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ISOLATION AND PURIFICATION OF RIBOFLAVIN BINDING PROTIEN FROM COOT EGG-YOLK (*Fulica atra)*

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ABSTRACT: Riboflavin-binding protein (RfBP) was isolated, purified and characterized from Coot (*Fulica atra*) egg. The RfBP was purified using DEAE-Sepharose ion exchange chromatography followed by gel filtration on Sephadex G-100. The purity of the proteins was judged by SDS- PAGE. The protein migrated as a single band on SDS gel with a molecular weight of corresponding to nearly 29 Kd.
Key words: Riboflavin binding protein (RfBP), DEAE-Sepharose, Fulica atra, Egg-yolk.

INTRODUCTION

Riboflavin-binding protein (RfBP) is present in both avian egg yolk and egg white, in contrast to reptilian eggs where it is only present in the yolk [1]. It has an important role in the uptake of riboflavin and delivering it to the developing oocyte. In mutants of the domestic fowl lacking RfBP, the developing embryos die of riboflavin deficiency at around 13 days of incubation [2]. It is at this stage that a rapid increase occurs in flavin kinase activity, required for the synthesis of FMN and FAD [3]. RfBPs from domestic fowl have been purified from the sera, egg-white and egg-yolk [4, 5]. All three proteins are the product of the same gene although plasma and egg-yolk RfBP are synthesized in the liver, and egg white RfBP in the oviduct. All three show polymorphism at position 14. Egg-yolk RfBP differs from the other two in having 11-13 fewer amino acids residues. When the plasma RfBP is taken up into the egg yolk the *C*-terminal peptide (11/13 residues) is cleaved. There are also some difference in the glycosylation pattern, although in all the three the oligosaccharides are linked by asparagines 36 and 147 [6].

Little detailed characterization has been carried out on any RfBPs from other avian sources. Walker *et al.,* (1991) [7] have shown that RfBP from quail egg white shows a distinct peptide map from that of domestic fowl after cleavage with thermolysin, chymotrypsin and also has differences in the tertiary structure as revealed by the near UV CD spectra, although the far UV CD spectra are similar, indicative of similar secondary structures. RfBP has been purified from duck egg white, and it is reported to have a quite distinct amino acid composition from that of the domestic fowl [8]. Duck egg-white RfBP is reported to lack praline, has a much lower proportion of methionine and arginine residues, but has higher proportions of valine, phenylalanine and histidine [8].

In contrast, the large amounts of lipids make yolk RfBP purification difficult and hence it is less thoughrly studied when compared with egg-white RfBP. The isolation of RfBP from hen egg yolk was first reported by Ostrowishi *et al.,* (1962) [9] and later by Miller *et al.,* (1981) [10] and Murthy *et al.,* (1979) [11]. Recently, the purification of pigeon egg-yolk RfBP was reported by Vijayalakshmi *et al.,* (2006) [12]. In the present study, RfBP was purified for the first time from Coot egg yolk and compared with hen egg white RfBP.

MATERIAL AND METHODS

Fulica atra eggs were procured from Mucharla lake which is located in Warangal district, Andhra Pradesh. The white and yolk were used immediately or stored at -12^{0}C. DEAE- Sepharose, Sephadex G-100 and Freund's complete adjuvant was obtained from Sigma Aldrich Chemical Company. St. Louis, USA. Bovine Serum albumin, acrylamide, N, N, N^1, N^1-Tetramethylethylene- diamine, N, N^1-methylene-bis-acrylamide and SDS were procured from Loba Chemical, Bombay, India.

Isolation and purification of Fulica atra egg yolk riboflavin binding protein (RfBP)

RfBP from *Fulica atra* egg yolk was isolated following the methods previously reported [13, 14, 15,] with a few modifications.

Fulica atra egg yolk was collected and homogenized with an equal volume of 0.1 M sodium acetate buffer (pH 4.5). To the clear supernatant DEAE- Sepharose previously equilibrated with 0.1 M Sodium acetate buffer, pH 4.5 was added. The DEAE- Sepharose with bound protein was washed with an excess of 0.1 M sodium acetate buffer, pH 4.5. Bound proteins were eluted with the same buffer containing 0.5 M sodium chloride by suction filtration. Fresh DEAE- Sepharose previously equilibrated with 0.1M sodium acetate buffer, pH 4.5 was packed into the column and then the partially purified RfBP was loaded onto the column. Riboflavin binding protein was eluted from the column with 0.1 M sodium acetate buffer, pH 4.5 containing 0.5 M sodium chloride. Fractions were collected and absorbances were measured at 280 nm and 455nm. Further purification of *Fulica atra* white RfBp was achieved by gel filtration column chromatography using Sephadex G-100.

