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Research Article

# Purification and characterization of Riboflavin carrier protein from egg yolk of South Indian spotted owlet (Athene brama)

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**ABSTRACT**

Riboflavin carrier protein has been isolated and purified from the Indian spotted owlet (Athene brama). Purification could be achieved by DEAE-Sepharose column chromatography and gel filtration chromatography on Sephadex G-100. Further the RCP was immunologically characterized and compared with the hen (Gallus gallus domesticus) egg yolk RCP (RfBP). The protein was characterized using absorption, fluorescence and CD spectral analysis. Comparison of the mobility of the purified proteins with the standard molecular weight marker proteins revealed that the spotted Owlet egg RCP had a molecular weight close to 29.2kDa and it was approximately 3 kDa less than the hen egg Yolk RCP.

**Keywords:** Spotted Owlet Eggs Yolk; DEAE-Sepharose; Sephdex G-100; fluorescence spectra; Circular dichroism (CD) spectroscopy

## INTRODUCTION

Normal fetal development requires adequate amounts of riboflavin; hence a specific carrier system of RCP or Riboflavin binding protein (RfBP) has evolved for the developing embryo. Antibodies against chicken RfBP caused termination of pregnancy in rats demonstrating the essential role of RfBP in the survival of the fetus (Krishnamurty *et al*., 1984), mice (Natraj *et al*., 1987) and the bonnet monkey (Visweswariah and Adiga, 1987). Increased levels of RBP were found in the serum of breast cancer patients and may be useful as a marker for breast cancer (Rao et al. 1999). RBPs from reptilian (Hamajima and Ono, 1995), amphibian (Storey et al., 1999), and fish (Wang et al., 2003) eggs of Indian python, painted turtle(Abrams et al., 1988) alligator (Abrams et al., 1989) goose (Stevens et al., 1994), Japanese quail (Walker et al., 1991), duck (Muniyappa and Adiga, 1980), and peacock (Rajender et al., 2007) Egg White Of Emu (*Dromaius novaehollandiae*) (Bindu et al., 2010) Hen (*Gallus gallus*) and Coot Egg-Yolk (*Fulica atra)* ( Rao et al.,, 2011 & 2012) have been purified and characterized. South Indian *Spotted Owlet* Egg White RCP was purified by Kudle et al., 2012. In continuation of this work, RCP was purified from egg yolk and the results are analyzed.

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## MATERIALS AND METHODS

South Indian Spotted Owl eggs were procured from Old City of Hyderabad, Andhra Pradesh. The yolk and white were used immediately or stored at -120C. DEAE- Sepharose and Sephadex G-100 were obtained from Sigma Aldrich Chemical Company. St. Louis, USA. Riboflavin binding protein from Owlet yolk was isolated by the following methods of Farell *et al*. 1969. SDS-PAGE was carried out according to the method of Leammli, 1979 using sodium phosphate buffer containing SDS. Protein content was estimated by the method of Lowry 1951. Antibodies against spotted Owlet and Hen egg white RCPs were produced adopting the method of Prasad and Adiga 1979. Ouchterlony double diffusion analysis was used for testing the presence of antibodies to the serum. Ouchterlony double diffusion analysis was carried out as follows: Agarose plates (1.2%) were prepared in 0.05M sodium phosphate buffer pH 7.8, containing 0.9% NaCl. The antiserum was placed in the central well and the proteins dissolved in the same buffer were placed in the adjacent wells. The appearance of precipitin white coloured lines indicated the presence of specific antibodies.

### Results and Discussion

The avian egg proteins have been studied thoroughly by many researchers. Most of these proteins serve the basic function of supplying adequate nutrients to the embryo, while some function as protease inhibitors; however, their physiological roles are not well understood. Among these proteins, the vitamin binding proteins from eggs have been extensively studied and their roles have been assigned with reasonable clarity. These include proteins like avidin, thiamin binding protein and riboflavin binding protein (or) RCP. This class

