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Characterization of γ-crystallins from eye lenses of shark: closer structural similarity to mammalian than other piscine γ-crystallins?

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Lens crystallins were isolated and characterized from sharks of the cartilaginous fishes. Four crystallin fractions corresponding to α-, β_H-, β_L- and γ-crystallins, similar to those of mammalian crystallins, were obtained. The native molecular masses and subunit structures of these purified fractions were analyzed by gel permeation chromatography, SDS gel electrophoresis and isolectric focusing, revealing the typical subunit compositions with various extents of heterogeneity in each orthologous crystallin class. Amino acid and N-terminal sequence analyses corroborate the identification and classification of crystallin classes based on electrophoresis. Unexpectedly, it was found that the amino acid composition and N-terminal sequence of shark γ-crystallin are more closely related to those of bovine than carp γ-crystallin. This finding may have some bearing on the divergence and specification of γ-crystallins between the phylogenetic lines of mammals and fishes.

Crystallin; Amino acid composition; Charge heterogeneity; Sequence comparison; Phylogeny; (Eye lens, Shark, Chondrichthyes)

1. INTRODUCTION

Understanding the evolutionary conundrum of various classes of proteins remains the Holy Grail of current research in protein chemistry and molecular biology. The abundant presence of various common and specific classes of structural proteins, i.e. lens crystallins, in different species of vertebrates provides a good model system to unravel the complex process of protein evolution [1,2]. Recent reports of sequence similarities between ε-crystallin and lactate dehydrogenase [3], δ-crystallin and argininosuccinate lyase [4], ϱ-crystallin and aldehyde/aldose reductase [5] plus bovine lung prostaglandin F synthase [6] have provided more interesting aspects of crystallin evolution regarding their possible enzymatic functions.

The modern chondrichthyes class of fishes, e.g. sharks and skates, are distinguished by their cartilaginous skeletons in contrast to the bony skeletons of osteichthyes (bony fishes). Sharks diverged from the Placodermi long before the appearance of modern bony fishes and amphibians [7]. The study of lens crystallins from the cartilaginous fishes such as shark is of particular interest from the evolutionary point of view because they constitute the early forms of fishes and are thought to have been ancestral to the land vertebrates. The characterization of shark crystallins is deemed very important for the phylogenetic comparison in light of the recent elucidation of the complete sequences of γ-crystallins from carp of the osteichthyes [8].

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2. MATERIALS AND METHODS

Sharks (*Scoliodon walbeemii*) provided by the local fishery company under a special contract for scientific research are usually present in the coastal waters of western Taiwan. The pooled lenses each weighing about 1.05–1.14 g (wet wt) were decapsulated and homogenized in 10–20 ml of 0.05 M Tris-Na bisulfite buffer (pH 7.5) containing 5 mM EDTA as described

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[9,10]. The supernatant from centrifugation at 27 000 × g was adjusted to give a concentration of about 30–50 mg/ml and the 3.0 ml aliquot was applied to Fractogel TSK HW-55 (Merck, Darmstadt). Cation-exchange chromatography of shark γ-crystallin fraction from the gel-permeation column was carried out further on a TSK CM-650(M) (2.5 × 15 cm) column. Lyophilized crystallins were dissolved in starting buffer (0.05 M ammonium acetate, 0.05 mM EDTA, pH 5.9) and centrifuged before applying to the column equilibrated in the same buffer. Elution was carried out in two steps: (i) with starting buffer and (ii) with a 500 ml linear gradient of 0.05–0.5 M ammonium acetate (pH 5.9). It resolved γ-crystallins into 6 subfractions, each of which was subjected to amino acid and N-terminal sequence analysis.

SDS-polyacrylamide slab gel electrophoresis (SDS-PAGE, 5% stacking/14% resolving gel) was performed as described [11] with some modifications. Isoelectric focusing in 7.5% polyacrylamide gel containing 2.8% (v/v) carrier ampholytes of pH 3.5–10 was carried out on a slab gel with incorporation of 0.1% 2-mercaptoethanol and 6 M urea.

