

## A STUDY OF THE ORGANIC MATRIX OF CUTTLEBONE: MOLECULAR WEIGHTS, CHARACTERIZED INFRARED SPECTRUM AND AMINO ACID COMPOSITION

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Abstract: Organic matrix of the shell of *Sepia esculenta*, the cuttlebone, was extracted by 10% acetic acid and double distilled water. It was analyzed using SDS-Polyacrylamide electrophoresis (SDS-PAGE), Fourier Transform Infrared spectrum (FT-IR) and the technique of analysis of amino acid composition. SDS-PAGE electrophoresis showed the number of bands of acid soluble matrix was lower than that of the aqueous soluble matrix, but the former protein concentration was higher than the latter. This may be attributed to two factors: The loss of the proteins of low molecular range weight in the process of dialyzing, and the poor resolution resulting from some very thick and broad bands. The FT-IR spectrum showed amide, amine, and carboxylic acid groups in the organic matrix of the cuttlebone, with high sugar/protein ratio and strong sugar bands. Moreover, the HCO<sub>3</sub><sup>-</sup> groups could be at the organic mineral interface. The results of amino acid analyses indicated a high content of aspartic acid (Asp) and glutamic acid (Glu) in both the soluble and the insoluble matrix, the sum of them respectively occupied close to 23% and 19%. Glycine (Gly) and Serine (Ser) were also present in a relatively high concentration. The total of Asp and Glu was obviously more than that of Ser and Gly in the soluble matrix, however, with the opposite in insoluble matrix. These data imply that acidic amino acids play an important role in the calcification of cuttlebone.

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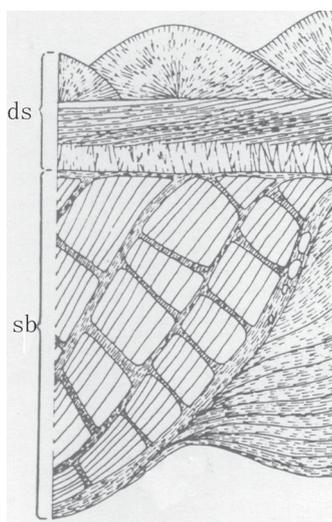
### INTRODUCTION

In cuttlebone, carbonate is mainly precipitated as the crystal of calcium carbonate in aragonite form, only a very small proportion in calcite form. For a long time, the mechanism of polymorphic formation of carbonate in organisms has been explored by many researchers in the fields of biochemistry, biomineralogy and biogeochemistry (Koji, 1966). Recently, particular attention was paid to the composition of the matrix of the shell or the nature of the protein. But in these studies, most authors were interested in such mollusks as gastropods or bivalves. As for the mechanism of shell formation, it was generally postulated that the elaborate fabrication of the biominerals arise from specific molecular interactions at the organic matrix-inorganic interface (Mann *et al.*, 1993), and that the organic matrix represents many of the important molecules involved in the interactions controlling crystal growth (*e.g.*, Watabe and Wilbur

1960; Lowenstam 1981; Weiner 1984; Lowenstam and Weiner 1989). Especially the organic matrix has been studied to a key element to unveil their roles in the mineralization processes. The matrix molecules have been classified conventionally into two types based on their solubility in weak acid solutions: the insoluble matrix is thought to be largely intercrystalline (Krampitz 1982) and provides a framework where mineralization is to occur, whereas the soluble matrix is known as intracrystalline or located on the intercrystalline matrix surfaces, but its functions are still poorly understood (Addadi and Weiner 1997). To understand the underlying mechanisms of the protein-mineral interactions, it seems essential first of all to know the primary composition, structure and properties of the proteins. However, few works have been done on cuttlebones. This study aimed to get the necessary information about the organic matrix of the cuttlebone and to supply some data for the shell formation mechanisms.

