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The amino acid analysis and gel electrophoresis of some components in the organic matrix of molluscan shells.

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Summary

The acid-insoluble organic matrix extracted from the nacreous layer of *Tectus conus* is solubilized with dilute alkali-solution and the solubilized component is fractionated by gel chromatography and SDS-PAGE. The matrix is composed of several components of proteins. The molecular weights of their components range between 80kD and 14kD. The amino acid composition of the alkali-soluble matrix is characterized with high proportion of aspartate, which is unlike that found in the unfractionated acid-insoluble matrix. The results can give a clarification of the structural composition of the acid-insoluble matrix and the elucidation of the role of that in the process of shell formation good aid.

Introduction

Two kinds of the organic matrices, a water-soluble matrix (S.M.) and a water-insoluble matrix (I.S.M.) are known to be contained in molluscan shells. Because of its high solubility in water, the S.M. has been analyzed as to the compositions using the biochemical procedures [1-3]. Moreover, since Ca^{2+}-binding glycoproteins were extracted out of the S.M. [4, 5], the S.M. was thought to have the possibility of accomplishing some important role during the formation of molluscan shells. On the other hand, the I.S.M. can not be dissolved in water, dilute acids and various buffers and therefore, the biochemical purification has not been able to be carried out. For these reasons, the I.S.M. has only been studied on the morphological observation of the matrix surface using electron microscope [6, 7], on the analysis of the high dimensional structure of the matrix by the infrared spectroscopic analysis and the X-ray diffraction analysis [8, 9]. Amino acid compositions were analyzed on the unfractionated I.S.M. [10, 11].

Meanwhile, Grégoire et al. [12] attempted to solubilize the I.S.M. in the nacreous layer, using borate buffer and concentrated alkali-solution. Tanaka *et al.* [13] also tried to dissolve the I.S.M. in the nacreous layer with CH_3COOH and NH_4OH, 4 pyridine covult-solution or Cu-EDTA solution. However, these tryings could not succeed to dissolve the matrix absolutely, and the biochemical analysis of the solubilized component also leave something to be desired.

It is generally recognized that the nacreous layer is formed by alternating deposition of the organic matrix and the aragonite crystals. Howerer, a role of the matrix during the formation of the nacreous layer has not been clarified. Bevelander & Nakahara [14] proposed the so-called "compartment hypothesis", in which they supposed that multiple layer of the organic matrix (so-called "sheet") are formed prior to crystal formation. Aragonite crystals begin to develop within the pre-formed organic components and grow up with being surrounded by this membrane-like material, so-called "envelope". The growth of the crystal continues untill it touch with "sheet".

As well Nakahara *et al.* [15, 16] found that Asp is concentrated in a component to be thought correspond to "envelope" in the nacreous and the prismatic layers and discussed that "envelope" could be a key substance in relation to the initiation and the acceleration of the crystal growth of calcium carbonate.

In this research, we attempted to solubilize the I.S.M. in the nacreous layer and originally fractionate the solubilized products by SDS-polyacrylamide gel electrophoresis. The method may serve as a good Fool for understanding the role of the I.S.M. in biomineralization.

Materials and Methods

The shells of *Tectus conus* were collected at Kuroshima, Okinawa, Japan. After the surface of the shells were cleaned with a dental drill, they were dropped in 1% NaClO to remove the organic contaminants and periostracum. The nacreous layer was separated from the prismatic layer with a dental drill, then dried, powdered and decalcified.

