Soluble Protein of the Organic Matrix of Mollusk Shells: A Potential Template for Shell Formation

Abstract. *A significant proportion of the soluble protein of the organic matrix of mollusk shells is composed of a repeating sequence of aspartic acid separated by either glycine or serine. This regularly spaced, negatively charged aspartic acid may function as a template upon which mineralization occurs.*

Mollusk shells are generally composed of calcium carbonate crystals enclosed in an organic matrix. Chemical analyses indicate that the organic matrix is primarily a glycoprotein, characteristically containing large proportions of acidic amino acids (*1*) and acid mucopolysaccharides (*2*). Lipids are also found in some organic matrices (*3*). The organization of these components in the matrix is not understood.

The organic matrix is observed to form prior to mineralization (*4*) and probably is involved in the formation of the shell (*5*). Two different functions have been ascribed to the matrix during mineralization (*6*). One theory envisages the organic matrix as a template which controls crystal growth (*7*), while the other proposes that the matrix functions as a compartment in which crystals grow (*4*). We report here a partial characterization of the protein component of the organic matrix. This information should be useful for obtaining a better understanding of the role of organic material in the process of mineralization.

Our strategy in this study was dictated by the observation that the organic matrix extracted from these shells contained from 15 to 43 mole percent of aspartic acid (Table 1). Accordingly, a mild acid hydrolysis procedure was employed that preferentially cleaves on both sides of aspartic acid (*8*). This procedure released significant quantities of additional free amino acids from all shell proteins, indicating that portions of the shell proteins are made up of simple repeating sequences with alternating aspartic acid residues (Fig. 1).

Shells of *Crassostrea virginica* and *Mercenaria mercenaria* (freshly collected) and *Crassostrea irredescens* and *Nautilus pompilius* (air-dried and stored) were physically cleaned and the periostraca removed. The shell portions and their mineralogy used for analysis are identified in Table 2. The shells were decalcified by dialysis at room temperature against phosphate-buffered 8 percent ethylenediaminetetraacetic acid, *p*H 6.9, containing 0.1 percent sodium azide. After complete decalcification the organic matrix was dialyzed against distilled water. The soluble and insoluble fractions were separated by centrifugation. The insoluble fraction was washed repeatedly with distilled water and then lyophilized. The soluble fraction was lyophilized and then desalted on a Sephadex G-25 column in distilled water in order to remove any adherent free amino acids. The observation that the soluble fraction of the organic matrix of *C. virginica* is partially excluded on Sephadex G-200 and included on Sepharose 4B suggests that it has a maximum molecular weight of the order of 1 million. The yields of soluble and insoluble fractions per unit of shell weight, given in Table 1, compare favorably with yields reported by Hare and Abelson (*5*). Weighed portions of the soluble fraction containing norleucine as an internal standard were hydrolyzed in vacuo with 0.5 ml of redistilled 6*N* HCl at 108°C for 20 hours. The hydrolysis products were analyzed on a Durrum 500 amino acid analyzer, and the amino acid compositions are given in Table 1. Shell proteins were also treated with 1.0 ml of 0.25*M* acetic acid in vacuo at 108°C for 48 hours. The resulting freed amino acids were analyzed directly on the Durrum 500 amino acid analyzer.

Cleavage on both sides of aspartic acid resulted in the release of significant quantities of glycine and serine in addition to the expected aspartic acid [see Table 2 and

Table 1. Amino acid compositions of the soluble fraction of the shell organic matrix and the proportions of soluble and insoluble organic matrix per unit shell weight.

Quantity	*Crassostrea virginica*			*Crassostrea irredescens*		*Mercenaria mercenaria* shell layers minus myostracum, aragonite	*Nautilus pompilius* shell layers minus myostracum, aragonite
	Foliated and chalky layers, calcite	Foliated layers, calcite	Chalky layers, calcite	Foliated and chalky layers, calcite	Adductor myostracum, aragonite		
Amino acid composition of soluble fraction (mole %)							
Aspartic acid + asparagine	32.53	36.75	33.22	42.96	15.10	25.60	26.13
Threonine	2.12	1.34	1.49	0.85	4.48	3.84	4.82
Serine	21.54	12.00	27.14	11.85	8.43	11.06	7.94
Glutamic acid + glutamine	5.25	7.08	5.31	6.60	13.70	7.16	6.59
Proline	2.78	2.43	1.53	0.70	6.43	8.11	4.58
Glycine	24.67	27.27	25.70	26.02	15.89	12.28	23.58
Alanine	1.47	1.52	1.15	2.80	4.85	4.78	4.44
Cysteine						2.08	
Valine	1.05	0.94		1.36	2.65	2.38	1.52
Methionine	0.33		0.29	0.64	1.64	1.22	0.57
Isoleucine	0.91	1.14	0.39	0.69	4.53	2.16	1.72
Leucine	0.89	0.96	0.43	0.98	6.45	3.23	2.21
Tyrosine	3.20	3.19	2.04	1.44	2.55	3.88	6.43
Phenylalanine				0.68	3.38	2.24	2.06
Histidine	0.46	0.87	0.14	0.44	2.26	0.99	2.12
Lysine	1.90	2.67	1.09	1.73	5.04	4.74	2.84
Arginine	0.90	1.77		0.87	2.54	4.18	2.38
Soluble and insoluble nondialyzable organic matter in shell (%)*	0.33	0.58	0.91	0.45	0.60	0.33 ± 0.04	4.05 ± 0.6
Soluble nondialyzable organic matter in shell (%)	0.12	0.29	0.85	0.11	0.07	0.09 ± 0.01	0.27 ± 0.05
Proportion of protein in soluble fraction (%)†	42.70	45.0	19.4	63.6	11.3	83.6	14.7

