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Purification and Characterization of ζ-Crystallin/Quinone Oxidoreductase from Camel Liver

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Abstract: ζ-Crystallin is a major protein in the lens of certain mammals. It has been characterized as a novel NADPH: quinone oxidoreductase, showing limited quinone substrate specificity. This study report for the first time purification of this protein from camel liver by a sequential procedure of batch adsorption chromatography using CM-Sephadex C-50, affinity chromatography using Blue Sepharose CL-6B and 2', 5' ADP-Sepharose 4B. The pure material was isolated in a yield of 2.5% and purification fold of 253 over homogenate, with specific activity of 22 units/mg protein. Kinetic and physical properties of this protein have been found to be identical with those of camel lens ζ-crystallin.

Key words: Purification, ζ-crystallin, NAPDH, quinone oxidoreductase, camel, liver

INTRODUCTION

Crystallins are structural proteins that are highly abundant in the cytoplasm of the lens fiber cells. They are responsible for lens transparency[1]. There are two major classes, the ubiquitous crystallins (α and (β/γ) present in all vertebrates and the taxon-specific enzyme-crystallins, which occur in phylogenetically restricted groups[2]. The enzyme-crystallins are either identical or related to metabolic enzymes. ζ-crystallin is one of the taxon-specific proteins, that was first reported as a novel 35 kDa crystallin of the guinea pig[3]. It constitutes about 10% of the soluble lens proteins of guinea pig and camel[3,4]. Complete amino acid sequence of ζ-crystallin deduced from cDNA of guinea pig showed that ζ-crystallin was distantly related to alcohol dehydrogenases, although it lacks alcohol dehydrogenase activity and the zinc binding site[5]. ζ-crystallin has been shown to bind NADPH specifically and to possess a reductase rather than dehydrogenase activity[6,7]. It was demonstrated that l-Crystallin is a novel NADPH: quinone oxidoreductase with substrate preference for orthoquinones[8,9]. A mutation in the gene for ζ-crystallin has been associated with autosomal dominant cataract in guinea pigs[10]. The mutant protein lacks both catalytic activity and the ability to bind NADPH[11]. It is of interest that ζ-crystallin has the ability to reduce xenobiotic compounds such as quinones, which are toxic to the cell[8] and is present in catalytic quantities in other tissues, especially liver and kidney from a variety of species[10]. ζ-crystallin from camel lens was purified and characterized[12], present study purified this enzyme from camel liver for the first time. The main objective of this study was the purification and characterization of ζ-crystallin/quinone reductase from camel liver and to find out how it compares with the lens ζ-crystallin.

MATERIALS AND METHODS

CM-Sephadex C-50, Blue Sepharose CL-6B, 2', 5' ADP-Sepharose CL 4B were purchased from Pharmacia LKB Biotechnology, Upsala Sweden. NADPH, 9, 10-phenantherenequinone, leupeptin were purchased from Sigma Chemical Company (St. Louis, MO.) USA. 2, 6-dichlorophenolindophenol, 1, 2-Naphthoquinone and 1, 4-Benzoquinone were purchased from Fluka AG, Buchs SG Switzerland. Menadione was from BDH Chemicals Ltd. Poole England. All other chemicals used were general grade reagents.

Enzymatic assays: The purification was monitored by assays of enzymatic activity, which relied upon the oxidation of NADPH by 9,10-phenanthrenequinone at 340 nm according to the method of Rao *et al.*[8]. The standard assay system contained in a final volume of 1 mL, 0.1 M Tris/HClbuffer pH 7.8, 0.2 mM Na_2 EDTA, 0.1 mM NADPH and 25 μM 9, 10-phenanthrenequinone and 0.5 ~ g of enzyme. Reactions were initiated by the addition of enzyme and the decrease in absorbance at 340 nm was monitored with a Perkin Elmer Lambda 3B

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spectrophotometer at 22°C. Blanks lacking either substrate or enzyme were run routinely. Assays with other quinone substrates were carried out under similar conditions. All quinones were dissolved in absolute ethanol, which was present at a concentration of 1% in all final reaction mixtures. A molar absorption coefficient of 6220 M^{-1} cm^{-1} for NADPH was used for the determination of enzyme activity. Assays with 2, 6-dichlorophenolindophenol were monitored by a decrease in absorbance at 600 nm; molar absorption coefficient of 21x103 M^{-1} cm^{-1} was used to calculate the enzyme activity. One unit of enzyme activity was defined as the oxidation of 1 μmole of NADPH per minute per mg protein under the specified condition.

