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# PURIFICATION AND IMMUNOLOGICAL CHARACTERIZATION OF RIBOFLAVIN BINDING PROTEIN (RFBP) FROM DIFFERENT AVIAN EGGS

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## ABSTRACT

Riboflavin binding protein (RfBP) was isolated first time in India from peacock eggs (*Pavo cristatus), Hen eggs* (Gallus gallus). The Rfbp was purified in two steps, DEAE-Sephadex A-50 ion exchange chromatography. The final purification of proteins (Rfbp) was achieved on sephadex G-100.The protein content was estimated with Lowry method. The purity of the proteins was judged by cylindrical and slab–gels, SDS-PAGE techniques. These proteins showed a single band on SDS gels and the molecular weight was 30 Kilodaltons. Antiserum was raised against these Rfbp's in rabbit. This antiserum showed Immunological cross reactivity (precipitation line) between the two compounds. This study proved that, these two birds (Peacock, Hen) are immunologically relative and phylogentically different from each other.

## KEYWORDS

Avian eggs, Rfbp, Purification, , Immunological characterization.

## INTRODUCTION

Water-soluble, yellow colored fluorescent pigments, now known to be identical or related to riboflavin (Rf), were first isolated form milk, eggs, and various animal tissues. These materials were initially named in relation to their origin were eventually recognized to be a single compound[1] which we know today as vitamin $B_2$.Riboflavin (7,8-dimethyl-10-(1`-D-ribityl isoalloxazine) in mammals is found predominantly in urine and milk it is also occurs in the eggs of reptiles and birds. All animals are incapable of synthesizing the isoalloxazine skeleton of Rf and require this vitamin in the range of 1-10 µg/g diet[2]. All flavins are 10-substituted derivatives of the isoalloxazine tricyclic ring system which is synthesized via a complex pathway from GTP[3]. The two co enzymatic derivatives of Rf, flavin mononucleotide (FMN; Rf 5`-phosphate) and flavin adenine dinucleotide (FAD; Rf 5`-adenine diphosphate) function as prosthetic groups in several mitochondrial oxidation-reduction enzymes. In most vertebrate tissues analyzed FAD predominant (ca. 75% of the total tissue flavin), followed by FMN (ca. 22%) and Rf (Ca. 2%)[4]. Conversely, mammalian[5] and avian[6] serum contains Rf as the predominant flavin with less FAD and only traces of FMN. In most instances the

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flavins are associated with specific proteins which serve a transport or sequestration function. In the case of Rf the complex is found in the blood or eggs, or in the case of FMN and FAD, as tightly bound prosthetic groups of oxidation-reduction enzymes.The specific binding proteins for fat soluble vitamins such as vitamin A and vitamin D are identified in normal serum in all vertebrates[7-10]. Binding proteins for water-soluble vitamins such as Riboflavin binding proteins[11, 12], Vitamin $B_{12}$ binding protein[13, 14], and Thiamin binding protein[15, 16] have been demonstrated in the sera and egg white and yolks of the egg laying hens. Riboflavin Binding Protein (RfBP) or Riboflavin Carrier Protein (RCP) was first isolated the chicken egg white[11]. Egg white RfBP is a phospho-glycoprotein having a molecular weight of 29,200 containing 219 amino acid residues[17]. The isolation of RfBP from egg yolk was first published[12] and improved methods were subsequently reported[18, 19]. The Riboflavin binding protein from peacock (*Pavo cristatus* ) egg-white was first isolated[20,21]and also domestic fowl (*Gallus gallus*) was purified.

As the aim of the present study was to prove the Riboflavin binding protein purification from different avian eggs and immunological characterization of the different avian Rfbp's antiserum.