## MATERIAL AND METHODS

In this paper, all the cuttlebones were from *Sepia esculenta* living in the water off Rizhao in North China. The cuttlebones were freshly collected, soaked in 5% NaOH, dried at room temperature, and crushed to fine powder. The powdered cuttlebones (25g samples) were suspended in 10% acetic acid and in double distilled water respectively, for 48 hr at room temperature (each containing 0.01% sodium azide) with continuous stirring. The acid suspension was dialyzed against 3 L of H<sub>2</sub>O with three changes. Then both suspensions were centrifuged for 20 min at 3,500 rpm. In this way, the acid-soluble matrix (acid-SM), the acid-insoluble matrix (for FTIR and amino acid analysis: the matrix was washed twice with H<sub>2</sub>O before centrifugation) and water-soluble matrix (WSM) was obtained. Then the acid-SM and WSM were analyzed by mini-SDS-PAGE. Infrared spectrometry: the powder of sponge body and the dorsal shield, which compose the whole cuttlebone (50–100µg) (Fig.1), the crystal of acid soluble and insoluble matrix, which were dried in an oven at 38°C overnight. Powdered samples and KBr were



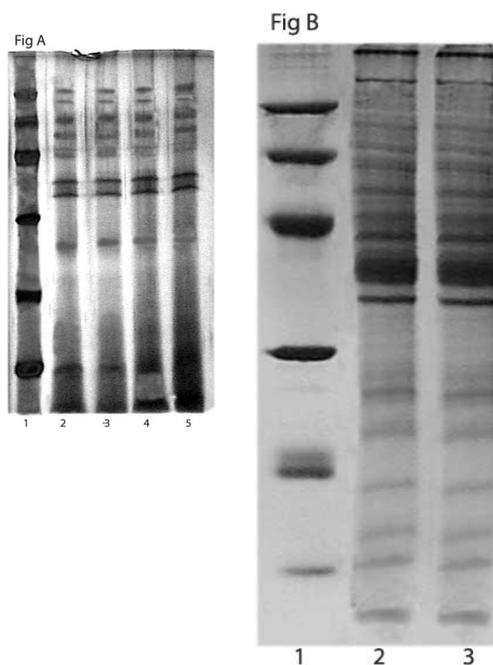
**Figure 1.** A cross section through the posterior part of the cuttlebone, showing the two components: dorsal shield (ds) and spongy (sb).

mixed (about 5% powdered samples in KBr) and loaded into the sample cup according to the method of Worms *et al* (1986). The samples for analysis of amino acid composition are the powders of sponge body, dorsal shield, whole cuttlebone, acid-soluble and insoluble matrix. The amino acid analysis was automatically performed using a HITA-CHI 835-50 Amino Acid Analyzer according to the method reported by Spackman *et al* (1958).

## RESULTS

### Results of SDS-PAGE

The acid-SM has nine major protein components and several minor components. The primary bands run at about 106, 87, 67, 53, 45, 38, 33, 26 and 14 kDa (Fig.2A). WSM (Fig.2B) show major bands at 170, 106, 85, 67, 53, 47, 40, 38, 33, 27, 23, 19, 16, 14 and 11kDa. The comparison of the two kinds of soluble matrix suggested that similar fractions were involved in them and more bands in WSM. The background under 20kDa in the gels in which acid-SM was run was stained darkly by silver, while the reverse occurred in WSM (Fig. 2B).



