STUDIES ON THE MINERALIZATION OF THE CALCIFIED
TISSUE IN MOLLUSCS—XII.
SPECIFIC PATTERNS OF NON-MINERALIZED LAYER
CONCHIOLIN IN AMINO ACID COMPOSITION*

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Conchiolin seems probably to be formed in the course of denaturation of mucous substances secreted by the glandular epithelium of shell-forming tissues and to be a scleroprotein with a small amount of mucopolysaccharides. One could find two groups of different features of conchiolin in the calcification of shells of molluscs; namely, the one conchiolin such as the horny plate of Cephalopoda and the periostracum of Gastropoda and Bivalvia is never mineralized themselves during the process of shell formation and may serve as a back-plate and substratum for the another which could induce nuclei formation and subsequent growth of mineral phase. How does the nucleation of mineral phase occur in organic phase in the mineralization of molluscan shells? To answer this question, one must first try to investigate the specific property of conchiolin which is capable for the opening of mineralization *in vivo*.

It is possible that critical nuclei of mineral crystals may be induced in specialized sites of organic matrices through heterogeneous nucleation in calcified tissues of organisms (SOBEL¹⁾, NEUMAN and NEUMAN²⁾, GLIMCHER³⁾, WADA⁴⁾). Many workers have paid a great attention to certain amino acids of proteins themselves (NEUMAN and NEUMAN²⁾, GLIMCHER³⁾, HARE⁵⁾), to certain acid mucopolysaccharides bound with appropriate parts of proteins (SOBEL¹⁾, ABOLINS-KROGIS⁶⁾, WADA⁴⁾), or to acid mucopolysaccharide itself (RUBIN and HOWARD) in the mineralization of calcified tissues of vertebrates and invertebrates.

The present author^{4), 8), 9)} has found the characteristic differences in stainability and histochemical reaction between conchiolins of non-mineralized and mineralized layers of molluscan shells and demonstrated that the metachromatic substance which seems to be due to sulfuric ester may involve in some ways the initiation of nucleation of mineral crystals *in vivo*. It was furthermore suggested that the nature of protein matrix may differ between non-mineralized and mineralized layers. For exploring the functional role of protein in the initiation of shell mineralization, this paper will describe the characteristic pattern of amino acid composition of conchiolins from non-mineralized layer compared with those from mineralized layers of molluscan shells reported in the preceding paper¹⁰⁾.

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Materials and Methods

Materials were conchiolins from non-mineralized layers of some of the same specimens used in the preceding investigation¹⁰⁾ and of the other species, and isolated carefully from mineralized layers by a razor under a magnifying glass before or after decalcification. Prior to the isolation, periostracal conchiolins of Gastropoda and Bivalvia could be possibly cleared of sessile animals, weeds and mud.

Hydrolysis and amino acid analysis of conchiolin were carried in the same ways described in the preceding paper¹⁰⁾.

Results

Fig. 1 shows a typical example of automatic amino acid analysis curve of the periostracal conchiolin of *Bivalvia*. The peak C seen in Fig. 1 of the preceding paper¹⁰⁾ also appears in the hydrolyzate of non-mineralized layers of *Sepia esculenta*, *Haliotis discus* and *Hemifusus ternatanus*, the peak D in Fig. 1 in that of the perio-

Fig. 1. The automatic amino acid analysis curve of the periostracal conchiolin of *Hyriopsis schlegeli*.

stracum of *Hyriopsis schlegeli*, and the peak Z (not shown in Fig. 1) between the peaks of tryptophane and lysine in that of the periostracum of *Hemifusus ternatanus*. These peaks are not determined to correspond respectively to some kinds of amino acids in this paper. The results obtained are summarized as the number of amino acid residues per 1000 total residues in Table 1.

