Studies on Elastoidin—III. Protein Components of Soluble Elastoidin

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Elastoidin consists of at least two components, one a typical collagen and the other a noncollagenous protein rich in tyrosine.¹⁻⁴⁾ The weight ratio of collagen and noncollagenous protein components is approximately 8 : 2⁴⁾ and these two components are assumed to be tightly bound and are inseparable from each other except by a severe treatment which may cause the cleavage of covalent linkages.¹⁾

It has been known that fresh collagenous tissues contain appreciable amounts of soluble collagen which can be extracted by neutral salt, dilute alkali, and dilute acid and that the soluble collagen is assumed to be a precursor of collagen. In our previous paper³⁾, it was shown that a small amount of soluble protein, rich in tyrosine and cystine but poor in hydroxyproline, was extracted from elastoidin fiber by cold dilute acid and that this protein seemed to be identical with an insoluble protein component of elastoidin fiber reported by some workers^{1,2)} in its amino acid composition.

While the major structural component of elastoidin fiber is the typical collagen, it is very possible that the noncollagenous protein can contribute to the stability of the fiber.³⁾ There is little knowledge about the interrelationship between these two components. The present paper describes the evidence of the presence of a collagen-noncollagenous protein complex in acid soluble protein fraction of elastoidin fiber.

Experimental

Preparation of soluble elastoidin. Elastoidin fiber was isolated from the pectoral fins of great blue shark, *Prionace glauca*, and purified as described previously.³⁾ The isolated fiber was suspended in 0.5 M acetic acid and shaken for 3 days at 5°C. The suspension was filtered through cheese-cloth and centrifuged for 2 hours at 40,000×g. The soluble protein in the supernatant was precipitated as a fiber by addition of NaCl to a final concentration of 5%. After standing in room temperature for a few hours, the fiber was separated by the centrifugation. The fiber thus obtained was redissolved in 0.5 M acetic acid under good stirring for 2 days, and then the viscous solution was centrifuged for 3 hours at 60,000×g. The soluble protein was precipitated in the same way as before. Purification was carried out by repeating the dissolving in acetic acid and the precipitation with 5% NaCl. The material extracted will be

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referred to as "soluble elastoidin" in this paper.

Treatment of soluble elastoidin with pepsin. The soluble elastoidin solution was incubated with pepsin* at 20°C for 48 hours in 0.5 M acetic acid (enzyme to protein, 1 : 100, w/w). After the incubation, the peptides split out by such treatment were collected by dialysis against distilled water on a shaker at 5°C for 48 hours. The pepsin was separated from the protein (pepsin-treated soluble elastoidin) by precipitation of the latter with 5% NaCl, and the precipitate was collected by the centrifugation.

Analytical methods. Amino acid analysis was performed by a Hitachi Model KLA-2 automatic analyzer. Hydroxyproline was assayed by the method of ONIZUKA.⁵⁾ Tyrosine derivatives were separated by paper chromatography in the solvent system of n-butanol-acetic acid-water (4 : 1 : 2) and developed with α -nitroso- β -naphthol-nitric acid reagent.

Viscosity and optical rotation were measured by Ostward-Fenske viscometer and Lippich's polarimeter, respectively. UV-absorption spectra were obtained by a Hitachi Model EPU-2 spectrophotometer and sedimentation diagrams by a Hitachi Model E ultracentrifuge at 60,000 r. p. m. and 15°C. Denaturation temperature was measured as described previously.³⁾

Results

Some general properties of soluble elastoidin. The amount of soluble elastoidin was about 0.2% of the original elastoidin fiber and the most of the elastoidin fiber remained as a sticky fibrous mass. The intrinsic viscosity and the specific rotation were 4.0 dl/g and -216 in 0.5 M acetic acid, respectively, and both values are about half of those values of so-called collagen. As shown in Fig. 1, the sedimentation diagram of soluble elastoidin showed a sharp peak corresponding to a soluble collagen having a sedimentation constant of about 2.9S and the existence of a large amount of aggregated materials which were centrifuged down at early stage of ultracentrifugation. The soluble collagen content estimated by the sedimentation diagram is about 20% of the soluble elastoidin. The UV-absorption spectrum is shown in Fig. 2 which has a maximum absorption at 277 m μ and a minimum at 250 m μ in acid medium, suggesting the high content of tyrosine in the soluble elastoidin.