Protein determination: Determinations of protein concentrations were made by applying the equation protein (mg/mL) = 1.55xA280 -0.76xA260 to measurements of absorbances at 280 and 260 nm[13].

Electrophoresis: SDS-PAGE was performed according to Laemmli[14] in vertical slab gel apparatus using 15% separating gel, with 0.3% cross-linking, 4.5% stacking gel and the gels were stained with Coomasie Blue R-250.

Isoelectric focusing (IEF):
IEF was performed on a 7% acrylamide gel (8x8 cm) containing 10% glycerol and an ampholine of pH 3.5-10. 1 M phosphoric acid was used at the anode and 1 M NaOH was used at the cathode end as described earlier by Duhaiman *et al.*[15]. The gel was focused at constant 6 W for 1 h. The pH gradient was determined by cutting one square cm pieces and immersing into 2.5 mL of distilled water and after vigorously vortexed, the pH was determined using pH meter (Metler, Delta 340).

Purification of ζ-crystallin:quinone reductase
Homogenization: Camel liver from a freshly killed animal was obtained from local abattoir and transported on ice bath to the laboratory. The tissue was cleaned of fat, blood vessels and connective tissue. It was then chopped into small pieces, washed with 20 mM sodium phosphate buffer (pH 6.5) containing 1 mM Na_2 EDTA and dried with filter paper. The washed tissue was minced using a meat grinder. The minced liver (300 g) was homogenized in 1.5 L of previously mentioned buffer containing 0.25 mM leupeptin using a Warring blender for 2 min at low speed and 1 min at high speed. The homogenate was centrifuged at 4°C for 30 min at 15, 000xg. The supernatant was filtered through a muslin cloth and centrifuged again as above. The resultant supernatant was subjected to the next step.

Batch adsorption chromatography: The supernatant fraction from step one was added slowly with gentle stirring to CM-Sephadex C-50 resin (500 mL) that had been fully equilibrated with 20 mM sodium phosphate buffer pH 6.5, containing 1 mM Na_2 EDTA, which was contained in a sintered glass funnel attached to a Buchner flask. After a gentle stirring for 1 h, the unbound proteins were removed from the damp cake of the resin by suction at a vacuum pump. The resin was washed with the equilibration buffer (4 L) until the absorbance at 280 nm of the filtrate was less than 0.05. The sintered glass funnel and its contents were transferred to another Buchner flask. The adsorbed material was then removed by gradual washing with 300 mL of 20 mM sodium phosphate buffer pH 6.5, containing 1 mM Na_2EDTA and 1 M NaCl at a vacuum pump. The concentration of the salt in the filtrate was reduced to 0.05 M NaCl by dialysis against 20 mM Tris/HCl buffer pH 7.8, containing 1 mM Na_2 EDTA, using an Amicon ultrafiltration cell fitted with a Diaflo PM 5 membranes.

Blue Sepharose affinity chromatography: The dialyzed extract was centrifuged at 27, 000 xg for 15 min at 4°C and the clear supernatant was divided into 4 aliquots and each aliquot was farther purified by affinity chromatography. In a typical run, 30 mL (466.2 mg) of the sample was loaded onto a column (1.6x12 cm) of Blue Sepharose CL-6B which had been equilibrated with 20 mM Tris/HCl buffer pH 7.8, containing 1 mM Na_2 EDTA and 0.05 M NaCl, at a flow rate of 10 mL/h collecting a 2.5 mL fraction every 15 min. The column was washed with 10 bed volumes of equilibration buffer. The adsorbed proteins were eluted by application of a linear gradient composed of 100 mL of equilibration buffer as starting buffer and 100 mL of 20 mM Tris/HC1, pH 7.8, 1 mM Na_2 EDTA and 1 M NaCl as finishing buffer. Fractions were collected every 15 min at a flow rate of 10 mL/h. The elution profile obtained is shown in Fig. 1. Fractions (12-30) with highest l-crystallin activity were pooled together and concentrated by ultrafiltration in an amicon cell fitted with a PM 5 membrane. The concentrated sample was dialyzed against 20 mM Tris/HC1, pH 7.8 containing 1 mM Na_2 EDTA and further concentrated.