Tables 1 and 2 indicate that all non-mineralized layers consist of the conchiolin having similar composition which differ conspicuously from that of mineralized layer conchiolin, though the pattern modifies rather widely with taxonomical groups or species. The conchiolin of non-mineralized layers has relatively higher concentrations

of proline but consistently lower serine contents than the conchiolin of mineralized layers has, regardless of taxonomical patterns. In a single group, the smaller amount of aspartic acid and the larger amount of histidine are always present in non-mineralized layer conchiolin. Although there is found at least one exception of the perio-

Table 1. Amino acid composition of non-mineralized layer conchiolin
(amino acid residues per 1000 total residues)

Materials Amino acid	Cephalopoda	Gastropoda		Bivalvia	
	<i>S. esculenta</i> horny plate	<i>H. ternatanus</i> periostracum	<i>H. discus</i> periostracum	<i>M. meretrix</i> <i>lusoria</i> periostracum	<i>H. schlegeli</i> periostracum
CySO ₃ H	8.8	4.7	1.5	24.5	4.1
Asp	142.2	158.5	178	50.8	45.9
Thr	56	43.4	24.1	15.4	8.1
Ser	59.3	51.1	14.2	16.3	26.1
Glu	82.5	107.8	111.4	19.7	18
Pro	82.7	46	80.9	61	52.3
Gly	65.6	92.7	140	322.5	343.7
Ala	87.8	48.1	16.9	10.9	5.7
Val	76	67.1	23.6	23.6	25.7
Met	2.2		+	66.8	
Ileu	33.7	58.7	33.7	17.3	29.3
Leu	62.7	71.5	11.8	21.1	24.8
Tyr	62.5	25.8	199	174.5	244.1
Phe	31.3	59.8	48.2	64.9	58.7
Lys	49.1	75.2	38.1	19.6	23
His	64.2	15.5	14.3	11.6	43.8
Arg	33.3	74	62.7	79.5	46.7
NH ₃	(29.5)	(17.0)	(17.4)	(8.3)	(10.9)

* Corrections for the destruction of threonine, serine and tyrosine were not made in this study.

stracal conchiolin of *Hemifusus ternatanus*, 25.8 residues per 1000, the tyrosine content of non-mineralized layer conchiolin is in remarkably high concentrations; 174.5 and 244.1 residues in Bivalvia, 199 residues in *Haliotis discus* (Gastropoda), and 62.5 residues in *Sepia esculenta* (Cephalopoda) per 1000, respectively. One could find that the number of tyrosine residues may increase with increasing amount of melanin. Glycine is similarly more abundant in non-mineralized layer conchiolins in a species, especially puts up about one-third of the total residues in the periostracum of the *Hyriopsis* and *Meretrix* shells (both Bivalvia) in which the aspartic acid and alanine contents are generally lower compared with mineralized layer conchiolins, though both amino acids are much higher in the conchiolin from the periostracum of Gastropoda and from the horny plate of *Sepia esculenta* than in that of the periostracum of Bivalvia. These differences of amino acid composition between the proteins of non-mineralized

and mineralized layers appears to occur most largely in the periostracum of Bivalvia, as shown in Table 2 and as illustrated in Fig. 2. Besides it is evident that the relatively high numbers of methionine and cysteic acid residues are contained in the periostracal conchiolin of *Meretrix meretrix lusoria* (Table 1). The amino acid com-

Table 2. The comparison of several amino acids between non-mineralized and mineralized layer conchiolins of a shell of the same species (amino acid residues per 1000 total residues).

Materials Mineral phase*	<i>Sepia esculenta</i>		<i>Haliotis discus</i>		<i>Hyriopsis schlegeli</i>	
	horny plate non	Spongy body arag.	periostracum non	nacre arag.	periostracum non	nacre arag.
Amino acid						
Asp	142.2	178.5	178	267.2	45.9	116.4
Ser	59.3	96	14.2	80.9	26.1	81.5
Pro	82.7	51.5	80.9	33.4	52.3	19
Gly	65.6	41.9	140	129	343.7	170.4
Ala	87.8	43.5	16.9	124	5.7	216.6
Tyr	62.5	33.3	199	22	244.1	22.2
His	64.2	22.4	14.3	4.1	43.8	8.3
Acidic amino acids	224.7	331.3	289.4	325.2	63.9	165.1
Basic amino acids	146.6	150.7	115.1	118	113.5	114.4
Acidic/basic residues	1.533	2.198	2.514	2.756	0.563	1.443

* non: non-mineral, arag.: aragonite.

position of the protein from a horny plate of *Sepia esculenta* appears to differ from that of the periostracal protein of the other shells used in this study.

