Jellyfish Mesogloea Collagen

CHARACTERIZATION OF MOLECULES AS $\alpha_1\alpha_2\alpha_3$ HETEROTRIMERS*$

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The mesogloea collagen of a primitive animal, the jellyfish Stomolophus nomurai, belonging to the class Scyphozoa in the Coelenterata, was studied with respect to its chain structure. Most of the mesogloea collagen was solubilized by limited digestion with pepsin and isolated by selective precipitation at 0.9 M NaCl in 0.5 M acetic acid. Upon denaturation, the pepsin-solubilized collagen produced three distinct $\alpha$ chains, $\alpha_1$, $\alpha_2$, and $\alpha_3$, in comparable amounts which were separable by CM-cellulose chromatography. The nonidentity of these $\alpha$ chains was confirmed by amino acid and carbohydrate analyses and peptide mapping. Furthermore, the introduction of intramolecular cross-links into native molecules by formaldehyde yielded a large proportion of $\gamma_{123}$ chain with chain structure $\alpha_1\alpha_2\alpha_3$, as judged by chromatographic behavior and peptide maps. We concluded that mesogloea collagen is comprised of $\alpha_1\alpha_2\alpha_3$ heterotrimers and is chemically like vertebrate Type V collagen. On the other hand, sea anemone mesogloea collagen from the class Anthozoa (Katzman, R. L., and Kang, A. H. 1981) like vertebrate Type V collagen. On the other hand, sea anemone mesogloea collagen from the class Anthozoa (Katzman, R. L., and Kang, A. H. 1981) was extensively dialyzed against 0.02 M NaHPO$_4$ in order to inactivate the pepsin. The pepsin digests were then examined by SDS-polyacrylamide gel electrophoresis and CNBr peptide mapping.

Preparation of Jellyfish Mesogloea—A large jellyfish (S. nomurai) weighing 37.6 kg was caught in Korean waters, salted with 30% NaCl on the basis of its body weight, and then transported to the laboratory. Mesogloea tissue, which occupies the major part of the umbrella, was dissected away from the skin tissues called exumbrella and subumbrella. The mesogloea was desalted by washing with cold distilled water at 4 °C for 3 days with two changes/day and lyophilized.

Preparation of Acid-soluble Proteins from Mesogloea—The mesogloea, 31.2 g, dry weight, was cut into small pieces and extracted twice with 2 liters of 0.5 M acetic acid for 3 days. The extracts, collected by filtering and squeezing the insoluble mesogloea through cheesecloth, were extensively dialyzed against 0.02 M NaHPO$_4$. The resultant precipitates were harvested by low-speed centrifugation and dissolved in 0.5 M acetic acid. After centrifugation at 15,000 g for 1 h, solid NaCl was added to the supernatant to a final concentration of 0.9 M. The 0.9 M NaCl-precipitable fraction (acid-soluble collagen) was redissolved in 0.5 M acetic acid, dialyzed against 0.1 M acetic acid, and lyophilized. The 0.9 M NaCl-soluble fraction (acid-soluble non-collagenous protein) was precipitated by dialyzing against 0.02 M NaHPO$_4$, dissolved in 0.1 M acetic acid, and lyophilized.

Pepsin Digestion of Acid-soluble Proteins—The acid-soluble proteins, collagen and non-collagenous protein, were digested with 4 or 10% pepsin, w/w (Sigma, 2X) at 4 °C in 0.5 M acetic acid. After 24 h, the mixtures were dialyzed against 0.02 M NaHPO$_4$, in order to inactivate the pepsin. The pepsin digests were then examined by SDS-polyacrylamide gel electrophoresis and CNBr peptide mapping.

Isolation of Pepsin-solubilized Collagen from Mesogloea—The insoluble mesogloea, 600 mg, dry weight, obtained after extraction of acid-soluble proteins was suspended in 600 ml of 0.5 M acetic acid and incubated with 30 mg of pepsin at 4 °C for 24 h. Most of the mesogloea collagen was solubilized; the viscous solution was clarified by centrifugation at 15,000 g for 1 h and extensively dialyzed against 0.02 M NaHPO$_4$. The resultant precipitate was washed several times with 0.02 M NaHPO$_4$ and dissolved in 0.5 M acetic acid. The pepsin-solubilized collagen was precipitated by addition of solid NaCl to a final concentration of 1.0 M NaCl; the majority of the collagen precipitated at 0.9 M NaCl. The collagen precipitate was dissolved in 0.5 M acetic acid, dialyzed against 0.1 M acetic acid, and lyophilized. This collagen preparation could not be fractionated further by subsequent salt precipitation at neutral pH.

