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A novel nacre protein N19 in the pearl oyster *Pinctada fucata*

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Abstract

A novel 19 kDa protein, which was named N19, was isolated from the nacreous layer of the pearl oyster *Pinctada fucata*. N19 is one of predominant proteins found in the water-insoluble fraction of the nacreous layer. MALDI–TOF/TOF analysis indicated that the three trypsin-digested peptides (791.45, 824.42, and 1118.65 *m/z*) corresponded to the amino acid sequences predicted from a cDNA isolated from a mantle cDNA library of *P. fucata*. Northern blot analysis revealed that the N19 mRNA was a little more abundant in the pallial region than the edge region, in the mantle. In $CaCO_3$ precipitation assay, the recombinant N19 protein inhibited the crystallization of $CaCO_3$. These results indicate that N19 is localized in the nacre and plays a negative regulatory role in calcification in the pearl oyster.

Keywords: Biomineralization; Mollusc; Pearl oyster; Shell; MALDI–TOF/TOF

Biominerals are formed as endoskeleton in vertebrates and as exoskeleton in invertebrates. In molluscs, the shell is a conspicuous exoskeletal biomineral that is calcified, and it is almost unanimously agreed that the shell is a genetically determined biomaterial and its formation is regulated by organic matrix proteins, which are found in shells as minor components [1–7]. Many of the organic matrix protein genes are transcribed specifically in the mantle and secreted into the extrapallial space, in which calcification occurs, and the secreted proteins are thought to be localized in pertinent places of the shell in the process of shell formation. The mantle is one of common traits in the Mollusca and plays crucial roles in calcification of the shell. Therefore, it is fair to say that analyzing the genes and the corresponding proteins expressed in the mantle contributes to deciphering the common mechanism of the shell formation in molluscs. Then, we have constructed a cDNA library using the mRNA purified from the mantle of the pearl oyster *Pinctada fucata* and sequenced cDNAs isolated from the library [8].

In some molluscs including the Pteriidae, two types of calcified microstructure are observed: the prismatic layer which is calcite and the nacreous layer which is aragonite [9–11]. The nacreous layer, which is composed of calcium carbonate crystals, shows characteristic shape constructing compact tablets of several hundreds nanometer in diameter [12], and has drawn much attention in respect of biomineralization in invertebrates and its industrial effect on pearl cultivation. Particularly, it is of note that the nacreous layer endows the shell with toughness inconceivable from the inorganic aragonitic calcium carbonate crystals, of which cause is attributed to the organic matrix proteins included in it. In vitro studies explicitly show that organic matrix macromolecules isolated from the nacreous layer regulate calcification leading to the growth of aragonite crystals, and several proteins found in the nacreous layer

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have been reported [13–25]. However, according to analyses of the proteins of the nacreous layer, many proteins remain to be identified [26].

In this study, we have identified a novel 19 kDa protein N19 in the nacreous layer of the shell in the pearl oyster *P. fucata*. We show that N19 could be extracted effectively with 8 M urea from the nacreous layer and demonstrate that the peptide sequences obtained by MALDI–TOF/TOF analysis are in accord with a predicted protein sequence of a cDNA derived from the mantle of *P. fucata*.

Materials and methods

Isolation of water-insoluble proteins from the nacreous layer of shells. The nacreous layer of shells of *P. fucata* was rinsed in diluted sodium hypochlorite overnight and washed with water. After drying, the nacreous layer was powdered and incubated in 20 volumes of 8 M urea and 50 μg/ml (*p*-AmidinoPhenyl)methanesulfonyl fluoride (APMSF) at 37 °C for 3 days, and then centrifuged to remove insoluble shell materials. The supernatant was dialyzed in H_2O overnight and then centrifuged. The water-insoluble proteins precipitated in this process were then separated by SDS–PAGE, followed by visualization with CBB R-250.

