

Isolation and Characterization of the N-linked Oligosaccharides in Nacrein from *Pinctada fucata*

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Abstract We analyzed the structure of the N-linked oligosaccharides enzymatically liberated from the organic matrix (OM) component in the nacreous layer of Japanese pearl oyster: *Pinctada fucata*. The lectin-blot analysis of the soluble OM after separation by SDS-PAGE, four components, with sizes of approximately 55 kDa, 35 kDa, 25 kDa, and 21 kDa were detected with GNA lectin, which recognized terminal mannose of high mannose and hybrid types of N-glycan. The 55-kDa component of the soluble OM detected by lectin blotting was identified as nacrein by using liquid chromatography/mass spectrometry (LC/MS). LC/MS analysis of the N-glycan liberated from nacrein detected a hybrid-type N-glycan, which contained sulfite and sialic acid at its terminus. The data strongly imply that nacrein, a sulfated OM glycoprotein, participates in molluscan biomineralization by creating a favorable environment for calcium ion uptake through sulfonic acid and sialic acid.

Keywords Glycosylation · Organic matrix · Shell formation

Introduction

Molluscan shells are made of CaCO₃ crystals in the form of aragonite and/or calcite together with a small amount of

organic matrix (OM), which is composed of macromolecules, such as proteins, glycoproteins, and polysaccharides. Investigations of genes encoding OM proteins have made significant advances during the last 10 years, revealing many aspects of molluscan shell biomineralization particularly in the nacreous and prismatic layers (Miyamoto et al. 1996; Sudo et al. 1997; Samata et al. 1999; Kono et al. 2000; Marin et al. 2000, 2005; Weiss et al. 2001; Tsukamoto et al. 2004; Suzuki et al. 2004; Gotliv et al. 2005; Yano et al. 2006). Consequently, common occurrence of potential N-glycosylation sites in the OM components, which can be determined with the consensus sequence Asn-Xaa-Ser/Thr (the middle amino acid can not be Pro) in their amino acid sequences, suggests that carbohydrate moieties fulfill key functions in the mineralization process of mollusks. To date, at least three mollusk shell proteins, mucoperlin (Marin et al. 2000), which is a mucin-like glycoprotein, dermatopontin (Marxen et al. 2003), and calprism (Marin et al. 2005) have been shown to be glycosylated. Of these three components, calprism was suggested to contain negatively charged carbohydrates of approximately 2 kDa by its positive reaction with Alcian blue stain (Marin et al. 2005). Despite the common occurrence of carbohydrates as glycoproteins and their intimate association with the mineral phase, their structures remain speculative and the precise identification of carbohydrate components and their functions have been a subject of intense research.

Nacrein, the first OM component isolated from molluscan shells (Miyamoto et al. 1996), possesses a highly specific structure composed of a carbonic anhydrase (CA) domain that is separated into two subdomains by an asparagine-glycine (NG)-repeat domain. Originally, the nacrein transcript was thought to be specifically expressed at the mantle pallial tissue, which is solely responsible for

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the nacreous layer formation. However, recent quantitative analysis using real-time PCR revealed its expression at whole regions of the mantle epithelium tissue of *Pinctada fucata* (Takeuchi and Endo 2006). The additional finding of nacrein not only in the nacreous and prismatic layers but also in the foliated layer implies that it is universally distributed in molluscan shells, suggesting its key role in shell formation (Norizuki and Samata 2007).

The work described herein was designed to elucidate the structure of the carbohydrate covalently attached to nacrein and to examine its function in molluscan shell formation. The results offer new insight into the extracellular events mediated by the OM, particularly spotlighting the carbohydrate moieties of nacrein.

Materials and Methods

Extraction of the Organic Matrix

Live specimens of the pearl oyster, *P. fucata*, were collected from Hatcheries in Ehime, Japan. The nacreous layer of the shells was immersed in a 5% solution of sodium hypochlorite to remove organic contaminants. The organic constituents were obtained by decalcification with 0.5 M EDTA (pH 8.0) by continuous stirring for 72 h at 4°C. After dialysis against distilled water, the entire extract was centrifuged at $15,000 \times g$ for 30 min, which separated the supernatant soluble OM (SM) and precipitated insoluble OM. The collected SM was then lyophilized and stored at -30°C .