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Decalcification was carried out in 5% CH_3COOH. The decalcified solution was centrifuged (10,000 x G, 20min.) and only the precipitate was used for further treatment. The precipitate was washed repeatedly with distilled water on a filter paper (Whatman No.3) and then homogenized in chloroform-methanol solution (chloroform/methanol=2/1, v/v) to separate lipid components. The insoluble layer thus obtained was lyophilized and then treated in diluted NH_4OH solution adjusting pH at 8.5 under stirring for 6 hours. The alkali-extracted solution was centrifuged (10,000 x G, 20min.) and only supernatant (alkali-soluble matrix) was fractionated by gel chromatography and gel electrophoresis. The matrix was loaded onto a 100 x 1,5cm, Bio-Gel A 1.5m column (Bio-Rad Lab., Tokyo, Japan) preequilibrated with 0.1 M NH_4HCO_3, pH 8.4. The absorbance of the eluate was measured at 235 nm with a double beam spectrophotometer UV 150-02 (Shimazu Co., Kyoto, Japan). The main fraction eluted from Bio-Gel A 1.5m was hydrolyzed in a hydrolysis tube under vacuum, at 110℃ for 24 hours, in 5.7 N HCl. The hydrolysate was then analyzed on an ATTO MLC-703S automatic amino acid analyser (ATTO Co., Tokyo, Japan).

SDS-PAGE was carried out in 8.5% gels as described by Anderson et al. [17]. Gels were stained with 0.4% Coomassie Brilliant Blue R-250, with stains-all by the method of Campbell et al. [18] and silver-stained, essentially by the method of Morrisey [19] with a kit for silver-staining (Wako Pure Chemicals Co., Osaka, Japan).

Results

1) Gel chromatography

As shown in Fig. 1, three main peaks are observed in the profile of gel-filtration of the alkali-soluble matrix on a Bio-Gel A 1.5m. Among them, the major fraction is eluted at the position of 70-90ml of the buffer and accounted for 70% of total protein loaded. Besides the major fraction, a peak approximately at the position V_o of the gel and rather large fraction at the buffer volume of 130-140ml are also observed.

2) SDS-PAGE

After gel electrophoresis, three kinds of staining procedure were employed for the gel, as shown in Fig.2. In the case of C.B.B. staining (Fig.2-Ⅰ), a clear band can be seen almost at the position of BSA, which possesses the molecular weight of 68kD. Two thin bands which cannot be stained well are also observed at the position of slightly higher and lower than BSA. molecular weights of these

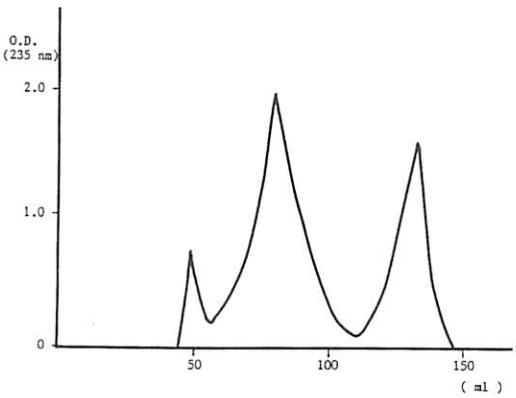


Fig 1. ; Gel chromatogram on Bio-Gel A 1.5m of the alkali-soluble matrix in the nacreous layer of *Tectus conus* shells.

components can be supposed to be approximately 80kD and 57kD. Moreover, a wide smear is observed at the position of molecular weight between 20kD and 10kD, though it is unvisible in the photograph.

By stains-all staining, the colour of each band changes drastically after taking out the gel from the staining solution. According to Fig.2-Ⅱ to Fig.2-Ⅳ, three steps of colour change are obvious. Fig.2-Ⅱ shows the gel still remaining in the staining solution, Fig.2-Ⅲ immediately after taking out from the staining solution and Fig.2-Ⅳ exposing in 20% isopropylalchohol for about 10 minutes. In Fig.2-Ⅱ, two faint bands which are stained to red and to blue are visible at the region of fairly low molecular weight. The molecualr weight of the red band is estimated approximately 14kD and that of the blue one as lower than 10kD. Because of the pinkish colour of the backgroud, the main 68kD-band after C.B.B. staining is very difficult to visualize. On the other hand, the 68kD-band is distinguishable from the background in Fig.2-Ⅲ, in which two bands with lower molecular weights turn to almosut invisible. It is characterisstic for stains-all stainng that the red colour of the simple protein band becomes fade and turns to white when the gel is exposed in isopropylalchohol, water or left in the air for about 10 minutes, after taking the gel from the staining solution. Fig.2-Ⅳ shows the white band of the 68kD component which is well comparable with that of BSA. Moreover, an additional faint white bands is visible just below the 68kD band.

