PRELIMINARY CHARACTERIZATION OF ORGANIC NUCLEATOR AND FRAMEWORK MACROMOLECULES IN MOLLUSC SHELLS

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ABSTRACT

The nacreous (mother-of-pearl) sections of molluscan shells are natural laminates of aragonitic calcium carbonate platelets and an organic matrix. The multi-edged platelets are sub-micrometer thick and surrounded on all sides by 10-20 nm-thick organic matrix that contains several proteins and polysaccharides. Our goal is to identify the components and structure of the organic matrix and determine their function in shell formation and organization. We have extracted organic material from red abalone-*Haliotis rufescens*, and chambered nautilus-*Nautilus pompilius*. From the soluble portion, we have partially purified two proteins and determined their amino acid compositions.

INTRODUCTION

Recent years have seen a resurgence of interest in the study of biological materials. Nacre (mother-of-pearl) has been appreciated for its beauty and used for ornamental purposes for millennia. Whole shells have been used as currency by several cultures. The desirability of pearls and nacre fragments as ornament depends partly on the durability of the nacreous structure. Studies of nacre as a material have indicated that it has more than 10 fold higher fracture toughness than geological calcium carbonate[1]. This compares favorably to some of the best ceramic materials. The increase in fracture toughness of nacre compared to geological calcium carbonate is due to the fact that it is a laminate of a relatively small amount (0.2 - 5%) organic material (proteins and polysaccharides) and mineral at the sub-micron scale (Figure 1). Layers of approximately hexagonal tablets are surrounded by organic matrix[1-4]. The layers are laid down parallel to the outer surface of the shell. Fracture propagation through the mineral phase is interrupted at the interface with the organic layer as tablets either slide horizontally within a layer, crack branching occurs, and energy is absorbed[1,5]. Tablets of aragonite at the growing edge assemble in stacks inside preformed compartments of organic material[4]. The c axis of the aragonite tablets is oriented perpendicular to the face of the tablets. Twinning of crystals is observed[6,7] which takes place at three levels. Adjacent tablets frequently show identical orientation of the a and b axes. Individual tablets are made of sectors, perhaps separated by thin organic layers. Twinning of sectors on opposite sides of a platelet is frequently observed. A third order of twinning occurs between grains within a sector.

Production of the molluscan shell, including the nacreous layer, is an extracellular process. Growth of the shell is controlled by the organic components, which are laid down prior to the mineral phase[4]. Thickening of the nacreous layer occurs over the entire inner shell surface during much of the life of the organism.

Proteins are thought to have major roles in the assembly of the shell. Proteins exist as discrete species of linear polymers of amino acids with different amino acid sequences. Individual protein species undoubtedly play different roles in the structure. The amino acid composition of the bulk matrix of either whole shells or isolated prismatic and nacreous layers has been determined for a number of species[8-11]. Amino acid composition of some individual proteins from *Mytilus* and *Haliotis* has been reported[12,13]. The most important data for determining function and evolutionary relationships are the sequences of amino acids in individual proteins. A recent report has determined a portion of the amino acid sequence from three shell proteins isolated from *Mytilus*[13]. Shell proteins are frequently rich in aspartic acid, this has led to the suggestion that adjacent aspartate residues combine to bind Ca^{++}[11].

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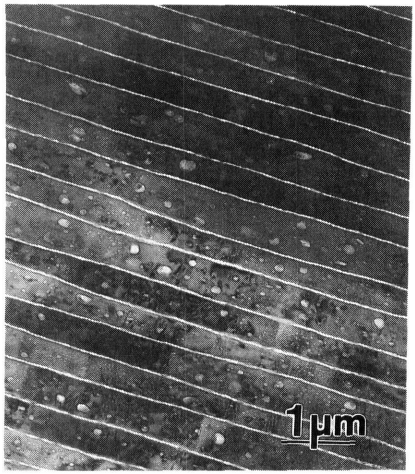

Figure 1. TEM image of nacre section of *Nautilus* showing brick and mortar organization of aragonite (orthorhombic $CaCO_3$) platelets and organic matrix, respectively, in edge-on orientation.