SDS-PAGE and Lectin Blot Analysis

The components in the SM were analyzed by SDS-PAGE by using 10–20% ready-made gels, Pagel (ATTO), using the buffer systems of Laemmli (1970). After electrophoresis, the gels were stained with Coomassie brilliant blue R250 (CBB) to visualize proteins and with periodic acid and Schiff's (PAS) reagent to visualize carbohydrates (Holden et al. 1971).

The glycoproteins were screened with five kinds of lectins in SM bound to a PVDF membrane using the DIG Glycan Differentiation kit (Roche Diagnostics) according to the manufacturer's instructions.

Two-dimensional Gel Electrophoresis

The samples were separated in the first dimension by isoelectric focusing (IEF) in agar gel (pH 3–10, ATTO) of the disc gel system according to the manufacturer's instruction. After IEF, SDS-PAGE was conducted using

the same system described above. The gels were stained with the Silver Stain MS kit (Wako).

In-gel Digestion of the OM Proteins and LC/MS Analysis

Excised gel spots were destained with a buffer included in the Silver Stain MS kit (Wako). The disulfide bonds were reduced with 10 mM DTT for 45 min at 56°C in 25 mM NH_4HCO_3 buffer, and sulfhydryl groups were subsequently alkylated with 55 mM of iodoacetic acid. After washing, the gel pieces were dehydrated with acetonitrile (ACN), reswelled in digestion-buffer containing 10 ng/ μl trypsin, 25 mM NH_4HCO_3 , and incubated overnight at 37°C . The cleaved peptides were extracted three times from the gel pieces by intermittent sonication for 10 min in 50% ACN containing 1% trifluoroacetic acid. The extracted samples were combined and dried by vacuum centrifugation and then purified and concentrated using ZipTip $_{\mu\text{C}18}$ (Millipore) according to the manufacturer's instructions. Purified digests were subjected to LC/MS analysis on a Paradigm MS4 LC System coupled to a model LCQ ion trap mass spectrometer (Thermo Fisher Scientific) equipped with an electrospray interface utilizing a C18 column (Michrom Bioresources). The digests were eluted at 2 $\mu\text{l}/\text{min}$ by using a 5–45% gradient of Solvent B (10/90/0.1 $\text{H}_2\text{O}/\text{ACN}/\text{HCOOH}$ (v/v/v)) over 30 min. The composition of solvent A was 98/2/0.1 $\text{H}_2\text{O}/\text{ACN}/\text{HCOOH}$ (v/v/v). MS/MS spectra were analyzed using the Bioworks software package (Thermo Fisher Scientific). Individual MS/MS spectra were searched against the nonredundant database utilizing the SEQUEST program. The output files were filtered by the Xcorr filter (Xcorr vs. charge state).

SM Analysis by Western Blotting

The blotted SM was blocked with 5% (w/v) skim milk powder in phosphate-buffered saline (PBS) overnight and then incubated for 120 min with antibody against CAII (Rockland Immunochemicals). Before and after incubation with peroxidase-labeled secondary antibody for 60 min, the membrane was extensively washed with PBS/0.1% (v/v) Tween 20 and incubated with diaminobenzidine solution for 5 min.

Glycosylation and Deglycosylation Studies

The SM component ($\sim 100 \mu\text{g}$) was dissolved in 10 μl ultra-pure water and incubated with 0.5% (v/v) Nonidet P40 and 2 U of *N*-glycosidase F (PNGase F) (Roche Diagnostics) at 37°C for 17 h. Analyses of the proteins before and after PNGase F digestion were performed by both SDS-PAGE and two-dimensional gel electrophoresis (2-DE), in which CBB and silver staining, respectively, were used to visualize the protein bands.

