Chemical taxonomy of the hinge-ligament proteins of bivalves according to their amino acid compositions

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The proteins in the hinge ligaments of molluscan bivalves were subjected to chemotaxonomic studies according to their amino acid compositions. The hinge-ligament protein is a new class of structure proteins, and this is the first attempt to introduce chemical taxonomy into the systematics of bivalves. The hinge-ligament proteins from morphologically close species, namely mactra (superfamily Mactracea) or scallop (family Pectinidae) species, showed high intraspecific homology in their compositions. On the other hand, inconsistent results were obtained with two types of ligament proteins in pearl oyster species (genus *Pinctada*). The results of our chemotaxonomic analyses were sometimes in good agreement with the morphological classifications and sometimes inconsistent, implying a complicated phylogenetic relationship among the species.

INTRODUCTION

The two shells of molluscan bivalves are connected with each other by the organic ligament at the hinge. The hinge ligament is elastic and functions to open the shells: the ligament is strained when the shells are closed by the adductor muscles, and when the muscles relax the spring-like action of the elastic ligament opens the valves. The morphological type of the hinge ligament is one of the essential elements in the classification of bivalve species (Habe, 1977; Habe & Ito, 1977; Abbott & Dance, 1982). The main components of hinge ligament are protein and calcium carbonate, of which the protein is presumably responsible for the elastic properties of the ligament. The protein is insoluble, so far as has been tested, in usual protein solvents such as 6 M-urea, 4 M-guanidinium chloride and dimethylformamide. The amino acid compositions of the ligament proteins from a mussel (Hare, 1963) and scallops (Kelly & Rice, 1967) have been reported. They are distinct from each other and also different from those of other known structural proteins such as collagen, elastin, resilin and silk fibroin.

In previous studies we observed an unusual amino acid composition of the hinge-ligament protein of the Sakhalin surf clam (*Pseudocardium sachalinensis*) (Kikuchi & Tamiya, 1981). About 50 mol% and 20 mol% of its constituent amino acids were glycine and methionine sulfoxide respectively. The composition is different from those reported for the hinge-ligament proteins of other species. In the present work we have compared the amino acid compositions of the ligament proteins from various bivalve species in order to study their chemotaxonomic relationships.

MATERIALS AND METHODS

Bivalves and their hinge ligaments

The bivalve species whose hinge ligaments were collected are shown in Table 1. The classification and the common names of the species are given according to Habe (1977), Habe & Ito (1977) and Abbott & Dance

(1982). The ligaments were removed from the shells and dried *in vacuo*.

Amino acid compositions of the ligament proteins

Small pieces (about 2 mg) of the ligament were heated in 6 M-HCl (0.3 ml) at 105 °C for 24 h *in vacuo*. The amino acids in the hydrolysate were analysed with a JLC amino acid analyser (models 10-D and/or 200A; JEOL Co., Tokyo, Japan). As methionine sulfoxide is slowly converted into methionine, homocysteic acid and some other minor compounds during the acid hydrolysis (Floyd *et al.*, 1963; Morihara, 1964), the contents of methionine, methionine sulfoxide and homocysteic acid were combined and taken as methionine content. The methionine sulfoxide content was determined on the alkali hydrolysate prepared from the ligament pieces (about 2 mg) and 2.5 M-NaOH (0.3 ml) at 105 °C for 15 h *in vacuo*. No correction was made for losses that occurred during the acid or alkali hydrolysis. Hydrolysis of the hinge ligaments of Mactracea (surf clam) species with 3 M-toluene-*p*-sulphonic acid at 110 °C for 22 h *in vacuo* (Hayashi & Suzuki, 1985) yielded 10% and 90% of methionine and methionine sulfoxide respectively of the total amount of methionine. The results are essentially similar to those obtained by NaOH hydrolysis (Table 2), showing that almost all of the methionine is in its sulfoxide form. But the small differences between them have not as yet been explained.

