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A docile method of isolation and purification of riboflavin binding protein (Rfbp) from peahen (*Pavo cristatus*) and domestic fowl (*Gallus gallus*)

G. Rajender, G. Benarjee, M.S.K. Prasad, B. Laxma Reddy and B. Narayana Rao Dept of Zoology, & Dept of Bio- chemistry, Kakatiya University, Warangal-506009, India.

dr.grajender@gmail.com

Abstract: Riboflavin binding protein (Rfbp) was isolated from domestic fowl (Gallus gallus) and peahen (Pavo cristatus) egg-white and egg-yolk. The protein was purified in two steps, DEAE-Sephadex A-50 ion exchange chromatography and eluted with phosphate buffer pH 7.3 containing 0.5 M sodium chloride. The final purification of protein was achieved on Sephadex G-100. The purity of the protein was judged on cylindrical and slab gel electrophoresis, SDS-PAGE technique. Sephadex G-100 fraction Rfbp moved as a single band both on the Slab and Cylindrical gels. Comparison of the mobility of Rfbp with that of the standard molecular weight marker proteins revealed with that the Rfbp had a molecular weight close to 29,000 kd. Interestingly, hen egg-white Rfbp and peahen egg- white, yolk Rfbp had the same molecular weight as revealed by the SDS-PAGE. This is a novel approach for the study of riboflavin binding protein purified from different avian eggs in two steps and studied electrophoretic characterization with standard molecular weight marker.

Keywords: Rfbp purification method, peahen, hen, egg white-yolk, SDS, PAGE/Native.

Introduction

Water-soluble, yellow fluorescent pigments, now known as 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 (vitamin G; Booher, 1933) which we know today as vitamin B₂. All animals are incapable of synthesizing the isoalloxazine skeleton of Rf and require this vitamin in the range of 1-10 μ g/g diet (Dadd, 1985). All flavins are 10-substituted derivatives of the isoalloxazine tricyclic ring system which is synthesized via a complex pathway from GTP (Young, 1986). Riboflavin (7, 8-dimethyl-10-(1`-D-ribityl isoalloxazine) in mammals is found predominantly in urine and milk, and it is also occurs in the eggs of reptiles and birds. The two coenzymatic 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%; Yagi, 1971). Conversely, mammalian (Burch et al., 1948) and avian (Common et al., 1947) serum contains Rf as the predominant flavin with less FAD and only traces of FMN.

The specific binding proteins for fat soluble vitamins such as vitamin A and vitamin D are identified in normal serum in all vertebrates (Edelstein *et al.*, 1973; Abe *et al.*, 1975; Thomas Jr. *et al.*, 1959; Kanai *et al.*, 1968). Binding proteins for water-soluble vitamins such as Riboflavin binding proteins (Rhodes *et al.*, 1959; Ostrowski *et al.*, 1962), Vitamin B_{12} binding protein (Grasbeck, 1969, Sonneborn *et al.*, 1970), and Thiamin binding protein (Naber *et al.*, 1954; Coates, 1971) have been demonstrated in the sera and egg white and yolk of the egg laying hens. Riboflavin Binding Protein (Rfbp) or Riboflavin Carrier Protein (RCP) was first isolated from the chicken egg white (Rhodes *et al.*, 1959). Rfbp from Peahen (*Pavo cristatus*) egg-white was first isolated and characterized (Rajender *et al.*, 2007).

The essential role of Rfbp has been demonstrated from a study of the homozygous recessive mutant (rd rd) of domestic fowl (Winter *et al.*, 1967). Developing embryos having this genetic constitution die at around 13 days of incubation, from riboflavin deficiency. Subsequently, it was shown that the homozygous recessive (rd rd) hens were unable to synthesize riboflavin binding protein (Winter *et al.*, 1967).

The aim of the present study was to purify Riboflavin binding protein from different avian eggs- hen *(G. gallus)* and Peahen (*P. cristatus*) and also to compare with standard molecular weight markers in SDS PAGE/native electrophoresis pattern.

Materials and methods

Fresh hen (Gallus gallus) eggs were obtained from the poultry farm, Gopalapuram, Warangal (A.P) Peahen (Pavo cristatus) eggs were obtained from Vana Vignana Kendram, Warangal (A.P0). DEAE-Sephadex A-50 used in the present study was obtained from Pharmacia Fine Chemicals, Uppsala, Sweden, Sephadex G-100 and Freund's complete adjuvant was obtained from Sigma-Aldrich Chemical Company, St. Louis, USA. Bovine Ν. Serum albumin. acrylamide, N. Ν'. N'-Tetramethylethylene- diamine, N, N¹-methylene-bisacrylamide, and SDS were procured from Loba Chemical, Bombay, India.

Isolation and purification of hen egg-white riboflavin binding protein (Rfbp)

Riboflavin Binding Protein (Rfbp) from hen egg-white was isolated following the methods previously reported (Rhodes *et al.*, 1959; Farrell *et al.*, 1969; Hamazume *et al.*, 1984) with a few modifications.