Preparation of N-linked Oligosaccharides

N-linked oligosaccharides were recovered by in-gel PNGase F digestion (Itoh et al. 2006). The released oligosaccharides were reduced with sodium borohydride and desalted with Supelclean Envi-Carb (Supelco) as described by Kawasaki et al. (2003).

Structural Analysis of Carbohydrate

The detailed carbohydrate structure was deduced with a Finnigan LTQ mass spectrometer equipped with an ESI source utilizing a C18 column eluted at 1 μ l/min with a gradient of 5–65% Solvent B over 20 min. To measure all types of glycans including neutral glycans and sulfated or sialylated acidic glycans, mass spectrometric glycan analysis was performed by using both positive and negative ion modes.

FT-IR

Infrared analyses were performed on released N-linked oligosaccharides. All spectra were recorded at 4 cm^{-1} resolution with 64 scans with a string Magna-IR™ spectrometer 750 (Thermo Fisher Scientific) from 4000 cm^{-1} to 400 cm^{-1} . Before a sample spectra were run, a background spectrum was measured for pure KBr. N-linked oligosaccharides and KBr were mixed and loaded on the sample cap.

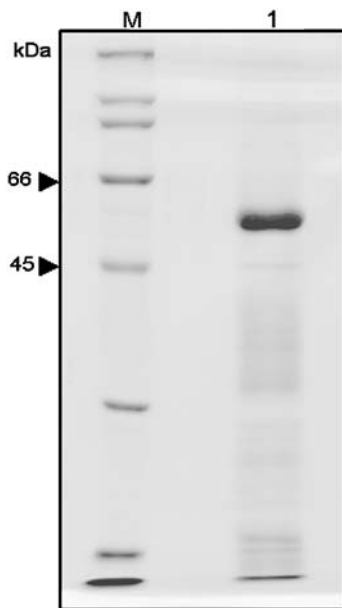


Fig. 1 SDS polyacrylamide gel electrophoretogram of the SM in the nacreous layer of *P. fucata*. Lane M, molecular mass standard; lane 1, SM. Gels were stained with Coomassie

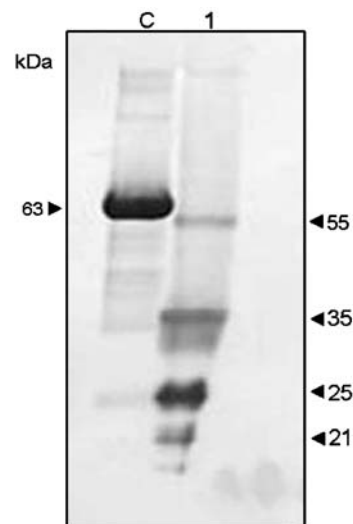


Fig. 2 Result of the lectin blot analysis for GNA lectin of the glycoproteins in the SM of *P. fucata*. Lane C, control glycoprotein (carboxypeptidase Y); lane 1, SM. The bands were visualized with NBT (4-Nitro blue tetrazolium chloride)/BCIP (5-Bromo-4-chloro-3-indolyl-phosphate)

Results and Discussion

Detection of SM Component Linked to Mannose

In our study to determine the carbohydrate structure, the targeted glycoprotein in the SM was first screened by lectin

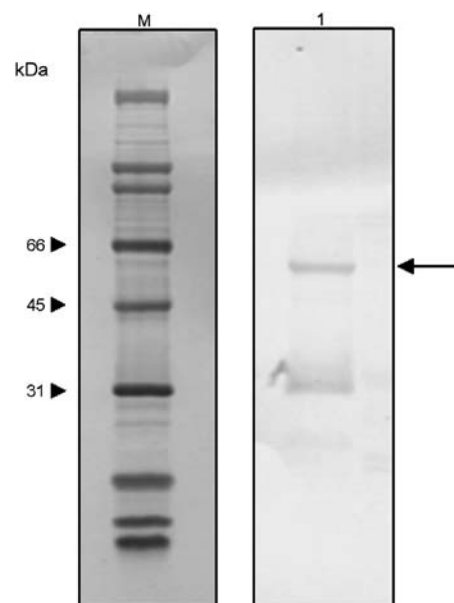


Fig. 3 Characterization of nacrein in the nacreous layer of *P. fucata* by western blotting. Lane M, molecular mass standard; lane 1, SM. Western blotting was conducted with anti-CAII to detect nacrein. The arrow on the right side of the blot indicates the position of an immunopositive band corresponding to nacrein