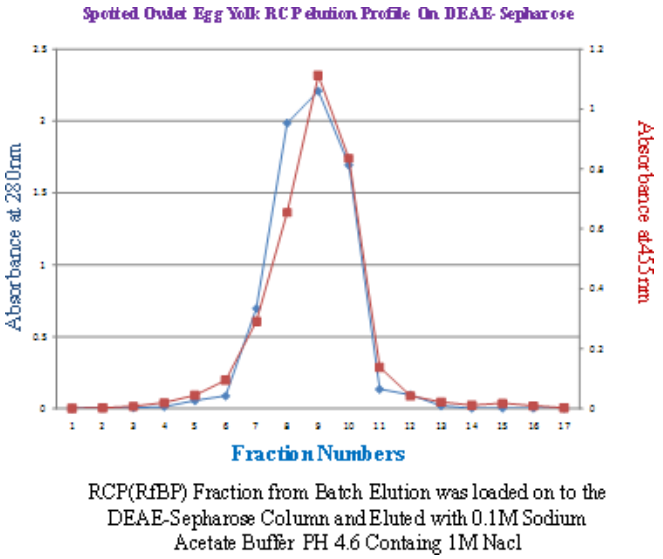


**Figure 1: Spotted Owlet egg RCP (RfBP) elution profile on DEAE-sepharose and study absorbance at 280nm and 455nm**

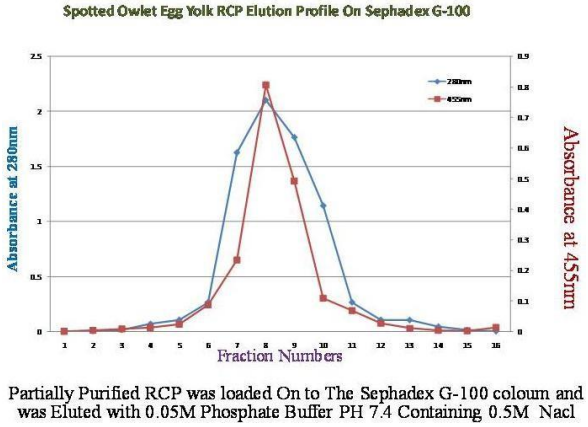


**Figure 2: Partial purified spotted Owlet RCP(RFBP) elution profile on Sephadex G-100 and study absorbance at 280nm and 455nm**

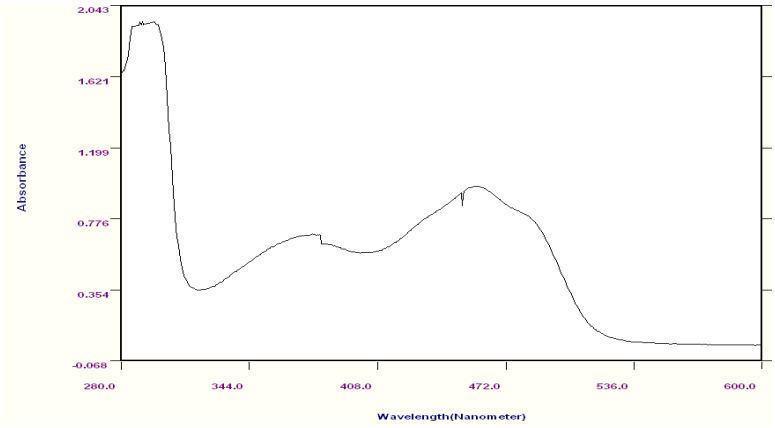


**Figure 3: Fraction absorbance Spectrum of SPOTTED OWLET Egg -Yolk RCP (Sephadex G-100 Fraction)**

of proteins is believed to be important in maintaining the supply of vitamins to the developing embryo (White, 1987; 1988). The essential role of RfBP has been demonstrated from a study on the homozygous recessive mutant (rd rd) of the domestic fowl (Winter et al., 1967). Riboflavin deficiency in developing embryos with this genetic constitution dies at around the13$^{th}$ day of incubation.

In the present study for the first time RCP was purified from Owlet egg yolk. The isolation of RCP, purification and characterization of the flavoprotein apoprotein system of chicken egg white was first reported by Rhodes et al., 1958 & 1959. Since then, several variations in the isolation procedures were based on the tight binding with the protein to DEAE-Cellulose at pH 4.3. The apoprotein was isolated using either CM-

