10) of this mixture was similar to that of the previous study.[25]

After the total nacre protein extraction, the method of cation-exchange chromatography was used for the first time to purify

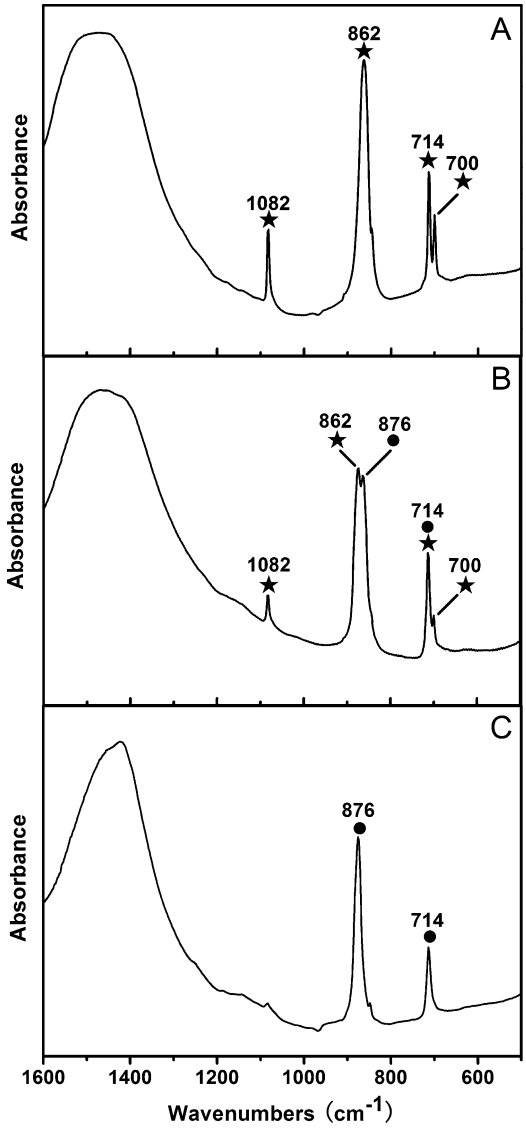

Figure 6. FTIR spectra of the crystals collected from the cover glass in crystallization experiments. The crystals were induced in the presence of N40 at concentrations of 30 μg/mL BSA (A, dominated by aragonite), 10 μg/mL BSA (B, mixture of calcite and aragonite), and 30 μg/mL BSA (C, control experiment, dominated by calcite). The characteristic peaks of calcite (indicated by circles) are at 876 and 714 cm^{-1}, and that of aragonite (indicated by stars) are at 1082, 862, 714, and 700 cm^{-1}.

shell matrix proteins. This was based on findings that a few matrix proteins were shown to be basic.[27] As shown in Figures 1 and 2, a protein designated N40 was purified. The amino acid composition analysis of N40 was performed subsequently, and the results (Table 1) showed that the basic amino acid residues (Lys and Arg, 11.2 mol %) were slightly greater than the acidic amino acid residues (Asx and Glx, 9.6 mol %). Furthermore, the acidic amino acid residues of N40 were much lower than those of the aragonitic proteins extracted from shells in previous studies.[28] In addition, the N40 protein could not enter the stacking gel when detected by native PAGE (data not shown). Therefore, we can conclude that the N40 protein is indeed a nonacidic shell protein.

Matrix proteins are often classified as soluble acidic matrix proteins (known as Asp-rich) and insoluble framework matrix proteins (said to be Gly- and Ala-rich) on the basis of their low and high solubility in aqueous solution after the mineral has been removed by chemical methods.[1] In a comparative study[29]

on the technique of extraction (extraction with ultrapure water alone vs decalcification with EDTA), the soluble proteins (extracted with ultrapure water) were quite different from those extracted with the conventional "soluble matrix" method (extracted with decalcified reagents) in the aspects of amino acid compositions and SDS−PAGE separation profiles. On the contrary, the water-soluble proteins were similar to conventional "insoluble matrixes" in amino acid compositions (Gly- and Ala-rich). In the present study, the N40 protein is also Gly- and Ala-rich (26.6 mol %, Table 1). Thus, we inferred that N40 might be a component of the conventional insoluble matrix protein of nacre on the basis of its extraction method (with Tris-buffered Milli-Q water) and amino acid compositions (Gly- and Ala-rich).

Because of the crucial roles they play in polymorphic transition, the aragonite-associated proteins have received a great deal of attention. Hitherto, some aragonitic proteins have been isolated from molluscan shell, and their influences on growth and precipitation of $CaCO_3$ crystals have been extensively investigated.[16,18−23,30−32] In our opinion, these proteins can be classified as (1) inhibitors of growth and precipitation of calcite (such as nacrein,[20] AP7, AP24,[19] AP8,[33] N16,[4] perlwapin,[34] and ACLS40[12]), (2) promoters of the precipitation of calcite (such as perlucin[35]), and (3) inducers of aragonite nucleation (such as N16,[4] mixture of N66 and N14,[16] total proteins extracted from aragonitic layers[2,21,23]). This is based on the results of in vitro crystallization experiments. In the third classification, a majority of the proteins induced aragonite formation with the need for adsorption to a substrate.[4,16,21] An exception[2,23] is the total proteins extracted from the aragonitic composite of abalone. It was found that these proteins alone were sufficient to induce aragonite formation. The total proteins of the abalone aragonitic layer contain at least six fractions,[2] while, in the present study, the N40 protein could nucleate aragonite singly. As far as we know, among the shell-extracted proteins, N40 is the first protein that can nucleate aragonite by itself, without the need for adsorption to a substrate or mixture with other macromolecules. Therefore, it is logical to assume that valuable information about the mechanism of aragonite nucleation is kept in its protein sequence. Related studies are currently in progress.

Recently, a two-compound model that accounts for both structural and functional aspects of the organic matrix has been proposed. It considers the insoluble organic matrix to function as a structural framework onto which the soluble acidic macromolecules, which present an active nucleating surface to the external solution, are anchored,[1] so the soluble acidic proteins of the shell are generally believed to function as nucleation sites[9,36,37] in biomineralization. The present study has proposed another possibility: that the nonacidic shell protein (perhaps a conventional "insoluble framework protein") may also participate directly in aragonite nucleation, even acting as a nucleation site.

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