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Diastereomers of Methionine *S*-Oxide in the Hinge-Ligament Proteins of Molluscan Bivalve Species

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The diastereomer ratio was analyzed on methionine *S*-oxide residues formed by in vivo posttranslational oxidation of methionine residues in a protein. The hinge-ligament protein of molluscan bivalves is distinct from usual proteins in containing a large amount of methionine *S*-oxide. The methionine *S*-oxide was released from the protein by proteolytic digestions and was found to be a mixture of approximately equall amounts of two diastereomers, (5*S*)- and (5*R*)-L-methionine *S*-oxide.

Molluscan bivalve species have ligaments at their hinges. The hinge-ligaments are elastic and function to open the shells: When the shells are closed by the contraction of adductor muscles, the hinge-ligaments are strained, and when the adductor muscles relax, the shells are opened by the elastic properties of the ligaments.

The hinge-ligaments are composed of proteins and aragonite crystals of calcium carbonate,[1,2] of which the protein may be primarily important for the elastic property. The hinge-ligament proteins contain cross-linking components, desmosine and isodesmosine,[3] and are insoluble unless hydrolyzed.[1] In the previous papers[1,4] we reported unusual amino acid compositions of hinge-ligament proteins from various bivalve species, such as surf clams (superfamily Mactracea), pearl oysters (superfamily Pteriacea) and a mussel (superfamily Mytilacea). They contain methionine (Met) at extremely high levels (20—40 mol% of the total amino acids) as compared to the Met contents of usual proteins, and almost all of the Met is found in an oxidized form, methionine *S*-oxide (MetO).[5] Since free MetO cannot be utilized as such in protein synthesis,[6] the MetO residues must have been derived from Met residues incorporated in the precursor form of the hinge-ligament proteins. It is known that the Met residues in proteins are oxidized slowly into MetO in various biochemical treatments. In the case of bivalves hinge-ligament proteins, however, the possibility of the artefactual oxidation is excluded by the observation that the intact hinge-ligaments are resistant to BrCN treatment[1] and by the non-destructive analyses of the hinge-ligament in solid-state ^{13}C NMR[7] and IR[2] spectrometries. The hinge-ligament protein is the first and the only instance of the MetO-containing proteins normally functioning in animals. The other instances of MetO-containing proteins are associated with some pathologically defective symptoms such as cataract[8] or rheumatoid arthritis.[9] Moreover, the in vitro oxidation of Met residues resulted in the loss of biological activities of various proteins.[10]

The conversion of Met into MetO introduces another asymmetric center into the amino acid molecule, namely *S*-oxide group of *S*- and *R*-configurations; they are designated as (5*S*)-MetO and (5*R*)-MetO, respectively.[5] Only one of the diastereomers can be expected when the Met-oxidation is catalyzed by enzymes, and even when the oxidation is non-enzymatic, the molecular size of the oxidant and the three-dimensional environments of the target Met residues may affect the stereochemical specificities in the Met oxidation. But the absolute configurations of MetO residues formed in vivo or in vitro have not been examined. The information on the composition of MetO diastereomers is essential for the understanding of the mechanism of Met-oxidation reaction and the roles of MetO residues in the elastic properties of hinge-ligaments. In the present paper we describe the analyses of MetO diastereomers released from the intact hinge-ligament proteins and those from the chemically reduced and reoxidized preparations.

Experimental

Bivalve Species and Their Hinge-Ligaments. Table 1 shows the bivalve species and their hinge-ligament layers subjected to the present study. The classification and the common names of the species are given according to Habe[11] and Abbott and Dance.[12] The hinge-ligaments of the species in superfamilies Mactracea and Limacea are a big rubber-like mass called 'resilium' (internal hinge-ligament). The ligaments of Veneracea species are composed of two histologically distinct layers, outer and inner layers. The ligaments of species in superfamilies Pteriacea and Ostracea are also composed of two layers, fibrous layer and lamellar layer. The resiliums, inner layers and fibrous layers were employed in the present study. They are ontogenetically homologous to one another.[13]

Methionine *S*-Oxide. An authentic MetO was prepared from Met (Wako Pure Chem. Ind. Co. Ltd., Osaka, Japan) and hydrogen peroxide as described by Toennies and Kolb.[14] Two diastereomers were resolved by fractional crystallization of the picrates as described by Lavine.[15] Their absolute configurations were determined by Christensen and Kjær.[16] Found (5*S*-diastereomer): C, 36.23; H, 6.89; N, 8.35%. Found (5*R*-diastereomer): C, 36.10; H, 6.91; N, 8.40%. Calcd for $C_5H_{11}NO_3S$: C, 36.35; H, 6.71; N, 8.48%.