Comparison of the amino acid compositions

Comparison of the amino acid compositions of ligament proteins from various species was made as follows. The difference index (D_{AB} , mol% in dimension) between the two species (*A* and *B*) was calculated by the equation (Metzger *et al.*, 1968):

$$D_{AB} = \frac{\sum_{i=1}^{17} |X_{iA} - X_{iB}|}{2} \quad (1)$$

where X_{iA} and X_{iB} represent the contents (mol%) of

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Table 1. Bivalve species whose hinge ligaments are subjected to chemotaxonomic analysis in the present work

Mactracea species	
(1) Sakhalin surf clam*	<i>Pseudocardium sachalinensis</i> (Schrenck, 1862)
(2) Atlantic surf clam†	<i>Spisula (Hemimactra) solidissima</i> (Dillwyn, 1817)
(3) Solid mactra‡	<i>Spisula solidia</i> (Linnaeus, 1758)
(4) Chinese mactra§	<i>Mactra chinensis</i> (Philippi, 1846)
(5) Keen's graper*	<i>Tresus keenae</i> (Kuroda & Habe, 1950)
(6) Chinese anapella clam‡	<i>Coecella chinensis</i> (Deshayes, 1855)
Pectinidae species	
(7) Yesso scallop§	<i>Patinopecten (Mizuhopecten) yessoensis</i> (Jay, 1857)
(8) Asian moon scallop	<i>Amusium pleuronectes</i> (Linnaeus, 1785)
(9) Farrer's scallop§	<i>Chlamys (Azumapecten) farreri</i> (Jones & Preston, 1904)
(10) Noble scallop¶	<i>Chlamys (Mimachlamys) senatoria nobilis</i> (Reeve, 1852)
(11) Atlantic deepsea scallop**	<i>Placopecten magellanicus</i> (Gmelin, 1791)
(12) Carolina bay scallop**	<i>Argopecten irradians concentricus</i> (Say, 1822)
(13) Japanese baking scallop††	<i>Pecten (Notovola) albicans</i> (Schröter, 1802)
Pinctada species	
(14) Golden-lip pearl oyster‡‡	<i>Pinctada maxima</i> (Jameson, 1901)
(15) Black-lip pearl oyster	<i>Pinctada margaritifera</i> (Linnaeus, 1758)
(16) Japanese pearl oyster §§	<i>Pinctada martensii</i> (Dunker, 1850)
(17) Fragile pearl oyster‡	<i>Pinctada albina</i> (Lamarck, 1819)
(18) Chemnitzian pearl oyster‡	<i>Pinctada chemnitzii</i> (Philippi, 1847)
(19) Maculated pearl oyster‡	<i>Pinctada maculata</i> (Gould, 1850)
* From Fukushima, Japan.	
† From Woods Hole, MA, U.S.A.	
‡ Provided by Dr. T. Habe (National Scientific Museum, Tokyo, Japan).	
§ From Miyagi, Japan.	
From Okinawa, Japan.	
¶ From Mie, Japan.	
** Amino acid composition data taken from Kelly & Rice (1967).	
†† From Fukuoka, Japan	
‡‡ From the Philippines.	
§§ Provided by Mikimoto Pearl Co. (Mie, Japan).	

amino acid *i* in the proteins from species *A* and *B* respectively. The calculation was made on 17 amino acids in the HCl hydrolysate. Asparagine and glutamine were combined with aspartic acid and glutamic acid respectively. The tryptophan content was not taken into account; in most cases it was negligibly small or not detected in the alkali hydrolysate.

RESULTS

The bivalve species in the superfamily Mactracea are called 'surf clams' or 'mactra species' [Table 1, (1)–(6)]. A hinge of this group consists of hinge teeth and an

internal hinge ligament, which is a big rubber-like mass, called the 'resilium'. The resilium consists of protein (40% by weight) and aragonite crystals of calcium carbonate (60% by weight) (Kikuchi & Tamiya, 1984). Fig. 1 shows the amino acid compositions of the resilium proteins from six Mactracea species. All of them are similar to one another in containing glycine and methionine to the extents of 45–50 mol% and 20–25 mol% respectively of the total amino acids. Almost all the methionine was detected as methionine sulphoxide in alkali hydrolysates of the resiliums [Table 2, (1)–(6)]. Non-destructive analyses by i.r. spectrometry and solid-state ¹³C-n.m.r. spectrometry confirmed the

Fig. 1. Amino acid compositions of the resilium proteins of Mactracea species

(1) *Ps. sachalinensis*; (2) *S. (H.) solidissima*; (3) *S. solidia*; (4) *M. chinensis*; (5) *T. keenae*; (6) *Coe. chinensis*.

