Studies on Elastoidin—II. Dialysable Peptides Released by Pepsin Digestion of Elastoidin

Shigeru KIMURA and Minoru KUBOTA*

(Received Nov. 14, 1966)

Elastoidin, a collagen complex found in the shark fins, consists of only two main components a collagen and a non-collagenous protein rich in tyrosine, and is highly insoluble¹⁻³⁾.

In the previous paper³⁾, it was shown that the elastoidin was solubilized by pepsin and the collagen molecule which was insoluble as bound in elastoidin were dispersed into each molecule having the comparative size and shape with other soluble collagens, and that the non-collagenous protein component and the probable terminal regions of the collagen molecule were digested into small peptides permeable to dialyzing membrane. Furthermore, the high shrinkage temperature, the reversibility of thermal shrinkage and the insolubility of elastoidin indicate the possibility of the presence in elastoidin of cross linkages that do not exist in other collagens. Amino acid analysis showed that elastoidin has an unique composition with a high content of tyrosine and with a significant amount of cystine, but failed to give evidence for the presence of any unusual components which could function as cross linkages between the peptide chains.

In the present paper, the amino acid composition of the dialysable peptides released by pepsin digestion of elastoidin and the presence of unknown amino acids will be described, and the presence of some cross linkages in pepsin solubilized elastoidin (PSE) are discussed.

Experimental

Elastoidin fibers of the pectoral fins of the great blue shark, *Prionace glauca*, were prepared as described previously³⁾.

The procedure of pepsin treatment of the elastoidin is shown in Fig. 1. One gram of the elastoidin, after extraction with acetic acid, was placed in 300 ml of 0.01 N HCl containing 10 mg of pepsin and incubated for 3 days at 17°C with good stirring. The solubilized solution was dialysed against 500 ml of distilled water at 5°C for 3 days, the distilled water being changed once a day. The dialysate was concentrated to 100 ml on a vacuum evaporator at 30°C and stored at -20°C. The dialysant was then dialysed against 0.02 M Na₂HPO₄ to precipitate the collagenous component (PSE)

* Tokyo University of Fisheries, Konan, Minato-ku, Tokyo, Japan (木村茂, 久保田穰, 東京水産大学)

and to denature and remove the pepsin. The collagenous component precipitated was dissolved in 0.01 M acetic acid. This procedure of precipitation and solution was repeated twice. About 300 mg of purified PSE was dissolved in 90 ml of 0.01 M HCl containing 3 mg of pepsin, incubated and precipitated as mentioned above. The precipitates (PSE and pepsin-treated PSE) were washed with distilled water and lyophilized.

Fig. 1. Flow sheet for the solubilization of elastoidin by pepsin

The total hexose content was determined by the method of MOSS⁴⁾, hexosamine content by the method of BOAS⁵⁾; hydroxyproline content by the method of ONIZUKA⁶⁾. Amino acid analysis was performed by an automatic instrument (Hitachi Co. Ltd., Type KLA-2) using columns of Amberlite CG-120 as described previously³⁾.

Tyrosine and its derivatives are separated by paper chromatography in the solvent systems *n*-butanol-acetic acid-water (4 : 1 : 2 and 4 : 1 : 5) and *n*-butanol-2N ammonia (1 : 1). Samples are hydrolyzed with 6N HCl for 24 hours, and the residues, after evaporation, was dissolved in distilled water and subjected to paper chromatography on Toyo-roshi No. 51A filter paper. After the run, the paper was dried and developed with α -nitroso- β -naphthol-nitric acid reagent of ACHER's which is specific for tyrosine and some of its derivatives¹¹. Synthetic 3-chlorotyrosine was prepared by the method of ZEYNEK⁸⁾.

Sedimentation velocity pattern was obtained by Hitachi ultracentrifuge Model UCA-1 at 59870 r.p.m. and at 18°C. The solvent used in the ultracentrifugal analysis was 0.15 M citrate buffer (pH 3.6) in the native state and 0.15 M citrate buffer containing 1.2 M KCNS (pH 3.6) in the denatured state. The sample (in the denatured state of pepsin-treated PSE) was prepared as follows. The pepsin-treated PSE was dissolved in phosphate buffer containing 2M KCNS (pH 7.8) at 40°C for 10 minutes in order to inactivate the pepsin completely, and the solution was dialysed against 0.15 M citrate buffer containing 1.2 M KCNS at 5°C.