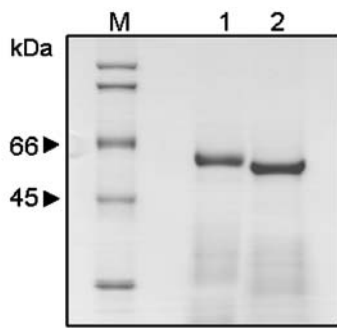


Fig. 4 Comparison of the electrophoretic mobility between samples treated with PNGase F. *Lane M*, molecular mass standard; *lane 1*, SM; *lane 2*, SM treated with PNGase F. The gel was stained with Coomassie

blot analysis after separation by SDS-PAGE (Fig. 1). The four prominent signals in the SM fraction with apparent molecular masses of 55 kDa, 35 kDa, 25 kDa, and 21 kDa were detected only with GNA (*Galanthus nivalis* agglutinin) lectin, which recognizes terminal mannose (Fig. 2). This result implied that almost all of the glycoprotein localized in the nacreous layer of *P. fucata* has “high mannose” or “hybrid” types of N-glycan. Of these four components, we selected the 55 kDa component, predominant band on SDS-PAGE, as the target molecule for further analysis of the carbohydrate structure.

55 kDa SM Protein is Nacrein

Two independent approaches were taken to identify the 55 kDa component. First, the protein was subjected to 2-DE and the target spot was analyzed by LC/MS/MS followed by a SEQUEST database search. With 39% sequence coverage, these analyses suggested that the 55 kDa com-

ponent corresponds to nacrein with a probability value of 6.22×10^{-15} . Second, western blot analysis with anti-human CAII was conducted to confirm the identity of the 55 kDa component as nacrein, which is commonly agreed to be one of the homologs of the CA family (Miyamoto et al. 1996). Two immunologically positive signals with molecular masses of approximately 55 kDa and 31 kDa were detected (Fig. 3), indicating that the 55 kDa glycoprotein was related to nacrein. Unlike the 55 kDa component, the 31 kDa component shared no distinctive similarity with the OM proteins reported so far, according to a SEQUEST database search.

Nacrein Contains a ~2 kDa N-glycan

Although both nacrein and its mRNA have been characterized, we found that the apparent molecular mass by SDS-PAGE of the protein was higher than that predicted from the amino acid sequence. This size discrepancy was originally thought to be attributed to phosphorylation or sulfation but not to glycosylation (Miyamoto et al. 1996). However, the size difference between the two forms is incongruent even when we take these two kinds of posttranslational modifications into consideration. Furthermore, the amino acid sequence of nacrein contains two potential N-glycosylation sites, suggesting that nacrein could be a glycoprotein.

To evaluate any ambiguities regarding putative glycosylation of nacrein, enzymatic deglycosylation with PNGase F was performed and the result was analyzed by SDS-PAGE and 2-DE. These analyses showed an apparent decrease of the molecular mass from approximately 55 kDa to 53 kDa and a distinct shift in the pI of the spot corresponding to nacrein from approximately 5.9 to 6.3 after treatment with PNGase F (Figs. 4 and 5). The result