MALDI–TOF/TOF analysis. A protein band corresponding to 19 kDa was excised and subjected to MALDI–TOF MS analysis. Picking of gel spots, destaining of the gel pieces, in-gel trypsin digestion, and sample loading onto MALDI plates were performed automatically using an Xcise robotic protein processing system (Shimadzu Biotech). The gel spots were cut into pieces and transferred into 96-well microtiter plates. The pieces were washed with 100 μl of 50% acetonitrile in 50 mM ammonium bicarbonate twice for 20 min and then with 100 μl of 100% acetonitrile for 20 min. Following trypsin (Promega) digestion of proteins in the gel pieces in 30 μl of 25 mM ammonium bicarbonate at 30 °C overnight, the resulting peptides were purified and concentrated using ZipTip^{TM} μC18 (Millipore), and subsequently spotted onto the MALDI sample plate with 2.5 mg/ml solution of α-cyano-4-hydroxycinnamic acid in 50% acetoni-

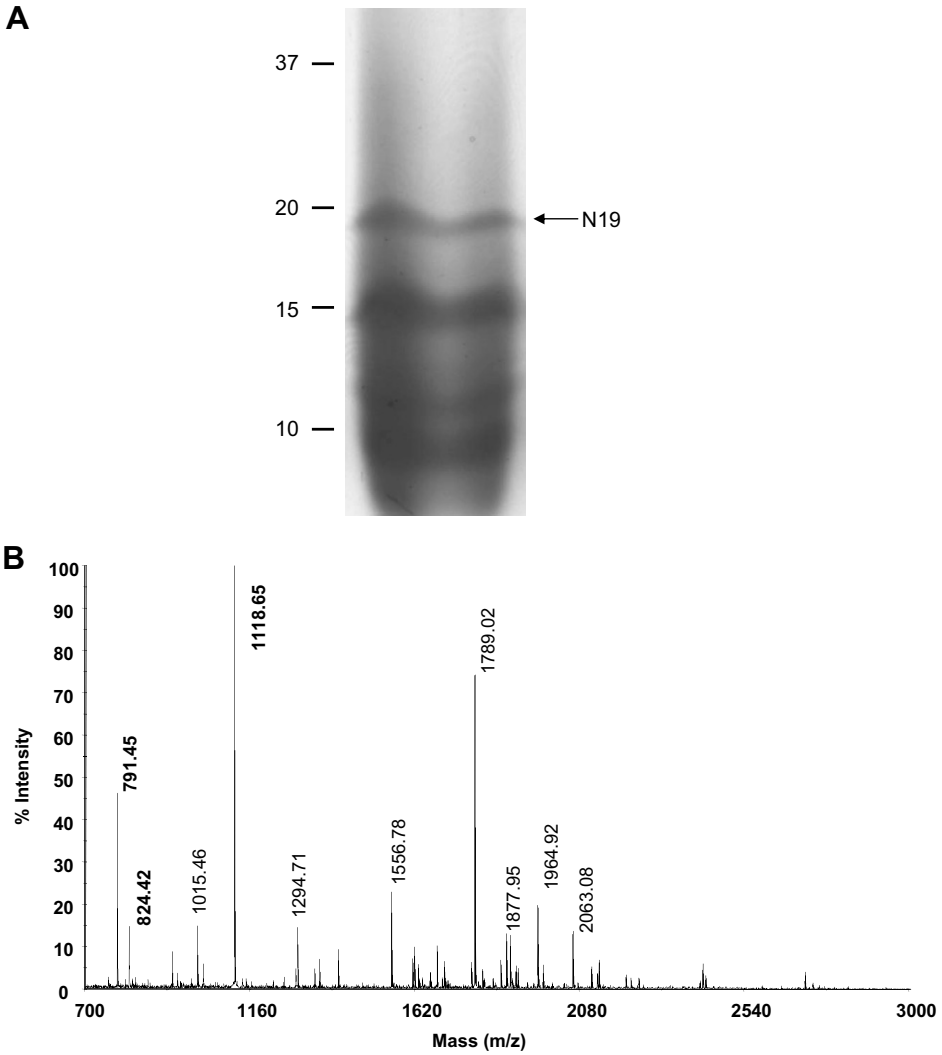

Fig. 1. Isolation and MALDI–TOF MS analysis of the N19 protein. (A) SDS–PAGE of water-insoluble proteins isolated from the nacreous layer of the shell. Water-insoluble proteins isolated from the nacreous layer were subjected to SDS–PAGE and stained with CBB R-250. Molecular mass standards are shown in kDa on the left side. (B) MALDI–TOF MS analysis of the 19 kDa protein N19 separated by SDS–PAGE. The N19 protein band was excised and digested overnight with trypsin at 30 °C. Following digestion, peptides were desalted and concentrated using ZipTip^{TM} μC18, and were subjected to mass spectrometry analysis. Three peaks corresponding to amino acid sequences predicted from a cDNA isolated from a mantle cDNA library were shown in bold.

trile/0.1% trifluoroacetic acid. MS or tandem MS spectrometric analysis of the tryptic digests was performed using a 4700 MALDI–TOF/TOF mass spectrometer (Applied Biosystems). MS spectra were measured in the reflector mode with a mass range from 700 to 3500 Da. External calibration was performed using five standard peptides. MS/MS spectra were measured in CID-off mode. Prediction of amino acid sequence from MS/MS data was carried out using denovo explorer ver. 1.22 (Applied Biosystems) with MS/MS tolerance of 0.2 Da.