presence of methionine sulphoxide in the intact resilium protein (Kikuchi *et al.*, 1982; Kikuchi & Tamiya, 1984). The two diastereoisomers of methionine sulphoxide were in the ratio 1:1 (Y. Kikuchi, unpublished work). The matrix shown in Fig. 2 represents the differences among these amino acid compositions. The values are not more than 10, showing that these compositions are very close to one another (Woodward, 1978). The chemotaxonomic differences among these amino acid compositions were simulated into distances in three-dimensional space and

	(1)	(2)	(3)	(4)	(5)	(6)
(1)	0	4	5	5	6	6
(2)	4	0	5	5	8	5
(3)	5	5	0	6	7	7
(4)	5	5	6	0	6	6
(5)	6	8	7	6	0	10
(6)	6	5	7	6	10	0

Fig. 2. Difference matrix for the amino acid compositions (given in D_{AB} values, see the text) of the resilium proteins of Mactracea species

The numbers for species are the same as those in Fig. 1.

Fig. 3. Chemotaxonomic relationship among the resilium proteins of Mactracea species

The three-dimensional scale represents 1 D_{AB} unit (mol%, see the text). The averaged error in the simulation is less than 9% of the values. The numbers for species are the same as those in Fig. 1.

illustrated in Fig. 3. The Mactracea species are thus homologous in their resilium protein composition as well as in morphology.

The scallop species belong to the family Pectinidae in the superfamily Pectinacea [Table 1, (7)–(13)]. They have no hinge teeth and have a big rubber-like internal resilium at the centre of their straight hinge-line. The resiliums of scallops consist mostly of protein and contain only small amounts of calcium carbonate. Fig. 4 shows the amino acid compositions of the resilium proteins from seven scallop species. Glycine is the most

Table 2. Methionine and methionine sulphoxide detected in alkali hydrolysates of hinge-ligament proteins

Abbreviation: Met(O), methionine sulphoxide.

Bivalve species	Protein source	Percentage	
		Met(O)	Met
Mactracea species			
(1) <i>Ps. sachalinensis</i>	Resilium	100	0
(2) <i>S. (H.) solidissima</i>	Resilium	100	0
(3) <i>S. solida</i>	Resilium	100	0
(4) <i>M. chinensis</i>	Resilium	100	0
(5) <i>T. keenae</i>	Resilium	100	0
(6) <i>Coe. chinensis</i>	Resilium	92	8
Pectinidae species			
(7) <i>Pa. (M.) yessoensis</i>	Resilium	41	59
(8) <i>Ch. (A.) farreri</i>	Resilium	40	60
(9) <i>Ch. (M.) senatoria</i>	Resilium	40	60
(10) <i>Pe. (N.) albicans</i>	Resilium	50	50
<i>Pinctada</i> species			
(11) <i>Pi. martensii</i>	Fibrous layer	99	1
(12) <i>Pi. albina</i>	Fibrous layer	85	15
(13) <i>Pi. chemnitzii</i>	Fibrous layer	99	1
(14) <i>Pi. maculata</i>	Fibrous layer	99	1

Fig. 4. Amino acid compositions of the resilium proteins of Pectinidae species

(1) *Pa. (M.) yessoensis*; (2) *A. pleuronectes*; (3) *Ch. (A.) farreri*; (4) *Ch. (M.) senatoria*; (5) *Pl. magellanicus*; (6) *A. irradians*; (7) *Pe. (N.) albicans*.

abundant amino acid (about 60 mol% of total amino acids) in all of them. But they are distinct from Mactracea resilium proteins in containing phenylalanine and methionine as predominant hydrophobic amino acids. The combined contents of the two amino acids are

	(1)	(2)	(3)	(4)	(5)	(6)	(7)
(1)	0	8	6	12	6	8	6
(2)	8	0	7	7	7	8	7
(3)	6	7	0	9	6	7	4
(4)	12	7	9	0	14	6	9
(5)	6	7	6	14	0	11	7
(6)	8	8	7	6	11	0	6
(7)	6	7	4	9	7	6	0

Fig. 5. Difference matrix for the amino acid compositions (given in D_{AB} values, see the text) of the resilium proteins of Pectinidae species

The numbers for species are the same as those in Fig. 4.

