J. Biosci., Vol. 5, Number 2, June 1983, pp. 147-154.

Physico-chemical characterisation and molecular organisation of the collagen from the skin of an air-breathing fish *(Ophiocephalus striatus)*★

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MS received 24 July 1982; revised 7 May 1983

Abstract. Collagen has been prepared from the skin of an air-breathing Indian fish *(Ophiocephalus striatus)* by extraction with cold 0.5M acetic acid and purification by alternate precipitation with NaCl and dialysis against 0.02M Na_2HPO_4. The purified collagen was characterised with respect to physico-chemical properties, amino acid composition and chromatography of the denatured collagen. The fish collagen has a higher shrinkage temperature and denaturation temperature compared to that of the allied teleosts living in exclusively aquatic medium. These differences could possibly be reflections of the response to the rigours of the environment. As found for other vertebrate collagens, the fish collagen contains two kinds of single chains the α_1 and α_2 chains as revealed by sodium dodecyl sulphate–polyacrylamidegel electrophoresis and carboxymethylcellulose chromatography.

Keywords. Fish collagen; characterisation of collagen; chain composition.

Introduction

Previous work on the properties of the skin collagen of fishes indicates that they show variations in amino acid composition and physical properties correlated with the temperature of the environment (Takahashi and Tanaka, 1953; Gustavson, 1953, 1956). The shrinkage temperature of the skin collagen of fishes living in warmer waters was found to be higher than that of fishes living in colder waters (Gustavson, 1955). Shrinkage temperature of 63°C has been reported for the skin collagen of Australian lung fish *Neoceratodus* (Eastoe, 1957). Such a correlation between the shrinkage temperature and environmental temperature suggests that the properties of skin collagen are attributable to the mode of life. In the light of the above observations, the nature of the skin collagen of a mud-dwelling fish *Ophiocephalus* is of interest. Like *Neoceratodus* it is air-breathing and possesses accessory air-breathing organs. The nature of the biochemical adaptations of collagen in a semi-terrestrial environment is interesting. Hence a study of the physico-chemical properties and molecular organisation has been made with reference to the skin collagen of *Ophiocephalus striatus.*

★ The work reported forms part of the thesis approved by the University of Madras for the award of the Ph.D. degree to the first author.

Materials and methods

Ophiocephalus striates used in the present investigation were collected from ponds in Madras.

Sodium dodecyl sulphate (SDS) and ammonium persulphate used were obtained from Sigma Chemical Company, St. Louis, Missouri, USA. Whatman CM-32 microgranular cellulose was obtained from Whatman Biochemicals, Kent, UK All other chemicals were of analytical reagent grade.

Isolation and purification of collagen

The skin of *Ophiocephalus striatus* was freed of scales and adhering tissues and stirred overnight with 0.5M sodium acetate at 4°C. It was washed well with cold distilled water and extracted with 0.5M acetic acid. Purification of the extract was carried out according to the method of Piez *et al.* (1963) as outlined below. The extract was centrifuged at 18,000 rpm in a Sorvall refrigerated centrifuge for 30 min and collagen from the supernatant was precipitated by the addition of solid NaCl to a concentration of 5%. The precipitate was collected by centrifugation at 10,000 rpm and redissolved in 0.5M acetic acid. The process of precipitation with NaCl and dissolution in 0.5M acetic acid was repeated thrice and collagen in solution was dialysed against 0.02M Na_2HPO_4. The precipitate was centrifuged at 18,000 rpm for 30 min, dialysed against large volumes of 0.1M acetic acid and lyophilised.

Chemical analyses

Collagen sample was hydrolysed in 6N HCl in sealed tubes at 110°C for 24 h. Amino acid composition was determined in an amino acid analyser, Beckman Spinco model 120 C (Spackman *et al.,* 1958). Hydroxyproline content of the sample was determined by the method of Neuman and Logan (1950) and tyrosine content by the method of Ottaway (1958).

Physical properties

The shrinkage temperature (Ts) of the skin collagen was measured with a micro shrinkage meter; viscosity was measured in Ubbelhode viscometer. The denaturation temperature (Td) was obtained from the midpoint of viscosity *vs* temperature profile in the range 20 to 40°C. For determination of intrinsic viscosity, viscosity of solutions in the concentration range 0.025 to 0.15% was determined and the value was obtained by extrapolation of the specific viscosities to zero concentration.

Polyacrylamide gel electrophoresis

The procedure of Laemelli (1970) was followed using 5% (W/V) acrylamide gels. Samples were run for 4 h at 3 mA per gel and protein bands were stained with 0.1 % coomassie blue in 50% TCA at 37°C for 1 h. Destaining was carried out with 7% TCA and the destained gels were scanned by using a Beckman scanning densitometer at 540 nm.