The amino acid composition is shown in Table 1. The soluble elastoidin was rich in tyrosine, cystine and histidine but poor in hydroxyproline, hydroxylysine and glycine in contrast to those of the elastoidin fiber. The evident characteristics were the contents of high tyrosine (15.3%) and low hydroxyproline (4.0%). In addition, the paper chromatography of acid hydrolysate of the soluble elastoidin revealed the existence of tyrosine derivatives reported in the previous paper.⁴⁾

* The pepsin used in this experiment was the two times recrystallized preparation purchased from Sigma Co.

Fig. 1. Sedimentation diagram of soluble elastoidin. The photograph was taken 192 minutes after the speed of 60,000 r.p.m. was reached. The solvent was 0.5 M acetic acid, at a concentration of 0.31%; temperature, 13°C; phase plate angle, 65°.

Table 1. Amino acid composition of soluble elastoidin.

Amino acid	Residues/1,000 residues
Hydroxyproline	35.0
Aspartic acid	77.5
Threonine	29.6
Serine	53.0
Glutamic acid	75.7
Proline	91.0
Glycine	214
Alanine	89.6
CyS (half)	8.2
Valine	39.2
Methionine	11.3
Isoleucine	20.8
Leucine	30.8
Tyrosine	85.8
Phenylalanine	13.0
Tryptophan	trace
Hydroxylysine	4.9
Lysine	28.1
Histidine	34.0
Arginine	58.3
Tyrosine derivatives	present
Amide ($-\text{NH}_2$)	(59.6)

Fig. 2. Ultraviolet absorption spectra of soluble elastoidin (SE) and pepsin-treated soluble elastoidin (PTSE). The solvent was 0.5 M acetic acid.

—: SE (0.043%)

-----: PTSE (0.056%)

Pepsin treatment of soluble elastoidin.

In order to separate the collagen and the noncollagenous protein components, the soluble elastoidin was treated by pepsin as described above. During the pepsin treatment, no significant changes in relative viscosity could be observed. After the incubation with pepsin, the reaction mixture was extensively dialyzed against distilled water. The dialysate was quantitatively collected and concentrated by a vacuum evaporator. The dialysable peptides corresponding in weight to approximately 55% of the soluble elastoidin were obtained. These peptides due to the noncollagenous protein contained a remarkable amount of tyrosine (27.0%) and a little hydroxyproline (0.95%).

After the pepsin treatment, the soluble elastoidin showed altered properties. Spectral change observed as the result of pepsin treatment (Fig. 2) showed that the characteristic absorption maximum at 277

$m\mu$ was greatly reduced. This change reflects the low content of tyrosine (0.91%) in the pepsin-treated soluble elastoidin. The hydroxyproline content was found to be 9.51% which agreed with the value of collagen fraction obtained by pepsin treatment of the native elastoidin fiber.³⁾ Some physicochemical properties of the pepsin-treated soluble elastoidin are assembled in Table 2 in contrast to those of the soluble elastoidin. The values of intrinsic viscosity, specific rotation and sedimentation constant are similar to the corresponding values obtained for soluble collagens,⁶⁾ and therefore the pepsin-treated soluble elastoidin is assumed to be identical with so-called collagen. These results suggest that the contents of collagen and noncollagenous protein components in the soluble elastoidin are 45% and 55%, respectively.

Effect of temperature on fiber formation of soluble elastoidin and thermal stability of its reconstituted fiber.

Below the denaturation temperature, the soluble elastoidin in 0.5 M acetic acid was completely precipitated as a fiber by addition of 5% NaCl. When the soluble elastoidin solution was kept at 65°C for 5 min., this solution lost its ability to form the fiber. However, the heat-treated solution increased in turbidity by addition of 5% NaCl and a part of the soluble elastoidin was precipitated as a fine particle. A control experiment was carried out under the same condition with acid soluble collagen from the skin of great blue shark. While the soluble collagen could be precipitated as a fiber by addition of 5% NaCl below the denaturation temperature (T_d : 27°C), the fiber formation could not be observed by addition of NaCl when heating the collagen solution at 65°C for 5 min.