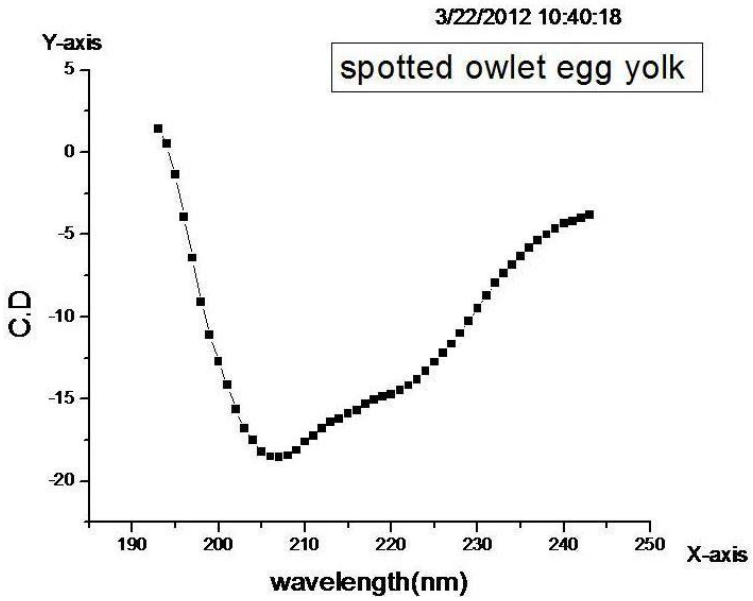


**Figure 4: Far U.V.C.D Spectra of Riboflavin Carrier protein from and spotted owlet egg yolk**

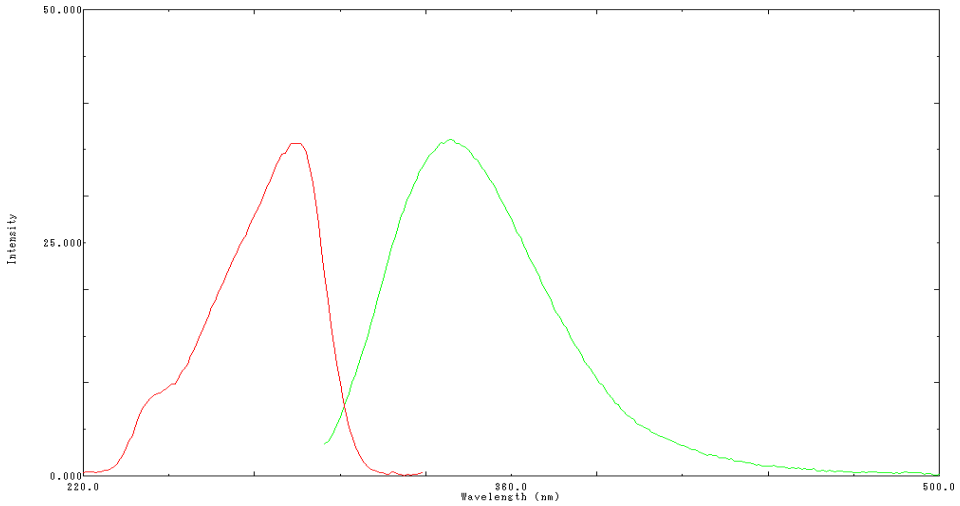


**Figure 5: Spotted owlet Egg Yolk RCP Florocesence spectra red colour line indicate excitation and green line called as Emission**

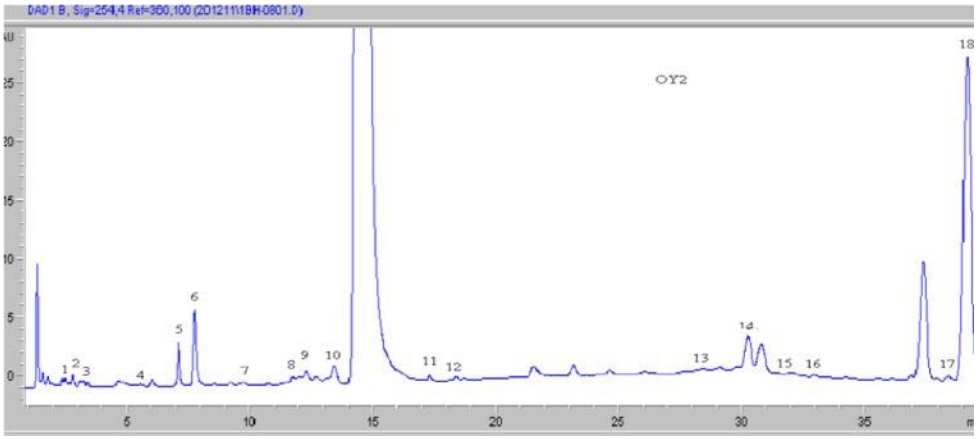


**Figure 6: Amino acid content of purified spotted owlet Egg yolk riboflavin carrier protein**

Cellulose or DE-Sephadex A-50 column chromatography at a pH of 3.8. (Hamazume et al., 1984) isolated RfBPs from hen eggs at pH 5.5 using DEAE-Sephadex and gel filtration chromatography. Purification of Riboflavin carrier protein was achieved successfully by Sepharose column chromatography and gel filtration. The protein was subjected to Ion-exchange column chromatography through a DEAE-Sepharose column as described earlier under Materials and Methods. The bound protein was eluted with 0.1M sodium acetate

