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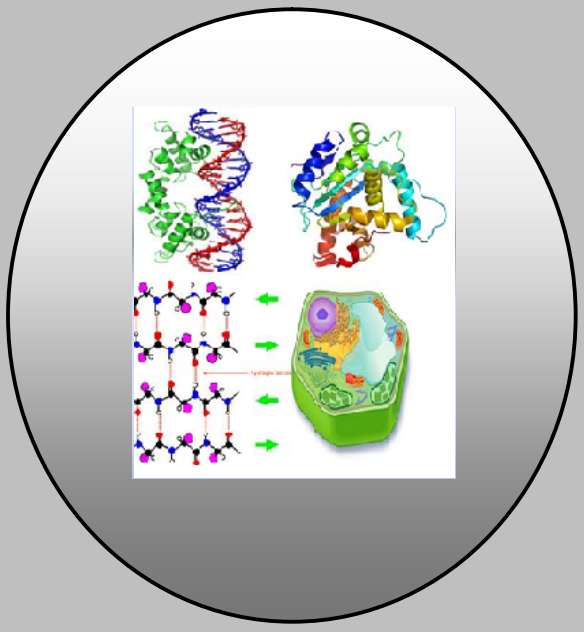

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RESEARCH PAPER

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Isolation and Immunological Characterization of Riboflavin Binding Protein from the Eggs of Coot (*Fulica atra*) and Hen (*Gallus gallus*)

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ABSTRACT

Riboflavin binding protein (RfBP) was isolated from the eggs of Fulica atra and Gallus gallus. The protein was purified in two steps, DEAE- Sepharose ion exchange chromatography followed by gel filteration on Sephadex G-100. The holoprotein had an absorption spectrum characteristic of flavoproteins. The purity of the protein was judged by SDS-PAGE technique. A single band on the slab and cylindrical gels revealed that the protein was pure. Comparison of the mobility of RfBP with that of the standard molecular weight marker proteins suggested that RfBP from the egg white and yolk of Fulica atra had a molecular weight close to 29 Kd. Ouchterlony double diffusion analysis revealed that the antibodies raised against Coot egg RfBPs could cross react with RfBPs isolated from Hen eggs, suggesting that the RfBPs from these two birds were immunologically identical.
Keywords: Riboflavin Binding Protein (RfBP), DEAE-Sepharose, Electrophoretic characterization, Molecular weight, Immunodiffusion analysis.

INTRODUCTION

Animal are incapable of de novo synthesis of the isoalloxanine skeleton of riboflavin and hence the vitamin should be supplemented in the diet (Dadd, 1985). Fuether it was discovered that the riboflavin binding protein which ensure deposition of adequate amount of the vitamins in the avian eggs was indispensable for a normal hatch of the fertilized eggs. Inadequate deposition of the vitamin in the eggs due to a splice mutation of riboflavin binding protein gene led to the embryonic mortality. (Mac Lachlan et. al, 1993).

In addition, the demonstration that immunoneutralization of riboflavin binding protein result in the abrupt termination of pregnancy in animals such as rats and monkeys, (Adiga et. al., 1988) clearly establish the functional significance of this vitamin binding /carrier protein. Additional functions of RfBP include storage transport and prevention of rapid loss of this vital nutrient due to excretion and /or metabolic degradation.

In the present study RfBP was isolated from the egg white and egg yolk of Hen (*Gallus gallus*) and Coot (*Fulica atra*) for a comparative study of the molecular characteristics such as molecular weight and immunological properties of this highly evolutionarly conserved protein. *Fulica atra* belongs to Rallidae family, Gruci forms and can be seen swimming on open water or walking across waterside grasslands. The Indian Coot is 32-42cm long and weighs around 585 to 1100 grms. It is largely black, aggressive and strongly territorial.