Fig. 6. Chemotaxonomic relationship among the resilium proteins of Pectinidae species

The three-dimensional scale represents 1 D_{AB} unit (mol%, see the text). The averaged error in the simulation is less than 10% of the values. The numbers for species are the same as those in Fig. 4.

almost equal to the methionine content in the Mactracea resilium proteins. The extents of oxidation of the methionine residues are lower than those in the Mactracea proteins [Table 2, (7)–(10)]. The difference matrix (Fig. 5) and the three-dimensional illustration of their chemotaxonomic relationship (Fig. 6) show that the scallop species are homologous with respect to their resilium proteins.

The genus *Pinctada* is a member of the family Pteriidae in the superfamily Pteriacea. The *Pinctada*

Fig. 7. Amino acid compositions of the hinge-ligament proteins of *Pinctada* species

(a) Lamellar-layer proteins; (b) fibrous-layer proteins. (1) *Pi. maxima*; (2) *Pi. margaritifera*; (3) *Pi. martensii*; (4) *Pi. albina*; (5) *Pi. chemnitzii*; (6) *Pi. maculata*.

(a)	(1)	(2)	(3)	(4)	(5)	(6)
(1)	0	3	10	10	8	12
(2)	3	0	10	11	10	13
(3)	10	10	0	7	8	7
(4)	10	11	7	0	6	7
(5)	8	10	8	6	0	9
(6)	12	13	7	7	9	0

(b)	(1)	(2)	(3)	(4)	(5)	(6)
(1)	0	7	35	31	35	26
(2)	7	0	29	26	29	20
(3)	35	29	0	10	6	11
(4)	31	26	10	0	6	7
(5)	35	29	6	6	0	11
(6)	26	20	11	7	11	0

Fig. 8. Difference matrix for the amino acid compositions (given in D_{AB} values, see the text) of hinge-ligament proteins of *Pinctada* species

(a) Lamellar-layer proteins; (b) fibrous-layer proteins. The numbers for species are the same as those in Fig. 7.

species [Table 1, (14)–(19)] are commonly known as pearl shells or pearl oysters, although they are not true oyster species (superfamily Ostracea). The hinge ligament of a *Pinctada* species is composed of two histologically distinct layers: a brownish 'fibrous layer' is found at the central position of the hinge-line, and a dark-greenish 'lamellar layer' at the posterior and anterior ends of the hinge-line. The terms 'fibrous' and 'lamellar' were proposed by Newell (1938) and supported by Trueman (1969) to describe the ligament layers. Fig. 7(a) shows the amino acid compositions of the lamellar-layer proteins from six *Pinctada* species. Although some small variations are observed in the contents of hydrophobic amino acids, they are quite similar to one another. The differences among these compositions are shown in Fig. 8(a). The values are small enough to support the morphological observations that classify these species into one group, genus *Pinctada*. Their close chemotaxonomic relationship is simulated into three-dimensional space and illustrated in Fig. 9(a). In contrast, two distinct types of amino acid compositions were observed among the fibrous-layer proteins. One of them is similar to those of the resilium proteins of mactra and scallop species in its high glycine content (60 mol%), but its predominant hydrophobic amino acid is isoleucine instead of methionine or phenylalanine [Fig. 7(b), (1) and (2)]. The other one [Fig. 7(b), (3)–(6)] has a lower glycine content (about 30 mol%) and remarkably higher contents of methionine (20 mol%) and aspartic acid (10 mol%) than the former type. In the latter type of fibrous-layer proteins most of the methionine is present in its sulphoxide form [Table 2, (11)–(14)]. The difference matrix for these amino acid compositions (Fig. 8b) and

the three-dimensional expression of their chemotaxonomic relationship (Fig. 9b) suggest that the *Pinctada* species are heterogeneous with respect to their fibrous-layer proteins.