Sequence analysis. The sequences of 3214 cDNAs derived from the mantle were searched for peptide sequences that were obtained by MALDI–TOF/TOF analysis using GENETYX-PDB database software (Genetyx). This resulted in identification of one cDNA, which was followed by sequencing using a Big-Dye terminator kit and an ABI 3100 DNA sequencer (Applied Biosystems).

Northern blot analysis. The mantle edge and pallial from the pearl oyster *P. fucata* were isolated in artificial sea water (ASW) and homogenized in 10 volumes of Trizol reagent (Invitrogen). Total RNA was extracted according to the manufacturer's instructions. RNA samples (10 μg) were separated by electrophoresis in a 1% agarose gel containing formaldehyde, and were then transferred to a Hybond-N filter (GE Healthcare). Hybridization was performed using the ^{32}P-labeled N19 cDNA probe in 6× SSC, 5× Denhardt's solution, 0.5% SDS, 4 mM EDTA and 100 μg/ml of salmon sperm DNA at

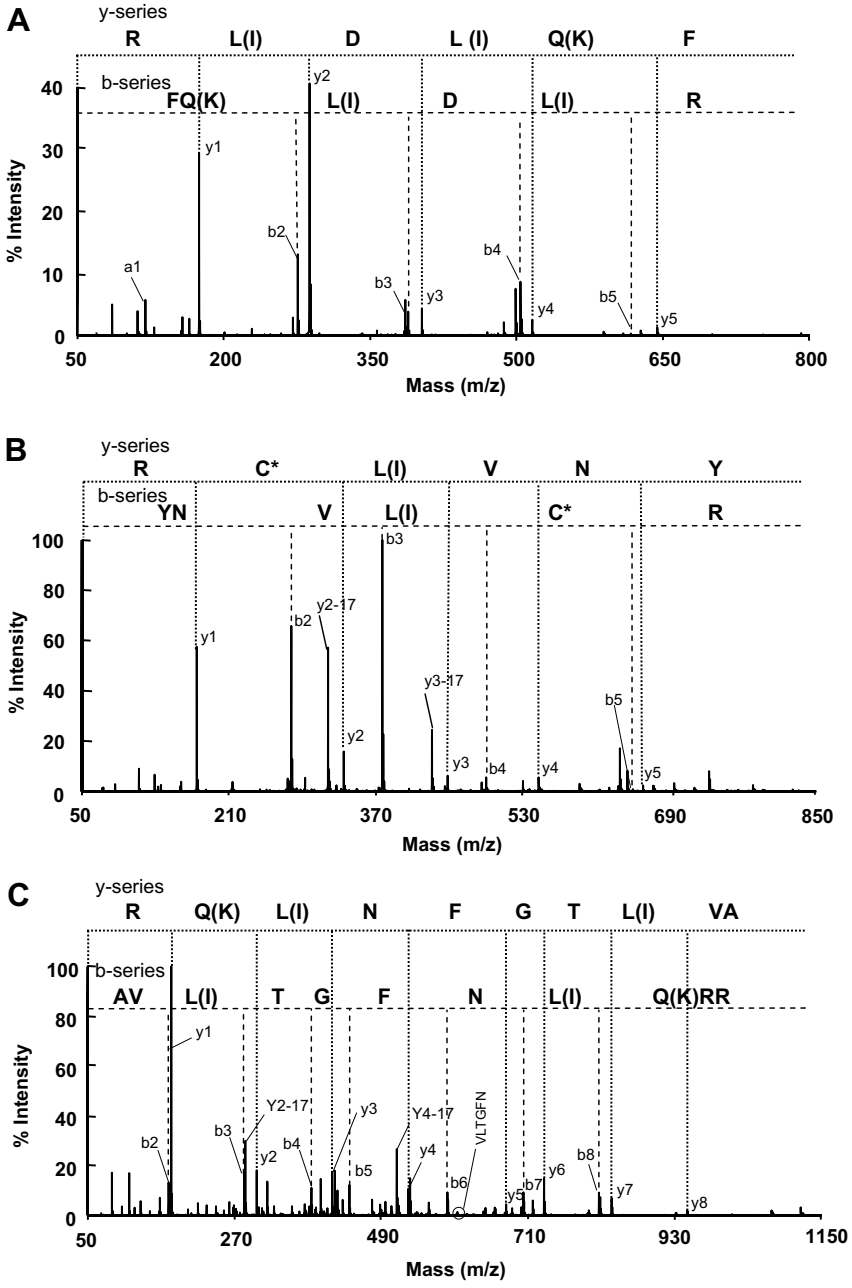

Fig. 2. Tandem mass (MS/MS) spectra of tryptic digests derived from the N19 protein with an amino acid sequence produced by de novo interpretation. In each spectrum, b- and y-ions are labeled, and the resulting sequence is given in the inset. (A) an MS/MS spectrum acquired from the precursor ion 791.45 *m*/*z* matched to the sequence FQL/IDL(I)R. Series of y-ion species (y-1 to y-5) and b-ion species (b-2 to b-5) for which the sequence is indicated could be assigned. (B) An MS/MS spectrum of peptide 824.42 *m*/*z* matched to the sequence YNVL/IC*R (C* means carbamidomethylated cysteine). Series of y-ion species (y-1 to y-5) and b-ion species (b-2 to b-5) could be assigned. (C) An MS/MS spectrum of peptide 1118.65 *m*/*z* matched to the sequence AVL/ITGFNL/IQR. Series of y-ion species (y-1 to y-8) and b-ion species (b-2 to b-8) and an internal fragment ion, VL/ITGFN could be assigned.