2', 5 'ADP-Sepharose CL 4B Affinity chromatography: The dialyzed sample was applied to a column (Ix5 cm) of 2', 5' ADP-Sepharose 4B that had been equilibrated with 20 mM Tris/HCl buffer pH 7.8, containing 1 mM Na_2 EDTA at a flow rate of 10 mL h^{-1} collecting a 2.5 mL fraction every 15 min. The column was washed with 10 bed volumes of the equilibration buffer. The adsorbed proteins were eluted by application of a linear gradient

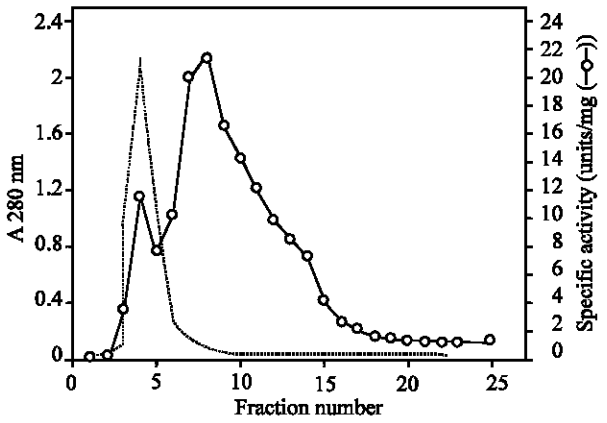

Fig. 1: Fractionation on Blue Sepharose CL-6B. ζ-Crystallin fraction obtained from batch adsorption chromatography was dialyzed with 20 mM Tris/HCl buffer pH 7.8 containing 1 mM Na_2 EDTA and fractionated on the column. (o) absorbance at 280 nm (----) distribution of quinone reductase activity

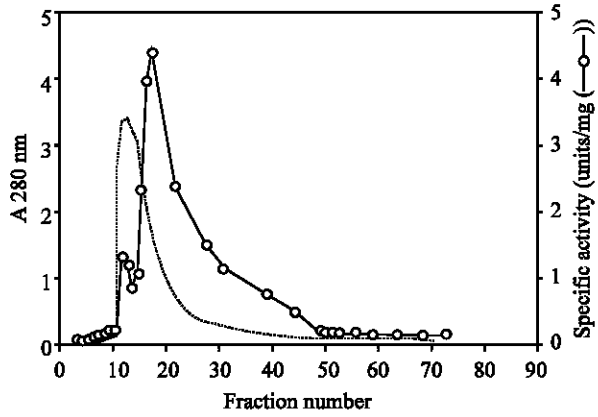

Fig. 2: Fractionation on 2, 5 ADP-Sepharose CL 4B. ζ-Crystallin fraction obtained from Blue-Sepharose CL-6B was dialyzed with 20 mM Tris/HCl buffer pH 7.8 containing 1 mM Na_2 EDT A and fractionated on the column. (o) absorbance at 280 nm (----) distribution of quinone reductase activity

composed of 100 mL of equilibration buffer as starting buffer and 100 mL of 20 mM Tris/HCl, pH 7.8, 1 mM Na_2EDTA and 1 M NaCl as finishing buffer. Fractions were collected every 15 min at a flow rate of 10 mL/h. The elution profile obtained was shown in Fig. 2. Fractions 7 and 8 had ζ-crystallin activity of 21.0 units/mg protein, these were pooled together, dialyzed and concentrated by ultrafiltration in an amicon cell fitted with a PM 5 membrane. The sample was kept at -20°C in small aliquots until required.