CM-cellulose Chromatography of Pepsin-solubilized Collagen—Chromatography of the collagen under non-denaturing conditions was performed at 10 °C as follows. The sample was dissolved at a protein concentration of 1 mg/ml in 0.06 M sodium acetate buffer, pH 4.8, at 4 °C. Approximately 6 mg of collagen was applied to a 0.9 × 11.5-cm column of CM-cellulose (Whatman CM52) and eluted with a linear gradient of 0–0.5 M NaCl over a total volume of 200 ml in the same buffer. The column was operated at a flow rate of 54 ml/h, and the effluent was monitored by an LDC Spectromonitor III at 230 nm. Pooled regions of the chromatogram were desalted on Sephadex G-25 in 0.1 M acetic acid and lyophilized.

1. The abbreviations used are: SDS, sodium dodecyl sulfate; CB, CNBr treated.

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dissolved at a concentration of 5 mg/ml in 0.02 M sodium acetate, pH 4.8, containing 6 M urea (starting buffer), warmed to 45 °C for 30 min, and applied to the column. When the sample contained more than 50 mg of collagen, the column dimensions were 1.5 x 11 cm, and when it contained up to 10 mg of collagen, a column of 0.9 x 10 cm was employed. The larger column was operated at a flow rate of 120 ml/h and the smaller one at 40 ml/h. Elution in the initial buffer was achieved with a linear gradient from 0-0.15 M NaCl over a total volume of 800 ml for the larger column and of 200 ml for the smaller one. The effluent was monitored at 230 nm.

Amino Acid Analyses—Amino acid composition was determined with a JEOL amino acid analyzer (JLC 3BC) using a single-column system; protein samples were hydrolyzed under vacuum in 6 N HCl at 110 °C for 24 h or in 4 N methanesulfonic acid containing 0.2% 3- (2-aminomethyl)indole at 115 °C for 22 h (8). Cystine and cysteine were converted to S-sulfocysteine by treating the methanesulfonic acid hydrolysates with dithiothreitol and tetrathionate. Glycosylated hydroxylysines were analyzed according to the method described previously (9).

Other Analytical Methods— Sepharose CL-4B gel filtration, SDS-polyacrylamide gel electrophoresis, CNBr peptide mapping, and formic aldehyde treatment of collagen or its subunits were performed as described (5).

RESULTS

Homogeneity of Mesogloea Collagen—The jellyfish mesogloea contained a small amount of acid-soluble protein; the yields of collagen and noncollagenous protein were only 2.2 and 1.4%, respectively, on the basis of washed dry weight. The collagen fraction was selectively precipitated at 0.9 M NaCl in 0.5 M acetic acid. When examined by SDS-gel electrophoresis, denatured collagen is seen at the top of gel (Fig. 1, gel 2), and α and β chain-sized components appear after proteolytic cleavage of native collagen with pepsin (Fig. 1, gels 3 and 4). The relative proportion of α chains was increased with increasing amounts of pepsin and the electrophoretic pattern, with α chains predominating, of 10% pepsin digests is very similar to that of pepsin-solubilized collagen from the insoluble collagen of mesogloea (Fig. 1, gels 1 and 4). Amino acid analyses suggest that the overall patterns indicative of collagen rich in hydroxylysine-linked carbohydrates are common to both soluble and pepsin-solubilized collagen fractions (Table I), while the contamination by non-}

![Fig. 1. SDS-polyacrylamide gel electrophoresis of protein fractions derived from jellyfish mesogloea. Samples were resolved on 5.5% gels in 0.1 M sodium phosphate buffer, pH 7.2, containing 0.1% SDS and 5.5 M urea at 8 mA/gel for 4 h and visualized with Coomassie Brilliant Blue.](image)

![Fig. 2. CNBr peptide maps of acid-soluble and pepsin-solubilized collagens from jellyfish mesogloea. CNBr peptides were resolved on 10% polyacrylamide gels in 0.1 M sodium phosphate buffer, pH 7.2, containing 0.1% SDS at 8 mA/gel for 5 h and visualized with Coomassie Brilliant Blue. The α1(1) chain of calf skin was cleaved with CNBr in parallel with acid-soluble mesogloea collagens, and one of the major peptides, CB-8, is identified.](image)

![TABLE 1](image)

<table>
<thead>
<tr>
<th>Amino acid and carbohydrate composition of protein fractions derived from jellyfish mesogloea</th>
<th>Acid-soluble proteins</th>
<th>Noncollagenous protein</th>
<th>Pepin-solubilized collagen</th>
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<td>Pepin-solubilized collagen</td>
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![Collagenous protein in the former fraction is apparent from the relatively low content of glycine (309 residues/1000).](image)