Results and Discussion

The chemical and physicochemical properties of native elastoidin, acid soluble protein (ASP) and pepsin solubilized elastoidin (PSE) were studied in the previous paper. The ASP was obtained only one to two per cent of the total amount of the elastoidin fiber and its amino acid composition which is greatly differed from that of elastoidin or collagen seems to be identical with the non-collagenous protein reported by GROSS and DUMSHA²⁾. The major part of the non-collagenous protein accounting for 20-25% of the elastoidin fiber could not be extracted with dilute acetic acid, because it is tightly bound to the collagen moiety in elastoidin. The PSE resembles typical collagen in its amino acid composition and molecular structure, indicating that the collagen moiety of elastoidin is readily released by pepsin treatment in the native state. The studies on chemical natures of the dialysable peptides released by pepsin digestion of elastoidin, therefore, will make some properties of the non-collagenous protein clear.

Quantitative fractionation of the dialysable peptides. The elastoidin fiber were completely solubilized by pepsin to give a strikingly viscous solution. The solubilized solution after centrifugation was dialyzed against distilled water, and the dialysate was collected quantitatively and concentrated on a vacuum evaporator. The dialysable peptides were obtained about 23% of the elastoidin by weight. After separation of the dialysable peptides, the dialysant then dialyzed against 0.02 M Na₂HPO₄ to precipitate the collagenous component (PSE). No nitrogen could be detected in the supernatant after precipitating the PSE. The electron micrograph of the PSE revealed the typical 650Å period similar to that of typical collagen as reported in the previous

paper³⁾. These results indicate that the collagen moiety of elastoidin was completely precipitated as native type collagen fiber, and non-dialysable materials do not exist in the supernatant.

Carbohydrate content and amino acid composition of the dialysable peptides fraction. The contents of carbohydrate and certain amino acids of native elastoidin and each fraction are shown in Table 1. Hexose and hexosamine contents of native elastoidin and PSE were of the same level as that for other collagens. The hexose components, glucose and galactose were identified by the paper chromatography in two solvent systems, namely, *n*-butanol-acetic acid-water (4 : 1 : 5); *n*-butanol-ethanol-water (4 : 1 : 1). About 14% of the hexose and 32% of the hexosamine contained in the whole fiber appeared in the dialysable peptides released by pepsin digestion of elastoidin, and the rest of the carbohydrate remained with the PSE fraction. This seems to indicate that most of the carbohydrates are contained in the PSE and are not present as impurities.

Table 1. Analysis of whole fiber (Elastoidin) and the PSE* and dialysable peptides fractions g/100 g dry weight

	Percent of whole fiber	Hexose	Hexosamine	Cystine	Tyrosine	Hydroxyproline
Elastoidin	100	0.39	0.40	0.18	7.52	7.93
PSE	77	0.56 (86)	0.35 (68)	0.00 (0.0)	0.94 (10)	9.76 (97)
Dialysable peptides	23	0.32 (14)	0.54 (32)	0.45 (100)	29.1 (90)	1.04 (3)

Values in parentheses are given in % of carbohydrate or amino acid in each fraction to that of in the whole fiber.

* PSE: Pepsin solubilized elastoidin

Amino acid composition of the dialysable peptides is given in Table 2. The composition exhibits the main features considered as characteristic of acid soluble protein (ASP) reported in the previous paper³⁾, but a little difference from the ASP is the higher tyrosine content of the dialysable peptides (29%) as compared to that of the ASP (19%). The dialysable peptides were rich in tyrosine, cystine, histidine, serine and acidic amino acids, but poor in hydroxyproline, hydroxylysine and glycine in contrast to that of the collagen moiety of elastoidin (PSE). These features appears to suggest that the dialysable peptides correspond mainly to the non-collagenous protein in elastoidin. Hydroxyproline content of the dialysable peptides was about 1% by weight and corresponds to 3% of the total hydroxyproline content of elastoidin. NISHIHARA and MIYATA⁹⁾ and HORMANN and HAFTER¹⁰⁾ found small amounts of hydroxyproline in hydrolysate of the dialysable peptides released by pepsin digestion of calf skin collagen, but RUBIN et al¹¹⁾ could not find any amount of hydroxyproline. It is therefore not possible at present to decide whether the hydroxyproline observed

Table 2. Amino acid composition of dialysable peptides, acid soluble protein and pepsin solubilized elastoidin

Amino acid	Dialysable peptides	ASP*	PSE**
	residues amino acid/1000 residues		
Hydroxyproline	9.8	24.2	76.0
Aspartic acid	75.4	78.0	41.7
Threonine	14.3	27.1	20.1
Serine	65.0	60.0	30.7
Glutamic acid	92.3	76.7	65.3
Proline	80.3	74.6	109.0
Glycine	144.0	172.0	329.0
Alanine	83.8	76.5	115.0
Cystine/2	10.0	8.0	0.0
Valine	47.6	42.5	17.0
Methionine	6.5	8.4	7.2
Isoleucine	17.6	19.8	21.1
Leucine	16.2	23.6	17.8
Tyrosine	203.0	118.3	5.0
Phenylalanine	17.0	10.5	10.9
Tryptophan	0.0	0.3	0.0
Hydroxylysine	1.1	2.1	7.7
Lysine	26.0	31.1	24.4
Histidine	30.4	31.8	4.8
Arginine	59.4	53.2	45.6
-NH ₂	90.0	57.5	46.0

* Acid soluble protein³⁾** Pepsin solubilized elastoidin³⁾

in the dialysable peptides is one of the constitutive amino acid in the non-collagenous protein or whether it is due to that in the terminal region of collagen molecule from elastoidin. However, by comparison with the above facts, it can be safely concluded that the non-collagenous protein in elastoidin contains very little or no hydroxyproline.