*Represents the proportions of soluble and insoluble organic matrix after decalcification and exhaustive dialysis against water. †Calculated from moles of amino acid per unit weight soluble fraction obtained on complete hydrolysis assuming 25 percent of the "dry" weight to be water.

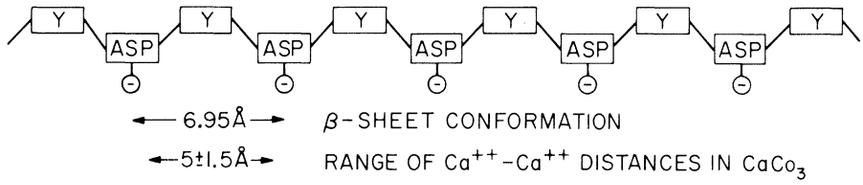

Fig. 1. Schematic illustration of the $(Asp\text{-}Y)_n$ sequence, where Y represents serine or glycine. The distance from one aspartic acid residue to the next in the β-sheet conformation is 6.95 Å. The Ca-Ca distance in the unit cells of aragonite and calcite ranges from about 3.0 to 6.5 Å.

Table 2. Proportions of free amino acids released after 48 hours of hydrolysis with 0.25*M* acetic acid at 108°C. After 48 hours the maximum yield of aspartic acid (Asp) was obtained. Yields are calculated from the amounts of the particular amino acids present in an equivalent, completely hydrolyzed sample of soluble organic matrix. The yields of gylcine (Gly) and serine (Ser) are significantly greater than yields obtained as a result of random cleavage (*9*). Values in the last column represent the moles released assuming an Asp-Y-Asp-Y-Asp-Y. . . sequence divided by the total moles present in an equivalent, completely hydrolyzed sample of soluble organic matrix.

Species and shell parts analyzed	Mineral	Yield (%)			Minimum proportion Asp-Y-Asp-Y sequence (%)
		Asp	Gly	Ser	
Crassostrea virginica					
Foliated and chalky layers	Calcite	82.1	28.8	20.0	29.9
Foliated layers	Calcite	58.9	25.7	13.0	17.8
Chalky layers	Calcite	89.9	33.3	27.7	37.8
Crassostrea irredescens					
Foliated and chalky layers	Calcite	80.2	36.8	28.8	26.4
Adductor myostracum	Aragonite	65.4	14.7	17.1	8.3
Mercenaria mercenaria					
Shell layers minus myostracum	Aragonite	65.0	26.5	18.3	13.3
Nautilus pompilius					
Shell layers minus myostracum	Aragonite	68.0	26.7	10.0	14.3

(*9*)]. Threonine, glutamic acid, and alanine are released in such small quantities that quantitation is not reliable.

The release of serine and glycine together with aspartic acid implies that sequences of the type $(Asp\text{-}Y)_n$, where Y is predominantly serine or glycine, are found in the protein of all the organic matrices examined (Fig. 1). The minimum proportion of $(Asp\text{-}Y)_n$ sequence is calculated, assuming one continuous Asp-Y-Asp-Y-Asp-Y. . . sequence, from the moles of glycine and serine released on partial acid hydrolysis compared to the total number of moles of amino acids present in a completely hydrolyzed equivalent portion of soluble organic matrix. The results are shown in Table 2. In all the species examined the $(Asp\text{-}Y)_n$ sequence comprises a significant part of the soluble organic matrix protein. In no case could it account for more than about 55 percent of the protein present. These results do not, however, distinguish between long continuous or short discontinuous sequences of the $(Asp\text{-}Y)_n$ type. Furthermore, it is not known whether such sequences comprise a majority of certain shell polypeptides or whether they are interspersed among a heterogeneous collection of proteins. This sequence, because of its repeating nature and the predominance of glycine and serine in the repeating unit, is similar to that of other structural proteins, such as collagen and silk.

