The Characterization of Collagen from the Skin of the Dogfish Shark, Squalus acanthias

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The dogfish shark, Squalus acanthias, is one of a number of cold water animals that have collagen characterized by a very low denaturation temperature (1). Because of this property and because this species belongs to one of the lowest classes of the vertebrates, Elasmobranchii, it was chosen for comparative study. Initial experiments which have been reported (2) indicate that the skin collagen is similar to collagens from higher vertebrates. The present report extends these studies. The presence of large amounts of γ component (3-5) in denatured samples has also allowed a study of this component in relation to native collagen and its α and β components.1

EXPERIMENTAL PROCEDURE

Preparation of Collagen—Members of the species S. acanthias, commonly called the spiny dogfish shark, were obtained from the Marine Biological Laboratory, Woods Hole, Massachusetts, and from the Mount Desert Island Biological Laboratory, Mount Desert Island, Maine. Two types of samples were prepared, one from the skin of immature animals and the other from the skin of a full grown female. The skin was dissected free of muscle, cut into small pieces, and suspended in about 10 volumes of 0.5 M acetic acid at 0-5°C. All operations were conducted below 5°C because of the low denaturation temperature of the collagen. After soaking several days with stirring, the swollen pieces of skin were disintegrated with a glass homogenizer. After several more days of stirring, the suspension was filtered through cheesecloth and centrifuged at 15,000 × g for 1 hour. Protein was precipitated by the addition of sodium chloride to give a concentration of about 0.5%. The solution was dialyzed against a large volume of 0.02 M sodium formate, pH 3.8, I'/2 = 0.15. The sample was dissolved in the solvent by stirring overnight to give a concentration of about 0.5 M acetic acid, dialyzed exhaustively against a large volume of 0.02 M disodium phosphate. It was then dissolved in 0.5 M acetic acid, dialyzed exhaustively against the same solvent, centrifuged, and lyophilized. Before lyophilization, the collagen solution from the young dogfish was slightly cloudy, as is characteristic of most collagen preparations. The collagen solution from the adult female was very cloudy and could not be readily clarified by centrifugation. To accomplish this, the lyophilized preparation was extracted twice at 5° with large volumes of 1 M sodium chloride, pH 7, by stirring overnight. That portion of the collagen which dissolved was separated by centrifugation, dialyzed exhaustively against 0.5 M acetic acid, and lyophilized. The samples were stored at 5° over calcium chloride. For the experiments described below, samples were dissolved by stirring overnight at 5°. If native collagen was desired, it was clarified by centrifugation at 100,000 × g for several hours. Denatured samples were prepared by warming the solution to room temperature and then filtering through a fine porosity sintered glass filter.

Concentrations were determined by micro Kjeldahl measurements on triplicate samples, assuming a nitrogen content of 18.6% for collagen.

Viscosity—Viscosity measurements were made in coiled capillary viscometers with flow times for water of about 60 seconds at 7°. The temperature was controlled to ±0.01°C. The solvent was 0.15 M citrate (sodium salt), pH 3.5.

Polarimetry—Measurements of optical rotation were made in a Rudolph model 80 photoelectric spectropolarimeter. A 10-cm jacketed cell was employed. Either a xenon arc lamp or a mercury lamp was used as the light source. The value of λ, obtained by fitting the data to a one-term Drude equation. The solvent was 0.15 M citrate, pH 3.5.

Sedimentation Velocity—Samples of collagen and fractions isolated by chromatography were examined by sedimentation velocity at 59,780 r.p.m. and at 5° and 25°, respectively, in the ultracentrifuge. Schlieren optics was employed. The solvent was sodium formate, pH 3.8, Γ/2 = 0.15.

Sedimentation Equilibrium—The procedure of Richards and Schaeiman (9), as previously adapted for α and β components (10), was employed to determine the molecular weight of native collagen. The column length was 1.5 mm. The equilibrium speed was either 4,069 or 5,563 r.p.m. at 7°. The solvent was sodium formate, pH 3.8, Γ/2 = 0.15. The sample was dissolved in the solvent by stirring overnight at 5° to give a concentration of about 0.5%. The solution was dialyzed against the solvent and then centrifuged at 100,000 × g for 16 hours; only the solution in the upper third of the tube was taken for study. The partial specific volume, measured at 20° in a 7-ml pycnometer on solutions with about 0.5% protein, was 0.705 ± 0.010 ml per g.

Denaturation Curves—These were obtained by measuring viscosity and optical rotation of a 0.1% solution of the collagen...
in 0.15 M citrate, pH 3.5. The temperature was raised stepwise and maintained at a constant level until no further change in viscosity or rotation could be seen. This required as long as 5 days at temperatures near the midpoint of the melting curve.

**Chromatography**—Denatured samples were chromatographed on carboxymethyl cellulose (Bio-Rad Laboratories) by a modification of the procedure described (2). The column, 2.5 × 20 cm, was kept at room temperature and eluted at a rate of 100 ml per hour. Gradient elution was employed, beginning with sodium acetate buffer, pH 4.8, \( r / 2 = 0.08 \), and ending with the same buffer containing sodium chloride, \( r / 2 = 0.12 \). A linear gradient was established by the use of an equal level, two-chamber device, each chamber containing 400 ml. The effluent was monitored continuously at 230 nm, and the absorbance was recorded on a strip chart recorder with a linear presentation. Protein was isolated from selected fractions and rechromatographed when desired as previously described (2).