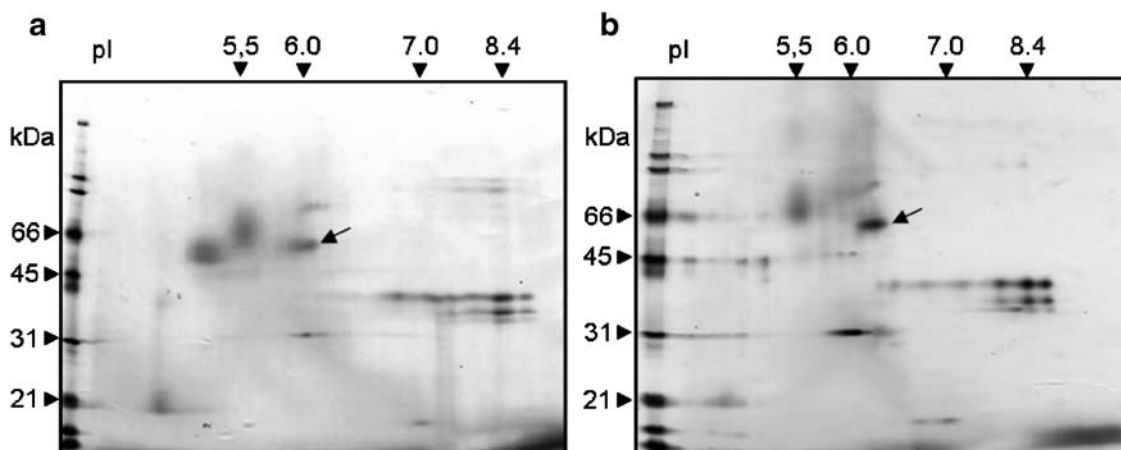


Fig. 5 Comparison of the isoelectric points of nacrein before and after treatment with PNGase F. The pI of the spot corresponding to nacrein was calculated as 5.9 (A); the pI of the spot shifted to 6.3 after

treatment with PNGase F (B). The *arrows* indicate the position of nacrein. The gels were stained with silver

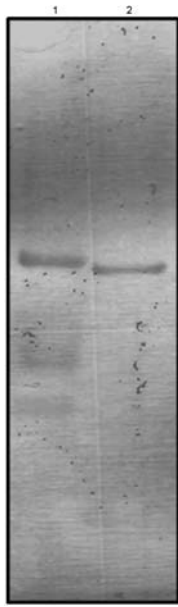


Fig. 6 SDS polyacrylamide gel electrophoretogram of the SM in the nacreous layer of *P. fucata*. Lane 1, SM; lane 2; SM treated with PNGase F. The membrane was stained with PAS

clearly indicates that nacrein contains an acidic N-glycan of about 2 kDa. On the other hand, the size discrepancy between the calculated and the apparent molecular masses by SDS-PAGE remains even if inferred mass of N-glycan is

taken into consideration. Although it remains a matter of speculation, the O-linked oligosaccharide would covalently attach to nacrein. Commonly recognized motifs of O-glycosylation sites are associated with three Thr-glycosylation sites and one Ser-glycosylation site (Pisano et al. 1993). According to the motifs, 12 potential O-glycosylation sites are found in the amino acid sequence of nacrein. In addition, the fact that nacrein is still visualized with PAS, which stains neutral and acidic sugars even after the complete release of the N-glycan can partly support our estimation (Fig. 6).

Structure of the N-glycan on Nacrein

The detailed carbohydrate structure of N-glycan was inferred by MS/MS analysis. Figure 7 shows the product ion spectra and structure of the N-linked oligosaccharide liberated from nacrein in the negative ion mode. In the carbohydrate fragmentation by collision-induced dissociation (CID)-MS/MS, fragment ions generated by this cleavage are represented as B-ion (nonreducing end) and Y-ion (reducing end). We detected the characteristic Y-ions, such as m/z 778 ($Y_{3/4\alpha''/6}$, $Y_{4\alpha''/4\beta/5\alpha'}$), 812 ($Y_{4\alpha}$), 883 ($Y_{1/4\beta/5}$), 978 ($Y_{4\alpha'}$), and 1078 ($Y_{5'}$), which are useful for the structural characterization of oligosaccharides, as well as a ladder of several Y-ions with intervals corresponding to N-acetylhexoseamine, N-acetylneuraminic acid (sialic acid),