RESULTS AND DISCUSSION

Using a three-step procedure (Ion exchange, Blue Sepharose affinity and 2', 5' ADP-Sepharose CL 4B affinity chromatographies), camel liver ζ-crystallin was purified in a yield of 2.5% (Table 1) and purification fold of 253. The pure material had quinone reductase activity of 22.0 units/mg protein. Two peaks of quinone reductase activity were present in the Blue Sepharose affinity chromatography profile (Fig. 1), the first in fractions 8-11 and the second in fractions 12-30. Fractions in the second peak had higher quinone: reductase activity with also higher protein recovery. These were pooled together, dialyzed against 20 mM Tris/HCl, pH 7.8, 1 mM Na_2 EDTA and concentrated by ultrafiltration in an Amicon cell fitted with a PM 5 membrane. The final step of the purification utilizing 2', 5' ADP-Sepharose CL 4B affinity column chromatography produced an apparently homogenous material with very high oxidoreductase activity (Fig. 2). The purified material migrated as a single band on native slap gel comparable to camel lens ζ-crystallin (Fig. 3). Under reducing condition the enzyme migrated as single band of 35 kDa and a faint band of about 65,000 which could be a contaminating albumin, attempt to remove the contaminant was not successful. (Fig. 4). This molecular weight is comparable to that of guinea pig liver[16] and lens ζ-crystallin[3] as well as ζ-crystallin from camel lens[12]. Gel filtration of the protein on Sepharose CL-6B gave a single peak with molecular mass of approximately 140 kDa (data not shown). Thus the purified ζ-crystallin seemed to be a homo-tetramer enzyme, an observation consistent with other reports[12,16]. The isoelectric point of native ζ-crystallin as determined from a non-denaturing system in an acrylamide slab gel was found to be 8.0. Camel lens ζ-crystallin was reported to have a pI of 7.6[12], therefore the lens enzyme appears to be slightly more acidic. The low yield from the purification reported in Table 1, however an underestimation. This is because the assay for quinone reductase activity is non-selective, meaning that the total activity reported or the homogenate (initial liver extract) represented activity from several different enzymes that can use 9, l0-phenantherenequinone as a substrate.

Thus, when the relative amount of ζ-crystallin was separated from the other quinone reductases, the apparent yield was low. Similar low yield was reported for purification of ζ-crystallin from guinea pig liver[16]. However, the specific activity of the purified camel liver enzyme is about 3.5 and 2.5 times higher than that of purified ζ-crystallin from guinea pig liver and lens respectively and about 3 units lower than that of camel lens. The difference between the values of specific activity of the lens and liver ζ-crystallin may result from

Table 1: Summary of results for purification of camel liver ζ-crystallin

Steps	Protein (mg/mL)	Total Protein (mg)	Sp. Activity (μ moles/min/mg)	Total activity (μ moles/min)	Purification fold	% yield
Extraction (Homogenate)	14.28	19278.00	00.087	1677.2	01.0	100.0
IEC(CM-Sephadex C-50)	15.54	1927.00	00.37	713.0	04.0	42.5
AC (Blue Sepharose CL-6B)	01.34	105.20	05.00	526.0	57.0	31.4
AC (2', 5'ADP-Sepharose CL-4B)	00.21	001.94	22.00	042.7	253.0	02.5

IEC: Ion Exchange Chromatography AC: Affinity Chromatography

Table 2: Kinetic constants of NADPH: quinone oxidoreductase activity of camel liver ζ-crystallin

Substrates	Km (μM)	V_{max} (units/mg)	V_{max}/Km (units/mg /μM)
9,10-phenantherenequinone	13.3	32.7	2.50
NADPH[a]	18.2	28.1	1.54
1,2-naphthoquinone	3.4	10.6	3.12
1,4-benzoquinone	54.1	2.4	0.04
2,6-dichlorophenol Indophenol[b]	18.2	1.6	0.09

[a] -with 9, 10-phenantherenequinone as the second substrate
[b] -activity was determined by following DCIP reduction at 600 nm