**Table 1: Concentration (pmol) of various amino acids present in egg yolk**

| Sl.No. | Name of Amino acid | Sample Conc. (pmol) |
|---|---|---|
| 1 | P. Serine | 0.004248736 |
| 2 | Aspartic acid | 0.004533489 |
| 3 | Glutamic acid | 0.002544961 |
| 4 | Amino adipic acid | 0.001216168 |
| 5 | Serine | 0.02919648 |
| 6 | Glycine | 0.059356004 |
| 7 | Taurine | 0.003388527 |
| 8 | Alanine | 0.009550426 |
| 9 | β-amino butyric acid | 0.007977644 |
| 10 | Proline | 0.029209495 |
| 11 | Carnosine | 0.003397737 |
| 12 | Arginine | 0.002601288 |
| 13 | Tyrosine | 0.02451368 |
| 14 | Valine | 0.072725779 |
| 15 | Methionine | 0.0170351 |
| 16 | Cystathionine | 0.017435622 |
| 17 | Cysteine | 0.005991033 |
| 18 | Isoleucine | 0.524874663 |

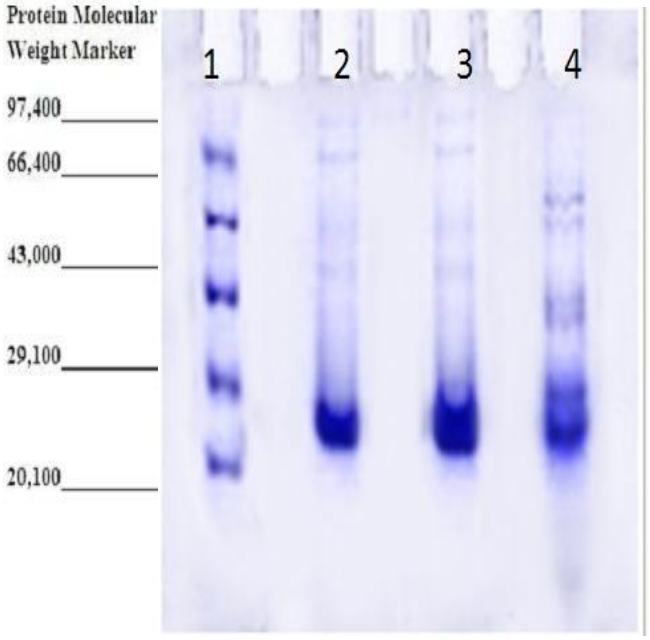


**Figure 7: SDS-page pattern of spotted owlet egg yolk ribofliven carrier protein**

1. Protein Molecular Weight Marker (20,000 to 97, 400Da); 2. Spotted Owlet egg-yolk RCP DEAE-Sephadex, G-100 Fraction; 3.Spotted Owlet egg-yolk RCP DEAE-Sepharose Column elution Fraction; 4. Spotted Owlet egg-yolk Crude homogenate

buffer, pH 4.6, containing 0.5M sodium chloride. Seventeen fractions of 2ml each was collected. Protein concentration is shown in Figure.1. The protein in each tube was also estimated by the method of Lowry et al., 1951. The peak fraction was dialyzed and lyophilized and the protein was further purified by gel filtration on Sephadex G-100. Elution of the column was done with sodium phosphate buffer at pH 7.4 and sixteen fractions (2ml each) were collected and the absorbance was recorded at both 280nm and 455nm (Figure 2). The protein in each tube was also estimated. The fractions having high absorbance at both 280nm & 455nm were pooled and take Absorbance spectra record 280nm to 600nm RCP absorbance peak at 458nm (Figure 3). Dialyzed against distilled water and lyophilized.