Amino Acid Analyses. The amino acid compositions of the hinge-ligament proteins were determined on the HCl-hydrolysates prepared from ligament pieces (2—3 mg) in 6 M

Table 1. Bivalve Species and Their Hinge-Ligament Layers Subjected to the Present Work

Sample No.	Bivalve species	Ligament layer
	Mactracea species	
1	*Pseudocardium sachalinensis* (Sakhalin surf clam)[a]	Resilium
2	*Spisula (Hemimactra) solidissima* (Atlantic surf clam)[b]	Resilium
3	*Spisula solidia* (Solid mactra)[c]	Resilium
4	*Mactra chinensis* (Chinese mactra)[d]	Resilium
5	*Tresus keenae* (Keen's graper)[a]	Resilium
6	*Coecella chinensis* (Chinese anapella clam)[c]	Resilium
	Veneracea species	
7	*Saxidomus purpuratus* (Purplish Washington clam)[e]	Inner layer
	Pteriacea species	
8	*Malleus (Malleus) albus* (White hammer oyster)[c]	Fibrous layer
	Limacea species	
9	*Acesta (Acesta) goliath* (Giant lima)[d]	Resilium
	Ostracea species	
10	*Crassostrea gigas* (Giant pacific oyster)[d]	Fibrous layer

a) From Fukushima, Jpn.; b) from Woods Hole, MA. U. S. A.; c) provided by Dr. T. Habe (National Scientific Museum, Tokyo, Jpn.); d) from Miyagi, Jpn.; e) from Mie, Jpn.

(1 M=1 mol dm^{-3}) HCl (0.3 ml) at 105 °C for 24 h in vacuo. The amino acid analyses were carried out with JLC 200A amino acid analyzer (JEOL Co. Ltd., Tokyo, Japan). The contents of MetO, Met and homocysteic acid, the latter two of which were derived from MetO during the HCl-hydrolysis,[17,18] were combined and taken as Met content. The MetO contents were determined on NaOH-hydrolysates prepared from ligament pieces (2—3 mg) in 2.5 M NaOH (0.3 ml) at 105 °C for 15 h in vacuo. No correction was made for hydrolytic losses.

Decalcification of the Hing-Ligaments. The dried hinge-ligament pieces (about 50 mg) were swollen in H_2O (5 ml) at 4 °C for 18 h and powdered in an agate mortar with a pestle. The ligament powder was collected by centrifugation at 3000 *g* for 10 min and stirred in 2% (v/v) acetic acid (3 times, 2 ml each for 2, 4, and 18 h, at 4 °C). The decalcified ligament powder was washed with H_2O thoroughly and freeze-dried.

Proteolytic Solubilization of the Hinge-Ligament Protein. A suspension of decalcified ligament powder (4 mg) in 0.1 M sodium borate buffer solution (pH 8.8) (1 ml) and one drop of chloroform as preservative was added with 0.1 ml aliquots of crystalline bacterial alkaline protease (Katayama Chem. Ind. Co. Ltd., Osaka, Japan) (4 mg ml^{-1} in the same buffer solution) at the time intervals of 24 h and the mixture stirred at 25 °C for 120 h. The extent of the solubilization was monitored by the combined contents of Met, MetO and homocysteic acid in the HCl-hydrolysates of the supernatant aliquots (30 μl) taken out from the mixture at every 24 h.

Proteolytic Liberation of MetO. The above mixture was stirred at 25 °C and added with 0.1 ml aliquots of leucine amino peptidase (cytosole) (Sigma) (2 mg ml^{-1}) at the incubation times of 0, 27, 50, and 90 h. The contents of free MetO in the supernatant aliquots (30 μl) were determined with amino acid analyzer at the incubation times of 27, 50, 90, and 120 h.