The ratio of acidic to basic residues is smaller in the protein of non-mineralized layers than in that of mineralized layers of the same species investigated but does not always appear to show such the tendency on the whole of molluscs (Table 2).

The periostracum covered on the external surface of shells of Gastropoda and Bivalvia, especially in the nappy periostracum of *Hemifusus ternatanus*, however, will be in the larger danger of contamination of the rest of sessile animals, weeds and other organic matters.

Discussion

After FREMY⁽¹¹⁾ had found the presence of the protein "conchiolin" in molluscan shells, amino acid analysis of conchiolin was mainly made in Bivalvia by many researchers (VOIT⁽¹²⁾, WETZEL⁽¹³⁾, FRIGA⁽¹⁴⁾, TANAKA *et al.*⁽¹⁵⁾) who were and are trying to determine the nature of the protein. Recently, biologists, biochemists and biogeologists are actively investigating the nature and functional roles of conchiolin on the fields of them (ROCHE *et al.*⁽¹⁶⁾ GRÉGOIRE *et al.*⁽¹⁷⁾, ABELSON⁽¹⁸⁾, BEEDHAM⁽¹⁹⁾, PIEZ⁽²⁰⁾,

HARE⁵⁾, TANAKA *et al.*²¹⁾, AKIYAMA²²⁾, DEGENS and LOVE²³⁾, WADA¹⁰⁾. Their results indicated that conchiolin may differ in amino acid composition from collagens and keratins, and that they undergo some modifications associated with the mineralization (HARE⁵⁾, WADA¹⁰⁾) and with the locally specialized function of periostracum and

Fig. 2. Histograms showing the relative amounts of 8 amino acids between the proteins of non-mineralized and mineralized layers. The number of amino acid residues per 1000 total residues were plotted on the ordinate. The comparison was made between *Sepia esculenta* and *Argonauta argo* in Cephalopoda, between *Haliotis discus* and *Cellana nigro-lineata* in Gastropoda, and between *Hyriopsis schlegeli* and *Ostrea gigas* in Bivalvia, respectively.

ligament (BEEDHAM¹⁹⁾). BEEDHAM¹⁹⁾ and HILLMAN²⁴⁾ reported that the conchiolin of periostracum of some Bivalvia contains the large concentration of phenolic amino acids, especially tyrosine, and glycine, which is characteristic of a quinone-tanned protein. As described above, it would be noted that the periostracum of Bivalvia

consists mainly of a quinon-tanned protein produced by the epithelial and secretory cells on the inner surface of an outer mantle fold and differ in composition from the protein formed by the cells on the other regions (BROWN²⁵), BEEDHAM¹⁹), HILLMAN²⁴).

The most interest discussion for the mechanism of nucleation of mineral crystal in the opening of mineralization in molluscan shells has been made by HARE⁵⁾ who obtained based on his comparative biochemical investigation of amino acid composition of conchiolin from non-mineralized and mineralized parts of the *Mytilus* shell the result that the non-mineralized units of the valve and ligament have the very low content of the acidic residues, and stated that the side chains of the acidic residues such as aspartic and glutamic acids could provide within protein itself negatively charged sites to would attract calcium ions, the basic side chains could similarly act as sites for the concentration of carbonate ions, and thus these side groups in the protein matrix provide appropriate initial concentration of Ca^{++} and CO_3^{--} for the nucleation of shell minerals.

The findings demonstrate conclusively that the protein of non-mineralized layers of molluscan shells may differ in amino acid composition from that of mineralized layers, and also that some modification of composition appears to occur in relation to the taxonomical significance. For example, the high concentrations of proline and tyrosine in non-mineralized layer conchiolin appear to be probably brought about under biochemical conditions locally specialized in the process of formation of the layer but may not be the specific pattern of the layer as suggested by the low tyrosine content of the periostracal conchiolin of *Hemifusus ternatanus*. Serine is consistently lower in all groups, and the lower aspartic acid content and the higher histidine content are found in single groups and species. The number of glycine, alanine and glutamic acid residues, on the other hand, may be correlated with the taxonomical pattern.