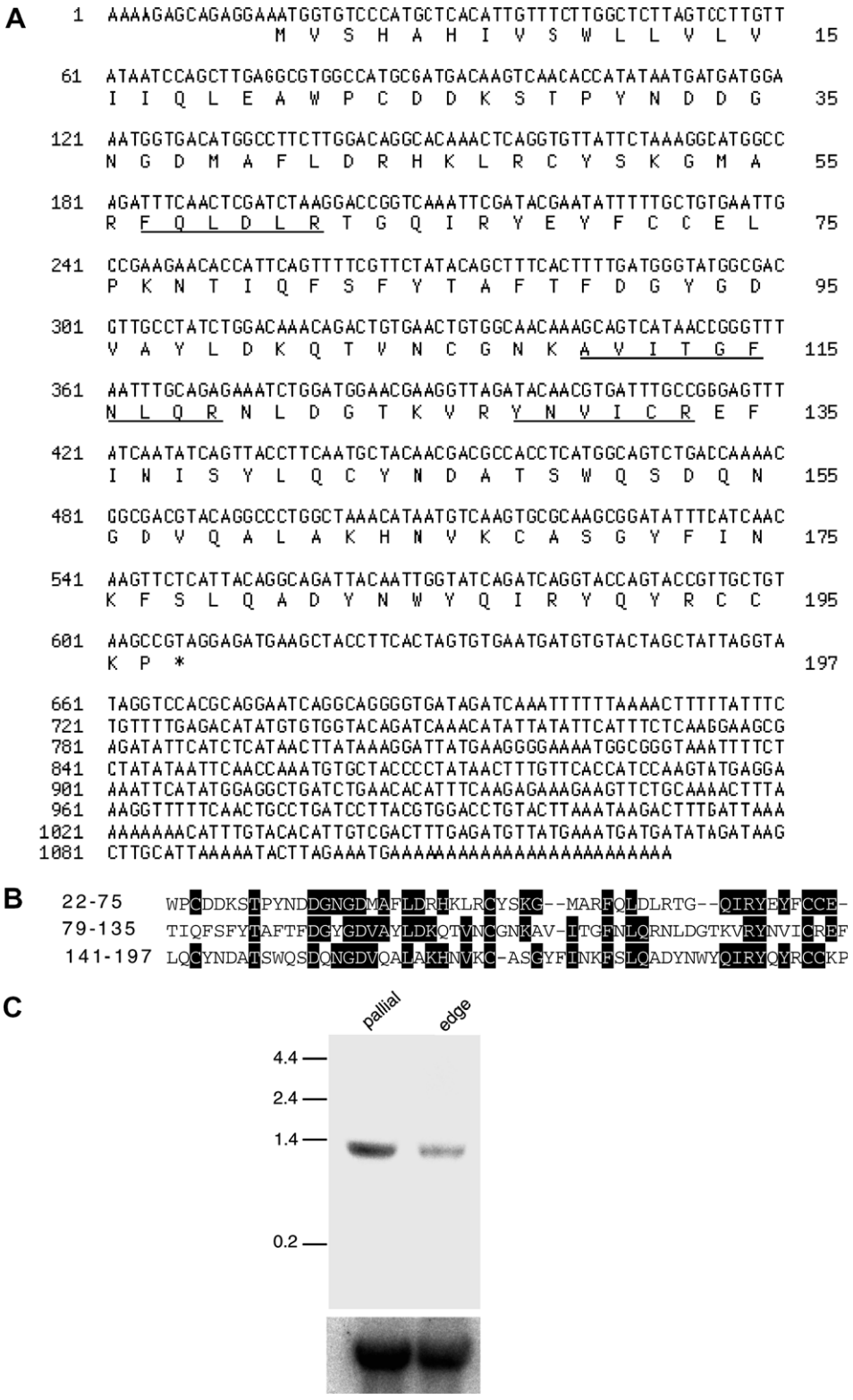

Fig. 3. Molecular characterization of N19. (A) Nucleotide and deduced amino acid sequences of a cDNA encoding the N19 protein. Peptides identified by MALDI–TOF/TOF analysis are underlined. The stop codon is marked with asterisk. (B) Sequence alignment of three repeat domains found in N19. Conserved amino acid sequences are highlighted. (C) Differential expression of the N19 protein in the mantle. Total RNAs were prepared from the pallial and the edge of the mantle and hybridized with the ^{32}P-labeled N19 protein cDNA. The lower panel shows the result of a control experiment with an actin probe. Nucleotide sequence data reported are available in the DDBJ/EMBL/GenBank databases under the Accession No. AB332326.

65 °C. The filter was washed in 0.5× SSC at 65 °C. Signals were detected using a BAS2500 Image analyzer (Fuji film).

Production of the recombinant N19 protein and calcium carbonate precipitation assay. The coding region excluding a signal peptide was cloned

into the expression vector pCold TF (Takara Bio). The ligated pCold TF was introduced into *Escherichia coli* BL21, and the fusion protein with trigger factor was induced with 1 mM IPTG. After 24 h of culture at 15 °C, cells were pelleted and resuspended in 25 ml of sodium phosphate buffer (pH 7.4). Following sonication, the soluble fraction was applied onto a HisTrap chelating HP column (GE