By silver staining method (Fig.2-Ⅴ), two well stained bands appear at the position of comparable with those of BSA and ovalbumin. In these bands, the former

corresponds to the 68kD band seen after C.B.B. and stains-all stainings and the latter to the faint band seen just below the 68kD band of Fig.2-Ⅰ and fig.2-Ⅳ. Moreover, several additional faint bands are stained at the position of molecular weight lower than 40kD. In them, the lowermost one, whose molecular weight is estiamted approximately 14kD corresponds to the red band after stains-all staining (Fig.2-Ⅱ).

3) Amino acid analysis

In Table 1, amino acid compositions of the unfractionated acid-insoluble matrix (I.S.M.) and of the fractionated alkali-soluble matrix (Al.S.M.) are shown. The I.S.M. after decalcification contains a large amount of Ala and Gly, and these two amino acids reach approximtely 44% of the total amount. Moreover, Ser shows also high content, while total amount of the acidic amino acids is not so high.

On the other hand, amino acid composition of the main peak fractionated by Bio-Gel A 1.5m in the Al.S.M. is characterized by the large amount of Asx. Though rich in the acidic amino acids, the basic amino acids are much less prevalent, resulting in a very high ratio of an acidic/basic amino acid value. Though Gly is also the main amino acid, it is contained lower by comparison with the I.S.M.. Moreover, in Al.S.M., Ala and Ser contents are lower than 50% of that in the I.S.M.. As a whole, the proportion of the polar amino acids in the Al.S.M. increases 10% compared with that in the I.S.M..

	unfractionated water-insoluble matrix	alkali-soluble matrix
Asx	13.04	34.47
Thr	3.00	4.73
Ser	12.07	5.08
Glx	6.09	9.40
Pro	1.79	4.32
Gly	17.37	11.53
Ala	26.91	11.72
Cys	-	0.64
Val	4.02	3.48
Met	0.25	-
Ile	1.17	2.01
Leu	3.60	5.28
Tyr	1.33	-
Phe	3.61	4.04
Lys	2.13	2.05
His	0.29	0.53
Arg	3.36	0.72
Acidic A.A.[1)]	19.13	43.87
Basic A.A.	5.78	3.30
Acidic/Basic	3.31	13.30
Asx/Glx	2.14	3.67
Hydroxy A.A.	15.07	9.81
Ser/Thr	4.02	1.07
Gly/Ala	0.64	0.98
Asx/Gly	0.75	2.99
Polar A.A.	58.68	69.15

[1)], A.A. : Amino acid

Tabel 1 ; Amino acid compositions of the unfractionated water-insoluble matrix and the alkali-soluble matrix in the nacreous layer of *Tectus conus* shell (residues per 100).

Discussion

In the nacreous layer of molluscan shells, the I.S.M. is present between the lamellar and the crystals as an "inter-lamellar matrix" and "intercrystalline matrix", respectively. Also within crystals, additional matrix is found as "intracrystalline matrix" [20].

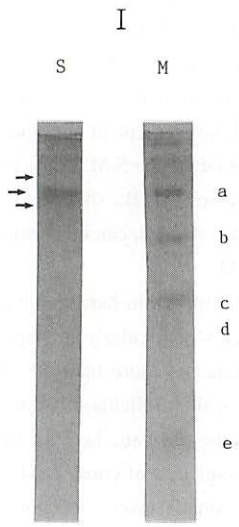
On the other hand, solubilization of the I.S.M., fractionation and analysis of the solubilized products have almost never been attempted, so that the differences on the composition of the morphologically distinguished parts in the I.S.M. have never been clarified. Tough up to now, Grégoire [12] and Tanaka *et al.* [13] tried to solubilize the I.S.M. in the nacreous layer by different procedures, as in those cases the condition for the solubilization were very severe, it might be possible that peptide bonds of the protein were broken.

