Studies on Marine Invertebrate Collagens—I. Some Collagens from Crustaceans and Molluscs

Shigeru KIMURA, Yoshihiro NAGAOKA, and Minoru KUBOTA*

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Collagenous connective tissues are widely distributed throughout the animal kingdom, even in arthropods which have an extracellular system dominated by a chitinous exoskeleton. Recently, invertebrate collagens were investigated by many workers in view of the evolutionary development or comparative studies of collagen¹⁻³⁾. However, the low solubility of collagens from many invertebrates as reported by PIKKARAINEN *et al.*³⁾ prevented further studies on the molecular structure of invertebrate collagens. It is widely known that insoluble collagen fibers are solubilized by pepsin treatment and that the pepsin-solubilized collagen have the same comparative size, shape, and amino acid composition as tropocollagen^{4,5)}. In the previous paper,⁶⁾ the authors reported the isolation and partial characterization of an insoluble collagen obtained by the pepsin treatment of the body of abalone, *Haliotis discus*. This paper is intended to extend the comparative studies of collagens from wider range of marine invertebrates such as crustaceans and molluscs.

Experimental

Materials. The following samples were collected for the preparation of marine invertebrate collagens: the mantle of pearl oyster, *Pinctada martensii*, the body walls of squid, *Todarodes pacificus* and octopus, *Octopus vulgaris*, and the subcuticular tissues of spiny lobster, *Panulirus japonicus* and blue crab, *Portunus trituberculatus*. Fresh frozen pearl oyster was kindly provided by Mikimoto Pearl Co.

Preparation of invertebrate collagens. The fresh collagenous tissues were purified from the adherent foreign bodies and thoroughly washed twice with 0.5 M sodium acetate and with 0.5 M acetic acid at 5°C. Soluble collagens were not obtained in appreciable amounts from all the tissues. Thus, the insoluble residues were digested with pepsin (enzyme : substrate = 1 : 100) in 0.1 M acetic acid at about 18°C for 2 days with good stirring. Nearly 100% of the tissues was solubilized to give the highly viscous solution. This solution was centrifuged at 40,000 × g for 2 hours and then the supernatant was dialyzed against 0.02 M disodium hydrogen phosphate at 5°C for 3 days. As the results, the pepsin was inactivated and the pepsin-solubilized collagen was precipitated as fibrous

* Tokyo University of Fisheries, Konan-4, Minato-ku, Tokyo, Japan (木村 茂, 長岡義弘, 久保田 穰: 東京水産大学)

mass. The precipitate thus obtained was dissolved in 0.1 M acetic acid and the precipitation was performed with 0.02 M disodium hydrogen phosphate in the same way as before. This procedure of precipitation and solution was repeated twice.

Analytical methods. Hexose content of collagens was determined by the method of MOSS,⁷⁾ hexosamine by the method of BELCHER⁸⁾, and hydroxyproline by the method of ONIZUKA⁹⁾. Amino acid analyses were performed by a Hitachi Model KLA-3B automatic analyzer.

Shrinkage temperature (Ts) was measured by heating the collagen fiber suspended in water and denaturation temperature (Td) was obtained by measuring the viscosity of collagen solution at various temperatures as described previously.¹⁰⁾ Viscosity and optical rotation were measured by Ostward-Fenske viscometer (an average shear gradient of about 1000 sec⁻¹ for water at 20°C) and Lippich's polarimeter, respectively. Acrylamide gel electrophoreses were performed according to the method reported by NAGAI *et al.*¹¹⁾ The solvent used in these physical analyses was 0.1 M acetic acid.

Results and Discussion

Physical properties. Some physical properties of the invertebrate collagens are assembled in Table 1. The intrinsic viscosity of these collagens in 0.1 M acetic acid fell in the range of 11–16 dl/g and the levo specific optical rotation in the range of 375°–420°. These values are in good agreements with those of collagens from vertebrates.¹²⁾ The thermal denaturation curve of the collagens was obtained by the measurement of reduced viscosity in 0.1 M acetic acid at various temperatures. The denaturation temperature (Td) at which the change in viscosity was half complete was relatively constant around 27–34°C and the shrinkage temperature (Ts) in distilled water ranged from 49–58°C. The difference between these two values, (Ts-Td), is considerably constant at about 20–25°C. The values of Ts and Td for these collagens corresponded to those for collagens from warm-water fishes.¹³⁾

Acrylamide gel electrophoretic patterns. The denatured collagens were separated into their subunits by disc electrophoresis of 7.5% polyacrylamide gel at pH 4.2. Their typical patterns are shown in Fig. 1. The invertebrate collagens examined contained always two main components, presumably α - and β -components. In all the collagens

Table 1. Some physical properties of marine invertebrate collagens.