Analyses of MetO Diastereomers. Two columns (0.8 cm×70 cm each) of sulfonated polystyrene resine (JLC-R-2, JEOL Co. Ltd. Tokyo, Japan) were tandemly connected and equipped on an amino acid analyzer, JLC 8AH (JEOL Co. Ltd.). The analysis was carried out at 54 °C with 0.067 M sodium citrate-NaCl buffer solution (pH 2.85, I[5]=0.2) at a flow rate of 0.5 ml min^{-1}. Under these conditions the diastereomers, (5*S*)- and (5*R*)-MetO, were equally sensitive to ninhydrin and eluted at 4.4 and 4.6 h, respectively. The peak areas were proportional to the amounts of materials up to 230 nmol and the routine analyses were carried out with 50—80 nmol each of the diastereomers.

Chemical Reduction of MetO Residues in the Hinge-Ligament Proteins. The decalcified ligament protein powder (about 150 mg) was stirred in 50% (w/v) aqueous *N*-methylmercaptoacetamide[19] (10 ml) at room temperature for 11 days. The protein powder was washed with H_2O by centrifugation and freeze-dried.

Chemical Reoxidation of Met Residues in the Reduced Hinge-Ligament Protein. Oxidation with Hydrogen Peroxide: A suspension of reduced ligament protein (8 mg) in H_2O (5 ml) was adjusted to pH 2 with 0.1 M HCl, added with 30% (w/v) hydrogen peroxide (0.5 ml) and stirred at room temperature for 18 h. The protein powder was washed thoroughly with H_2O and freeze-dried. The reduced ligament protein (8 mg) was also reoxidized in 0.1 M sodium borate (pH 8.8) (5 ml) in the same procedures as described above.

Oxidation with Chloramine T or *N*-Chlorosuccinimide: The Met residues in the reduced protein (12 mg) were oxidized with chloramine T or *N*-chlorosuccinimide (5 mol/mol of Met residues) in 0.033 M sodium citrate-NaCl buffer solution (pH 2.2, I=0.1) or 0.1 M Tris-HCl (pH 8.5) (40 ml) for 1 h at room temperature as described by Shechter et al.[20] The protein powder was washed with H_2O and freeze-dried.

Chemical Oxidation of Free Methionine. Oxidation with *N*-Chlorosuccinimide: A solution containing L-Met (75 mg) and *N*-chlorosuccinimide (80 mg) in 0.033 M sodium citrate-NaCl buffer solution (pH 2.2, I=0.1) (2 ml) was stirred for 1 h at room temperature, adjusted to pH 7 with 1-butylamine and added with methanol (4 ml) and acetone (40 ml). The precipitated amino acid was collected by centrifugation (3000 *g* for 10 min), washed with acetone (3 times, 30 ml each) and dried in vacuo (83 mg, 100%). The product agreed with the authentic MetO on TLC analysis using 0.1 mm precoated cellulose plates (Merck) and 1-butanol/water/acetic acid (5/2/1, by vol) as developing solvent.

Oxidation with Chloramine T: A solution containing L-Met (75 mg) and Chloramine T (170 mg) in 0.1 M Tris-HCl buffer solution (pH 8.5) (4 ml) was stirred for 1 h at room temperature and added with methanol (4 ml) and acetone (100 ml). The precipitated MetO was collected and characterized as described above (45 mg, 54%).

Results and Discussion

The compounds containing *S*-oxide moieties are

widely distributed in the natural products. Since their S-oxide groups are not racemic as reviewed by Kjær,[21] the enzymatic sulfide oxidation may be common in their biosynthetic pathways. On the other hand, the non-enzymatic mechanism was suggested for the in vivo oxidation of Met residues in the proteins from pathologically defective tissues, although the absolute configuration of the MetO residues was not analyzed.[22] In contrast to the above proteins, the ligament proteins are from non-defective and normally functioning hinge-ligaments of the bivalve species. They contain Met in extremely high contents and the extent of Met-oxidation in them is almost complete.

The amino acid compositions of the hinge-ligament proteins are shown in Fig. 1. The resilium proteins of the Mactracea species (1—6) are similar to one another in containing Gly and Met to the extents of 50 and 20 mol%, respectively, and almost all of their Met residues are present in the S-oxide form, MetO (Table 2, column 2). The ligament proteins from other species (7—10) also contain Met as predominant constituents and their Met residues are mostly in S-oxide form.