In the present research, we attempted to solubilize the I.S.M. and originally succeeded in separation of at least four components in it electrophoretically, whose molecular weights could be estimated to be approximately 80kD, 68kD, 57kD and 14kD. Judged from the pinkish-red colour from stains-all staining, the former three components can be eatimated to be simple proteins which do not contain sugars or sialic acids. The preliminary staining by PAS resulted no visible band and it supports the above estimation. On the other hand, the component with molecular weight of 14kD is stained to dark-orange by stains-all, together with the fairly poor staining by silver, implying that it can be estimated to be mixture of protein and phospholipid [18]. Moreover, several additional components may be contained in the I.S.M.. Weiner *et al.* [21] have already reported the result on SDS-PAGE of the organic matrix in molluscan shells. They have electrophoresed the EDTA-soluble matrix from 10 molluscan species by Laemmli procedure [22] and stained them by C.B.B., and clarified the discrete

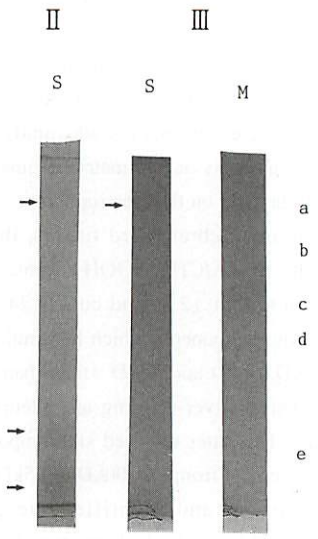
components with molecular weight up to 100kD. However, the result varied widely such as the different positions of the stained bands among species analyzed, perfect undetection of the band among some species and so on. It is difficult to compare their result with ours, since different matrix in the different species was analyzed and they performed the analysis on the matrix of unseparated shell layers. As to the data on the electrophoretic analysis of the matrix in the invertebrate hard tissues, there are some reports on the EDTA/CH_3CHOOH-soluble matrix in the spicule of sea urchin [23] and coral [24]. The former fractionated the components which had molecular weights of 64kD, 57kD, 50kD and 47kD (main bands were 50kD and 47kD), using silver-staining and identified those as glycoproteins. The latter resolved six components ranging in molecular weights from 99/100kD to 25kD using C.B.B. and PAS staining and identified the glycoprotein component of 80kD band. Venkatesan and Simpson [25] also observed six bands in the range of molecular weights between 117kD and 19.5kD in the soluble matrix of sea urchin spicules. These data shows apparent differences with indicated in the present paper. If the reasons for these differences are caused by animal species analyzed, matrix analyzed and/or analytical methods used, is not known at present. In spite of the diverse molecular weights of the components in the matrix, they showed similar reaction against C.B.B. staining. In the case of the matrix in the sea urchin spicules, it was difficult to visualize any bands after C.B.B. staining and they could only be seen by silver staining of when the matrix was radio-iodinated. The matrix in the coral spicules was stained weakly by C.B.B.. Similarly as these examples, the solubilized matrix in the molluscan shells is stained intensively only when the sample is loaded onto the gel with large amount of proteins. Moreover, intensity of the 80kD, 68kD and 57kD bands varied from gel as in the case of the coral spicules. These may be due to the low content of the basic amino acid residues [26].