**Acrylamide Gel Electrophoresis**—This procedure, as adapted to denatured collagen (11), separates \( \alpha \), \( \beta \), and \( \gamma \) components on a microscale and allows an approximate measure of the relative amounts of each component.

**Amino Acid Analysis**—Samples were analyzed on an automatic instrument as previously described (12).

**RESULTS**

The physical chemical properties of skin collagen from the dogfish shark are assembled in Table I. These results all were obtained with the sample of collagen from young dogfish sharks. The intrinsic viscosity, specific rotation, rotatory dispersion parameter (\( \lambda_o \)), partial specific volume, and sedimentation coefficients of the native and denatured protein are similar to values obtained for other collagens. Harrington and von Hippel (13) have summarized typical data.

The weight average molecular weight, obtained by sedimentation equilibrium on several samples (Fig. 1), was 348,000. This is undoubtedly a maximal value, since in every case the plot of \( \ln c \) versus \( x^2 \) showed concavity upwards, indicating heterogeneity. The recent investigation of Rice et al. (14) indicates a value near 350,000 for calf skin collagen. Studies on the \( \alpha \) and \( \beta \) components of denatured rat skin collagen yielded a value of 294,000 for the native molecule, assuming three \( \gamma \) chains with a molecular weight of 98,000 each (10). A value of about 300,000 is consistent with our data on the assumption of the presence of 10 to 15% aggregates. Further investigations are in progress to evaluate the degree of heterogeneity of this type of sample.

The distinctive property of skin collagen from the dogfish shark is its low denaturation temperature. Melting curves obtained by measurement of changes in viscosity and optical rotation are shown in Fig. 2. The temperatures at which the change was half complete (\( T_w \)) was 14.3° when measured by viscosity, and 16.0° when measured by optical rotation. The difference presumably indicates that the asymmetry of the molecule is lost at a faster rate than helix under the conditions employed here.

A sedimentation velocity pattern and chromatogram of the skin collagen from young dogfish sharks have been published (2). This sample consisted largely of the single chain \( \alpha_1 \) and the double chain \( \beta_1 \), while the sedimentation velocity pattern of the collagen from the adult dogfish shark (Fig. 3) shows that it contains, in addition, a large amount of the triple chain \( \gamma \) component.

This was confirmed by chromatography on carboxymethyl cellulose (Fig. 4). The various chromatographic peaks were identified by acrylamide gel electrophoresis of small portions (0.1 ml) taken from the effluent (Fig. 4). Peaks corresponding to \( \alpha_1 \), \( \alpha_2 \), \( \beta_1 \), and \( \gamma \) were observed in the expected positions. The large shoulder on the leading edge of the \( \beta_1 \) peak was \( \gamma \) component (\( \gamma_1 \); see below). With these results as a guide, fractions were taken from several chromatograms containing the partially resolved components. These were rechromatographed separately, and fractions were again taken. Protein was isolated
from each and examined by gel electrophoresis and sedimentation velocity in the manner applied to other collagens (2, 11). α1 and β12 contained no measurable amounts of other components. β11 contained about 40% α1 component, presumably α1. α2 was estimated to be 80 to 90% pure and to contain small amounts of heavier components, presumably γ112 and β12. γ112 was also 80 to 90% pure, the contaminant being β12.

These five preparations and the unfractionated collagen were analyzed for their amino acid content (Table II). These data confirm the identification of β11 and β12 expected by analogy with results obtained with other collagens (2). That is, β12 has a composition equivalent to a 1:1 mixture of α1 and α2, and β11 has a composition identical with α1. γ112 has a composition which does not differ significantly from the unfractionated collagen and which is consistent with the expected chain composition of two α1 and one α2 chains.

The major factors which determine the chromatographic position of a component in the present experiments are apparently charge density and molecular weight. Thus, α1 and α2 separate because the latter is more basic, and α1 and β11 separate because of their different molecular weights. Similarly, β12 chromatographs between α1 and α2 because it is composed of these two components, but is closer to α2 because of its higher molecular weight. γ112 then would be expected to appear in the effluent shortly after α1 on the basis of charge density, but would be delayed somewhat more because of its higher molecular weight. That it does show this behavior confirms the expected chain structure.

The degree to which the molecular weight affects the chromatographic position varies with the carboxymethyl cellulose preparation. Previous experiments (2) resolved α1 and β11 to a greater extent, but β12 and α2 to a lesser extent, than in the present studies. The γ component also appeared later in the effluent in the earlier studies, coinciding in position with α2.