Since the hinge-ligament proteins are insoluble in usual protein solvents, they were solubilized by the proteolysis with crystalline bacterial alkaline protease (Fig. 2). The extents of solubilization were 80% or more in most cases examined. Amino acids were released from the solubilized peptides by the action of an exopeptidase, leucine amino peptidase (Fig. 3). About 80% of MetO was detected as a free amino acid in the digests of the ligament proteins from Sakhalin surf clam (*Ps. sachalinensis*) and Giant pacific oyster (*C. gigas*) (Fig. 3, A and E, respectively), but only 30—

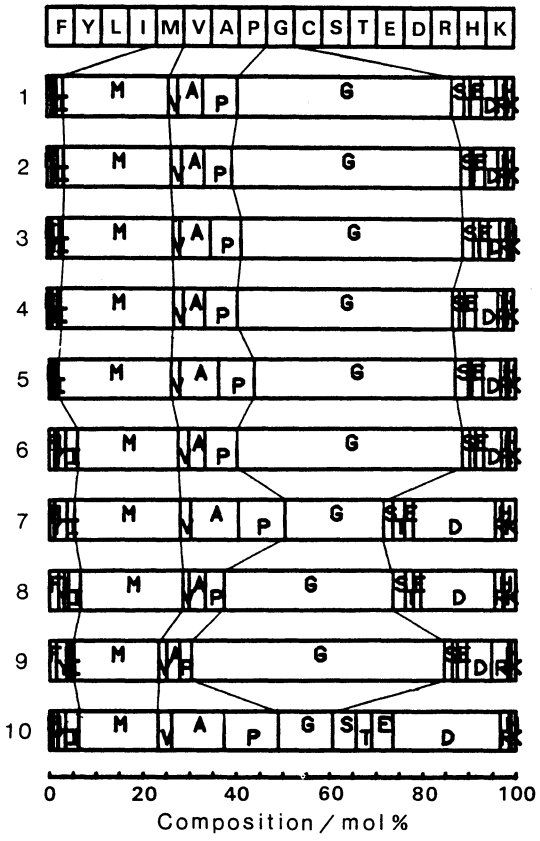

Fig. 1. Amino acid compositions of the hinge-ligament proteins of bivalve species.[5] The numbers for species and ligament layers are the same as those in Table 1.

Table 2. Methionine S-Oxide in the Hinge-Ligament Protein of Various Bivalve Species

Species and ligament layer[a]	Content: MetO/(Met+MetO) ratio in the NaOH-hydrolysate/%	Diastereomer ratio: (5S)-MetO/(5R)-MetO
Mactracea species		
1	100	1.1
2	100	1.2
3	100	1.2
4	100	1.2
5	100	1.2
6	92	1.2
Veneracea species		
7	100	1.1
Pteriacea species		
8	100	1.1
Limacea species		
9	100	1.5
Ostracea species		
10	100	1.1

a) Numbers for the bivalve species and the ligament layers are the same as those in Table 1.

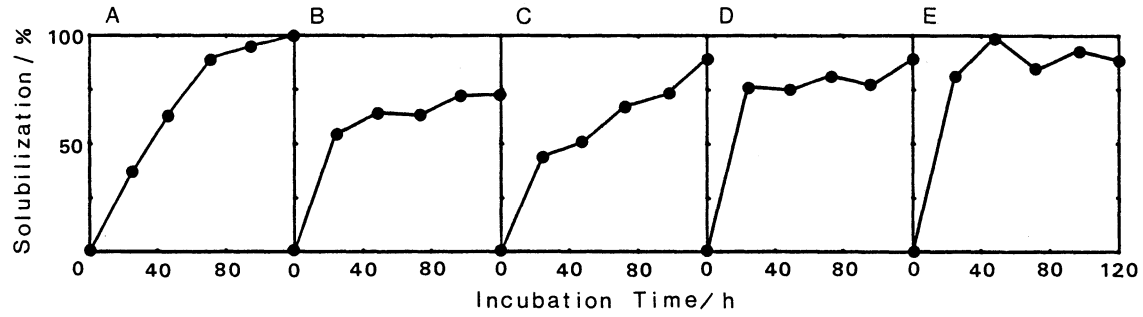

Fig. 2. Time-courses of the proteolytic solubilization of the hinge-ligament proteins. The samples in A, B, C, D, and E are those introduced as 1, 7, 8, 9, and 10 , respectively, in Table 1.