MATERIAL AND METHODS

Fulica atra eggs were collected from Nagaram Lake which is located in Warangal district, Andhra Pradesh, and Hen eggs were procured from local shayampet Hanamkonda. The white and yolk were separated and used immediately or stored at -12^{0}C. DEAE- Sepharose and Sephadex G-100 were 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 riboflavin binding protein (RfBP)

RfBPs from *Coot* egg white and yolk were isolated following the methods previously reported with a few modifications. *Coot* egg white or 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, to remove the unbound proteins. 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 for proteins and 455nm for bound riboflavin. Further purification of *Coot* egg white and yolk RfBPs were achieved by gel filtration column chromatography using Sephadex G-100. The almost pure *Coot* egg white RfBP was loaded onto the column previously equilibrated with 0.02 M phosphate buffer pH 7.3 containing 0.5 M sodium chloride and immediately eluted with the same buffer. Fractions were collected and the protein in each fraction was determined by the method of Lowry et.al. (1951).

The same steps were followed for purification of RfBP from *Hen (Gallus gallus*) egg white and yolk.SDS-PAGE on cylindrical and slab gels were carried out as described earlier (Madhukar Rao and Prasad, 2011, Madhukar Rao and Prasad, 2012a, 2012b) following the method of Lemmli (1979).

SDS– PAGE SILVER STAINING

The proteins were also stained by the silver staining method to obtain a more sensitive image of the protein bands. A Silver staining kit (Bangalore Genei Ltd.) was used and the following reagents were prepared.

Reagent preparation

1) Fixing solution: 25ml of the given fixing solution was made up to 50ml with distilled water.

2) Sensitizing solution: 14 ml of 25% Glutaraldehyde, 1ml of 10% sodium thiosulphate and 11.6ml of 10% sodium acetate were made up to 50ml with distilled water.

3) Silver solution: 620µl of 20% Silver Nitrate and 20µl 37% formaldehyde were made up to 50ml with distilled water.

4) Developing solution: 12.6ml of sodium carbonate and 20µl of 37% formaldehyde were mixed and made up to 50 ml.

5) Stop solution: 5ml of the stop solution was made up to 50 ml with distilled water.

RESULTS AND DISCUSSION

The electrophoretic pattern obtained on the cylindrical gels (Fig .1) and slab gels (Fig.2) using purified *Coot* egg white RfBP and Hen egg white RfBP along with protein molecular weight markers revealed that the RfBPs from both the bird eggs had a molecular weight close to 29 kDa. Similarly, the mobilities of RfBPs isolated from *Coot* egg yolk and *Hen* egg yolk on both cylindrical (Fig.3) and slab gels (Fig.4) clearly indicated that these RfBPs also had a molecular weight close to 29kDa. Further, silver staining of purified RfBPs from *Coot* egg white & yolk as well as Hen egg white and yolk after SDS- PAGE on slab gels established that these RfBPs had similar electrophoretic mobilityes having the same molecular weight of 29kDa. (Fig. 5).

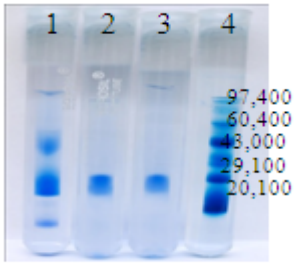

Fig.1. Cylindrical Gel Electrophoresis (SDS Polyacrylamide) pattern of Coot egg white and Hen egg white RfBPs
1.Coot egg white crude
2.Coot eggwhite G-100 eluted fraction
3.Hen egg white G-100 eluted fraction
4.Protein Molecular weight marker(20.100to97.400Kd.)

The absorption spectra of the isolated pure *Coot* egg white and egg yolk RfBPs were shown in Figs.6 and 7. These proteins showed absorption maxima at 375 nm and 458nm, characteristic for flavoprotein as reported earlier (Choi, Mc Comic, 1980).

The Ouchterlony immunodiffusion analysis revealed that the antibodies raised against Coot egg white and yolk RfBPs could cross react with *Coot* egg white and yolk RfBPs (Figs. 8 and 9). Further the antibodies could also show cross reactivity with Hen egg white and yolk RfBPs (figs. 8 and 9).