In addition to common amino acids, a ninhydrin positive peak appeared in a position between the peaks of lysine and histidine in the elution pattern of automatic amino acid analysis. This component could not be identified in paper chromatography, as the amount of material was too little.

Presence of tyrosine derivatives in the dialysable peptides. Paper chromatography of hydrochloric acid hydrolysate of the dialysable peptides is shown in Fig. 2. In addition to tyrosine, four unidentifiable spots reacting with ACHER's reagent were present. As α -nitroso- β -naphthol-nitric acid reagent of ACHER's is specific for tyrosine and some of its derivatives, these compounds are assumed to be tyrosine derivatives. These compounds were also present in the acid hydrolysate of native elastoidin, but the PSE and skin collagen of the same individual each gives only one spot due to

tyrosine. Thus it can be concluded that the presence of these compounds are due to the non-collagenous protein component of elastoidin. Furthermore, these compounds were found in alkaline hydrolysate (1.5 N KOH, 110°C, 20 hours) of the dialysable peptides, and thus they are presumably built into the peptide chains in the same manner as the other amino acids.

Fig. 2. Paper chromatogram of tyrosine derivatives in hydrochloric acid hydrolysate of native elastoidin, PSE and dialysable peptides. The solvent system is *n*-butanol-acetic acid-water (4 : 1 : 5).

- A: Tyrosine
- B: Native elastoidin
- C: Pepsin solubilized elastoidin (PSE)
- D: Dialysable peptides
- E: Synthetic 3-chlorotyrosine

Recently JOSEPH et al.¹²⁾ reported the presence of 3-chlorotyrosine and other three tyrosine-positive materials in addition to tyrosine in the hydrolysate of elastoidin. R_{tyrosine} values of the tyrosine-positive materials reported by JOSEPH et al.¹²⁾ are different from those of the tyrosine derivatives found in the present paper. It has been known that 3-chlorotyrosine is considered to arise from di-*p*-(2-amino-2-carboxylethyl)-phenyl peroxide residues formed by oxidation of two tyrosine residues^{13,14)}. Thus they suggested the possibility of peroxide linkage involving tyrosine residues in elastoidin. The dialysable peptides are markedly rich in tyrosine and might therefore be expected to contain a large amount of 3-chlorotyrosine. In the present paper, 3-chlorotyrosine could not be detected in the dialysable peptides and the PSE, but a very faint spot (not conclusive) corresponding to 3-chlorotyrosine was obtained in the native elastoidin. The 3-chlorotyrosine found are probably due peroxide linkage that might have been formed during subsequent oxidation of the elastoidin on standing in air, or due to decomposed product formed during preparation of the hydrolysate of elastoidin, and are therefore negligible. Thus it is reasonable to conclude that the peroxide linkage formed by two tyrosine residues do not exist in elastoidin.

On the other hand, ANDERSON¹⁵⁾ reported that two new amino acids which are built into the peptide structure, in such a way as linking the chains in a three-dimensional

network, have been isolated from the hydrolysate of resilin in elastic ligament of insects. These compounds give a brownish-red color with ACHER's reagent and show a bright blue fluorescence in ultra-violet light, and one of them (designated compound II) had an R_f value of 0.18 similar to that for S-2 fraction in the present paper. In view of the fact that very few types of cross linkage are probably present in elastoidin, it is interesting to elucidate the nature of the four compounds reacting with ACHER's reagent in the dialysable peptides. Ion-exchange chromatographic separation of these compounds are now in progress.