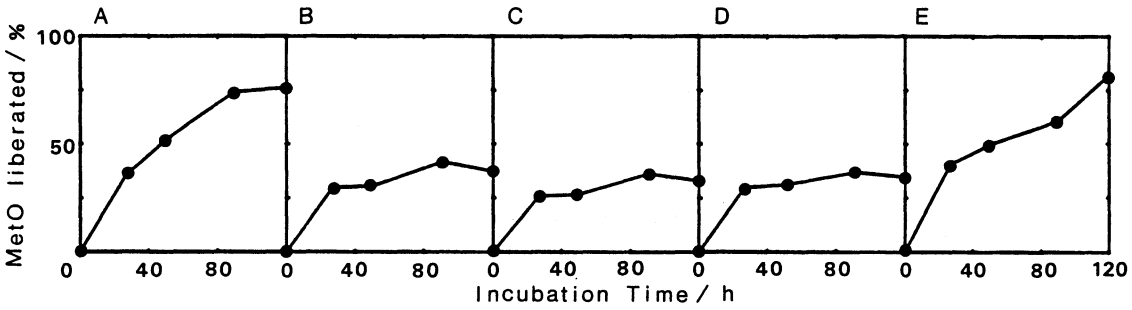

Fig. 3. Time-courses of proteolytic liberation of methionine *S*-oxide from the solubilized fragment-peptides of the hinge-ligament proteins. The samples in A, B, C, D, and E are those introduced as 1, 7, 8, 9, and 10, respectively, in Table 1.

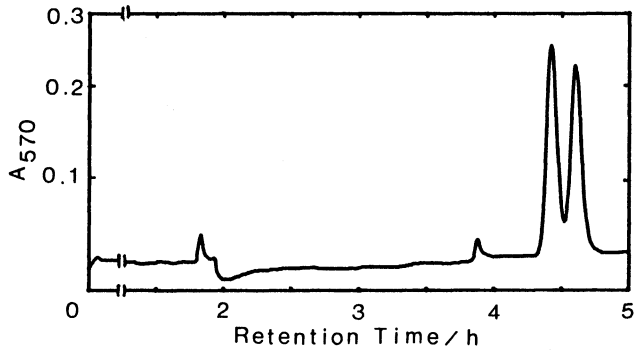

Fig. 4. Separation of MetO diastereomers on amino acid analyzer. For detailed chromatographic conditions see experimental section.

40% of MetO was liberated from the peptides in the other cases (Fig. 3, B, C, and D). The difference in the extent of the MetO liberation might be due to the difference in amino acid sequence of the protein.

The ratio of MetO diastereomers was determined by amino acid analysis. Figure 4 shows the results for the MetO from the resilium protein of Sakhalin surf clam (*Ps. sachalinensis*). The peaks at 4.4 and 4.6 h were assigned to (5*S*)- and (5*R*)-MetO, respectively. From their peak areas, the (5*S*)-MetO/(5*R*)-MetO ratio was determined as 1.1. Table 2 (column 3) shows the ratios of MetO diastereomers in the ligament proteins from various bivalve species. The values are 1.1—1.2 in most cases and 1.5 for the MetO from the resilium protein of Giant lima, *A.* (*A*). *goliath*. Since no interconversion was observed between the two diastereomers during the above proteolytic treatments (data not shown), the values show the ratios of MetO diastereomers in the ligament proteins. The results show that the two diastereomers are present in approximately equal amounts in the hinge-ligament proteins from bivalve species of a wide variety of the taxonomic classes.