Property	Lobster	Blue crab	Octopus	Squid	Pearl oyster
Ts (°C)	54	52	49	49	58
Td (°C)	33.6	31.8	27.4	27.2	32.8
$[\eta]$ (dl/g)	11.0	12.0	16.0	12.5	14.0
$[\alpha]_D$	-420°	-376°	-381°	-379°	-375°

Fig. 1. Disc electrophoretic patterns of denatured collagens from marine invertebrates. Denatured collagen (40°C, 15 min.) was separated by 7.5% polyacrylamide gel at pH 4.2. A current of 4 mA per tube was applied for 60 min.

except octopus, the first component corresponding to α consisted of several thin ring-shaped bands, though α -components in vertebrate collagens migrate as a single band.¹¹⁾ This may be due to the digestion of the terminal regions (telopeptides) of the collagen molecules by pepsin.^{4,5)}

Considering from the physical properties and electrophoretic patterns, it can be said that the invertebrate collagens reported here have the same fundamental molecular properties as collagens from warm-water fishes.

Amino acid compositions. In Table 2, the amino acid compositions of the collagens, together with the composition of abalone collagen reported previously,⁶⁾ are presented in terms of residues of amino acid per 1000 total residues. The mean values of the compositions of these six invertebrate collagens are also given in Table 2. Though the general patterns of the compositions were similar to those of 28 vertebrate collagens summarized by Gross,¹⁴⁾ there are significant and interesting differences, particularly in the contents of acidic amino acids and hydroxylysine. Acidic amino acids, especially glutamic acid, are rich in the invertebrates except octopus than in vertebrates. The content of amide nitrogen for these invertebrates is two or three times as large as that for vertebrates, suggesting that considerable amounts of acidic amino acids for these invertebrates exist in amide form.

The hydroxylysine contents of these collagens are several times as large as those for vertebrates. Particularly the value of blue crab collagen (34.9 residues/1000) is much higher than that of any collagens thus far known. On the other hand, the lysine contents of these collagens are one half to one-third the values of vertebrates. The total contents of lysyl residues (lysine+hydroxylysine) in the molluscan collagens are relatively con-

Table 2. Amino acid composition of marine invertebrate collagens.
(Residues/1000 residues)

Amino acid	Mean	Crustacean		Mollusc				Vertebrate* (reference)
		Lobster	Blue crab	Octopus	Squid	Pearl oyster	Abalone ⁶⁾	
3-Hydroxyproline	2.4	1.3	1.0	5.3	3.8	0.5	2.6	
4-Hydroxyproline	82.3	73.6	77.5	89.8	85.5	83.3	84.6	90.5**
Aspartic acid	58.8	47.5	65.8	52.8	57.7	62.5	66.5	48.0
Threonine	25.0	30.9	23.2	28.4	26.2	22.2	18.7	20.6
Serine	48.6	49.9	50.4	51.5	46.9	39.4	53.7	39.1
Glutamic acid	93.3	97.4	96.8	63.5	86.4	112	104	73.5
Proline	102	121	101	101	96.0	97.0	94.4	118.1
Glycine	314	318	314	324	308	325	296	329.5
Alanine	71.9	49.0	46.5	100	88.8	65.4	80.7	108.2
Cystine (half)	3.4	0.9	3.9	7.6	3.6	2.6	2.8	0
Valine	20.0	21.2	19.4	18.6	21.1	23.1	16.8	21.4
Methionine	9.0	11.8	5.2	6.0	7.7	13.5	9.7	6.9
Isoleucine	20.0	21.2	23.2	21.8	20.9	20.0	13.2	12.7
Leucine	37.8	45.2	46.5	29.5	33.9	36.8	34.9	24.5
Tyrosine	5.2	4.7	3.9	4.5	4.5	6.3	7.3	3.6
Phenylalanine	10.1	9.5	11.6	7.8	11.5	11.5	9.3	14.3
Hydroxylysine	20.6	21.2	34.9	15.7	16.1	15.7	20.0	6.6
Lysine	13.4	16.5	15.5	11.3	15.3	10.0	11.8	28.2
Histidine	4.3	7.1	3.9	3.3	7.4	1.3	2.7	5.2
Arginine	58.8	52.2	58.2	57.6	59.0	53.9	70.4	48.4
Amide nitrogen	(79.2)	(92.7)	(65.8)	(76.0)	(77.4)	(70.8)	(91.7)	(35.4)

* Mean value of 28 vertebrate collagens from the table compiled by Gross¹⁴⁾.