Although it is probably expected that conchiolins of non-mineralized and mineralized layers of all groups of molluscs are respectively characterized by specific patterns, in the comparative study based only on amino acid composition of them it is difficult to suppose whether certain amino acid groups themselves provide in the protein appropriate sites for initiating the nucleation of mineral crystals and then involve the mechanism of crystal nucleation in such ways described by HARE⁵⁾. But it is likely that some segments of conchiolin charge negatively or positively and then attract Ca^{++} or CO_3^{--} . According to WADA's histological and histochemical observations^{4), 8)} of the organic matrix of molluscan shells, the matrix of non-mineralized layers (or eosinophil granules) shows affinity for acidic dye and is not metachromatic with toluidine blue, whereas the matrix of mineralized layers (or organic crystals) affinity for basic dye and metachromatic. He⁹⁾ demonstrated histochemically that the metachromasia of the organic crystal is due to the presence of sulfuric ester. Further the present amino acid analysis seems to suggest that conchiolin may be classified under at least two groups of non-mineralized and mineralized layer

ones in the stereochemical structure. Considering the significant difference of serine and aspartic acid contents between the conchiolins, it is probable that these residues may involve in the mechanism of attachment of acid mucopolysaccharides to conchiolin, as has been known in chondroitin sulfate-protein complexes^{26), 27)}, and acid mucopolysaccharides must be in the active configuration which can capture basic dyes and cations in a surrounding solution, in the process of mineralization of conchiolin. Therefore the present author proposes the hypothesis that when any stable nuclei of mineral crystals do not exist in shell mineralization *in vivo*, the segment of conchiolin, chondroitin sulfate-protein complex or acid mucoprotein with sulfate groups, which is positive to the metachromatic reaction may be endowed with the more effective affinity depended on thermodynamics or electrostatics for nucleation of calcium carbonate crystals, and then either locally reduces the value of the critical supersaturation or locally increases the concentration of $\text{Ca} \times \text{CO}_3$ under some physicochemical and enzymatic factors of the extrapallial fluid. Accordingly, it seems that any stable nuclei of aragonite or calcite could not be induced in the conchiolin of non-mineralized layers and eosinophil granules under lower supersaturation provided by animals. In all cases, this problem will be resolved in the further experiments of *in vivo* and *in vitro* mineralization of conchiolin.

The proteins localized in non-mineralized layers appear to be distinguishable in amino acid composition from collagens and keratins, as in the proteins of mineralized layers, because of the specific patterns of conchiolin. The conchiolin of the periostracum is probably hardened by quinone-tanning, except the nappy one of *Hemifusus ternatanus*.

Summary

1. Amino acid analysis was made on the conchiolin of non-mineralized layers of molluscan shells, and the results obtained were compared with those in mineralized layer conchiolin.

2. The relatively lower serine content as well as the higher tyrosine and proline contents were contained in the conchiolin of non-mineralized layers, irrespective of the taxonomical pattern, though there was at least one exception of the periostracal conchiolin of *Hemifusus ternatanus* in which tyrosine was very low.

3. As to aspartic acid, glycine and histidine residues, the similar differences of these amino acids between the conchiolins of non-mineralized and mineralized layers appeared to occur respectively in single species and groups. Similarly, the sum of acidic amino acids was consistently smaller in non-mineralized layer conchiolin in single species, though such the tendency was confused on the whole of molluscs.

4. The pattern of amino acids of the most periostracum indicates the characteristic of a quinone-tanned protein.

5. The conchiolin of mineralized layers and organic crystals probably appears to

be chondroitin sulfate-protein complex or acid mucoprotein with sulfate groups.

6. The characteristic pattern of the composition of non-mineralized layer conchiolin may suggest the specialized physiological conditions of formation of conchiolin in the regions of these layers.

7. The taxonomical significance also appeared to be found in some modification of amino acid composition of the proteins.

8. The mechanism of crystal nucleation in shell mineralization *in vivo* was discussed in the comparison of the nature of the organic matrix between non-mineralized and mineralized layers.

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