Infrared Spectroscopic Studies on the Resilium of a Surf Clam, *Spisula (Pseudocardium) sachalinensis*

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Sulfinyl group was detected in the resilium (internal hinge-ligament) of surf clam by IR spectrometry. The results reconfirm the previous reports on the presence of methionine *S*-oxide in the intact resilium protein. Aragonite structure was deduced for $CaCO_3$ in the resilium because the IR spectrum of the resilium was reproduced from those of decalcified resilium and aragonite ($CaCO_3$). The IR spectrum of intact protein component in the resilium was simulated from those of the resilium and aragonite ($CaCO_3$).

The hinge-ligaments of molluscan bivalves are elastic and function to open the shells. The resilium (internal hinge-ligament) of surf clam species (Mactridae family) consists of protein (40% of the dry weight) and calcium carbonate ($CaCO_3$). The resilium protein contains 50 mol% of glycine and 20 mol% of methionine of total amino acids.[1,2] Methionine in the resilium protein was suggested to be in an oxidized form, methionine *S*-oxide, by amino acid analysis of the alkaline hydrolysate of the resilium[1] and by solid-state ^{13}C-NMR spectroscopy on powdered resilium preparation without any chemical treatment.[2] It was the first solid evidence for the presence of methionine *S*-oxide in the intact natural proteins.

In the present study, an additional evidence for the presense of methionine *S*-oxide was obtained by infrared (IR) spectrometry and the aragonite structure of $CaCO_3$ was deduced by simulation method applied on IR spectra.

Experimental

Materials *Resilium*: Sakhalin surf clams, *Spisula (Pseudocardium) sachalinensis*, were collected at the north east coast of the main island of Japan. The resiliums were removed from the shells, dried *in vacuo* and stored at −20 °C.

Reduced Resilium: The powdered resilium (8.9 g) was stirred in 50% (v/v) aqueous *N*-methylmercaptoacetamide[3] (100 ml) at room temperature for 7 d, washed successively with water (50 ml×5), 50% (v/v) methanol (50 ml×5), methanol (50 ml×5) and acetone (50 ml×5) and dried *in vacuo* (7.1 g). The amino acid compositions of the resilium before and after the reduction are shown in Table 1.

TABLE 1. AMINO ACID COMPOSITION OF THE RESILIUM[a] BEFORE AND AFTER THE REDUCTION WITH *N*-METHYLMERCAPTOACETAMIDE

Amino acid[12]	Composition/mol%	
	Resilium	Reduced resilium
Met (O)	16	1
Met	0	19
Gly	53	52
Others	31	28

a) The resilium (about 2 mg) was hydrolyzed with 2.5 mol dm^{-3} NaOH at 105 °C for 15h. Amino acids were analyzed on JLC 10-D amino acid analyzer (JEOL Co. Ltd., Tokyo). No correction was made for hydrolytic losses.

Decalcified Resilium: The powdered resilium (2 g) was stirred in 10% acetic acid (20 ml) for 15 h, washed with water (20 ml×5) and dried *in vacuo* (0.8 g).

Calcite and Aragonite: The authentic specimens of $CaCO_3$, calcite and aragonite, were provided by Dr. M. Akizuki of the Geology Department of this faculty.

Infrared Spectra. Infrared spectra were measured on a Hitachi 260-10 IR spectrophotometer (Hitachi Co. Ltd., Tokyo, Japan) by KBr disk method.

Combination of IR Spectra. The spectral data were digitized into absorbance values at every 50 cm^{-1} between 4000 cm^{-1} and 2000 cm^{-1} and at every 10 cm^{-1} between 2000 cm^{-1} and 650 cm^{-1}. The combination of the spectra was accomplished according to the additivity of absorbance values. The combination ratio was determined to give the smallest squared deviation of the differences between the resilium spectrum and the artificially prepared spectrum.