Amino acid compositions were determined using a Beckman (model 6300) high-performance amino acid analyzer with a dual-channel data system using a single column based on ion-exchange chromatography. The special procedure for the preparation of protein hydrolysates using microwave irradiation was essentially according to [12]. Custom-made Teflon-Pyrex reusable hydrolysis tubes (4 mm i.d. × 150 mm) resistant to high temperature and pressure in the microwave oven were ordered from a local glass-plastic shop (Continuity Enterprise, Taipei). The basic designs of the tubes are based on inert-gas flushing for removal of oxygen inside the tubes with a specially designed Teflon cap and plunger to ensure leak-free operation under microwave irradiation [13,14]. Constant-boiling 6 M HCl and 4 M methanesulfonic acid containing 0.2% 3-(2-aminoethyl)indole in 1-ml ampoules were obtained from Pierce (Rockford, IL). We have shown the applicability of short-time (5 min) microwave irradiation in achieving comparable amino acid composition data similar to those obtained by the tedious conventional 110°C/24 h protocol [12]. The content of half-cystine and tryptophan could be determined with accuracy by hydrolysis with 4 M methanesulfonic acid as in [14]. The N-terminal sequences of γ-crystallin fractions from the cation-exchange column were determined on automated Edman degradation with a pulsed liquid-phase sequenator (model 477A, Applied Biosystems, Foster City, CA).

3. RESULTS AND DISCUSSION

Most physicochemical studies on characterization of crystallins have placed more emphasis on species of higher vertebrates with relatively few reports on lenses from the lower aquatic vertebrates, i.e. various classes of fishes [15]. The piscine lenses are usually spherical and hard as compared to the more flexible and soft lenses found in the avian class, which may be related to the state and content of water, i.e. degree of hydration, in the different lenses of vertebrate species [16]. The poor solubility and susceptibility of lens proteins from most fishes to denaturation have hampered detailed biochemical characterization of piscine crystallins under nondenaturing conditions [17,18]. Here, the characterization of shark lenses is of special interest and significance in our systematic study of the evolutionary relationship of lens crystallins from vertebrates and invertebrates [1,9,10]. Shark lenses also provide large quantities of lens proteins even on the basis of a single lens of lens weight similar to that of bovine lens, a rich source for current crystallin research.

Fig.1 shows the elution pattern of lens extract from a single shark lens separated on TSK gel-permeation column. The reason for using lenses from a single shark in each case is in order to circumvent the heterogeneity problem caused by the presence of allelic variants of crystallin polypeptides arising from the pooled lenses of several animals. Four peaks were obtained for this species in contrast to three major peaks from carp [1], which belongs to the osteichthyes. Identification of each crystallin fraction from the column was initially based on subunit analysis by SDS gel electrophoresis in fig.2. The native molecular masses of 4 eluted peaks were estimated from the column calibration with standard proteins. The molecular masses were determined to be 620, 190, 105 and 19 kDa for peaks 1–4, respectively. In contrast to previous characterizations [19–21] of crystallins from dogfish, also belonging to a group of cartilaginous fishes, shark lenses seemed to contain a much higher proportion of γ-crystallin (> 50%) as compared to dogfish lenses. The subunit molecular masses for these fractions were analyzed by SDS-PAGE as shown in fig.2A. Peaks 1–4 show exactly the same well-defined subunit compositions similar to those of mammalian crystallin classes [22,23].

Apparent charge heterogeneity could be demonstrated by high-resolution isoelectric focusing gel analysis as shown in fig.2B. The γ-crystallin fraction showed at least 12 bands with p*I* values ranging from pH 5.9 to 9.2. By contrast, the γ-crystallins of carp and calf lenses showed much simpler banding patterns under the same conditions of isoelectric focusing (not shown). In a study of γ-crystallins from the dogfish, Siezen et al. [21] also reported more than 10 γ-crystallin components over the pH range from below 6 to above 10 by isoelectric focusing. However, we could not