Healthcare). After washing the column, the fusion protein was eluted with imidazole buffer (10–500 mM). Fractions containing the fusion protein were collected and subjected to dialysis in H_2O. Calcium carbonate precipitation assay was performed using 10 μg/ml of protein as described [24].

Results and discussion

In our previous experiments [8,27], we tried to extract proteins from the prismatic layer of the shell of *P. fucata* using 8 M urea, in which we succeeded in identifying several novel matrix proteins: glycine-rich shematirns and tyrosinase-like proteins that corresponded to cDNAs isolated from a cDNA library of the mantle. The same strategy was applied to proteins in the nacreous layer. To identify water-insoluble proteins that are present in the nacreous layer of the pearl oyster *P. fucata*, we employed solubilization with 8 M urea at 37 °C. As shown in Fig. 1A, there found several protein bands, of which the 19 kDa protein, N19, was excised and submitted to MALDI–TOF MS analysis following the treatment with trypsin. When three peaks (791.45, 824.42 and 1118.65 *m*/*z*) in the resulted MALDI–TOF MS spectrum (Fig. 1B) were further analyzed by MALDI–TOF/TOF instrument, in which sequences indicated by y and b series ions were identical, reliable peptide sequences were obtained: FQL/IDL/IR from 791.45 *m*/*z*, YNVL/ICR from 824.42 *m*/*z* and AVL/ITGFNL/IQR from 1118.65 *m*/*z* (Fig. 2).