![Definitive evidence for identity of both collagen fractions was obtained by CNBr peptide mapping. Fig. 2 (gels 2 and 3) clearly shows no significant difference between 10% pepsin digests of soluble collagen and the pepin-solubilized protein](image)
Characterization of Jellyfish Mesogloea Collagen

of insoluble collagen. As indicated by the arrow in Fig. 2, one of the main peptides corresponds in its migration rate to \( \alpha_1(1)CB-8 \) of calf Type I collagen. These results indicate that the soluble collagen is derived from a molecular species identical to the insoluble collagen predominating in the mesogloea tissue and is present as highly cross-linked polymers susceptible to limited proteolysis with pepsin. Thus, the pepsin-solubilized collagen of mesogloea, referred to hereafter as mesogloea collagen, was utilized for examining chain structure.

The chemical nature of mesogloea collagen was estimated by calculating \( R \) values, the ratios of polar (p), hydroxy (OH), and hydrophobic (e) amino acids to the total amino acids and graphing them according to Matsumura's technique (10). The \( R \) values of pepsin-solubilized collagen were calculated to be \( R_p \), 522; \( R_{OH} \), 313; and \( R_e \), 165, indicating that mesogloea collagen has a resemblance to interstitial (Types I-III) and pericellular (Type V) collagens, but not to basement membrane (Type IV) collagens (10, 11).

Incidentally, the 0.9 M NaCl-soluble fraction is quite different in its amino acid composition from collagen (Table I); the small amount of hydroxyproline and hydroxylsine is probably due to some collagen contamination. Furthermore, this noncollagenous protein fraction is shown by SDS-gel electrophoresis to consist of one main band appearing on the gel top, together with minor diffuse bands in a lower part of the gel (Fig. 1, gel 5). The main band with an extremely high molecular weight is characterized by its strong resistance to cleavage with pepsin (Fig. 1, gel 6). Presumably, this protein was polymerized by unique cross-links because it remained essentially unchanged after reduction with dithiothreitol.

Chain Structure of Mesogloea Collagen—When mesogloea collagen is subjected to CM-cellulose chromatography under nondenaturing conditions, its elutes as a single but tailing peak (Fig. 3A). This peak was divided into two fractions as indicated by bars (Fig. 3A) and was then examined by CM-cellulose chromatography under denaturing conditions to separate the subunits. Both fractions were found to be identical. A representative chromatogram is depicted in Fig. 3B; three distinct peaks appear, containing about equal amounts of protein. Various cuts, as indicated by numbers (Fig. 3B), were analyzed by SDS-gel electrophoresis. A doublet with \( \alpha \) chain-sized components is seen in each peak (Fig. 4); the slow moving band of the doublet is always found in the shoulder preceding each peak (gels 1, 4, and 7). The relative proportion of fast and slow moving bands varied with the degree of pepsin digestion (data not shown), indicating that both \( \alpha \) chain bands in each peak were derived by pepsin cleavage of a single intact \( \alpha \) chain. Therefore we tentatively designate the \( \alpha \) chains as \( \alpha_1 \), \( \alpha_2 \), and \( \alpha_3 \) in the order of their elution positions. Furthermore, the \( \alpha_3 \) peak contains a considerable amount of \( \beta \) and \( \gamma \) chains (Fig. 4, gel 9). As will be described later, one of these chains was identified as an intermolecular cross-linked \( \alpha_3 \) dimer, \( \beta_3 \alpha_3 \), and the other as an \( \alpha_3 \) trimer, \( \gamma_3 \alpha_3 \), on the basis of CNBr peptide maps.