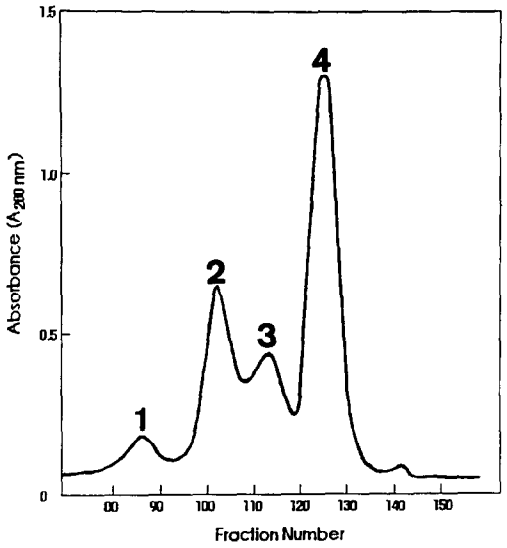

Fig.1. Isolation and fractionation of shark lens crystallins by gel-permeation chromatography on Fractogel TSK HW-55(S) (2.5 × 115 cm column). Column eluates (3.2 ml/tube per 4.1 min) were monitored for absorbance at 280 nm. The four crystallin fractions (1–4) labeled above each peak correspond to α-, β_H-, β_L- and γ-crystallins, respectively. The % yields for each crystallin class were estimated from the areas under each crystallin peak.

detect basic components of pI>10 even though sharks and dogfishes are supposedly closely related.

Some of the structural parameters for the four major fractions of shark lenses are tabulated in table 1 for comparison. It is noteworthy that the molecular sizes and subunit compositions of each orthologous crystallin in shark lens are found to be more similar to those of mammalian than carp crystallins. It is even more intriguing that shark γ-crystallin does not show a high methionine content in the amino acid composition (table 2) as do most bony fishes.

This evolutionary anomaly in the composition of shark γ-crystallin has prompted us to investigate its sequence in order to obtain a more defined and meaningful comparison. We have hence carried out separation of crude crystallin into its subfractions on cation-exchange chromatography (not shown) essentially according to the same procedure used for isolation and subsequent microsequencing of γ-crystallins from bovine and carp lenses [24,25]. N-terminal sequence analyses of 6 shark γ-crystallin subfractions isolated from TSK CM-650

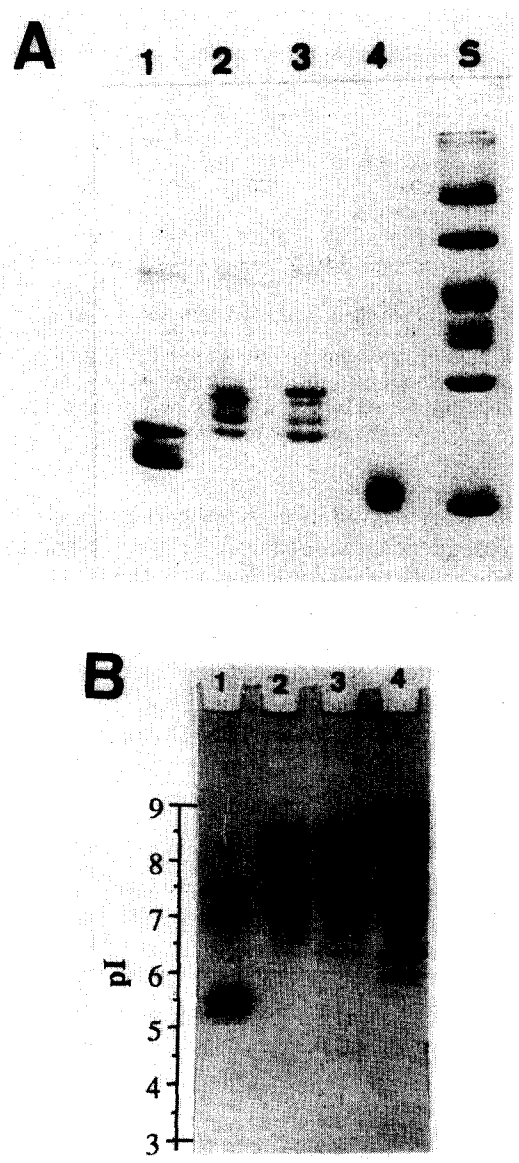

Fig.2. (A) Gel electrophoresis of fractionated shark crystallins under denaturing conditions (SDS-PAGE) in the presence of 5 mM dithiothreitol. Lanes S, standard proteins used as molecular mass markers (in kDa): transferrin (80), bovine serum albumin (66), ovalbumin (45), lactate dehydrogenase (37), carbonic anhydrase (30) and soybean trypsin inhibitor (20). Lanes 1–4 are the 4 fractions of fig.1 corresponding to peaks 1–4, respectively. The gel was stained with Coomassie blue. (B) Isoelectric-focusing gel analysis of shark crystallins under denaturing conditions. About 10 μg of each crystallin fraction in (A) were layered on a 7.5% polyacrylamide gel containing 6 M urea and 0.1% 2-mercaptoethanol for isoelectric focusing (basic end at the top). The pI was estimated via a pI calibration kit (3.6–10.2).