Carboxymethyl-cellulose chromatography

The method of Piez *et al.* (1963) was adopted. The collagen sample was heat denatured in 0.06M sodium acetate buffer pH 4.8 and then applied to a

corboxymethyl-cellulose column (1.8×15 cms) maintained at 40°C which had been equilibrated with the above buffer. Elution was carried out by applying a gradient of 0-0.16M NaCl. 10 ml fractions were collected and the absorbance was measured at 230nm in a Pye Unicam model SP 1800 UV spectrophotometer.

The distribution of α and β chains in the collagen sample was determined by molecular seive chromatography using 6% agarose (Piez, 1968). A column (2.3 × 120 cm) of 6% agarose (Bio-Gel A 1.5M 200-400 mesh) was prepared and equilibrated with 0.5M Tris/1M$CaCl_2$ buffer (pH 7.5). The sample (about 20 mg) was dissolved in 2-3 ml of $CaOl_2$/Tris buffer at 45°C for 5-10 min. The flow rate was maintained at 25 ml/h and 5 ml fractions were collected. The fractions were monitered at 230 nm.

Electron micrograph of the native reconstituted collagen was obtained by scanning in a Siemens Elmiskop I.

Results

About 90% of the collagen from the skin of *Ophiocephalus* could be extracted with 0.5M acetic acid which indicates a high degree of solubility. Previous work indicates that the solubility of the skin collagen of fishes varies widely. Pikkarainen (1968) observed that collagens of hag fish and ray fish could be extracted upto 80 to 90% of the total quantity. Among the bony fishes the solubility of the collagen was only 33% in pike whereas Young and Lorimer (1960) reported that about 62% of the collagen could be solubilized from full grown cod. In the case of *Ophiocephalus* the solubility of the skin collagen remains unchanged at all growth stages which is in contrast to the mammalian collagens in which the solubility decreases with age (Bailey, 1967).

Table 1. Aminoacid composition of skin collagen of *Ophiocephalus.*

Amino acid	*Ophiocephalus* skin collagen	Calf skin collagen
	Residues/1000	
3-Hydroxyproline	—	—
4-Hydroxyproline	85.0	99.1
Aspartic acid	42.0	46.1
Threonine	24.8	19.4
Serine	38.0	36.7
Glutamic acid	67.0	67.9
Proline	125.0	128.2
Glycine	323.0	322.3
Alanine	116.3	109.2
Valine	22.8	24.1
Methionine	15.1	6.7
Isoleucine	9.7	11.5
Leucine	22.5	24.5
Tyrosine	2.8	3.1
Phenylalanine	11.1	10.2
Hydroxylysine	7.1	7.5
Histidine	6.1	5.4
Lysine	27.8	28.7
Arginine	53.8	49.4

The results of the amino acid analysis are given in table 1. The amino acid content of 210 residues/1000 resembles that reported for the skin collagen of *Neoceratodus* (Eastoe, 1957). The value is on the higher side when compared to those of teleosts such as carp and pike and hake (Eastoe, 1967; Yamaguchi *et al.*, 1976). A similar accord with skin collagen of *Neoceratodus* in the values for serine and threonine was noted. The significance of the higher values for these residues is not clear although it has been suggested by Pikkarainen (1968) that the higher content of serine and threonine may provide more hydroxyl groups to make up for the deficiency in the content of hydroxyproline.

The physical properties of skin collagen of *Ophiocephalus* are given in table 2. A shrinkage temperature of 60°C was observed which is high when compared to the

Table 2. Physical properties of skin collagen of *Ophiocephalus.*

Properties	*Ophiocephalus* skin collagen
Shrinkage temperature °C	60.0
Denaturation temperature °C	32.9
Reduced viscosity dl/g	22.0
Intrinsic viscosity	15.2

other aquatic teleosts but resemble *Neoceratodus.* In the case of pike classified as a warm water fish, the shrinkage temperature has been reported to be 55°C while that of the allied type cod living in cold waters at a temperature of 15°C a lower shrinkage temperature such as 40°C has been observed (Gustavson, 1955). The denaturation temperature of 32.9°C (figure 1) is also higher than the values for aquatic teleosts. Rigby and Prosser (1975) observed that the melting temperature