Results and Discussion

Methionine S-Oxide. While only 20 amino acids are involved as monomer building blocks in the protein synthesis, more than 140 amino acids have been identified in natural proteins because some amino acids are modified after they are incorporated into the peptide chain of proteins.[4] Methionine *S*-oxide had been detected in some proteins,[5-7] but it was not included in the list of post-translational modification products[4] because its contents were low and methionine can be oxidized *in vitro* during the isolation procedures of proteins.[8] Therefore, the presence of methionine *S*-oxide in an intact protein should be demonstrated excluding the possibility of formation as an artifact or, preferably, without any chemical treatment. In the previous studies, about 90% of methionine in the resilium protein was detected in sulfoxide form after alkaline hydrolysis of the resilium.[1] To exclude the posibility that methionine was oxidized during the alkaline hydrolysis, the presence of methyl group attached to sulfinyl group was demonstrated by solid-state ^{13}C-NMR using cross-polarization and magic angle spinnig techniques.[2]

In the IR spectrum (Fig. 1-a) of the resilium of a surf clam, *S.* (*Ps.*) *sachalinensis*, the absorption band at 1020 cm^{-1} was assigned to S-O stretching vibration of sulfinyl group. The corresponding band was not observed in the spectrum (Fig.1-b) of the reduced resilium, in which methionine *S*-oxide had been converted into methionine with reducing reagent (Table 1). The S-O stretching vibration band of authentic methionine *S*-oxide appeared at 1030 cm^{-1} and 1020 cm^{-1} as splitted

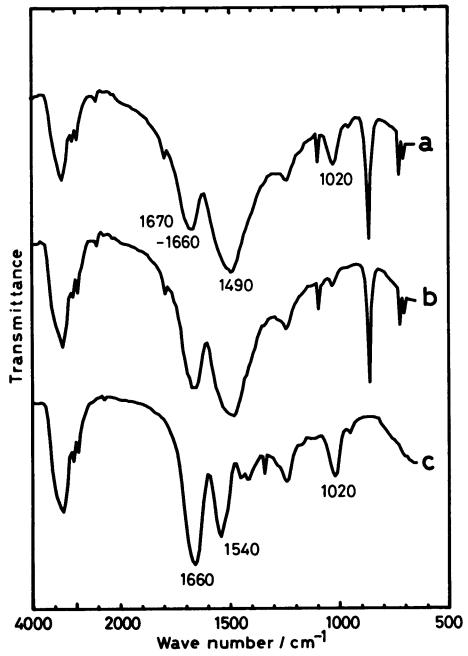

Fig. 1. IR spectra of (a) resilium, (b) reduced resilium, and (c) decalcified resilium.

peaks (not shown). These results indicate the presence of sulfinyl group in the resilium and recomfirm the presence of methionine *S*-oxide in the intact resilium protein.

Aragonite Structure of $CaCO_3$. In Fig.1-a, the absorption band at 1670—1660cm^{-1} is assigned to amide I band of the resilium protein, but that at 1490 cm^{-1} is unusual as a typical amide II band because it is more intense than amide I band and it is at lower wave number than 1500cm^{-1}. When the resilium was decalcified with 10% acetic acid, most of the protein remained insoluble. The decalcified resilium showed a typical amide II band at 1540cm^{-1} (Fig.1-c). Therefore, the unusual band at 1490cm^{-1} in Fig. 1-a was concluded to contain amide II band of protein and C-O stretching band of $CaCO_3$. Calcite and aragonite are the major crystal types of polymorphic $CaCO_3$ and they are distinguishable by IR spectrometry (Fig. 2-a and b) as reported by Adler and Kerr.[9] If a mixture containing the decalcified resilium and one of the $CaCO_3$ polymorphs could afford the IR spectrum of the resilium (Fig. 1-a), that type of $CaCO_3$ would be the one present in the resilium. In the present study, the spectra of decalcified resilium (Fig. 1-c) and $CaCO_3$ (Fig. 2-a or b) were combined to simulate the spectrum of the mixture. The combination ratio was determined to give the closest spectrum to that of the resilium (Fig. 1-a). Figure 3-a shows the spectrum prepared artificially from those of the decalcified resilium (Fig. 1-c) and calcite ($CaCO_3$) (Fig. 2-a). The amide II band of protein (1540cm^{-1}) and C-O stretching band of $CaCO_3$ (1430 cm^{-1}) were observed separately. On the other hand, the combination of the spectra of the decalcified resilium (Fig. 1-c) and aragonite ($CaCO_3$) (Fig. 2-b) gave a single absorption band (1490cm^{-1}) which contained amide II band and C-O band (Fig. 3-b). The IR spectrum of the resilium (Fig. 1-a) was reproduced with the spectra of the decalcified resilium and aragonite ($CaCO_3$). Calcite is the most stable form of $CaCO_3$ at normal temperature, but aragonite is more popular than calcite in the biologically derived structures such