Table 1

Structural parameters of shark crystallins

Properties	α	β_H	β_L	γ
(1) Yield (%)	5.1	24.2	17.1	53.7
(2) Native molecular mass (kDa)	620	190	105	19
(3) Subunit molecular mass (kDa)	23, 26	26–30	26–30	20–21
(4) Isoelectric points (p*I*)	5.2–5.6, 7.0–7.5	6.5–8.5	6.5–8.5	5.9–9.2
(5) N-terminal residue	blocked	blocked	blocked	glycine

α, β_H, β_L and γ crystallins correspond to the four peak fractions in fig.1. Methods used for determination of these parameters are described in section 2. Yield (%) was estimated from the peak areas of 4 fractions in fig.1

by Edman degradation were carried out in the microsequencing sequenator. All were found to be closely related to each other with almost identical sequences for their N-terminal segments except for some positions with more than two amino acids being identified. It appeared to indicate some sequence heterogeneities still exist in these γ-crystallin subfractions. The reasons for these microheterogeneities are still unknown. The possibility of genetic polymorphism due to allelic variants of different individual sharks can be ruled out, since single lenses from one shark were used throughout the isolation and purification.

Table 2

Comparison of amino acid compositions of carp, bovine and shark γ-crystallins

Amino acid	γII-Crystallin		
	Carp	Calf	Shark
1/2Cys	4.5	4.2	3.8
Asx	11.5	10.2	11.3
Thr	1.0	2.4	2.7
Ser	8.6	8.1	7.3
Glx	9.5	11.5	9.8
Pro	2.5	4.8	6.0
Gly	8.9	8.3	9.2
Ala	0.3	1.0	1.4
Val	2.2	3.1	3.3
Met	13.4	4.5	3.8
Ile	4.1	3.2	3.7
Leu	2.7	7.3	3.8
Tyr	8.2	8.1	8.3
Phe	6.1	5.0	5.6
His	2.9	2.7	3.2
Lys	0.8	1.4	1.9
Arg	11.6	12.1	12.4
Trp	1.4	2.0	2.4

Data are expressed as mol%. Each value represents the mean of duplicate determinations. The preparation of hydrolysates was by means of microwave irradiation for 5 min using 4 N methanesulfonic acid as described in section 2

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1        5        10       15       20
G-K-I-I-F-Y-E-D-R-N-F-Q-G-R-S-Y-D-C-M-S-  (Carp γII)
G-K-I-T-F-Y-E-D-R-G-F-Q-G-H-C-Y-E-C-S-S-  (Calf γII)
G-K-I-I-F-Y-E-D-R-G-F-Q-G-H-C-Y-E-C-S-S-  (Shark γII)
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Fig.3. Comparison of N-terminal sequences of γII-crystallins from carp, calf and shark. The sequences listed for carp and calf crystallins were taken from [8,23]. Note that the N-terminal sequences of 20 residues are identical for calf and shark proteins whereas 6 residues underlined at positions 4, 10, 14, 15, 17 and 19 differ between carp and shark crystallins. Amino acid residues are denoted according to the single-letter code.

Preliminary comparison of the N-terminal sequences of γII (the second peak eluted from the cation-exchange column, a major subfraction of γ-crystallins) from shark, carp and calf is shown in fig.3. Unexpectedly, no differences between the sequences of shark and calf were observed. In contrast, there are several conservative changes of amino acids along the N-terminal segments of shark and carp. Nonconservative changes of amino acids were also identified at residues 14 (Arg/His), 15 (Ser/Cys) and 19 (Met/Ser).

Elucidation of the mechanism of evolution of functionally related proteins from different species remains a general biological problem. Extensive characterization from the evolutionarily or developmentally unique animal species such as the cartilaginous shark described here may eventually provide some insight into the phenomenon of species diversification and the accompanying molecular origin of crystallins.

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