This characterization of the protein components does not reveal any consistent differences between the calcitic shells of the oyster species and the aragonitic shells of *Mercenaria* and *Nautilus*. However, the protein fractions of the separated shell layers of *C. virginica* contain different amounts of $(Asp\text{-}Y)_n$ sequence and the proportions of Gly and Ser released are also different.

Negatively charged aspartic acid residues are thus found to be present as approximately every second residue, forming a significant portion of the organic matrix proteins (Fig. 1). It is possible that this sequence, comprising regular repeating negative charges, could bind Ca^{2+} ions and thus perform an important function in mineralization. The distance from one aspartic acid residue to the next, if the protein is fully extended, is 7.27 Å (*10*). Most of the Ca^{2+}-Ca^{2+} distances in the crystal lattices of aragonite and calcite range from about 3.0 to 6.5 Å (*11*). As folding will reduce the aspartate-aspartate distances, the spatial requirements for interacting with crystals of calcium carbonate are consistent with an $(Asp\text{-}Y)_n$-type sequence, provided the charges are located on one side of the protein only. This latter provision could be accounted for if the $(Asp\text{-}Y)_n$-type sequence adopted a β-sheet conformation. Infrared spectroscopy indicates that at least part of the protein is present in the β-sheet conformation (*12*). Furthermore, when aspartic acid, glycine, and serine occur in proteins, more than 50 percent of the time they are most likely to be in the β-sheet conformation (*13*).

It is interesting to note that one of the calcium binding sites of carp and hake myogen has a sequence remarkably similar to the organic matrix protein, namely, Gly-Asp-Ser-Asp-Gly-Asp-Gly-Val-Asp-Glu (*14*). Although the mechanisms of binding are probably different, the known association of calcium with this type of sequence lends support to the hypothesis that the $(Asp\text{-}Y)_n$-type sequence itself binds calcium.

The $(Asp\text{-}Y)_n$-type sequence is shown to be present in the organic matrices from the five mollusks examined. This repeating sequence clearly plays an important functional role in the organic matrix and may function as a template for mineralization.

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9. Four control proteins, egg-white lysozyme, yeast glyceraldehyde-3-phosphate dehydrogenase, bovine ribonuclease, and bovine insulin A, were partially hydrolyzed under the designated experimental conditions in order to determine the degree of nonspecific cleavage. The average mole percentages of glycine and serine released nonspecifically are 4.4 ± 1.5 and 3.1 ± 1.7, respectively, despite the fact that they are present in different proportions in the original proteins. These values are well below those reported for mollusk shell proteins in Table 2. It was also noted that the mole percentage of aspartic acid released after partial acid hydroly-

sis of ribonuclease A, glyceraldehyde-3-phosphate dehydrogenase, and lysozyme corresponds to ± 10 percent of the known mole percentage of aspartic acid in these proteins. For insulin A, however, which contains only asparagine, 70.4 percent of the asparagine was released as aspartic acid, indicating that considerable deamidation had taken place. Thus, the mole percentage of aspartic acid released from shell proteins should be regarded only as an approximation of the amount of aspartic acid present relative to asparagine.
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Goldfish Retina: Functional Polarization of Cone Horizontal Cell Dendrites and Synapses

Abstract. *In serial electron micrographs we observed that dendrites of goldfish cone horizontal cells are either central or lateral in ribbon synaptic triads, depending on cone and horizontal cell type. The chromatic properties of cone horizontal cell responses may be explained if the cone horizontal cells act as interneurons, receiving from cones through their central processes but acting on cones through their lateral processes.*

The initial step in the processing of neural information by the vertebrate retina involves interactions between photoreceptor, horizontal, and bipolar cells at the outer synaptic (or plexiform) layer. It is known that photoreceptors make synapses on bipolar cells and that horizontal cells modify bipolar cell responses (*1*).

Studies of synaptic ultrastructure in the retina (*2, 3*) have shown that the synapses of photoreceptors are characterized by clusters of postsynaptic processes opposite presynaptic organelles (synaptic ribbons). In goldfish, the postsynaptic processes are arranged in groups of three (*4*). Such triads comprise a bipolar cell dendrite that is centrally located opposite the ribbon and a pair of horizontal cell processes that are lateral to the ribbon and bipolar cell dendrite. We now report on the relation of the structural arrangements in ribbon synapses to functional pathways that involve cones and horizontal cells.

Stell and Lightfoot (*5*) identified three morphologically distinct types of cone horizontal cells in the goldfish retina by silver chromate impregnation (Golgi method). By morphological identification of the cones that made contact with processes of these cells, and by correlation of cone structure with visual pigment content as determined by microspectrophotometry (*6*), they showed that type H1 cells make contact with red-, green-, and blue-sensitive cones, H2 cells make contact with green- and blue-sensitive cones, and H3 cells make contact only with blue-sensitive cones.