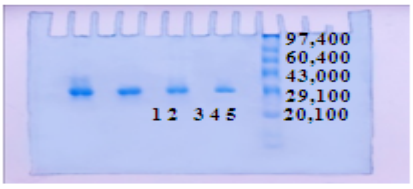

Fig. 2. SDS Polyacrylamide Gel Electrophores pattern of Coot egg white and Hen egg white RfBPs
1. Coot egg white DEAE Sepharose eluted fraction
2. Coot egg white Sephadex G-100 fraction
3 .Hen egg white DEAE Sepharoseeluted fraction
4. Hen egg white Sephadex G-100 fraction
5. Protein molecular weight marker (20,100to 97,400)

The present study clearly showed that the RfBP isolated from egg white and egg yolk of the flying and water born bird, Fulica *atra* (*Coot*) had an electrophoretic mobility similar to that of the RfBP isolated from the non flying bird, the *Hen* egg white and egg yolk, having a molecular weight close to 29kDa.

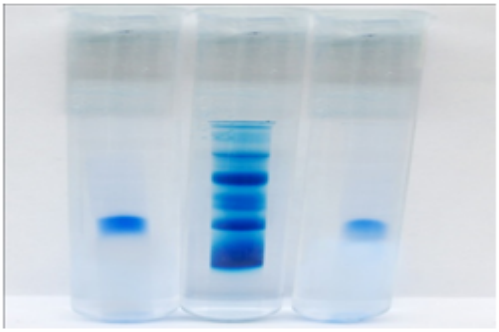

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Fig. 3. Cylindrical Gel Electrophoresis (SDS Polyacrylamide pattern of Coot egg yolk and Hen egg yolk RfBPs
1.Coot egg yolk G-100 eluted fraction
2. Protein molecular weight marker (20,100to 97,400)
3.Hen egg yolk G-100 eluted fraction

Further, studies on the immunological characterization of the RfBP from the eggs of Coot and from the eggs of Hen revealed that the two RfBPs were immunologically identical as immunological cross reactivity could be demonstrated in Ouchterlony double diffusion analysis.

These finding indicated that the RfBP remained unaltered and conserved in these bird eggs, though they appeared evolutionarily distinct.

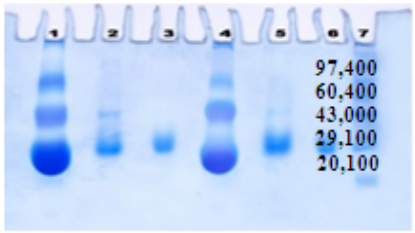

Fig. 4. SDS Polyacrylamide Gel Electrophoresis pattern of Coot egg white and egg yolkn RfBPs
1. Coot egg white crude
2. Coot egg white DEAE Sepharose eluted fraction
3. Coot egg white Sephadex G-100 fraction
4. Coot egg yolk crude
5. Coot egg yolk DEAE Sepharose eluted fraction
6. Coot egg yolk Sephadex G-100 fraction
7. Protein molecular weight marker (20,100to97,400kD)

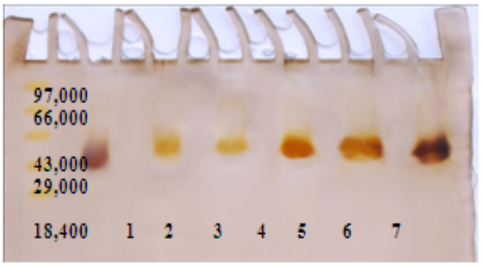

Fig. 5. Siliver staining on SDS Polyacrylamide Gel Electrophoresis pattern of Coot egg white and yolk RfBPs
1.Protein molecular weight marker (18,400 to 97,000Kd)
2.Coot egg white DEAE Sepharose eluted fraction
3.Coot egg white Sephadex G-100 fraction
4.Coot egg yolk Sephadex G-100 fraction
5.Coot egg yolk DEAE Sepharose eluted fraction
6. Hen egg white Sephadex G-100
7. Hen egg yolk Sephadex G-100

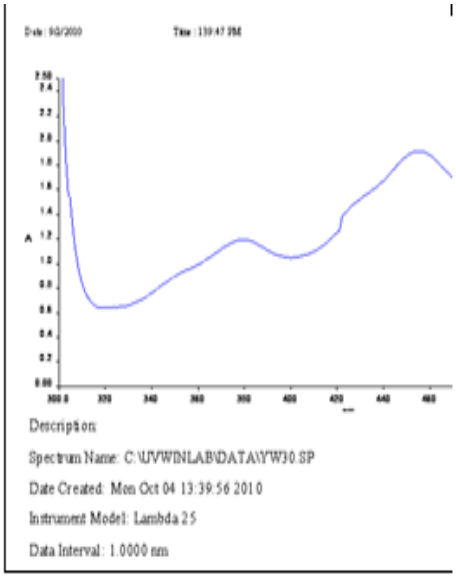

Fig. 6. Absorption Spectrum of Coot egg white Coot egg Spectrum of Riboflavin Binding Protein (Sephadex G-100).