## MATERIALS

Fresh hen (*Gallus gallus*) eggs were obtained from the poultry farm, Elkaturthy, Karimnagar.Peacock (*Pavo cristatus*) eggs were obtained from Vana Vignana Kendram, Warangal. DEAE-Sephadex A-50 used in the study was obtained from Pharmacia Fine Chemicals, Uppasala, (Sweden). Sephadex G-100 and Freund's complete adjuvant was obtained from Sigma-Aldrich Chemical Company, (St. Louis, USA). Bovine Serum albumin, acryl amide, N, N, $N^1$, $N^1$-Tetramethylethylene-diamine, N, $N^1$-methylene-bis-acrylamide, SDS were procured from Loba Chemical Industrial Company, Bombay, India. All other reagents used were of analytical grade. For immunological characterization rabbit obtain from University college of Pharmaceutical sciences, Kakatiya University, Warangal, Andhra Pradesh.

## METHODS

Riboflavin Binding Protein from hen egg-white was isolated by the following methods of[11, 17, 22] with a few modifications as described. Hen egg-whites were collected and homogenized with an equal volume of 0.1 M sodium acetate buffer pH 4.5. The homogenate was processed with stirring over night. To the crude yellow supernatant, DEAE-Sephadex, previously equilibrated with 0.1 M sodium acetate buffer pH 4.5, was added and stirred overnight at $4^0$C. The DEAE-Sephadex was washed extensively with 0.1 M sodium acetate buffer; pH 4.5.The bound protein was eluted with same buffer containing 0.5 M NaCl by suction filtration.

Eluted protein was loaded onto the DEAE-Sephadex column and washed with the 0.1 M sodium acetate buffer pH 4.5. The bound protein was eluted with same buffer containing 0.5 M NaCl. Fifteen fractions (10 ml each) were collected. Protein concentration in each fraction was estimated by measuring absorbance at 280 nm. **Figure: 1** shows the elution profile absorbance at 280 nm.. The absorbance at 455 **(Figure: 2)** is due to riboflavin bound to the protein. Peak fraction was dialyzed and lyophilized

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Peacock egg-white Riboflavin Binding Protein was purified to apparent homogeneity in two steps. Batch adsorption to DEAE-Sephadex and gel filtration column chromatography on Sephadex G-100 following the methods of[17, 19] with a few significant modifications as described. SDS-PAGE was carried out according to the method[23] using sodium phosphate buffer containing SDS .Protein content was estimated by the method of Lowry[24].

***Production of Antiserum to riboflavin Binding Protein (RfBP)***

Antibodies against RfBPs were produced adopting the method[25] briefly; the protein was emulsified with an equal volume of Freund's complete adjuvant (Sigma) and injected subcutaneously at weekly intervals for 4 weeks into the animals at multiple sites. The rabbits were bleed through the ear vein, 7 days after the last injection. The presence of antibodies in the serum was tested using Ouchterlony double diffusion analysis.

## RESULTS

In the present study crude egg-white solution was prepared from peacock eggs. The egg-white was processed as described under Materials and Methods. After centrifugation pale yellow egg-white solution was used for batch adsorption onto DEAE-Sephadex. The unbound protein was removed by washing the DEAE-Sephadex with excess of chilled buffer on a Buchner funnel. The bound protein was eluted with 0.1 M

Fig-1 : Hen egg white RfBP elution profile on DEAE-Sephadex absorbance at 280 nm

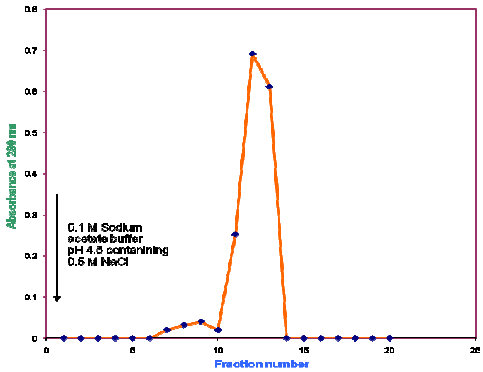
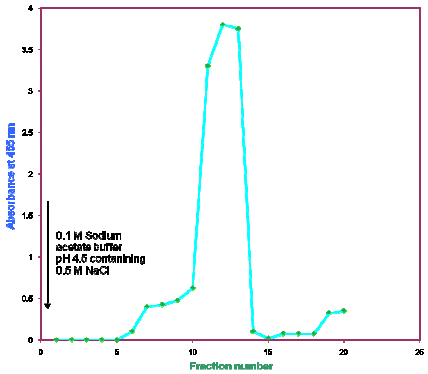