The MetO residues in the resilium protein of Sakhalin surf clam (*Ps. sachalinensis*) were reduced into Met with *N*-methylmercaptoacetamide,[19] and the Met residues in the reduced protein were reoxidized with various reagents. The MetO/(Met+MetO) ratios were less than 0.03 in the reduced protein and more than 0.95 in the reoxidized preparations. The ratios of MetO diastereomers in the reoxidized preparations are shown in Table 3. The chemical reoxidations of Met residues with various reagents in either acidic or alkaline solutions resulted in the formation of the two MetO diastereomers in approximately equal amounts. The results may suggest that the in vivo Met oxidation is a nonenzymatic reaction, although the active oxidizing species, which is not identified yet, can be produced by some enzymes as suggested by Wong and Travis,[23] Fliss et al.[24] and Kühn et al.[25] The participation of enzymes in Met oxidation was examined as follows. The soft body of Sakhalin surf clam (*Ps. sachalinensis*) was dissected into eight tissues; adductor muscles, gills, mantle, labial palp, viceral mass, siphon, crystalline style and body wall (foot). The tissues were homogenized in phosphate buffer saline and the homogenates centrifuged at 15000 *g* for 30 min. The supernatant fractions were taken and incubated with reduced resilium protein of Sakhalin surf clam at 25 °C for 20 h. But no significant and reproducible increase in MetO/(Met+MetO) ratio was observed in any protein preparations incubated with the tissue extracts. Since the addition of D-glucose and L-leucine (50 mM each) into the medium did not affect the results, the possibilities of participation of glucose oxidase or amino acid oxidase, which would release hydrogen peroxide into the medium, were also

Table 3. The Ratio of MetO Diastereomers Prepared by Chemical Oxidation of Met Residues in Reduced Resilium Protein of Sakhalin Surf Clam

Oxidant	(5*S*)-MetO/(5*R*)-MetO ratio	
	pH 2.2	pH 8.8
Hydrogen peroxide	1.1	1.1
N-Chlorosuccinimide	1.1	1.1
Chloramine T	n.d.[a]	1.2

a) n.d.: not determined because of the low yield due to low solubility of the reagent in the buffer solution.

Table 4. The Ratio of MetO Diastereomers Prepared by Chemical Oxidation of Free L-Methionine

Oxidant	pH	(5*S*)-MetO/(5*R*)-MetO ratio
Hydrogen peroxide	2	1.4
N-Chlorosuccinimide	2.2	1.7
Chloramine T	8.5	1.6

excluded. When free Met was incubated with freshly prepared resilium powder, a small portion of it was converted into MetO. The results can, however, be explained by chemical exchange of oxygen atom between free Met and MetO residues in the protein.[26] Although these results indicate no enzyme participation in the in vivo oxidation of Met residues, the examination might be too preliminary to exclude the possibilities of enzymatic formation of active oxidizing species.

The chemical oxidation of free Met was not highly stereospecific: The (5*S*)-MetO/(5*R*)-MetO ratios were 1.4—1.7 in the MetO preparations obtained from free L-Met and various oxidizing reagents under conditions similar to those described above (Table 4). On the other hand, in the oxidation of Met residues in proteins, the stereospecificity of the reaction can be largely affected by the three-dimensional protein structures. Since the MetO in the hinge-ligament proteins was found to be a mixture of two diastereomers in approximately equal amounts, the conformation of the hinge-ligament proteins might be random and the two MetO diastereomers might be formed with equal probabilities. Alternatively, the protein conformation might determine the stereospecificities at every Met-oxidation sites in the protein to form the two MetO diastereomers in equal amounts.

As the MetO is a predominant constituent of the hinge-ligament proteins of some bivalve species, and as the conversion of Met into MetO is almost complete in these proteins, the MetO residues are supposed to play some essential roles in the properties of the hinge-ligaments. The MetO side chains make the protein highly hydrophilic and promote the swelling of the hinge-ligaments (Y. Kikuchi et al., unpublished data). The relationship between the MetO residues and the elastic properties of the ligaments will be discussed elsewhere.

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5) Abbreviations for amino acids are used according to the recomendation of IUPAC-IUB Joint Commission on Biological Nomenclature: *Eur. J. Biochem.*, **138**, 9 (1984). All amino acids except for glycine are of L-configuration. The sulfur in methionine *S*-oxide was treated as carbon in the numbering. Other abbreviations: *I*, ionic strength; Tris, 2-amino-2-hydroxymethylpropane-1,3-diol.
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