The partially purified Coot egg-yolk RfBP was loaded onto the column previously equilibrated with 0.02M phosphate buffer, pH 7.3 containing 0.5M NaCl. The protein was eluted with the same buffer. Fractions were collected and the protein in each fraction was determined by the method of Lowrey *et al.*, (1951) [16]. Further the absorbances of each fraction were measured both at 280 nm and 455 nm using UV-Visible spectrophotometer. The peak fractions were pooled, dialyzed against distilled water and lyophilized. The purity was checked by SDS-PAGE.

SDS –PAGE

Sodium dodecyl sulphate - poly acrylamide gel electrophoresis was carried out according to the method of Leammli (1979) [17] using sodium phosphate buffer containing SDS. The following solutions were made:

1. Sodium phosphate stock buffer (pH 8.0): to 461ml of 0.2 M sodium hydroxide, 500 ml of 0.2 M sodium dihydrogen phosphate was added and made up to 1 liter with distilled water. To this 5mM EDTA and 1 gm SDS were added.
2. Electrode buffer: 500 ml of stock buffer was diluted to 1 liter with distilled water.
3. Acrylamide-bisacrylamide buffer: 30 gm of acrylamide and 0.8 gm of bisacrylamide were dissolved in 100ml of degassed water.
4. Ammonium persulphate solution: 150 mg of ammonium persulphate was dissolved in 25 ml of degassed water.
5. Sample buffer: 20ml of the electrode buffer was degassed and 600 mg of SDS added to it. To 1 ml of this buffer 30 mg SDS, 500 mg sucrose 20ml bromophenol blue were added.
6. Protein staining solution: Coomassi blue (0.2gm) was dissolved in a solution containing 50 ml of methanol, 7ml of acetic acid and 43 ml of distilled water.
7. Destaining solution: the gels were destained with the solution containing 50% methanol and 7 % acetic acid.

Gel preparation

The gels were prepared by mixing 4ml distilled water, 16ml of electrode buffer, 8ml acrylamide – bisacrylamide, 40µl TEMED and 4ml ammonium per sulphate solution.

Fulica atra egg - yolk samples were dissolved in 50µl sample buffer and kept in a boiling water bath for 2 minutes. Samples (20µl) were loaded into the slots. Initially electrophoresis was carried out at 15mA for 30 minutes after which the current was raised up to 30 mA. The plates were removed from the chamber and gel was detached by flushing distilled water between the plates. The gel was stained immediately at room temperature. Later the gel was destained using the destaining solution.

RESULTS AND DISCUSSION

In the present study it was found that better purification could be achieved by using two successive ion exchange binding steps using DEAE, Sepharose. Complete purification of Coot egg yolk RfBP was accomplished by gel filtration on sephedex G-100. The absorption spectrum of the riboflavin-apoprotein complex indicated that the flavoprotein had absorption maxima at 370 nm and 456 nm (Fig.1). The spectral data were in full agreement with that reported earlier for hen egg-white RfBP by Choi and Mc. Cormic (1980) [18].

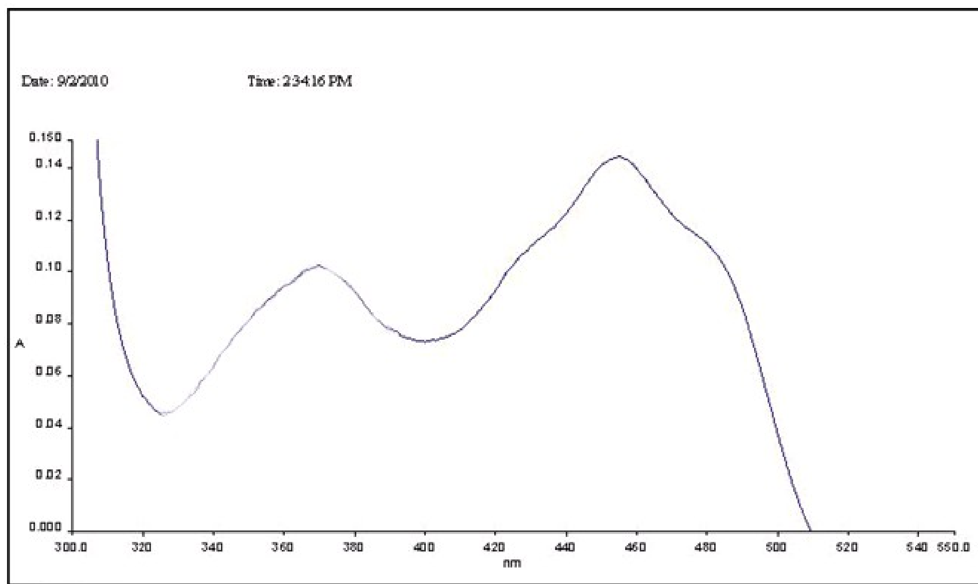

Fig- 1: Absorption Spectrum of purified Coot egg-yolk RfBP

(Sephadex G-100 fraction)