**Figure 2.** SDS-PAGE of the acid-SM and WSM.

### Infrared spectrometry

All three samples show similar strong amine (3500–3300 $\text{cm}^{-1}$ ). The powdered cuttlebone (Fig. 3) reveals the presence of a band at 2522.5  $\text{cm}^{-1}$ , which is usually conceived as a result of the vibration of the  $\text{HCO}_3^-$  at the organic-mineral interface (Balmain *et al*, 1999). The band at 1789  $\text{cm}^{-1}$  may point to amine or acid anhydride and the transmittance at 1457  $\text{cm}^{-1}$  to the vibration of N-H, which indicate amide. In addition, some apparent strong aragonite peaks appear at 712 and 699  $\text{cm}^{-1}$ . The acid-SM (Fig.4) and acid-ISM (Fig. 5) illustrate that the major polysaccharide absorption region is between 1000 and 1150  $\text{cm}^{-1}$ , and the content of polysaccharide in acid-ISM is obviously higher than in acid-SM. The 1654  $\text{cm}^{-1}$  amide <sup>2</sup> is the main band in acid-ISM. The amide bands for protein are near 1577  $\text{cm}^{-1}$  in acid-SM. This is in agreement with the findings of Dauphin and Marin (1995) for the organic matrix of several cephalopod skeletons. The band at 925  $\text{cm}^{-1}$ , which is usually interpreted as a sulfate band, is stronger in acid-SM. Amide III vibration is observed around 1415  $\text{cm}^{-1}$  (Daniele, 1986)

### Composition of Amino Acids

Both Table 1 and Table 2 indicate that spongy body and shield body as well as acid-SM and acid-ISM have relatively high concentrations of aspartic acid (Asp), glycine (Gly), glutamate (Glu) and serine (Ser) residues. The ratio of acidic to basic amino acid residues indicates a significant difference between spongy body and shield body, but no remarkable difference between acid-SM and acid-ISM. As is the case with the nonpolar amino acids. Table 1 also illustrates that the sum of Asp and Glu in spongy body is higher than shield body, and the content of Gly and Ser in spongy body is similar to that in shield body. But the sum of Ser and Gly in acid-SM is higher than that in acid-ISM. Otherwise, we still found high concentrations of alanine (Ala) in all four samples.

### DISCUSSIONS

We found that the components of the organic matrix of cuttlebone were very complex. It was difficult to separate them well by SDS-PAGE

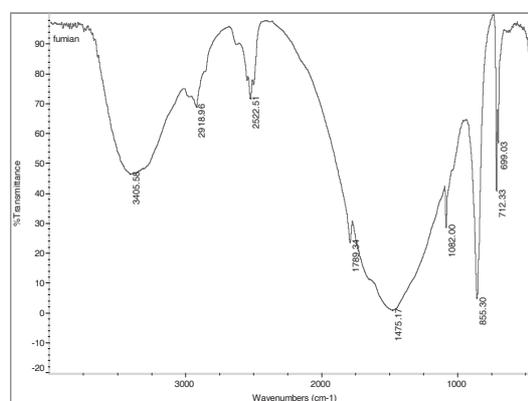


Figure 3. FT-IR spectra of the powdered cuttlebone.

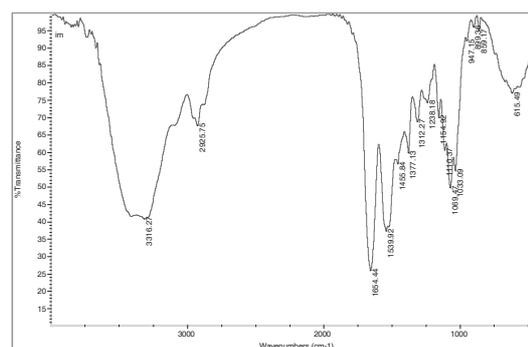


Figure 4. FT-IR spectra of the acid-insoluble matrix of the cuttlebone.

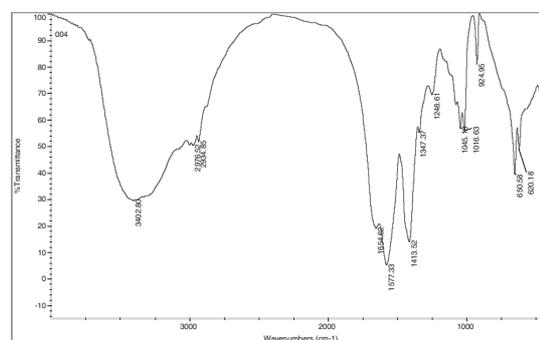


Figure 5. FT-IR spectra of the acid-SM.