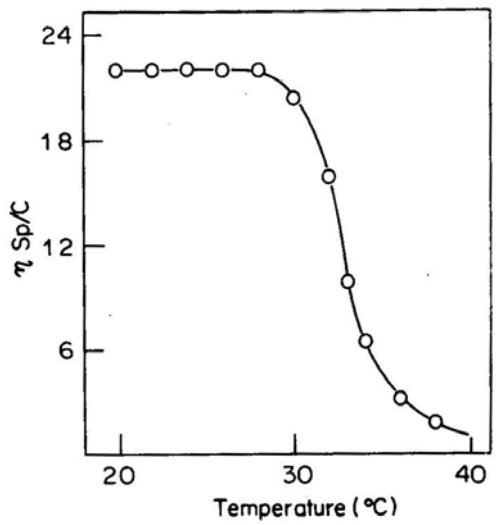

Figure 1. Denaturation curve of acid soluble collagen of skin of *Ophiocephalus* based on viscosity measurements at different temperatures.

of collagen in the skin of fishes from different depths have a correlation with the temperature of external medium of fish. The values for both reduced viscosity and intrinsic viscosity (figure 2) of the collagen resemble those of other fish collagens.

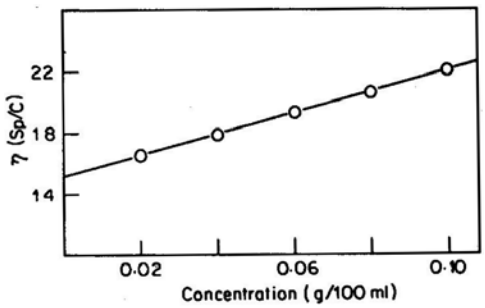

Figure 2. Reduced viscosity *vs* concentration plot for skin collagen of *Ophiocephalus* (in 0.1M acetic acid at pH 3.6).

The subunit composition was determined after denaturation of the samples and by subjecting it to polyacrylamide gel electrophoresis (figure 3). The pattern comprises of 2α chains corresponding to α_1 and α_2 chains of calf skin collagen

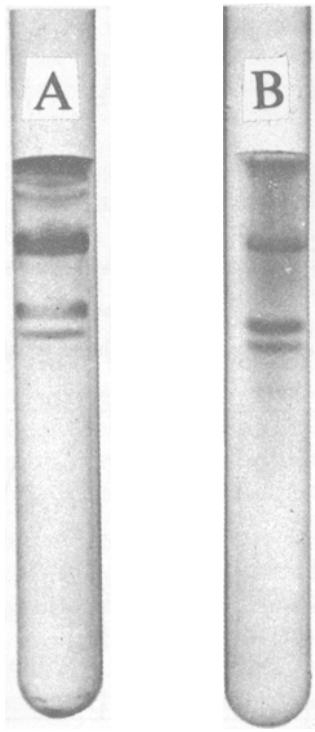

Figure 3. 5% Sodium dodecyl sulphate–polyacrylamide gel electrophoretic pattern. **A.** *Ophiocephalus* skin collagen. **B.** Calf skin collagen.

together with β and γ components. The results were confirmed by carboxy methyl cellulose chromatography (figure 4). The results obtained show the presence of both α_1, and α_2 chains. The trailing edge of α_1 peak was found to contain β_{11} and the peak preceding the α_2 peak was. found to be a mixture of β_{12} and α_2. It is seen that the chain composition of skin collagen of *Ophiocephalus* conforms to the pattern of Type I collagen of vertebrates.

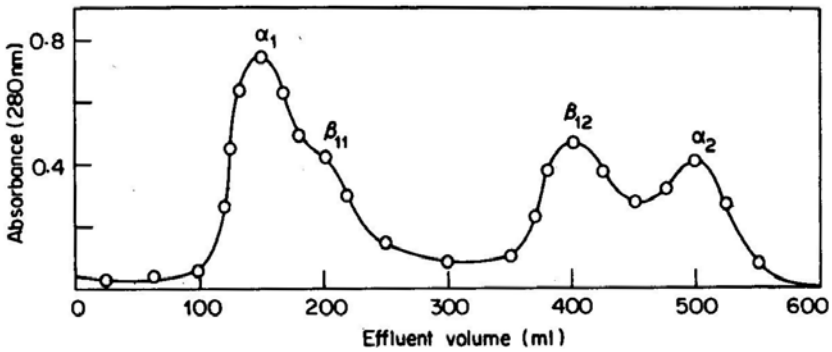

Figure 4. Carboxy methyl–cellulose elusion pattern of denatured acid soluble collagen of skin of *Ophiocephalus.*

The elution profile of the denature skin collagen of *Ophiocephalus* from 6% agarose is shown in figure 5A. The α-components as well as the dimeric and trimeric