Our studies, which are still in a very early stage, are directed toward understanding the role of each organic component in shell formation. It is hoped that when the mechanism by which nacre formation is understood at the molecular level, it will be possible to use these biomimetic principles to create new materials with better mechanical properties using ceramics other than calcium carbonate and polymers other than proteins and polysaccharides.

MATERIALS AND METHODS

Shells of red abalone (*Haliotis rufescens*) were collected from Sonoma County, California. The shells were broken into pieces and the periostracum and prismatic layers were removed by abrasion on a diamond grinder. Shells of razor clams (*Siliqua costata*) and chambered nautilus (*Nautilus pompilius*) were obtained from local curio shops with the periostracum and outer prismatic layers removed. Shells were broken into pieces about 0.5 cm on each side, and milled to a fine powder with a roller mill.

Shell powder was demineralized in 5% NaAcetate pH 3.0 under vacuum to assist in removal of CO_2. The insoluble material was precipitated by centrifugation at 7,000 rpm in a Sorvall GSA rotor for 10 minutes. The supernatant was filtered through an Amicon YM10 ultrafiltration membrane (10,000 nominal molecular weight cutoff). The retentate was washed with 5% NaAcetate pH 3.0, and subsequently with 10 mM Tris-Cl pH 8.0, 20 mM NaCl. The retentate (YM10 soluble extract) was resuspended in 10 mM Tris-Cl, 20 mM NaCl pH 8.0, filtered through a 0.2 m membrane and stored at 4°C.. For the abalone extract, the filtrate which was not retained by the YM10 membrane was filtered through a YM3 (3,000 nominal molecular weight cutoff) ultrafiltration membrane and filtered. This is termed a YM3 soluble extract. Residual insoluble material, which comprised the bulk of the organic material, was incubated with sodium dodecyl sulfate and 2-mercaptoethanol to extract additional protein. Alternatively, for some experiments, residual insoluble material was extracted with 0.5 M EDTA·

For attachment of dabsyl (4-dimethylaminoazobenzene-4-sulfonyl) and dansyl (5-dimethylaminonaphthalene-1-sulfonyl) tags, the chloride form of the dyes were dissolved in acetone at 3 - 10 mg/ml. Extracts were dialyzed against 0.1 M CO_3 buffer, pH 8.9. Extract (50μl) was heated to 70° C and 50 μl dye was added. Reactions were mixed periodically for 10

min. Acetone (60 µl) was added, the samples were spun briefly, and incubated at -20°C for a few minutes. Protein was precipitated by centrifugation at 15,000 g for 10 minutes. Protein was assayed by the bicinchoninic acid method[14].

Phenylthiocarbamyl amino acid analysis[15] and automated Edman degradation[16] were performed as previously described. Electrophoretic separation of proteins was performed in SDS-polyacrylamide gels. Laemmli discontinuous buffer system with 11% acrylamide separating gels, 4-20 % acrylamide gradient gels or the buffer system of Schagger and von Jagow with bicine substituted for tricine[17] were used. Electrophoretic transfer of proteins from acryalmide gels to polyvinylidenedifluoride (PVDF) membranes was performed in 25 mM Tris, 192 mM glycine, pH 8.3. Blots were stained with 0.1% Coomassie blue in 50% methanol.

RESULTS AND DISCUSSION

The aforementioned sample preparation scheme yields up to three solublized fractions from each species. A YM10 soluble extract (material retained by an Amicon YM10 ultrafiltration membrane) was prepared from abalone, razor clam, and chambered nautilus. Filtrate which passed through the YM10 membrane was passed through a YM3 membrane (YM3 soluble extract). Some proteins of apparent molecular weight of greater than 20,000 passed through the YM10 (10,000 nominal molecular weight cutoff) indicating performance alteration of the membrane under the conditions we have used. Some proteins not removed by sodium acetate were extracted with sodium dodecyl sulfate. Even under the most stringent extraction conditions the majority of organic material remained insoluble.