**Table 1.** Amino acid composition of the whole cuttlebone consisted of outer layer (dorsal shield) and inner layer (spongy body).

	Spongy body		Dorsal shield	
	$\mu\text{mol/g}$	mol%	$\mu\text{mol/g}$	mol%
<b>Aa</b>				
<b>ASP</b>	16.467	13.539	9.730	10.705
<b>THR</b>	6.712	5.519	3.356	3.692
<b>SER</b>	12.377	10.173	6.662	7.330
<b>GLU</b>	12.918	10.621	4.079	4.488
<b>GLY</b>	11.992	9.860	11.992	13.193
<b>ALA</b>	8.982	7.385	11.228	12.353
<b>CYS</b>	1.651	1.357	1.651	1.8166
<b>VAL</b>	11.102	9.128	8.540	9.395
<b>MET</b>	6.704	5.512	6.704	7.376
<b>ILE</b>	3.051	2.508	2.288	2.517
<b>LEU</b>	7.627	6.27	6.101	6.712
<b>TYR</b>	1.656	1.362	2.208	2.430
<b>PHE</b>	3.028	2.490	3.028	3.332
<b>LYS</b>	6.158	5.063	4.105	4.517
<b>HIS</b>	3.223	2.650	3.223	3.546
<b>ARG</b>	6.307	5.185	5.160	5.677
<b>PRO</b>	1.664	1.368	0.832	0.915
<b>Total</b>	121.622		90.89	
<b>Basic Aa</b>	12.901		13.741	
<b>Acid Aa</b>		24.107		15.193
<b>Ratio(A/B)</b>	1.873		1.106	
<b>Nonpolar Aa</b>	34.665		42.603	

Notes: amino acid (Aa), ratio (A/B) (the ratio of acid Aa to basic Aa)

Acid Aa (Asp+Glu), Basic Aa (Lys+His +Arg), Nonpolar Aa (Pro+Met+Val+Leu +Ileu+Ala+Phe)

(Dauphin, 2001), and the question of low repeatability still occurred in this experiment. But in our opinion, the repeatability may be related to the methods of sample treatment. In this study, the comparatively high resolution and repeatability both occurred in WSM, which came from treating powdered cuttlebone with ultrapure water (Milli-Q). It was impossible to obtain the accurate molecular weights of the separations through SDS-PAGE because of the different protein structure such as:  $\alpha$ -helical form,  $\beta$ -structures and random coil form. Especially the property of the acid-protein and the glycosylation of the protein both contributed to the poor separation and estimation of MW. Fig.2 shows more bands in WSM than in acid-SM. This may be attributed to two factors:

The loss of the proteins of low molecular range weight in the process of dialyzing, and the poor resolution resulting from some very thick and broad bands. In our SDS-PAGE studies, two different models and sizes were used in the gels of preparing. A large area of the gel was beneficial to resolution.

The results of FT-IR indicate that protein and polysaccharide are major components in the organic matrix of cuttlebone. There is a high content of sugar in acid-ISM. According to Dauphin and Marin (1995), components of these sugars are glucosamine, galactose, xylose, galactosamine, arabinose *etc.* But their functions in the shell for biomineralization remain questionable. These results are in accordance with Dauphin (1995), Dauphin and Denis (2000) and Dauphin (2001)

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**Table 2.** Amino acid composition of the acid soluble and insoluble matrix