Amino acid composition of the Al.S.M. shows especially Asx-rich and is throughly different from that of the I.S.M. before solubilization and of the remaining I.S.M. after solubilization. This indicates that Al.S.M. may be contained as a small amount of a different component from the main part of the I.S.M., which is characterized with the high amount of Ala and Gly. Although Ca-binding glycoprotein which is a main component of the S.M. in the nacreous layer of *Tectus conus* is also Asx-rich and acidic (Samata, unpublished data), the Al.S.M. contained a higher content of Asx and lower amount of basic amino acids than this component. Taking account of the undetection of visible band on SDS-PAGE of the S.M. in the nacreous layer of *T. conus* in all cases of C.B.B., silver and stains-all stainings (data not shown), Al.S.M. can be considered as a different unit from the S.M..

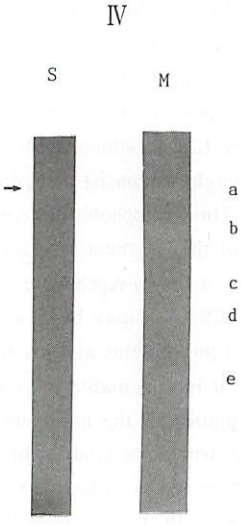
It is known that matrix in hard tissue is generally rich in acidic amino acids, particularly in Asp [2, 27]. Asp was highly concentrated of more than 50% followed with the very high ratio of the acidic/basic amino acids, in the case of the matrix in the prismatic layer of the molluscan shells [15] and in the spicuels of corals [24]. The highly acidic matrix in the prismatic layer was considered to constitute "envelope" by observation with electron microscope [15]. As the Al.S.M. showed highly acidic nature by its amino acid composition some specific components in it may be contained to constitute "envelope". According to Bevelander and Nakahara [14], since $CaCO_3$-crystal could be initiated and develope in "envelope", ion concentration into "envelope" is expected to happen. In addition, taking account of the extraction of a large amount of Ca-binding lipids from the I.S.M. of *T. conus* after decalcification (Samata, unpublished data), it might be possible that "envelope" consists of the complex of lipids and proteins and both two components are involved in ion concentration in connection reciprocity. On the other hand, the remaining main part of the I.S.M., which lipids and Al.S.M. were extracted is thought to consist of hydrophobic proteins, kitin and so on. This hydrophobic proteins are thought to be made mainly of the β-sheet structure [8] and to be estimated to have a simply repeated sequence consisting of Ala and Gly [28]. It may be possible that kitin is interwined with the proteins and the firmed structure is formed. Then, it is reasonable to consider that in the process of formation of the nacreous layer, the acidic proteins together with some kinds of lipids are involved in "envelope" which seems to have a role for the crystal-initiation and growth, however, hydrophobic proteins together with kitin are involved in "sheet" which seems to regulate the crystal growth.



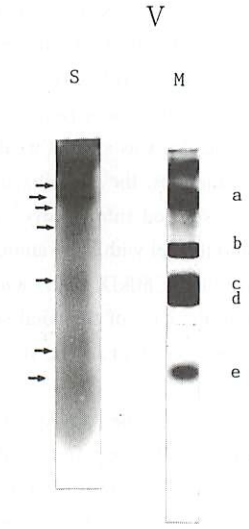
Ⅰ:Gels are stained by C.B.B..
S; Sample, M;Marker, a-BSA (68kD), b-Ovalbumin (43kD), c-CA (30kD), d-Chymotrypsinogen (25kD), e-Lysozym (14.3kD) ;Positions of the bands



Ⅱ-Ⅳ:Gels are stained by stains-all.
Ⅱ:A gel is remaining in the staining solution
Symbols are the same as described in Ⅰ
Ⅲ:Immediately after the gels are taken out from the staining solution
Symbols are the same as described in Ⅰ



Ⅳ:Gels are exposed in 20% isopropylalchohol for about 10minutes
Symbols are the same as described in Ⅰ



Ⅴ:Gels are stained by the kit for silver staining
Symbols are the same as described in Ⅰ

Fig 2 ; SDS-PAGE pattern of the alkali-soluble organic matrix in the nacreous layer of *Tectus conus*.

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