Proteins from the SDS soluble extract of red abalone shells were separated in preparative SDS-polyacrylamide gels and transferred to PVDF membranes. Bands of apparent molecular weight 56,000 and 41,000 were cut out of the membrane and subjected to microsequence analysis. No sequence was obtained suggesting that the amino termini were blocked. Blockage of the amino terminus is a common occurrence in eukaryotes and might be expected in a protein excreted from the organism as it would provide some protection from proteolysis by aminopeptidases. Amino acid compositions were obtained from the 56k and 41k bands and are shown in Table 1. Glycosylation, other posttranslational modification or anomalous mobility in SDS gels may have influenced the observed molecular weights in this preliminary study.

Table 1
Preliminary Amino acid composition of electroblotted protein bands

NaAcetate insoluble, SDS soluble extract from red abalone

	41K	56 K
Amino acid	Mole%	
Asp	20.7	9.0
Glu	7.4	11.3
Ser	8.7	12.3
Gly	25.0	22.8
His	1.1	2.3
Arg	5.1	4.7
Thr	3.6	4.9
Ala	6.4	6.7
Pro	4.1	3.9
Tyr	1.9	2.6
Val	3.1	3.8
Met	0.3	0.3
Ile	2.0	2.9
Leu	5.1	6.1
Phe	2.4	2.4
Lys	3.1	4.2

Cariolou and Morse[12] have reported purification of proteins of similar (43,000 and 54,000) molecular weight from adult and juvenile shells of red abalone. The amino acid compositions observed are similar but not identical to what we have seen, it is not yet clear whether we have characterized the same proteins purified by Cariolou and Morse. Our analysis is in agreement with the previous study in that a high amount of acidic residues, particularly aspartic acid is present in both proteins. Other studies of bulk amino acid compositons from molluscan shells have frequently found similar enrichment of acidic residues[8-13]. The acidic groups have been proposed to be involved in Ca^{++} binding[10]. The separation on a one dimensional gel means that our bands were not completely pure. The amino acid compositions reported by Cariolou and Morse are unusual in that histidine, tyrosine, methionine, valine, phenylalanine, isoleucine, and leucine were not found in the 43k protein protein. Histidine, threonine, arginine, tyrosine, methionine, isoleucine, , leucine, and lysine were not found in the 54k protein. It is extremely rare to see proteins of such high molecular weight with such a limited range of amino acids present. The limited amino acid composition observed would be compatible with low molecular weight monomers associating to form a higher molecular weight complex.

Binding of the cationic dye stains-all has been used as an indication of calcium binding by proteins[12]. Blue staining has been correlated with calcium binding while red staining is characteristic of most other proteins. Our 56k protein has been observed to stain red with stains-all while the previously reported 54k protein was reported to stain blue. We did our extraction with SDS in the presence of reducing agent. One plausible explanation for this discrepancy is association of EDTA with proteins might change their dye binding properties. EDTA is frequently used for demineralization, but is difficult to remove from proteins once they have been solubilized. One report of calcium binding by oyster shell extract was later found to be due to EDTA associated with the protein[18,19]. Binding of Ca^{45} to proteins from acetic extracts of *Mytilus* has recently been reported[13].We have made several attempts to further purify proteins from SDS-solubilized material by a variety of procedures other than electroblotting with little success. These failures are probably due to the low yield of protein, the failure of proteins to stain well with protein stains, and the formation of aggregates. We are exploring the use of dyes covalently attached to proteins as tags to follow proteins during purification and during mineralization experiments. Dabsyl is an orange dye which can easily be attached to amino groups. Dansyl is a fluorescent dye which also attaches to amino groups and can be removed if necessary. Excitation of dansylated proteins by 340 nm light results in strong fluorescence at 510 nm. Figure 2 shows results of dansylation of YM10 extracts from abalone and nautilus. Typically in silver stained gels of material soluble in sodium acetate we see a few somewhat diffuse bands with lesser amounts of less strongly staining bands and a background smear of staining. In the dansylated preparation we see low molecular weight bands which are much more clearly defined than silver stained material plus considerable background staining. Dansylation and dabsylation show promise as a method for reduction of aggregation as well as a tag allowing the monitoring of proteins during purification. Unlike silver staining, which results in material unsuitable for further use, dansylated material is not destroyed by the analytical process. Bands can be electroeluted from preparative gels and digested with proteases. Peptides isolated from dansylated material should be suitable for protein sequencing. One preliminary experiment suggests that dansylation and dabsylation may not prevent association of proteins with calcium carbonate during mineralization experiments. Tagged proteins may thus be useful for the study of protein location during mineralization. Once dansylated, however, the resolved proteins do not stain well with silver.

