Studies on the Mineralization of Calcified Tissues in Molluscs—XVI. Amino Acid Composition of Mucous Substance Secreted through Inner Surface of Mantle of Some Marine Bivalves*

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In biological mineralization, it has been supported theoretically and experimentally that organic matrices in such calcified tissues as bone and tooth have an important role in initiating the nucleation of mineral crystals under the physiologically controlled concentration of calcium phosphate. Different considerations appeared on the role which chemical fractions of the matrices are most available as the crystal-nucleating site. SOLOMONS and IRVING¹⁾ have found that dentine collagen differs probably from skin and tendon collagen on some properties of structural observation and chemical behaviors and paid attention to the ϵ -amino groups contained in the nucleation center of lysine and hydroxylysine in the calcifying collagen. It was proposed by NEUMAN and NEUMAN²⁾ that crystals of hydroxyapatite may be introduced onto the organic matrices by epitaxial growth without exceeding the solubility product of calcium phosphate. GLIMCHER³⁾ concluded that the nucleation and mineralization of collagen in non-mineralized tissues are inhibited by the negatively charged mucopolysaccharides and glycoproteins in the ground substance. Most of histochemical investigations,⁴⁻⁶⁾ on the other hand, have suggested that acid mucopolysaccharides may be important in calcification *in vivo*. SOBEL⁷⁾ has paid special attention to the functional role of acid mucopolysaccharides together with sulfate, which charges negatively and can bind with calcium, as a "local factor" in calcification. From a fine electron micrograph which showed clearly the microcrystals of apatite deposited at regular intervals along the striations of the collagen fibrils, PIEZ⁸⁾ has emphasized that the mechanism of nucleation in biological mineralization may probably depend on some sequential order of amino acid side chains which would constitute an "active" surface akin to the active site of enzymes.

In shell formation of molluscs, it has been already suggested that sulphated mucopolysaccharides in mucous substances of the mantle may combine an enormous amount of calcium and transport them to the site of mineralization of the shell throughout the mantle tissue (TANAKA and HATANO⁹⁾), and so occurrence of sulfatase in the mantle mucus assumes to liberate calcium by hydrolysing the sulphated mucopolysaccharides there (SOUHA¹⁰⁾). TANAKA and HATANO¹¹⁾ has thought, based on the above-mentioned concepts, that the sulphated mucopolysaccharides and mucoprotein

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may act as a calcium carrier and then calcium carbonate put out to precipitate crystals in molluscan shells. Mucous substances in the extrapallial fluid secreted between mantle and shell show metachromasia with toluidine blue (NAKAHARA,¹²⁾ WADA¹³⁾ and appear to be of acid mucopolysaccharides and mucoprotein (TANAKA and HATANO,⁹⁾ KOBAYASHI,¹⁴⁾ FURUHASHI and WADA¹⁵⁾). Although the mucous substances seem to be incorporated into the calcified nacre in *Pinctada martensii* and *Pinna attenuata*, (NAKAHARA¹²⁾) it is possible that the mucous substances may be denatured to form the organic matrix, conchiolin.^{15,16)} A part of organic matters in the fluid appears to be removed in the process of formation and mineralization of conchiolin. HARE¹⁷⁾ and WADA¹⁸⁾ have found that amino acid composition of the organic matrix in mineralized layers differs markedly in quantity but not in quality from that of the matrix in non-mineralized layers. That side chains of acidic and basic residues of the organic matrix are important in opening of nucleation has been reported by HARE.¹⁷⁾ Furthermore, WADA¹⁹⁾ has found mixing of mineralized and non-mineralized organic granules formed on a coverslip put between the mantle and shell and the incorporation of calcium-45 in only the organic granules, which show metachromasia with toluidine blue, when the isotope was injected into adductor muscle of the oyster with coverslips. That mineral crystals occur along distribution of the metachromatic substance in the organic granules may suggest that the segment of the organic matrix which is metachromatic involves in some ways in initiation of *in vivo* mineralization of shells without excess supersaturation of calcium carbonate, as has been discussed by WADA.^{18,19)} He²⁰⁾ found histochemically that metachromasia of the matrix is probably due to sulfate groups of acid mucopolysaccharides. ABOLINŠ-KROGIS²¹⁾ thought that the metachromatic granules which appeared in broken areas on repairing of the shell of *Helix pomatia* and were termed "organic crystal" by her, have a physiological function corresponded to the segment of the organic matrix to induce the nucleation in bones and teeth. On the other hand, an attention has been paid that organic and inorganic phosphates in metabolites and natural sea water behave as a crystal poison which inhibit the nucleation and subsequent growth of shell minerals (SIMKISS²²⁾).