When the soluble elastoidin fiber was heated in 0.5 M acetic acid containing 5% NaCl at different temperatures such as 65, 85, and 100°C for 5 min., it shrunk thermally and partially dissolved. On the other hand, the soluble collagen fiber was completely dissolved (gelatinized) under the same conditions. After the heat treatment of the soluble elastoidin fiber, the insoluble fiber was analyzed in the contents of tyrosine and hydroxyproline. As shown in Table 3, a small decrease of hydroxy-

Table 2. Some physicochemical properties of soluble elastoidin (SE) and pepsin-treated soluble elastoidin (PTSE).

Property	SE	PTSE
$[\alpha]_D$	-216	-391
$[\eta]$	4.0 dl/g	13.6 dl/g
$S_{20,w}^*$	2.9 S*	2.8 S
T_D	—	26.8°C

* This value represents the sedimentation constant of soluble collagen moiety of the soluble elastoidin.

Table 3. Changes in tyrosine and hydroxyproline contents of reconstituted fiber from soluble elastoidin after heat treatment

	Tyrosine	Hydroxyproline
	g/100 g protein	
Unheated	15.3	4.0
Heated at 65°C	16.8	3.4
Heated at 85°C	17.2	3.2
Heated at 100°C	18.5	3.0

Samples were heated 5 min. at each temperature in 0.5 M acetic acid containing 5% NaCl.

proline content and a considerable increase of tyrosine content as compared with those of the untreated fiber were observed. From the hydroxyproline content (3.0–3.4%), the ratio of collagen and noncollagenous protein components in the insoluble fiber is assumed to be about 1 : 2. These results indicate that about 20% of the soluble elastoidin is a soluble collagen which is easily gelatinized by the heat treatment and that about 80% is a collagen-noncollagenous protein complex having a higher thermostability than the soluble collagen.

Discussion

From these investigations, it can be concluded that the soluble elastoidin consists of a typical collagen (45%) and a noncollagenous protein (55%). There are two types of collagen in the soluble elastoidin, one a soluble collagen and the other a collagen complex which combined with a noncollagenous protein, and about half of the total collagen is present as the soluble collagen. The soluble collagen moiety which accounted for about 20% of the soluble elastoidin is found as a sharp peak in the sedimentation diagram, and its reconstituted fiber is easily gelatinized by the heat treatment (65°C, 5 min.). On the other hand, the collagen complex moiety is about 80% of the soluble elastoidin and the weight ratio of collagen and noncollagenous protein is about 1 : 2. The collagen complex is not found as a single peak in the sedimentation diagram, but a gel-like or aggregated material which is centrifuged down at early stage of ultracentrifugation, suggesting that the complex is a cross-linked protein with considerably high molecular weight.

The reconstituted fiber of the collagen complex is not gelatinized so easily as the soluble collagen fiber, but digested by pepsin and the collagen moiety of the complex is dispersed into each molecule having the size and shape comparable to those of the soluble collagen. The thermostability of the fiber is probably attributed to disulfide cross-links due to cystine and unusual cross-links due to tyrosine derivatives. The partial characterization of tyrosine derivatives and the discussion of their function in elastoidin will be reported elsewhere. As far as we are aware, this is the first demonstration of the natural occurrence of a collagen-noncollagenous protein complex in a soluble state.

These results suggest that the cross-links in the collagen complex would be responsible for the formation of highly thermostabilized fiber. The thermostability shown by the collagen complex fiber is similar to that of elastoidin fiber, suggesting that the function of the noncollagenous protein in elastoidin fiber must be to link the collagen molecules together in a stable collagenous fiber with high thermostability.

Summary

1. An acid soluble protein of elastoidin fiber is composed of two components a soluble collagen (20%) and a collagen-noncollagenous protein complex (80%).

2. The collagen complex is assumed to be a cross-linked protein with considerably high molecular weight, because of its high thermostability and the weight ratio of collagen and noncollagenous protein is about 1 : 2.

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