Cone horizontal cells of all three types were selected from Golgi preparations, remounted, and sectioned serially at 60 to 90 nm in the plane perpendicular to the long axis of the cones. Fifty-three cone pedicles (synaptic endings) were sectioned serially and recovered. Each section was examined and photographed in the electron microscope. The series of micrographs were evaluated with regard to (i) cone type, identified by continuing the series of sections to the characteristic cone inner segments; (ii) number of cone synaptic ribbons; (iii) number of ribbon synapses in which a stained horizontal cell process made contact (*7*); and (iv) position of that contact (central or lateral) in the synaptic ribbon complex. Representative synaptic complexes were reconstructed graphically as projection drawings on orthogonal planes.

Electron micrographs of cone pedicles contacted by Golgi-stained horizontal cell processes (Fig. 1) demonstrate that horizontal cells of different types contact cones of a given type in different ways. Figure 1A shows dendrites of an H1 cell that terminate as lateral elements in synaptic ribbon complexes of a green-sensitive cone. This

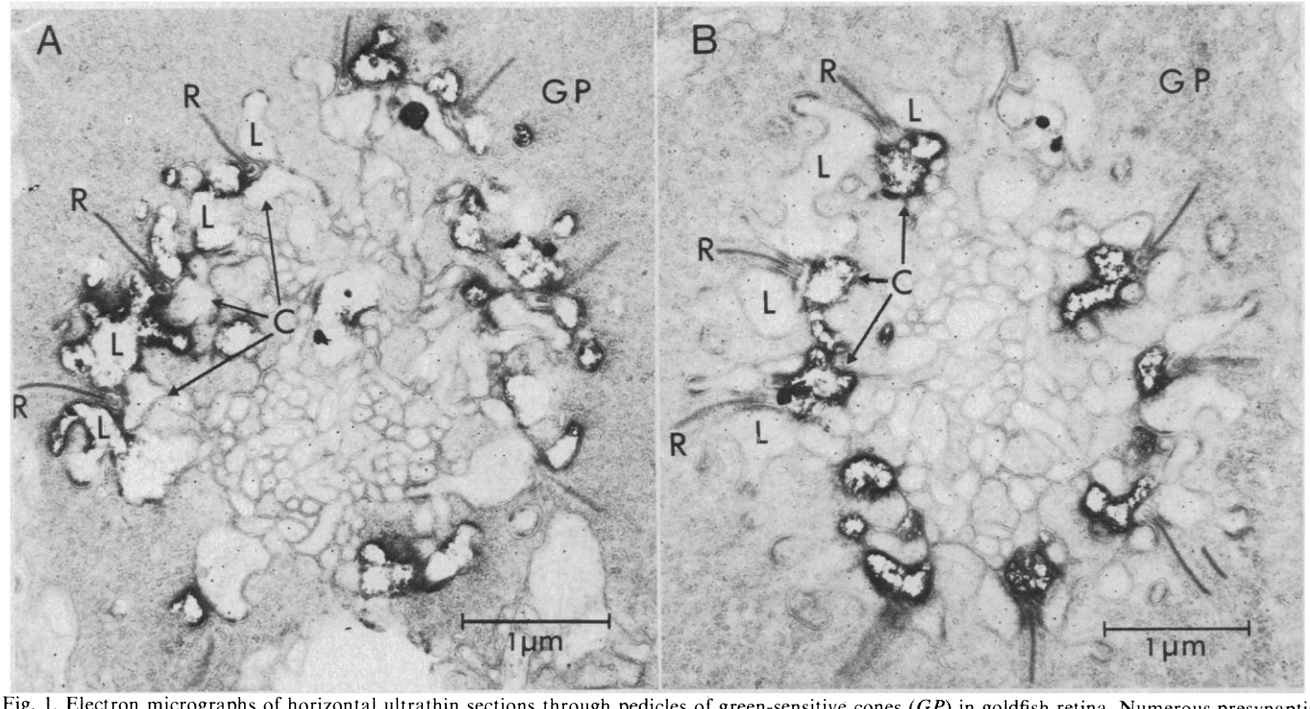

Fig. 1. Electron micrographs of horizontal ultrathin sections through pedicles of green-sensitive cones (*GP*) in goldfish retina. Numerous presynaptic ribbons (*R*) and postsynaptic triadic arrangements of central (*C*) and lateral (*L*) horizontal cell processes are shown. (A) Dendrites of Golgi-impregnated H1 cells are lateral in distribution. (B) Dendrites of Golgi-impregnated H2 cells are central in distribution.