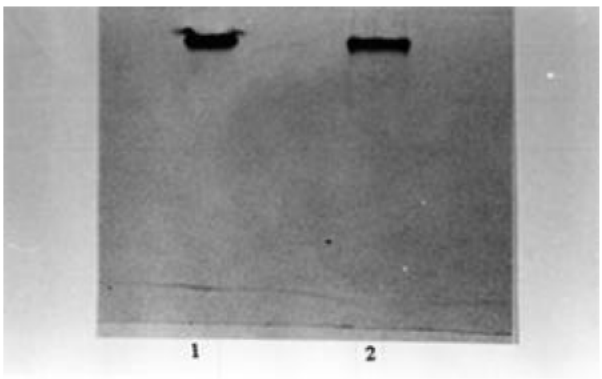

Fig. 3: Native polyacrylamide gel electrophoresis of camel liver ζ-crystallin. Lane 1 camel lens ζ-crystallin, lane 2 camel liver ζ-crystallin

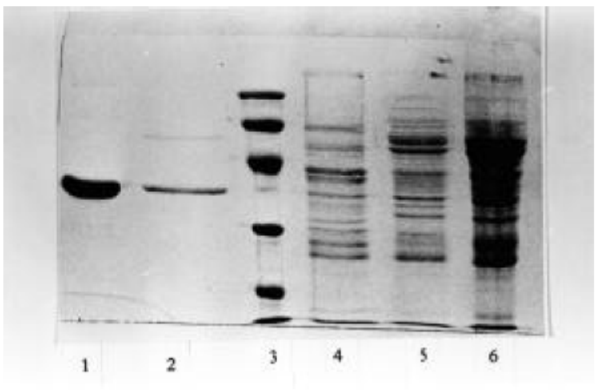

Fig. 4: SDS- polyacrylamide gel electrophoresis pattern of camel liver and lens ζ-crystallin. Lane l lens ζ-crystallin, lane 2 liver ζ-crystallin, lane 3 standard molecular mass markers, lane 4 Blue Sepharose fraction, lane 5 batch adsorption fraction and lane 6 liver homogenate

inactivation caused by additional steps required for purification of the liver enzyme or an inherent low activity of the liver enzyme. Similar difference was observed for the enzyme from guinea pig lens and liver[16].

A yield of 53.1% was obtained when ζ-crystallin was purified from camel lens[12], initial attempt (data not shown) to purify the liver enzyme using purification protocol of the lens enzyme produced an impure material with specific activity of 10 units/mg protein. This required a change of protocol with additional steps.

The purified liver ζ-crystallin was enzymatically active as a quinone reductase. The activity was dependent on NADPH. The enzyme also reduced 9, 10-phenantherene-quino fie, 1, 2-naphthoquinone, 1, 4-benzoquinone and 2, 6-dichlorophenolindophe-nol. To determine the substrate specificity of μM, various quinones were analyzed at the same enzyme concentration. Table 2 shows kinetic constants of the NADPH: quinone oxidoreductase activity of the liver ζ-crystallin. These values are very similar to those reported for camel lens ζ-crystallin[17]. The ratio of V_{max}/Km was taken as an indicator of catalytic efficiency. tested, wit] phenantherenequinone was the substrate of choice since the background NADPH oxidation was very low. The enzymatic activity followed Michaelis-Menten kinetics, the Km with 9, 10-phenantherene-quinone being 13.3 μM, a value slightly lower than that of camel lens ζ-crystallin (17.0 μM). As was the case with lens ζ-crystallin were found to be the best substrates among the quinones h 1, 2-naphthoquinone as the most active. However, 9, 10- crystallin, the liver enzyme did not reduce menadione. Over 80% of the enzymatic activity was also inhibited by 0.2 mM of both N-ethylmaleimide and dicumarol. Similar results were reported for both guinea pig liver ζ-crystallin[16] and camel lens ζ-crystallin[17]. All kinetic characteristics and the substrate specificity of the liver enzyme were essentially identical to those of lens ζ-crystallin. The function of ζ-crystallin and the nature of its active site are yet to be fully established. However, since ζ-crystallin possesses the ability to reduce certain quinones and present in small amounts in tissues like liver and kidney, it can be suggested that this enzyme plays a very important role in cellular metabolism. In conclusion, present study report the purification of ζ-crystallin from camel liver for the first

time and demonstrated that it is physically and catalytically identical to the lens ζ-crystallin.

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