![Fig. 3. Sedimentation velocity pattern of denatured dogfish shark collagen from the skin of an adult animal. The photograph was taken 160 minutes after a speed of 59,780 r.p.m. was reached. The boundaries, in order of increasing sedimentation rate (left to right), are designated α, β, and γ, representing monomers and covalently linked dimers and trimers. The solvent was sodium formate, pH 3.8, 1/2 = 0.15, at a concentration of approximately 0.5%; temperature, 25.0°C; phase plate angle, 60°.](image1)

![Fig. 4. Chromatogram of denatured dogfish shark collagen from the skin of an adult animal. Protein concentration in the effluent from a carboxymethyl cellulose column was measured by continuous recording of the absorbance. The dashed line indicates the approximate contributions of the individual components. The gel electrophoresis patterns (upper) show the composition of samples taken from the effluent at the numbered points. The γ component can be seen at the interface between the spacer gel (dark) and the running gel, and is particularly prominent in tubes 5 and 6.](image2)
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side chains (valine, isoleucine, and leucine) in cr2 than in al. The retention of these and other features during the evolution of cr2 is much greater than that of ot1, a difference which accounts for the higher content of amino acids with hydrophobic side chains (valine, isoleucine, and leucine) in cr2 than in al. The histidine content of cr2 and ot2 follow a pattern. For example, ot2 always has less histidine because of the small amounts present.

The gel electrophoresis patterns of samples taken from the carbohydrate effluent (Fig. 4) illustrate the pronounced tailing characteristic of this type of chromatography. For example, traces of β and γ component can be seen well into the region occupied by α2. α1 appears in the regions occupied by β and γ components. The doubling of the α-band in tubes 9 and 10 was not a consistent finding. It may represent a trace of α1.

The results of this study show that skin collagen from the dogfish shark is closely related to collagens of higher vertebrates—amino acid composition and cross-linking. A comparison of the amino acid data in Table I with similar data for other collagens (2) shows that the differences between α1 and α2 are present in the ratio 2:1 that all collagen molecules initially have the structure (α1)α2 (2, 7). The existence of molecules with different proportions of α1 and α2 seems highly unlikely from considerations of possible mechanisms of synthesis and from the fact that collagen behaves as a single species (for example, during thermal denaturation). The fact that γ1,2, and no measurable amounts of other γ components, were found provides direct evidence that this assumption is correct.

**DISCUSSION**

The results of this study show that skin collagen from the dogfish shark is closely related to collagens of higher vertebrates. There are, however, distinct species differences which permit further insight into two important aspects of collagen structure—amino acid composition and cross-linking.

A comparison of the amino acid data in Table I with similar data for other collagens (2) shows that the differences between α1 and α2 follow a pattern. For example, α2 always has less proline and hydroxyproline than α1. The histidine content of α2 is much greater than that of α1, a difference which accounts in large part for the greater basicity of α2. Another constant feature is the higher content of amino acids with hydrophobic side chains (valine, isoleucine, and leucine) in α2 than in α1. The retention of these and other features during the evolution of the vertebrates indicates that they play a significant role in collagen structure.

The relationship between the content of the imino acids proline and hydroxyproline and the denaturation temperature of the collagen has been studied extensively (see Harrington and von Hippel (13)). The recent experiments by von Hippel and Wong (15) and Josse and Harrington (16) establish the close correlation.

The collagen studied here is typical of the least stable collagen. The imino acid content is apparently near the minimum necessary for a functionally stable helical structure. α2 has a very low content of amino acids, a total of 120 residues per 1000, compared with 160 residues per 1000 in the whole molecule and about 220 residues per 1000 in mammalian collagens (2).

An unusual property of the collagen sample from the adult dogfish shark is the high content of γ component, estimated from the chromatograms to be 30 to 40%. Soluble collagen preparations from other species, e.g., calf skin (17), contain only a small percentage. The large amount of the collagen present permitted this component to be located readily on the chromatogram and isolated for amino acid analysis. Previous evidence, based on the sedimentation properties, shows that it is a collagen molecule in which the three chains are intramolecularly cross-linked. This is confirmed by its chromatographic behavior and amino acid composition.

It has been assumed implicitly that because α1 and α2 are present in the ratio 2:1 that all collagen molecules initially have the structure (α1)α2 (2, 7). The existence of molecules with different proportions of α1 and α2 seems highly unlikely from considerations of possible mechanisms of synthesis and from the fact that collagen behaves as a single species (for example, during thermal denaturation). The fact that γ1,2, and no measurable amounts of other γ components, were found provides direct evidence that this assumption is correct.

**SUMMARY**

Soluble collagen preparations from the skin of the dogfish shark, *Squalus acanthias*, were characterized with regard to physicochemical properties, chromatography of the denatured collagen, and amino acid composition. The collagen is unusual in that it has a melting point of 16.0º in solution as measured by optical rotation. This low value is consistent with the low content of proline and hydroxyproline. As found for other vertebrate collagens, dogfish shark collagen contains two kinds of single chains, the α1 and α2 chains, which are similar but have different amino acid compositions. They are present largely as the covalently linked dimers β1 (α1-α1) and β2 (α1-α2), with a pronounced preference for the latter. In samples from older animals, a large amount of trimer (γ component) was also present. The γ component was identified as γ1,2 (α1-α1-α2) by amino acid composition and chromatographic behavior. This confirms the proposed chain structure of native collagen as (α1)α2.

**REFERENCES**

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