Searching the sequence data of the mantle [8] for the above peptide sequences determined by MALDI–TOF/TOF analysis, we could identify a cDNA encoding the three peptide sequences. The nucleotide sequence is 1130 bp in length and encodes a predicted protein composed of 197 amino acids, which is calculated to be 23.0 kDa in molecular mass (Fig. 3A). The N-terminal sequence is hydrophobic, therefore, it is presumably explained that the difference between molecular mass in SDS–PAGE analysis and that predicted from the cDNA rests on cleavage of N-terminal signal peptide. In the predicted protein sequence, three peptide sequences determined by MALDI–TOF/TOF analysis were found, which suggests that the N19 protein detected in the nacreous layer of *P. fucata* is obviously encoded by the cDNA analyzed in this research. The predicted N19 protein sequence contains three similar domains: residues 22–75, 79–135 and 141–197 (Fig. 3B). When compared to known sequences, no significant similar sequence was found. However, weak homologous proteins were identified: hypothetical proteins in γ-proteobacteria, *Hahella chejuensis* and *Shewanella denitrificans* (UniPlot Accession Nos.: Q2SAH2 and Q12LD3). The function of both two proteins of γ-proteobacteria is not clarified, and it is yet to be determined whether their similarities have important meanings or not.

To confirm the expression of N19 in the mantle, we prepared a Northern blot of total RNA from the mantle pallial and edge, which are thought to be responsible for the formation of the nacreous layer and the prismatic layer, respectively. The N19 transcript was more abundant in the pallial region of the mantle (Fig. 3C), suggesting that the N19 protein is predominantly expressed in the pallial region and translocated into the nacreous layer, where N19 may play a role in calcification of the shell.

Several proteins identified so far in shells have a negative activity to crystallization of $CaCO_3$. To examine the effect of the N19 protein in crystallization of $CaCO_3$, we generated the recombinant N19 protein fused with trigger factor in *E. coli*. As shown in Fig. 4, the recombinant N19 protein inhibited the lowering of the pH in the $CaCO_3$ precipitation assay. The same amount of trigger factor had no effect on the lowering of pH.

The N19 protein found in this research has an inhibitory effect on the crystallization of $CaCO_3$ and can be isolated from the nacreous layer. In addition, its transcript is predominantly detected in the mantle pallial. These findings suggest that the N19 protein functions as one of negative factors predominantly in the nacreous layer formation.

Although the matrix protein is a minor component in mollusc shells, various primary structures are reported. A hallmark feature of functional analysis of these proteins is that opposite activities are found in calcification. Positive activity is found in perlucin [20], MSI7 [28] and p10 [25], all of which accelerate the growth of $CaCO_3$ crystals. On the other hand, nacrein [24], mucoperlin [19] and caspartin [29] inhibit the precipitation of $CaCO_3$ crystals, just as indicated in the assay using the N19 protein in this research. A key issue that remains unclear in biomineralization in mol-

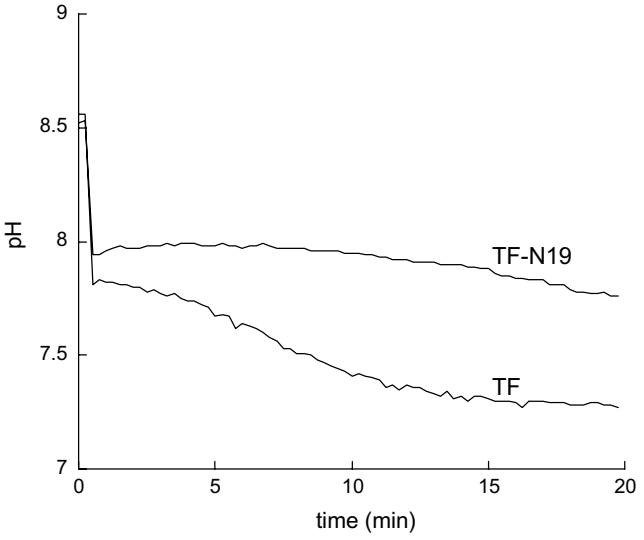

Fig. 4. Inhibition of the crystallization of $CaCO_3$ by N19. The pH of the solution containing $NaHCO_3$ and $CaCl_2$ was recorded every 15 s in the presence of the N19 recombinant protein or trigger factor. TF, trigger factor; TF-N19, recombinant N19 protein fused with trigger factor.

luscs is the molecular mechanisms by which matrix proteins having opposite effects on calcification are coordinated appropriately and function to create aragonitic or calcitic $CaCO_3$ crystals, producing distinct microstructures and shell morphologies. To understand the mechanism of shell formation, it will be important not only to determine what relationships among matrix proteins are, but also to investigate whether the relationships are conserved in diverse mollusc species.

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