The \( \alpha \) chains were successfully isolated by CM-cellulose chromatography on a preparative scale followed by Sepharose CL-4B gel filtration (data not shown). On the basis of their elution positions from a calibrated Sepharose column (5), the molecular weights of \( \alpha_1 \), \( \alpha_2 \), and \( \alpha_3 \) were roughly estimated to be 97,000, 87,000, and 86,000, respectively, suggesting the presence of equimolar amounts of these \( \alpha \) chains. The amino acid compositions are shown in Table II; as a whole, they resemble each other and are characterized by the very low content of histidine and the relatively high content of hydroxylsine and its glycosides. The degree of glycosylation of hydroxylsine ranged from 63–78%, and most of the carbohydrate moiety was glucosylgalactose. The main differences between these \( \alpha \) chains are the high levels of hydroxyproline and arginine and the low levels of threonine and alanine in \( \alpha_3 \). They were further examined by SDS-gel electrophoresis of their CNBr cleavage products. As shown in Fig. 5, the nonidentity of \( \alpha_1 \), \( \alpha_2 \), and \( \alpha_3 \) chains is evident, and the main peptides of the original collagen are accounted for by those of each \( \alpha \) chain. In addition, a mixture of \( \beta \) and \( \gamma \) chains in the \( \alpha_3 \) peak, which was isolated by Sepharose CL-4B gel filtration, was found to be identical in its CNBr peptide map to the \( \alpha_3 \) chain (Fig. 5). This finding, together with their elution positions just behind the \( \alpha_3 \) chain, suggests that these \( \beta \) and \( \gamma \) chains are composed only of \( \alpha_3 \) chains.

Fig. 3. CM-cellulose chromatography of mesogloea collagen under nondenaturating and denaturing conditions. The pepsin-solubilized collagen was applied to a column of CM-cellulose and eluted with a linear gradient of NaCl over a total volume of 200 ml. Panel A (native collagen): column dimensions, 0.9 x 15.5 cm; column temperature, 10 °C; starting buffer, 0.06 M sodium acetate (pH 4.8); limiting buffer, 0.06 M sodium acetate (pH 4.8) containing 0.5 M NaCl; flow rate, 54 ml/h; sample weight, 6 mg. Panel B (denatured collagen): column dimensions, 0.9 x 10 cm; column temperature, 31 °C; starting buffer, 0.02 M sodium acetate (pH 4.8) containing 6 M urea; limiting buffer, 0.02 M sodium acetate (pH 4.8) containing 6 M urea and 0.15 M NaCl; flow rate, 40 ml/h; sample weight, 10 mg. Several fractions indicated by numbers were examined by SDS-gel electrophoresis.

Fig. 4. SDS-polyacrylamide gel electrophoresis of several fractions recovered following CM-cellulose chromatography of denatured mesogloea collagen. Electrophoretic conditions were the same as shown in Fig. 1. Orig., pepsin-solubilized collagen; 1-9, chromatographic fractions as shown in Fig. 3.
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A mixture of β and γ chains (Fig. 4, gel 9) recovered following Sepharose CL-4B gel filtration of the α3 fraction was digested in parallel with pepsin-solubilized collagen (Orig) and its α chains. Electrophoretic conditions were the same as described in the legend to Fig. 2.

**Fig. 5.** CNBr peptide maps of three distinct α chains from mesogloea collagen. A mixture of β and γ chains (Fig. 4, gel 9) recovered following Sepharose CL-4B gel filtration of the α3 fraction was digested in parallel with pepsin-solubilized collagen (Orig) and its α chains. Electrophoretic conditions were the same as described in the legend to Fig. 2.

**Fig. 6.** CM-cellulose chromatography of formaldehyde-treated mesogloea collagen under denaturing conditions. The 0.02% solution of pepsin-solubilized collagen in 0.1 M acetic acid was treated with 3.4% formaldehyde at 4 °C for 3 days. The resulting cross-linked collagen was denatured at 40 °C for 30 min and resolved on a 0.9 × 10-cm column of CM-cellulose equilibrated with 0.02 M sodium acetate buffer, pH 4.8, containing 6 M urea at 31 °C. Elution was achieved with a linear gradient of 0–0.15 M NaCl at a flow rate of 40 ml/h over a total volume of 200 ml. Arrows indicate the elution positions of α1, α2, and α3 chains from pepsin-solubilized collagen. Three fractions as indicated by numbers were subjected to SDS-gel electrophoresis.

These composite results are taken as evidence for the existence of an α1α2α3 heterotrimer in mesogloea collagen. In order to further confirm this assumption, native collagens were treated with formaldehyde to introduce intramolecular cross-links according to the procedures described previously (5). The formaldehyde-treated collagen was denatured at 40 °C for 30 min and chromatographed on CM-cellulose under denaturing conditions; it eluted as a single peak at a position behind the α2 peak (Fig. 6). The protein recovered from the peak was found by SDS-gel electrophoresis to comprise largely γ chain with some intermolecular cross-linked polymers (Fig. 7, gels C, 1–3). Furthermore, CNBr peptide mapping revealed the identity of native collagen with the cross-linked protein (Fig. 7, gels D and E), although there are some differences due to intermolecular cross-linked polymers in the upper part of the peptide maps. Thus, the γ chain was reasonably identified as γ123 with chain structure α1α2α3 on the basis of its elution position from CM-cellulose and the CNBr peptide map.