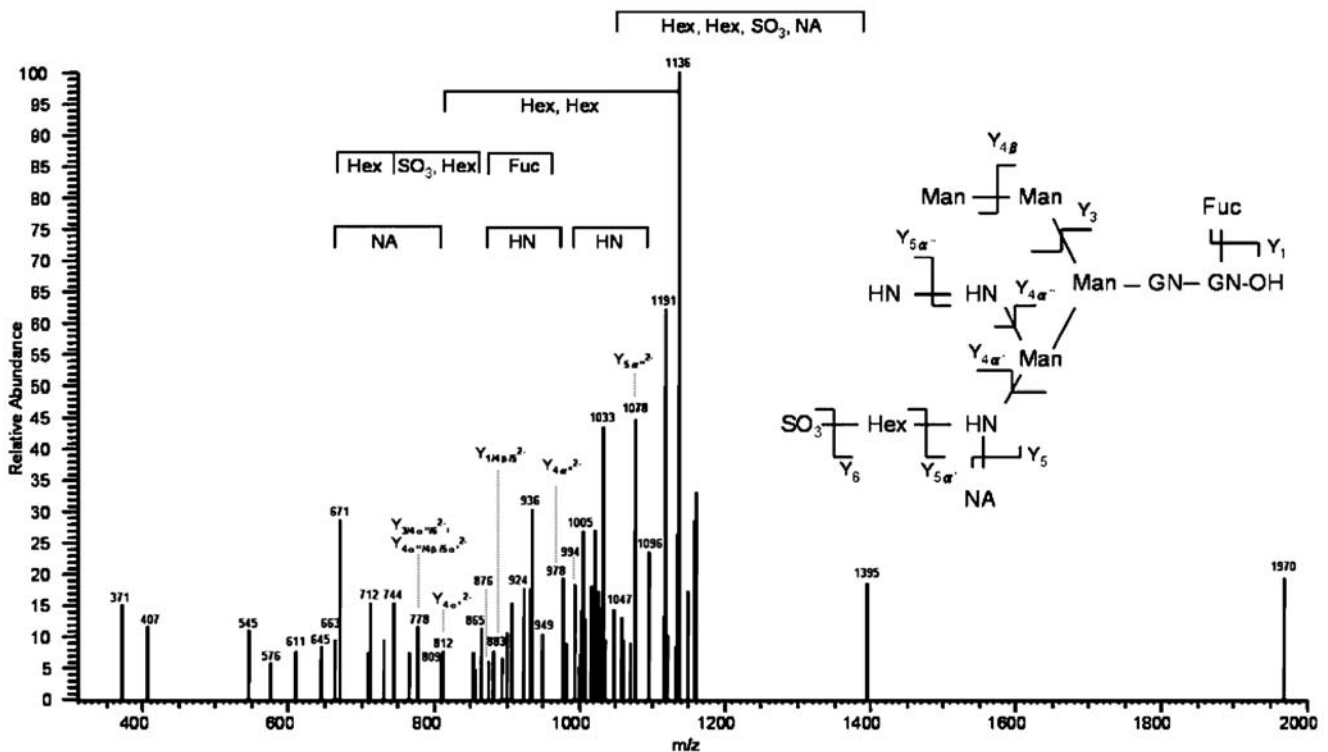


Fig. 7 MS/MS spectra of the N-glycan of nacrein (precursor ion: m/z 1179.7) in negative-ion mode. GN; N-acetylglucosamine, Fuc; fucose, Man; mannose, HN; N-acetylhexoseamine, NA; N-acetylneuraminic acid (sialic acid), Hex; hexose