	Acid-SM		Acid-ISM	
Aa	$\mu\text{mol/g}$	Mol%	$\mu\text{mol/g}$	Mol%
<b>ASP</b>	341.317	12.538	341.317	12.162
<b>THR</b>	144.3195	5.301	144.3195	4.402
<b>SER</b>	184.656	6.783	184.656	7.745
<b>GLU</b>	262.442	9.640	262.442	6.0498
<b>GLY</b>	274.483	10.082	274.483	12.091
<b>ALA</b>	257.13	9.445	257.13	12.202
<b>CYS</b>	9.0819	0.333	9.0819	2.585
<b>VAL</b>	222.905	8.188	222.905	6.926
<b>MET</b>	40.891	1.502	40.891	4.684
<b>IIE</b>	124.323	4.566	124.323	2.613
<b>LEU</b>	221.951	8.153	221.951	5.471
<b>TYR</b>	82.279	3.022	82.279	5.764
<b>PHE</b>	106.002	3.893	106.002	3.778
<b>LYS</b>	164.237	6.033	164.237	3.664
<b>HIS</b>	88.335	3.244	88.335	4.332
<b>ARG</b>	135.894	4.922	135.894	3.284
<b>PRO</b>	61.998	2.277	61.998	1.793
<b>Total</b>	2722.257		2722.257	
<b>Basic Aa</b>		14.270		1.280
<b>Acid Aa</b>		22.178		18.659
<b>Ratio(A/B)</b>		1.554		11.654
<b>Nonpolar Aa</b>		38.027		37.470

Notes: amino acid (Aa), ratio (A/B) (the ratio of acid Aa to basic Aa)

Acid Aa (Asp+Glu), Basic Aa (Lys+His +Arg), Nonpolar Aa (Pro+Met+Val+Leu +Ileu+Ala+Phe)

respectively. The intensity of amide <sup>2</sup> in acid-SM is stronger than that in ISM, and the amide I usually adopts the  $\alpha$ -helical structure. Precise interpretation of bands is difficult because there is significant overlap of the  $\alpha$ -helical structure with random structures. The amide II is stronger in acid-ISM, and adopts  $\beta$  structure, which is related to glutamic acid. The band at 925  $\text{cm}^{-1}$  which is usually interpreted as a sulfate band is stronger in acid-SM. Sulfated polysaccharides have been discussed as possible calcium-binding sites (Wilbur, 1976) and could play a role in the nucleation and the growth inhibition of the mineral (Crenshaw and Ristedt, 1976). The band at 2522.5  $\text{cm}^{-1}$ , is conceived as a result of the vibration of the  $\text{HCO}_3^-$  at the organic-mineral interface because the band only occurred in the spectrum of whole (organic and

mineral) cuttlebone, and no band at 2500–2650  $\text{cm}^{-1}$  was detected on the IR spectra of the acid-ISM or acid-SM. The results agree with Balmain *et al* (1999).

For a long time, it has been assumed that the proteins are the factor controlling a crystal form of calcium carbonate. Table 1 and Table 2 indicate that spongy body and shield body as well as acid-SM and acid-ISM have relatively high concentrations of aspartic acid (Asp), glycine (Gly), glutamate (Glu) and serine (Ser) residues. The abundance of these proteins in Asp residues was usually associated with their calcium-binding ability (Cariolou and Morse 1988), although some authors have noticed that the fractions actually capable of promoting calcification were not the ones with the highest levels of acidic amino acids (Samat and Kramipitz, 1982). In fact, an abundance

of Asp and Glu residues inhibited rather than promoted carbonate crystallization (Wilbur and Bernhardt, 1984). The ratio of acidic to basic amino acid residues indicated a significant difference between spongy body and shield body but also that no such difference existed in acid-SM and acid-ISM. These results agree with the assumption that ratio of acid and base residues are related to the phase adopted by calcium carbonate during mineralization. In addition, the high content of the Gly and Ser were found in shield body and acid-ISM, while Gly and Ser are important elements of fibrin. According to Levi *et al* (1998), the important function of  $\beta$ -chitin-silk fibroin in vitro system to calcification has been demonstrated and

they showed that the chitin framework was very porous when viewed by scanning electron microscopy.

#### ACKNOWLEDGEMENTS

The authors would like to thanks Dr. Zhao Jianming, Institute of Oceanology, Chinese Academy of Science for providing some important literatures. We are also grateful to Dr. Wang Pinhong, Institute of Oceanology, Chinese Academy of Science for her assistance in laborstory work.

This study is supported by Shandong Marine Sci-tech Plan (No.2002-8)

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