**DISCUSSION**

There are two types of chain structure, (α)3 and (α1)α2, in various interstitial collagens of invertebrates (2–6, 12–14). The present study has revealed the existence of a third type of chain structure in the mesogloea collagen of a primitive animal, the jellyfish. The bulk of our data suggest that jellyfish mesogloea collagen is composed of α1α2α3 heterotrimers. However, the finding of a small amount of β22 and γ233 chains in denatured mesogloea collagen (Fig. 4, gel 9) appears to contradict this conclusion and seems to suggest the possible presence of (α3)3 homotrimers. If the α3 chains originate from a single molecular (α2)3 species, intramolecular γ233 chains should be produced by treatment of the native molecules with formaldehyde. This was not the case, because we could not detect any such γ333 chain in denatured cross-linked collagen (Fig. 6). The most likely explanation for these findings is that the β22 and γ233 of the denatured original collagen are due to intermolecular cross-linked α3 chains derived from some dimer and trimer components of the native molecules. Upon denaturation, these native polymers may yield intermolecular cross-linked homomers such as β11, β22, γ111, and γ222 as well as β33 and γ333, but there was no evidence for the presence of any homomers containing α1 or α2. Probably, intermolecular cross-links are mainly present between α3 chains or those between α1 or α2 chains must be easily removed by pepsin digestion.

A recent preliminary electron microscope study showed that intact mesogloea collagen consists of very thin fibrils 10–30 nm in diameter with no apparent cross-striations. Occurrence of these thin fibrils must be related to their high contents of glycosylated hydroxylsines, 23–25 residues/1000 (Table II), which potentially can affect the polymerization of collagen molecules into fibrils. In fact, the diameter of collagen fibrils seems to vary reciprocally with their carbohydrate content.

S. Kimura and K. Uehara, unpublished data.
The high content of glycosylated hydroxylsines in the mesogloea collagen is reminiscent of vertebrate Types IV and V collagens; there has been some discussion concerning the unique in its high level of polar amino acids. As for the amino acid data, however, mesogloea collagen is apparently different from Type IV collagen, but similar to Type V collagen. When amino acid composition of other jellyfish collagens, there is only one hand, information on the chain structure of collagen in Protostomia and Deuterostomia (5). This is based on the observations that 

Eumetazoa of the animal kingdom may be subdivided into three groups, Coelenterata, Protostomia, and Deuterostomia. We have recently proposed that Type I or Type I-like collagen might have evolved along independent phylogenetic lines from Protostomia and Deuterostomia (5). This is based on the observations that \((\alpha_1)_{\alpha}2\) molecules homologous to the vertebrate Type I molecule are widely distributed not only in Deuterostomia but also in Protostomia (4–6). On the other hand, information on the chain structure of collagen in Coelenterata was limited to mesogloea collagen containing \((\alpha_3)\) molecules in sea anemones (class Anthozoa (2, 3)). Thus, our present finding that the mesogloea collagen of jellyfish of the class Scyphozoa comprises \(\alpha_1\alpha_2\alpha_3\) molecules is quite interesting from the viewpoint of collagen evolution. It is reasonable to postulate that the \(\alpha_1\alpha_2\alpha_3\) heterotrimer collagen arose with the divergence of Scyphozoa and Anthozoa, as a result of duplication and subsequent mutation of a single gene coding for the collagen \(\alpha\) chain of an ancestral coelenterate.

Jellyfish live a floating life in water and generally have a well-developed mesogloea with a gelled structure in which a large amount of water can be retained. This situation is in contrast with sea anemones which are sedentary and have a mesogloea with a rigid fibrous structure. Probably, the thin fibrils of collagen comprising three distinct \(\alpha\) chains are responsible for the formation of elastic gels, rich in water, in jellyfish mesogloea.

The possible presence of heterotrimers comprising three distinct \(\alpha\) chains was previously reported for some vertebrate collagens, such as the Type I collagen of codfish skin (18), the Type V collagen of human placenta (19) and the \(\alpha_2\alpha_2\alpha_3\) collagen of bovine hyaline cartilage (20). Recently, a fraction of the Type V collagen of human placentas has been established to contain \(\alpha_1\alpha_2\alpha_3\) molecules (21), while the majority of Type V molecules from vertebrate tissues has a chain structure of \((\alpha_1)_{\alpha}2\). However, our results appear to be the first example of such a heterotrimer collagen in invertebrates. Conceivably, it is distributed in other invertebrate tissues. Comparatively little information is available on the chain structure of invertebrate collagens.

**REFERENCES**