Fig - 2 : Hen egg white RfBP elution profile on DEAE-Sephadex absorbance at 455 nm




sodium acetate buffer pH 5.0 containing 0.5 M NaCl filtration by suction. The elutant was dialyzed and loaded onto a DEAE-Sephadex column. After washing the column the bound protein was eluted with 0.1 M sodium acetate buffer, pH 5.0 containing 0.5 M NaCl. Fractions (5 ml each) were collected. Protein concentrations in the elutes were estimated by measuring absorbance at $A_{280}$ using UV-visible recording spectrophotometer **Figure no:3**. Fractions eluted from column were also assayed for protein bound Riboflavin by measuring the absorbance at 455 nm. The peak fractions with the highest absorbance at 280

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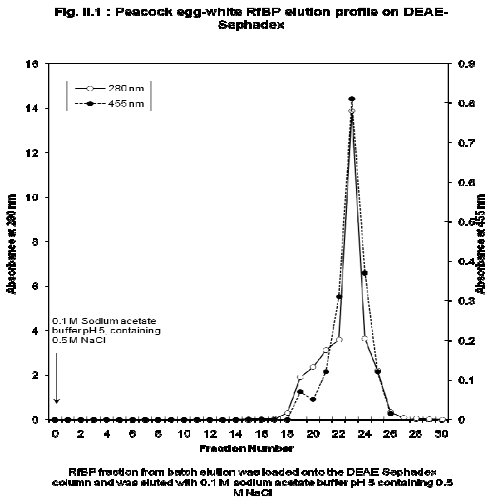
nm were pooled and dialyzed against distilled water. The protein content in all fractions was also estimated[23]. Polyacrylamide gel electrophoresis at pH 8.3 of the DEAE-Sephadex eluted protein fraction revealed the presence of contaminating proteins, suggesting partial purificationof RfB's at this stage




**Riboflavin binding protein Sephadex fraction**.

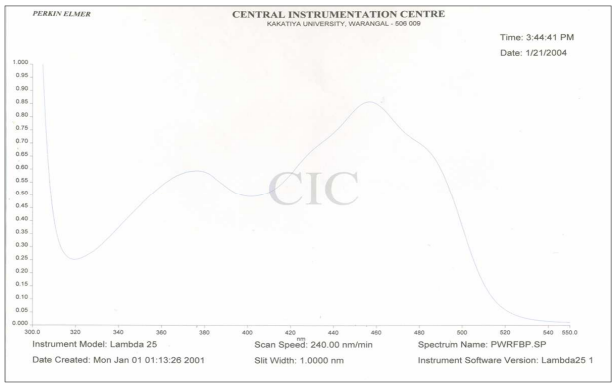



**Fig 3.*Absorption spectrum of Peacock egg white***

**Purification of Peacock (Pavo cristatus) Egg-White RfBP Using Gel –Filtration Chromatography on Sephadex G-100:**

The pooled fraction from DEAE-Sephadex were dialyzed against distilled water and lyophilized. The partially purified RfBP was dissolved in 1.0 ml of phosphate buffer and loaded on a Sephadex G-100 column (2 x 36 cm) pre-equilibrated with 0.05 M phosphate buffer, pH 7.4 containing 0.5 M NaCl. The protein was eluted with the same buffer. Twenty fractions (5 ml each) were collected. The fractions were monitored for absorbance at 280 nm and 455 nm. The protein in each tube was also estimated[23]. The elution profiles was presented in Figure: II.1. The peak fractions were yellow in color with highest absorbance at 280 nm were pooled and dialyzed against distilled water. For hen egg-white purification the same steps were followed.