All of the organic matrix which reacts metachromatically and has the higher ratio of acidic to basic amino acid residues, however, do not always mineralize in process of formation of shells.¹³⁾ For example,¹⁸⁾ amino acid pattern of conchiolin appears to be modified in dependence on taxonomic significance, and several non-mineralized conchiolin of Cephalopoda and Gastropoda has the similar ratio of acidic to basic residues as pointed out in mineralized conchiolin by HARE.¹⁷⁾ While young periostracum formed between outer and middle folds of mantle of several marine bivalves never be calcified itself *in vivo*, despite it shows metachromasia.¹³⁾ In addition, shell minerals occur from outer secretion, so-called "extrapallial fluid", excreted through the outer surface of mantle tissue but do not precipitate from inner mucous substances secreted through the inner surface of mantle tissue. In his histochemical

comparison of the mucous substances included in mucous cells on the inner and outer surfaces of *Anodonta*, BEEDHAM²³⁾ has reported that sulfated mucopolysaccharides are contained in only the inner mucous cells. According to the WADA's report,¹³⁾ it is evident that mucous substances in the mucous cells distributed in the mantle of fresh-water and marine molluscs appear to contain acid mucopolysaccharides, but the intensity of metachromasia depends upon molluscan species and local areas of a mantle. JODREY²⁴⁾ and Horiguchi,²⁵⁾ on the other hand, suggested that almost all of dynamic calcium concentrated in the mantle may be excreted in a narrow space between the mantle and the shell valve in some marine bivalves by using calcium-45. For exploring the mechanism of nucleation in mineralization of molluscan shells, it is important to consider how the shell could be formed only in outer secretion of the mantle.

Materials and Methods

This study was carried out with same individuals of *Pinctada martensii* and *Chlamys nobilis* used in the preceding works.^{16, 26)} Mucous substances secreted and adhered on the inner surface of the mantle were collected with an injector without a needle with scrupulous care so as not to be contained such contaminations as small fragments of the mantle tissue and organic matters in sea water. Hydrolyzates of pooled samples of each species with 6 N hydrochloric acid were offered in order to amino acid analysis. An KLA-3 Hitachi amino acid analyzer was used for automatic determination of amino acid contents.

Results

Table 1 shows amino acid composition of mucous substances covered on the inner surface of mantle of *Pinctada martensii* and *Chlamys nobilis* compared with that of extrapallial fluid of each species. In *Pinctada martensii*, amino acid patterns of the secreted mucous substances on inner and outer surfaces of the mantle appear to be quite similar each other. While the number of taurine, tyrosine, phenylalanine and histidine residues of the inner differs markedly from the outer mucous substance in *Chlamys nobilis*. That is, the inner mucus contains larger amounts of taurine and tyrosine, whereas about one-second of phenylalanine and histidine in the extrapallial fluid. Taurine is also higher in the inner mucus of *Pinctada martensii* than in the fluid.

The inner and outer mucous substances secreted from the mantle of *Pinctada martensii* have higher contents of taurine and non-polar amino acids excepts to phenylalanine and about twice to three times of arginine residue in those of the *Chlamys* mantle. On the contrary, larger amounts of glycine, phenylalanine and histidine residues are contained in the mucus of the latter species. Similar concentrations of aspartic acid, serine and glutamic acid, as seen in Table 1, are found between both of the species, respectively.

Table 1. Comparison of amino acid composition between the mucous substances secreted through the inner and outer surfaces of mantle of the same species (amino acid residues per 1000 total residues).