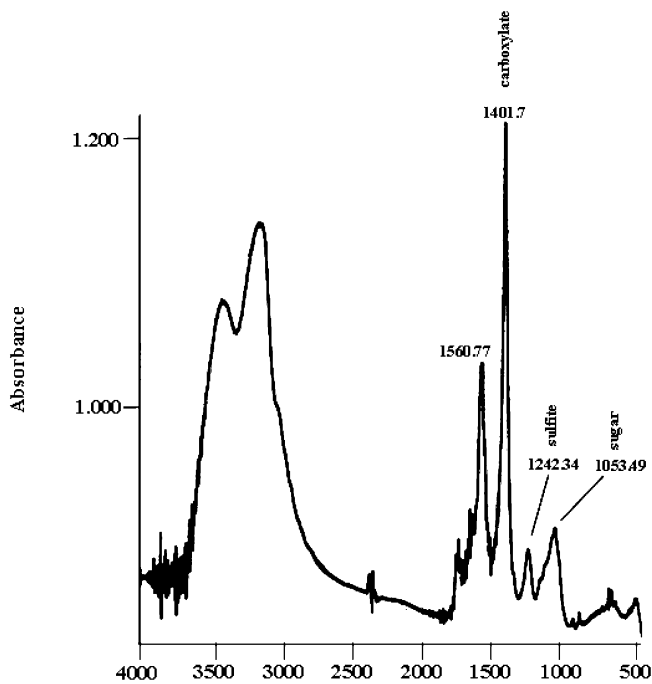


Fig. 8 FT-IR absorption spectra of the N-linked oligosaccharide liberated from nacrein

hexose, fucose, and sulfite. The presence of sulfite and sialic acid was revalidated by Fourier Transform Infrared spectroscopy (FT-IR), which showed the presence of absorption bands at 1242 cm^{-1} and 1401 cm^{-1} , corresponding to sulfite and sialic acid, respectively (Fig. 8).

It has long been maintained that OM components containing sulfate may be involved in molluscan biomineralization (Crenshaw 1972; Wada 1980). This ambiguous idea was validated for the first time in this study through characterization of the N-glycan covalently attached to an OM protein. Although our experiments do not directly address the role of a sulfite group in crystal nucleation, the estimated carbohydrate structure strongly suggests that this group indeed is involved in molluscan biomineralization.

Putative Functions of Nacrein Relevant to Molluscan Biomineralization

Originally, nacrein was believed to mediate both HCO_3^- and Ca^{2+} concentrations, and thus is deeply involved in molluscan shell mineralization, particularly in selective nucleation of aragonite crystal (Miyamoto et al. 1996). The authors tentatively proposed that Ca^{2+} would be concentrated with regular alignment of the carboxyl groups of Asp in the NG-repeat domain. However, we doubt whether the Ca-binding capacity is fulfilled by participation of the NG repeat domain, because contrary to their hypothesis, Asn is predominant over Asp in the protein N66, a relative of nacrein from *Pinctada maxima* (Kono et al. 2000). Our result suggests that instead of Asp in the

NG domain, sulfite and sialic acid may provide the necessary negative charge in the N-glycan to promote Ca^{2+} uptake of nacrein. Sulfite in the oligosaccharide structure can generate a strong field to which Ca^{2+} is drawn and consequently may create the supersaturation of Ca^{2+} necessary for nucleation of CaCO_3 crystals (Greenfield et al. 1984). Our idea is partly supported by the fact that the absorption bands corresponding to sulfite and sialic acid in N-glycans have been detected not only in nacrein but also in N66 by infrared analysis (data not shown).

Finally, we conclude that nacrein is a multi-functional glycoprotein that may be closely involved in CaCO_3 crystallization by supplying HCO_3^- and Ca^{2+} through the CA domain and N-glycan moieties, respectively. Furthermore, nacrein and its relatives are found in the aragonitic nacreous and calcitic prismatic (Takeuchi and Endo 2006) and foliated layers (Norizuki and Samata 2007), and thus another factor that is specific in shell microstructures may function as a template to control crystal polymorphism.

The mechanism of molluscan shell formation has been studied by using a variety of materials and methods. Nevertheless, the common role of macromolecules involved in mineralization processes has been explained only superficially. The characterization of carbohydrate structures in other glycoproteins present in molluscan OM will provide a new framework to elucidate the mechanism of molluscan mineralization.

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