**Production of Antiserium to Riboflavin binding proteins (RfBP):**

Antibodies against RfBPs were produced adopting the method[25]. The protein was emulsified with an equal volume of Freund's complete adjuant (Sigma) and injected subcutaneously at weekly intervals for 4 weeks into the animal at multiple sites. The rabbit was bleed through the ear vein, 7 days after the final injection. The presence of antibodies in the serum was tested using Ouchterlony double diffusion analysis.

Ouchterlony double diffusion analysis was carried out as follows: Agar / Agarose plates (1%)

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were prepared in 0.05M phosphate buffer, pH 7.9 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 precipitation line indicated the presence of specific antibodies. The following same steps were followed other avian eggs for protein purification and antibodies production.

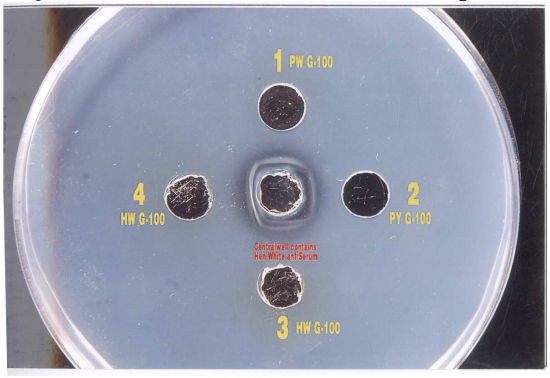


**Fig 4.** ***Ouchterlony double diffusion analysis (The central well contains hen egg-white antiserum)***

1. *Purified peacock egg-white RfBP (Sephadex G-100 fraction)*
2. *Purified peacock egg-yolk RfBP (Sephadex G-100 fraction)*
3. *Purified hen egg-white RfBP (Sephadex G-100 fraction)*
4. *Purified hen egg-white RfBP (Sephadex G-100 fraction)*

Ouchterlony double diffusion analysis using antiserum raised against peacock egg white RfBP established the immunological cross reactivity between RfBPs isolated from peacock and hen eggs. The antiserum gave precipitin line against (1) Purified peacock egg-white RfBP, Purified hen egg-white RfBP (3, 4) (**Figure no: 4**).

## DISCUSSION

Proteins from the eggs of different avian species have been studied by many researchers; the most extensive of all was the ovomucoid[26]. In the present study, RfBP's were purified from both the hen and peacock eggs for the first time and immunological characterization was carried out, as no study was undertaken earlier on RfBP from peacock eggs.

The isolation of RfBP, purification and characterization of the flavoprotein apoprotein system of chicken egg white was first reported[11, 27] . Since then, several variations in the isolation procedures were based on the tight binding of the protein to DEAE-cellulose at pH 4.3. The apoprotein was isolated using either CM-cellulose or SE-Sephadex A-50 column chromatography at a pH of 3.8. Later[17] isolated the hen egg-white and egg-yolk RfBPs using DEAE-Sephadex at a pH of 5.5, followed by gel

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filtration chromatography on Sephadex G-100.Immunodiffusion studies were carried out to determine whether there was immunological cross-reactivity between (1) Peacock egg-white RfBP and hen egg-white RfBP (2) Peacock egg-white RfBP and Hen egg-white, RfBP.

The antiserum produced an immunoprecipitation line when allowed to diffuse against peacock egg-white crude homogenate, partially purified peacock egg-white RfBP and purified peacock egg-white RfBP.These results indicate that hen RfBP of Hen(Gallus gallus) egg-white and peacock (Pavo cristatus) egg-white RfBP's are immunologically relative and phylogentically different from each other.

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