The purity of the isolated protein was judged by SDS-PAGE (Fig-2). A major band corresponding to RfBP along with a few minor bands was seen with DEAE- Sepharose column eluted fraction. Complete purification was achieved by gel filteration on Sephadex G-100, as a single band free from other contaminating proteins could be seen (Fig-2). Comparison of the mobility of Coot egg-yolk RfBP with that of the standard marker proteins revealed that the Coot egg-yolk RfBP had a molecular weight close to 29 Kd. Further, Coot egg-yolk RfBP and hen egg-white RfBP appear to have the same molecular weight, as these two proteins have the same electrophoretic mobilities on cylindrical gels (Fig-3). This suggests that, even though the two avian species (Hen, Coot) phylogenetically distinct, their RfBPs may be structurally similar.

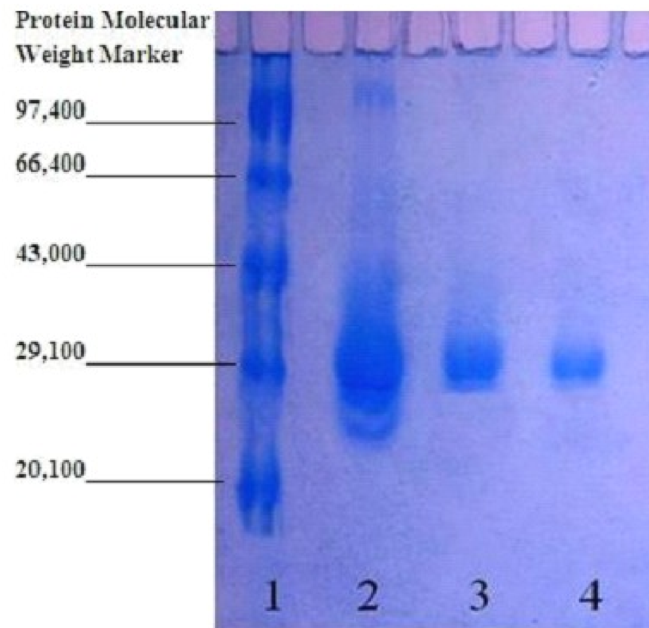

Fig-2: SDS-PAGE Pattern of coot egg-yolk RfBP

1. Protein Molecular Weight Markers (20,000 to 97,400 Da)
2. Coot egg-yolk crude homogenate
3. Coot egg-yolk RfBP DEAE-Sepharose column eluted fraction
4. Coot egg-yolk RfBP DEAE-Sephadex, G-100 fraction

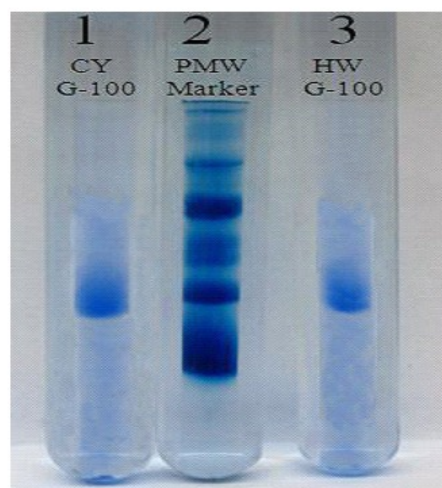

Fig- 3: Cylindrical Gel Electrophoretic pattern of the Coot RfBPs (SDS-PAGE)

1. Coot egg-yolk RfBP Sephadex G-100 fraction
2. Protein Molecular Weight Markers (20,000 to 97,400 Da)
3. Hen egg-white RfBP Sephadex G-100 fraction

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Abbreviations: RfBp-Riboflavin Binding Protein; DEAE-Diethyl Amino Ethyl; Kd-kilodalton; SDS-Sodium Dodecyl Sulfate; PAGE-Poly Acrylamide Gel Electrophoresis.

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