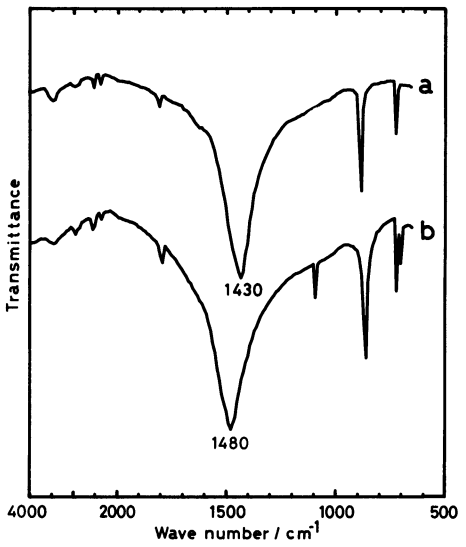

Fig. 2. IR spectra of (a) calcite and (b) aragonite.

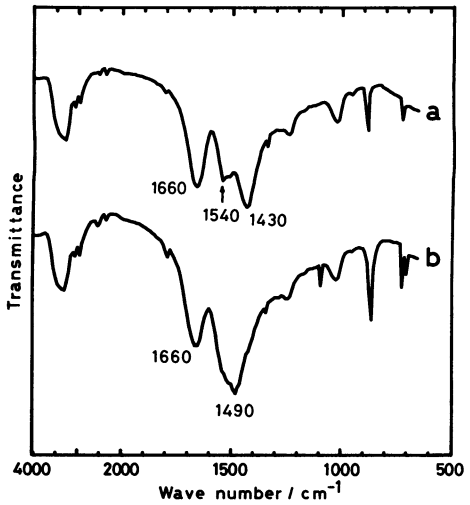

Fig. 3. IR spectra of resilium simulated with (a) decalcified resilium (Fig. 1-c) and calcite (Fig. 2-a) and (b) decalcified resilium (Fig. 1-c) and aragonite (Fig. 2-b).

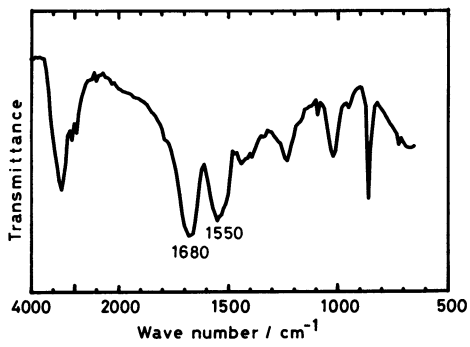

Fig. 4. Simulated spectrum of the protein component in the resilium. The spectrum of aragonite (Fig. 2-b) was subtracted from that of the resilium (Fig. 1-a).

as coral reefs and shells of molluscus.[10] Calcium carbonate in the resilium of surf clams appeared to be in the aragonite form as shown above.

The spectrum of aragonite ($CaCO_3$) (Fig. 2-b) was subtracted from that of the resilium (Fig. 1-a) to simulate the spectrum of the protein component in the resilium (Fig. 4). In Fig. 4, amide I and II bands are observed at 1680 cm^{-1} and 1550 cm^{-1}, while the corresponding bands of the decalcified resilium are at 1660 cm^{-1} and 1540 cm^{-1}, respectively (Fig 1-c). As the amide I and II vibrations are affected by the protein conformation,[11] the small shifts observed above might result from the conformational change of protein caused by decalcification. The amide I and II bands of the decalcified resilium (Fig. 1-c) are close to those assigned to the random-structured protein. The bands in the simulated spectrum of protein component of the resilim (Fig. 4) do not coincide with those proposed for α-helical or β-sheet structure. The protein conformation in the resilium is supposed to be stabilized with calcium ions and contribute to the elasticity of the resilium.

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12) Abbreviations for amino acids are according to the IUPAC-IUB Commission on Biochemical Nomenclature, *J. Biol. Chem.*, **247**, 977 (1972). Met(O) represents methionine *S*-oxide.