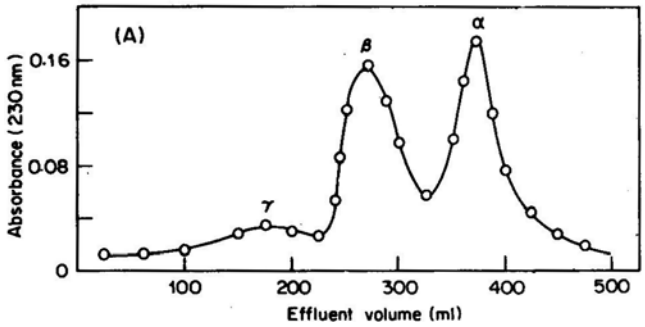

Figure 5A. Elution profile of denatured acid soluble collagen of skin of *Ophiocephalus* on 6% agarose.

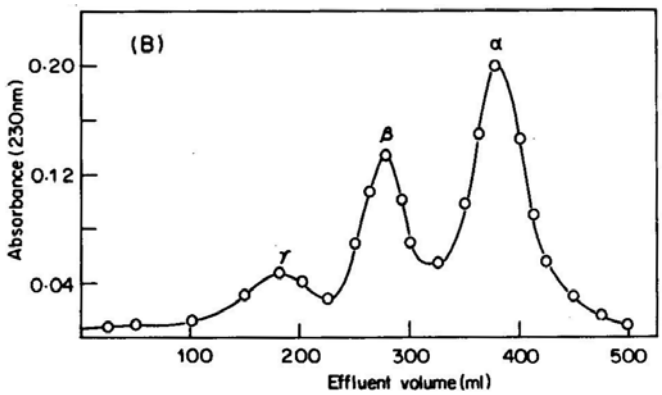

Figure 5B. Elution profile of denatured acid soluble calf skin collagen on 6% agarose.

components eluted in the region corresponding to those of calf skin collagen (figure 5B). The α-chains of skin collagen of *Ophiocephalus* thus have a molecular weight of 95,000-100,000 similar to those reported for other fish collagens.

The electron micrograph of the skin collagen of *Ophiocephalus* shows a banding pattern of 640 Å (not shown) which accords with the pattern reported for vertebrate collagens.

Discussion

The results reported above indicate that the skin collagen of *Ophiocephalus* is peculiar in a number of respects particularly in regard to the amino acid composition and some of its physical properties such as shrinkage temperature (Ts) and denaturation temperature (Td). A higher amino acid content (proline+ hydroxyproline) is noted on comparison with; skin collagens of teleosts living exclusively in aquatic medium. Correlated with this feature of amino acid composition are the physical properties of skin collagen with Ts and Td of 60°C and 32.9°C respectively. These values are also higher than those reported for skin collagen of allied teleosts such as pike, carp and cod. A higher Ts value has been related to environmental temperatures of fishes in which they live. Although a correlation between Ts values and temperature of the environment has been supported by the results of various studies carried out on skin collagen of fishes, it is seen that the Ts values extend over a range of 15-30°C above the maximal environmental temperature. It is therefore suggestive that it is not merely a question of approximating to the temperature of the environment but has probably a deeper biological significance associated with metabolic factors involving collagen synthesis and function.

In this context it is relevant to recall the data on the physical properties of *Neoceratodus* which is typically air-breathing (Eastoe, 1957). The Ts value approximates to that reported in the present study on the skin collagen of *Ophiocephalus.* The air-breathing habit is common to both fishes and may suggest that this may be one of the factors contributing to the high values for Ts noted in them. It may be suggested that the high content of iminoacids and the correlated high values for Ts and Td are of the nature of responses to the rigours of the environment and hence of adaptive value. The mode of life of this fish is unusual and lives burried under mud for prolonged periods. Necessarily such a mode of life for a fish would demand adjustments and modifications in the metabolic pattern which may be reflected in the biochemistry of skin collagen.

Notwithstanding the deviations in amino acid compositon and physical properties of skin collagen of *Ophiocephalus* reported above, there is seen a close similarity in its chain composition to that of other teleosts such as carp, pike and hake in conforming to Type I collagen of higher vertebrates. (Piez *et al.,* 1963; Paz *et al.,* 1967; Pikkarainen, 1968). In the case of cod fish, Piez (1965) reported the presence of three non-identical α chains. It is seen that the molecular organisation which is determined by genetic factors remains unaltered to a large extent and is not influenced by factors external to the organism but changes in amino acid composition and physical properties of collagens have been reported to occur in response to environmental demands.

Acknowledgement

The authors wish to thank Dr. N. Ramanathan for his generous support and encouragement during this investigation.

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