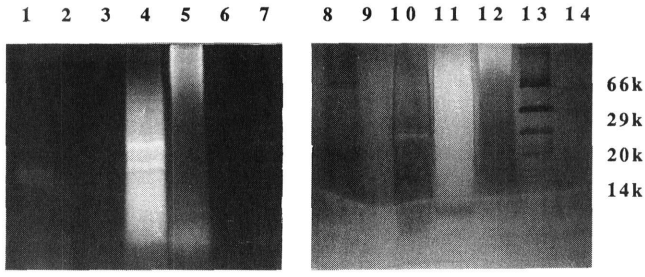

Figure 2 Comparison of dansylated and silver stained preparations. Lanes on left were photographed under ultraviolet light to show dansylated material. Lanes on right are the same gel silver stained. Lanes 1, 8 - 14 k region of dansylated abalone YM10 extract isolated from preparative gel. Lanes 2, 9 - as lane 1 except digested with trypsin. Lanes 3, 10 - Abalone YM10 extract. Lanes 4, 11 - Abalone YM10 extract, dansylated. Lanes 5, 12 - Nautilus YM10 extract, dansylated. Lanes 6, 13 - Molecular weight standards, Bovine serum albumin, carbonic anhydrase, trypsin inhibitor, lysozyme. Lanes 7, 14 - Molecular weight standards dansylated.

Mineralization experiments were carried out in 5 ml artificial seawater[20] with various amounts of mollusc shell extracts in 18 x 150 mm tubes. The solution was supersaturated with $CaCO_3$ by addition of 0.2 ml 0.1 M Na_2CO_3 while rapidly mixing the solution with a vortex mixer. In the absence of extract, hundreds of small particles formed on the side of the tube within 4 days. Addition of greater than 25 μg/ml protein of abalone or razor clam extract completely inhibited particle formation. Intermediate concentrations of extract produced particles with interesting properties which will be described in a separate manuscript. Inhibition of mineralization by organic extracts of shells has previously been reported, in these cases, the extracts were from shells which were calcitic rather that aragonitic[19,21]. Organic matter has been shown to bind to calcium carbonate[22]. It has been suggested that in the ocean environment, calcium carbonate free of organics may not exist. At first thought, the idea that modulation of growth of calcium carbonate occurs by inhibition rather than facilitation may seem questionable, however, incorporation of ions into crystals is usually inhibitory rather than stimulatory. The same principle may hold for organics. Ions such as Mg^{++} which are incorporated more readily into calcite favor growth of aragonite crystals, while ions such as Ba^{++} favor growth of calcite, but are more readily incorporated into aragonite[23,24]. Incorporation of anything other than the appropriate ion into a crystal will result in lattice distortion and subsequent slower growth of the crystal face.

Nacreous structures are found in some members of the three major groups of mollusks, the gastropods, cephalopods, and bivalves. These groups diverged over 500 million years ago. While the basic aspect of the nacreous structure has been conserved in these groups, the long time since their divergence could allow considerable variation in molecular components. We find size differences in the major extractable proteins from abalone and nautilus[25].

The ultimate goal of our research is the development of new materials based on the princliples governing the assembly of nacre. We plan to clone the genes coding for the proteins of nacre and express them in bacterial or eukaryotic cells. We are developing a protein-producing bioreactor which will be useful for making large quantities of proteins[25]. This work may ultimately allow artificial production of nacre.

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