Cross linkages in the PSE. The sedimentation diagram of the denatured PSE which has been reported in the previous paper³, showed both α - and β -components*, and their relatively proportion estimated by the areas in the diagram is about 60% and 40%, respectively. As shown by Fig. 3 where only α -components are appreciable with little β -components, the amount of β -components found in the denatured PSE is decreased by incubating the native PSE with pepsin. Sedimentation diagram of the pepsin-treated PSE in the native state showed a hypersharp peak similar to that of the PSE reported in the previous paper³. In addition, small amounts of dialysable peptides were obtained by pepsin treatment of the native PSE suggesting that the cross linkages which are present within the PSE are situated in the regions of this peptides. This peptides are probably due to a portion of the terminal regions which remained in the PSE because of the incomplete digestion of elastoidin by pepsin. It appears therefore that either the cross linkages involved in the PSE include a peptide chain or the chain close to the cross linkages contains pepsin-sensitive bonds. Furthermore it is assumed that some amounts of of the cross linkages in the collagen molecule of native elastoidin were removed by pepsin treatment during the preparation of the PSE. These results are in good agreement with the facts reported by some workers^{9,11,16} that the action of pepsin on native collagen is confined to the terminal regions of the collagen molecule and converts some of the β -components into two α -components each, thus showing that intramolecular cross linkages in

Fig. 3. Sedimentation velocity pattern of pepsin-treated PSE in the denatured state. The photograph was taken 128 minutes after the speed of 59870 r.p.m. was reached. The solvent was 0.15 M citrate buffer plus 1.2 M KCNS (pH 3.6), at a concentration of 0.8%, temperature 17°C; phase plate angle 65°.

$S_{20,w}$: 3.4

* Collagen molecule in the denatured state is dissociated into three randomly coiled polypeptide chains having a nearly equal molecular weight of about 100,000, and each chain is designated α -component. β -component is a covalently linked dimer of α -components.

β -components occur in the terminal region of two α -components.

Summary

1) About 23% of the elastoidin is digested by pepsin to small peptides permeable to dialyzing membrane. The dialysable peptides, consisting of the non-collagenous protein (about 20% of the elastoidin by weight) and the probable terminal region of the collagen molecule in elastoidin, have a characteristic amino acid composition, the remarkable amounts of tyrosine (29.1%) being one of the most unique characteristics of the non-collagenous protein.

2) Small amounts of hexose and hexosamine were present in the dialysable peptides, but most of these compounds found in the whole elastoidin fiber remained with the collagen fraction (PSE).

3) Although 3-chlorotyrosine could not be detected, four tyrosine derivatives in addition to tyrosine are found in hydrolysate of the dialysable peptides by paper chromatography. These results show that the peroxide linkages formed by oxidation of two tyrosine residues are not involved in the elastoidin, and the tyrosine derivatives derived from the non-collagenous protein are probably related to unusual cross linkages in elastoidin.

4) After successive treatment of the native elastoidin with pepsin, β -components appearing in the denatured state of the PSE were converted into α -components. This seems to indicate that the cross linkages in the PSE were situated in the terminal regions of the PSE.

The authors wish to thank Dr. M. SUYAMA, Ass. Prof. of Tokyo University of Fisheries for amino acid analysis, Mr. K. KANNA, Research Member of Tokai Regional Fisheries Research Laboratory for the ultracentrifugal analysis, and Mr. S. FUTAMI for his technical assistance.

References

- 1) L. V. S. SASTRY and L. K. RAMACHANDRAN: *Biochim. Biophys. Acta*, **97**, 281~287 (1965).
- 2) J. GROSS and B. DUMSHA: *Biochim. Biophys. Acta*, **28**, 268~270 (1958).
- 3) S. KIMURA and M. KUBOTA: *J. Biochem.*, **60**, 613~621 (1966).
- 4) J. A. MOSS: *Biochem. J.*, **61**, 151~156 (1955).
- 5) N. F. BOAS: *J. Biol. Chem.*, **204**, 553~559 (1953).
- 6) T. ONIZUKA: *J. Japan. Biochem. Soc.*, **32**, 13~19 (1960).
- 7) R. ACHER and C. CROCKER: *Biochim. Biophys. Acta*, **9**, 704~707 (1952).
- 8) R. ZEYNEK: *Z. Physiol. Chem.*, **144**, 246~254 (1925).
- 9) T. NISHIHARA and T. MIYATA: *Collagen Symposium (Japan)*, **3**, 84~93, (1962).
- 10) H. HORMANN and R. HAFTER: *Leder*, **15**, 293~299 (1964).
- 11) A. L. RUBIN, M. P. DRAKE, P. F. DAVISON, P. T. SPEAKMAN, and F. O. SCHMITT: *Biochemistry*, **4**, 181~190 (1965).
- 12) K. T. JOSEPH, V. M. RADHAKRISHNAN, and N. RAMANATHAN: *Leather. Sci.*, **10**, 413~414 (1963).
- 13) C. DEASY: *J. Am. Leather Chemists. Assoc.*, **57**, 576~580 (1962).
- 14) J. W. DAVIES and G. HARRIS: *J. Chem. Soc.*, 3193~3197 (1961).
- 15) S. O. ANDERSON: *Biochim. Biophys. Acta*, **69**, 249~262 (1963).
- 16) F. S. STEVEN: *Biochim. Biophys. Acta*, **97**, 465~471 (1965).