The visible absorption spectra revealed that the RfBPs & RCP isolated had absorption maxima at 376 and 459nm, characteristic of riboflavin-apoprotein forms (holoprotein). The free riboflavin showed absorption maxima at 363 and 446 nm. Binding of riboflavin to the protein (holoprotein) resulted in the shift of the absorption peak at 446 to 459 nm. At the same time the absorption at 366 nm showed remarkable hypochromism without a shift of band position. The spectral

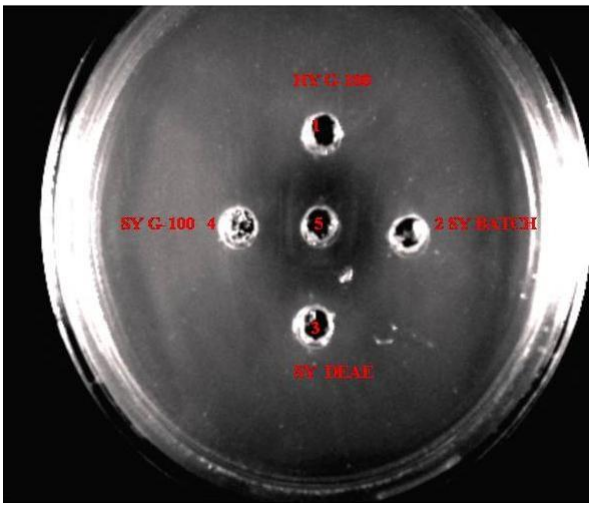


**Figure 8: Ouchterlony double diffusion analysis of Spotted Owlet RCP (Agarose) (The central well Contain Spotted Egg –Yolk RCP antiserum)**

1. Purified Hen egg yolk RCP (SephadexG-100); 2. Crude Spotted egg Yolk homogenate (Batch); 3. Partially Purified Spotted Owlet egg yolk RCP (DEAE Sepharose Fraction); 4. Purified Spotted Owlet egg yolk RCP (Sephadex G-100); 5. Spotted egg yolk RCP antiserum

changes were seen at 450nm as a red shift. The fact that the 370 nm band of the flavin did not shift to any significant extent when the flavin combined in the protein indicated the concomitant involvement of a hydrophilic or polar interaction. Exactly similar spectral data were reported earlier for hen egg white RfBP (Rhodes et al., 1958 and Choi & Mc Cormick, 1980). Far U.V.C.D spectrum (198 to 250 nm) of Owlet egg yolk RCP. It has a sharp minimum at 206 nm and a shoulder at 210nm. The near U.V.C.D of Owlet egg yolk RCP(Figure.4). Fluorescence spectra recorded, which showed a maximum at 340nm revealing the presence of the protein (Figure 5).

The amino acid analysis of the isolated pure Owlet egg yolk RCP was analyzed on a Beckman HPLC amino acid analyser (Figure 6). The amino acid composition of duck egg white RfBP was initially reported by Muniyappa and Adiga 1980. They found significant differences in the amino acid composition when compared with hen egg white RfBP. The amino acid analysis of quail egg white RfBP revealed close similarities not only between quail egg yolk RfBP but also between quail RfBPs and Hen RfBPs. In the present study, it was observed that the amino acid composition of Owlet egg showed the presence of higher amounts of leucine (Table 1). However final purification was achieved by gel filtration chromatography. SDS-PAGE analysis resolved the protein into two bands that could be due to micro heterogeneity of this glycoprotein. A comparison of the electrophoretic mobility's revealed that the Spotted Owlet egg yolk RCP (0r) RfBP had a molecular weight of approximately 27,000Da (Figure.7). In addition comparison with the SDS-PAGE pattern of hen egg yolk RCP and Spotted egg yolk clearly indicated that the Spotted Owlet RCP was approximately 3 kDa less than hen RCP (or) RfBP. This is the first time purification of RCP from Spotted Owlet eggs was investigated. It is to be noted that this range of molecular weights has been reported for other species like turtles and alligators and is unusual in the avians. Freund's adjuvants are used to produce water-in-oil emulsions, which stimulate high and long-lasting antibody responses. Ouchterlony double diffusion analysis was performed using the antiserum raised against spotted Owlet yolk and hen egg yolk RCP (or) RfBPs. The antiserum was placed in the central well and the purified and partially purified proteins (RCP) were placed in the adjacent wells. The antiserum raised against purified spotted owlet egg yolk RCP could show clear immunological cross reactivity with (1) Purified Spotted egg RCP (Sephadex G-100 fraction) (2) partially purified spotted egg RCP (DEAE-Sephadex fraction) and (3) crude spotted egg yolk RCP. However, the antisera failed to cross react with hen egg-yolk RCP (Figure.8).

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