DISCUSSION

The bivalves appeared 5×10^8 years ago, and there are about 10000 living species described as class Bivalvia of phylum Mollusca (Abbot & Dance, 1982). The identification of bivalve species is usually based on the morphology of shells, hinge ligaments and soft bodies. Chemical analyses of their proteins have not hitherto been introduced into the systematics of the bivalves. Chemotaxonomic studies usually employ sequence homologies in proteins or nucleic acids as measures of genetic differences among the species. Amino acid composition data were employed by Nishikawa *et al.* (1983a,b) and by Nakashima *et al.* (1986) to classify a wide variety of proteins into several groups. Some attempts have been made to detect correlations among proteins according to their amino acid compositions (Harris & Teller, 1973; Black & Harkins, 1977; Woodward, 1978; Cornish-Bowden, 1978). In the present work, the amino acid compositions of the hinge-ligament proteins were employed to examine the chemotaxonomic relationship among bivalve species. As the hinge ligaments are insoluble in any solvents so far tested, the parts used for the amino acid analysis were separated from the other parts by visual observation. The resiliums of Mactracea species (surf clams) and Pectinacea species (scallops) are easy to separate. With the

Fig. 9. Chemotaxonomic relationship among the hinge-ligament proteins of *Pinctada* species

(a) Lamellar-layer proteins; (b) fibrous-layer proteins. The three-dimensional scale represents 1 D_{AB} unit (mol%, see the text). The averaged errors in the simulations are less than 9% and 2% of the values respectively. The numbers for species are the same as those in Fig. 7.

fibrous and lamellar layers of the *Pinctada* species (pearl oysters), only the typical parts were collected. The hinge ligaments are characteristic to bivalve species, and their proteins play a common role in the opening of the shells. The comparison of the taxonomic correlations based on different criteria (morphology and composition of hinge ligament proteins in the present work) is a point of interest. It can provide novel viewpoints on the evolutionary history of the species when two taxonomic relationships are inconsistent.

As shown above, the amino acid compositions of the resilium proteins were indistinguishable among the species in superfamily Mactracea (Figs. 1, 2 and 3) or family Pectinidae (Figs. 4, 5 and 6). In these cases the two taxonomic correlations are consistent, although the hinge-ligament protein is one of the characteristics to be examined in elucidation of the genetic relationship among the bivalve species.

On the other hand, obvious heterogeneity was detected in the comparison of the hinge-ligament proteins from pearl oyster species, which are in the genus *Pinctada*. The proteins in fibrous layers of the hinge-ligaments of golden-lip pearl oyster (*Pi. maxima*) and black-lip pearl oyster (*Pi. margaritifera*) are very similar to each other and distinct from those of the other four species. However, the proteins in the lamellar layer of their hinge ligaments are similar among the six pearl oyster species, agreeing with the morphological similarities of the species. Although amino acid composition does not provide detailed information on protein structure, it was possible to distinguish the hinge-ligament proteins of the bivalve species in separate taxonomic groups, namely superfamily, family or genus. And the hinge-ligament proteins from closely related species were, in most cases in the present work, not distinguishable by comparison of their amino acid compositions. Therefore the differences observed among the pearl oyster species are significant in indicating that two types of species are included in the genus *Pinctada*. They are homologous in many elements but different in the fibrous-layer protein of their hinge ligaments. The origin of this heterogeneity is not clear at this moment. The two types of fibrous-layer proteins might come from different ancestor proteins that were introduced into the species by hybridization or some other gene-transfer mechanism, as suggested by Tamiya & Yagi (1985) in the non-divergence theory of evolution. Alternatively, the heterogeneity might be ascribed to highly specific mutations that caused extensive change of the fibrous-layer proteins and left other characters less changed. This is not the first instance that inconsistent relationships were deduced depending on the proteins subjected to the chemotaxonomic analyses. For example, three non-superimposable relationships were obtained from the amino acid sequences of snake-venom proteins, namely short neurotoxins, long neurotoxins and phospholipases; also, the relationships deduced from bacterial electron-carrier proteins did not coincide with one another (Tamiya & Yagi, 1985).

In the present work chemical taxonomy was for the first time applied on the bivalve species according to the amino acid compositions of their hinge-ligament pro-

teins. The results were sometimes supplementary and sometimes complementary to the morphological classifications, implying a complicated phylogenetic relationship among the species. As the material used in this work is physically intractable and insoluble under non-hydrolysing conditions, we must discuss the possibility that the material may be a mixture of several gene products in different relative amounts in each species. Although this factor remains to be clarified and may limit any further development of the method, the differences detected among the groups of molluscan bivalves in amino acid compositions seem to be sufficiently great to indicate that the results are valid.

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