Materials Amino acid	<i>Pinctada martensii</i>		<i>Chlamys nobilis</i>	
	inner mucus	extrapallial fluid*	inner mucus	extrapallial fluid*
Cysteic acid	12.4	13.8	4.2	5
Taurine	135.7	82.2	55	10.3
Aspartic acid	110.3	127.6	112.3	143.3
Threonine	50.2	60.4	39	39.4
Serine	40.2	49.9	40.3	37.7
Glutamic acid	110.5	116.4	106.1	113
Proline	44.9	36.7	24.9	22.4
Glycine	65.4	48	101.1	115.2
Alanine	43.8	39.8	29.2	34.5
Valine	44.1	51.9	28	24.8
Methionine	9	11.2	4	8.2
Isoleucine	38.7	42.7	23.3	27.2
Leucine	54.2	56.5	37.8	33.2
Tyrosine	44.7	53.7	192.8	22
Phenylalanine	37.8	55.6	64.3	160.6
Lysine	65.8	59.3	46.1	46.8
Histidine	28.2	33.4	63.4	122
Arginine	64.2	60.9	28.1	34.5
NH ₃	(7.6)	(9.9)	(2.4)	(2.5)

* extrapallial fluid=outer mucus.

The content of crude protein calculated from total nitrate which was determined by micro-Kjeldahl technique is about 4~8 mg/dl of the outer mucus and about 6 mg/dl of the inner one for *Pinctada martensii*, but varies more or less with conditions and seasons of the sampling.

Discussions

There are similar features in carbohydrate content between the mucous substances covered on the inner and outer surfaces of mantle of *Pinctada martensii* (FURUHASHI and WADA¹⁵⁾). Further the present results indicate that amino acid composition of the inner mucous substance appears to be essentially same from that of the outer one, extrapallial fluid, in *Pinctada martensii* at least. These facts seem to suggest that if both the mucous substances consist of the same organic constituents, available circumstances which will promote the occurrence of shell materials are provided in the extrapallial fluid, whereas factors which will inhibit the formation and mineralization of conchiolin exist around and in the inner mucus.

The following considerations may be made on the basis of the above-mentioned results and the pictures of calcification in molluscs described in the previous introduction.

The mantle and periostracum consisting of a quinon-tanned protein may form together the ground of crystal growth of shell minerals separated from surroundings between them. Consequently, it is probable that (1) ions and molecules which act as crystal poison will be removed from the extrapallial fluid, (2) some organic constituents of the extrapallial fluid will be able to denature to form premineralized matrix, and (3) the effective activity of calcium carbonate which are quite enough to induce the nucleation of shell minerals on the matrix will be controlled through the homeostatic mechanism of the organisms. When such the circumstances are created by molluscs and no shell mineral occurs there, metachromatic conchiolin which is synthesized in a biological system and shows the strongest affinity for shell minerals appears to act effectively in initiating of nucleation of mineral crystals. It is assumed, as suggested in the preceding papers,²⁶⁾ that the mineralizing matrix gets the stereochemical structure having the active surface to be available to the initiation of crystal nucleation in the course where proteinous substances contained in the extrapallial fluid are supposed to denature to the matrix in irreversible change. Especially, the present author has paid the most important attention to the combination of certain segments of serine and aspartic acid with acid mucopolysaccharides or with calcium itself in opening of mineralization of the organic matrix, since the amount of these residues is significantly larger in the conchiolin of mineralized layers than in that of non-mineralized ones. Non-mineralized matrix such as periostracum and horny plate may also serve as the substratum for the mineralized matrix.

Summary

1. Amino acids of the mucous substance covered on the inner surface of mantle of *Pinctada martensii* and *Chlamys nobilis* were determined by using a KLA-3 Hitachi amino acid analyzer.
2. In *Pinctada martensii* at least, the amino acid pattern of inner mucus where shell matters never occur *in vivo* did not differ from that of extrapallial fluid which is secreted on the shell side of a mantle and able to form mineralized shell.
3. The similar number of aspartic acid, serine and glycine residues which are the main amino acids of mineralized matrix were found between both the mucus of the two species, respectively.

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