** Sum of 3- and 4-Hydroxyproline.

stant of about 30 residues which are slightly lower than those in vertebrates (34.8 residues), while the contents in the crustacean collagens, especially blue crab (50.4 residues), are considerably higher.

As shown in Table 3, the ratios of hydroxylysine to lysine for these invertebrates range between 1.1–2.3 and are much higher than 0.23 for vertebrates.¹⁴⁾ It has been known that hydroxylysine in collagen arises from lysine than from free hydroxylysine.¹⁵⁾ Thus it can be concluded that the hydroxylation of lysine proceeds at higher degree in these invertebrates reported here than in vertebrates. Though this feature is common to most of all the invertebrate collagens reported previously,¹⁾ it is also observed in some vertebrate collagens from various tissues, such as vitrosin,¹⁵⁾ dentine,¹⁵⁾ and cartilage,¹⁶⁾ except skin. The ratios of hydroxyproline to proline are also listed in Table 3. The values for this ratio in the crustacean collagens are equal or somewhat lower than those in vertebrates (0.77), while the values in the molluscan collagens, as well as in coelenterate and porifera collagens reported by PIEZ *et al.*,¹⁾ are slightly higher.

Table 3. Degree of hydroxylations of lysine and proline in marine invertebrate collagens.

Ratio	Lobster	Blue crab	Octopus	Squid	Pearl oyster	Abalone ⁶⁾
Hyls/Lys	1.28	2.27	1.39	1.05	1.56	1.70
Hypro/Pro	0.62	0.78	0.99	0.94	0.86	0.93

Other features in the amino acid compositions of the invertebrate collagens which distinguish them from vertebrate collagens are the low content of alanine (particularly in the crustacean collagens) and the relatively high contents of cystine and tyrosine. In addition to 4-hydroxyproline, its isomer, 3-hydroxyproline was found in all the invertebrate collagens as well as some vertebrate collagens. The 3-hydroxyproline content of octopus (5.3 residues) is slightly higher than those of some vertebrates (1–4 residues).^{17,18)}

Carbohydrate contents. The contents of carbohydrate are given in Table 4. Most of the carbohydrate was neutral sugar determined as glucose and the content of hexosamine was very low. All the collagens examined were considerably rich in carbohydrate (2.1–7.0%), while purified collagens of vertebrates showed low values of 0.6–2.0%.¹⁹⁾ The carbohydrate that could not be separated even by the pepsin treatment is assumed to be firmly associated with the collagen molecules. GROSS *et al.*¹⁹⁾ reported that invertebrate collagens contained 5.0–15.5% of carbohydrate. Their high values may be due to impurities in their collagen preparations, because of their simple methods for the isolation of collagens.

Table 4. Carbohydrate content of marine invertebrate collagens

Carbohydrate	Lobster	Blue crab	Octopus	Squid	Pearl oyster	Abalone ⁶⁾
Hexose (%)	4.1	6.3	1.7	2.0	3.1	2.2
Hexosamine (%)	0.4	0.7	0.4	0.4	0.8	0.5

Summary

Collagens from marine invertebrates were obtained by pepsin treatment of the collagenous tissues such as the mantle of pearl oyster, the body walls of squid and octopus, and the subcuticular tissues of spiny lobster and blue crab, because of the low solubility of these collagens. The invertebrate collagens have the same fundamental molecular properties as vertebrate collagens. The thermal stability of the collagens corresponds to that of collagens from warm-water fishes and the denatured collagens contain both α - and β -components.

The invertebrate collagens are rich in hydroxylysine, acidic amino acids, amide nitrogen, and carbohydrate as compared with vertebrate collagens. The ratios of hydroxy-

lysine to lysine are always higher than those for vertebrates. 3-Hydroxyproline as well as 4-hydroxyproline was found in all the preparations.

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