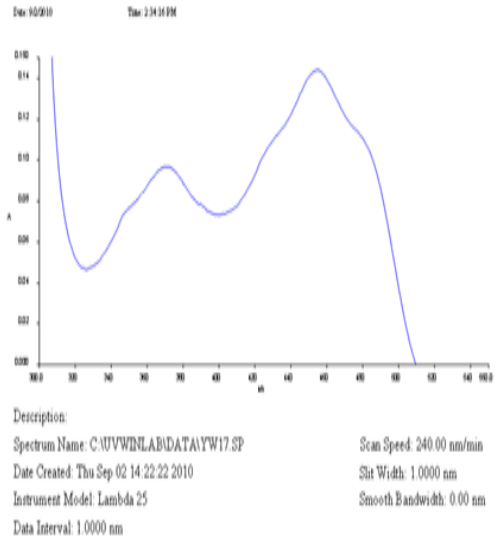

Fig.7. Abosorption Yolk Ribofavin Binding Protein (Sephadex G-100).

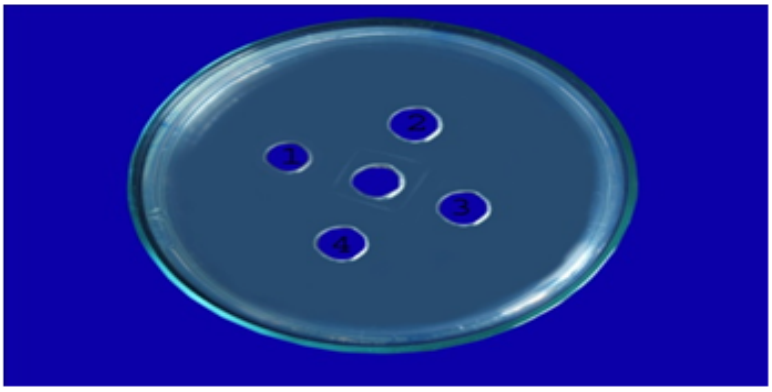

Fig. 8. Ouchterlony double diffusion analysis
(The central well contains Coot egg white antiserum)

1. **Purified Coot egg white RfBP Sephadex** G100 fraction,
2. **Purified Coot egg yolk RfBP Sephadex G100 fraction,**
3. **Purified Hen egg yolk RfBP SephadexG100 fraction,**
4. **Purified Hen egg white RfBP SephadexG100 fraction.**

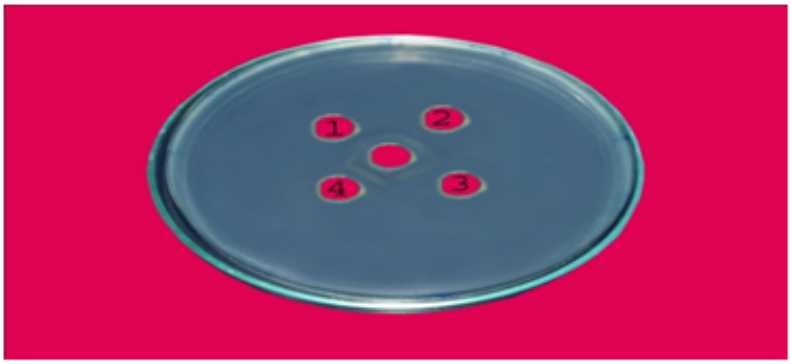

Fig. 9. Ouchterlony double diffusion analyses
(The central well contain Coot egg yolk antiserum)

1. **Purified Coot egg white RfBP DEAE Sepharose fraction,**
2. **Purified Coot egg yolk RfBP DEAE Sepharose fraction,**
3. **Purified Hen egg yolk RfBP DEAE Sepharose fraction,**
4. **Purified Hen egg white RfBP DEAE Sepharose fraction.**

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