Hen egg white was collected and homogenized with an equal volume of 0.1 M sodium acetate buffer pH 4.5. To the clear supernatant DEAE-Sephadex previously equilibrated with 0.1M sodium acetate buffer pH 4.5 was added. The DEAE-Sephadex with bound protein was washed with excess of 0.1M 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-Sephadex previously equilibrated with 0.1M sodium acetate buffer pH 4.5 was packed into Indian Journal of Science and Technology



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the column and then the partially purified Rfbp was loaded onto the column. Riboflavin binding protein was eluted from the column with 0.1M sodium acetate buffer, pH 4.5 containing 0.5M sodium chloride. Fractions were collected and absorbance measured at 280 nm, 455nm. Further purification of hen egg white Rfbp was achieved by gel filtration column chromatography using Sephadex G-100. The column was equilibrated with 0.025 M phosphate buffer pH 7.3 containing 0.5 M sodium chloride. Protein in each fraction was determined by the method of Lowry *et al.*, (1951). The same steps were followed for purification of protein from Hen (*G. gallus*) egg-yolk and Peahen (*P. cristatus*) egg-white, yolk. *SDS-PAGE*

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

1. Sodium phosphate stock buffer (pH 8.0): To 461 ml of 0.2 M sodium hydroxide, 500 ml of 0.2 M sodium die hydrogen phosphate was added and made upto 1 lit with distilled water. To this 5 mM EDTA and 1 gm SDS were added.

2. Electrode buffer: 500 ml of stock buffer was diluted to 1 lit with distilled water.

3. Acrylamide-bisacrylamide buffer: 30 gm of acrylamide and 0.8 gm of bisacrylamide were dissolved in 100 ml of degassed water.

4. Ammonium persulphate solution: 150 mg of ammonium persulphate was dissolved in 25 ml of degassed water.

5. Sample buffer: 20 ml 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, 20μ l bromophenol blue was added.

6. Protein staining solution: Coomassi blue (0.2 gm) was dissolved in a solution containing 50 ml of methanol, 7 ml 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. *Cylindrical gels*

The gels were prepared by mixing 2 ml of distilled water, 8 ml running buffer, 4 ml acrylamide/bisacrylamide solution, 20 μ l TEMED and 2 ml of ammonium persulphate solution. The samples were dissolved in 70 μ l of sample buffer and heated in a boiling water bath for 2 minutes. 50 μ l aliquot was loaded onto the gel tubes. *Slab gels*

Slab SDS-PAGE was carried out according to the method of Learnmli (1979) using Tris-glycine buffer containing SDS using the solutions described for SDS-PAGE. The gels prepared by mixing 4 ml distilled water, 16 ml of electrode buffer, 8 ml acrylamide - bisacrylamide, 40 µl TEMED and 4 ml ammonium per sulphate solution.

Peahen egg-white, yolk samples were dissolved in 50 μ l sample buffer and kept in a boiling water bath for 2 minutes. Samples (20 μ l) were loaded into the Research article "Riboflavin bin

slots.Initially electrophoresis was carried out at 15 mA 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.

Native gel electrophoresis

The following solutions were prepared for native gel electrophoresis:

1. Tris Glycine Electrode buffer pH 8.5: 0.6 g of Tris and 2.8g of Glycine was dissolved in 1 lit of distilled H_2O .The pH was adjusted to 8.3.

2. Tris HCl buffer pH 8.8 (sol. A): 36.6 g of Tris was dissolved in 50 ml of distilled H_2O and 48 ml of 1N HCl and 0.46 ml of TEMED added. The volume was made upto 100 ml with distilled water.

3. Acrylamide-bisacrylamide solution (sol. C): 30g of acrylamide and 0.8g of bisacrylamide were dissolved in 100 ml of distilled degassed water.

4. Ammonium Persulphate solution: 140 mg of ammonium persulphate was dissolved in 100 ml of distilled degassed water.

5. Protein staining solution: Coomassi blue (0.2 g) was dissolved in a solution containing 50 ml methanol, 7 ml of acetic acid and 43 ml distilled water.

6. Destaining solution: The gels were destained with the solution containing 50% methanol and 7% acetic acid. *Gel preparation*

The gels were prepared by mixing 2 ml of solution A, 4 ml of solution C, 2 ml of distilled water, 8 ml of ammonium persulphate solution. The Peahen, Hen eggwhite, yolk sample was dissolved in 50 μ l of Tris HCl buffer containing 40% sucrose and 20 μ l of bromophenol blue solution. 50 μ l of the sample was loaded on the gels. Electrophoresis was carried out at 2-5 mA / tube until the dye reached the end of the tube. This is a novel *Fig. 1. Native-PAGE gel pattern of peahen egg-white RfBP*



Peahen egg-white crude homogenate
Peahen egg-white DEAE-Sephadex batch eluted fraction
Peahen egg-white DEAE- Sephadex column eluted fraction
Peahen egg-white Sephadex G-100 fraction

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method for the purification of Rfbp binding protein in different avian eggs in Sephadex A-50, followed by Sephadex A-100.

Results

Electrophoresis on analytical polyacrylamide gels (7.5%) was conducted at pH 8.3. The purity of the isolated protein was judged by Native-PAGE. SDS-PAGE and cylindrical gels. The electrophoretic pattern obtained was shown in Fig.1, 2. A major band corresponding to Rfbp along with a few minor bands was obtained with the DEAE-Sephadex fraction. Complete purification was achieved by gel-filtration chromatography on Sephadex G-100, as single band free from other minor contaminating proteins. Rfbp moved as a single band both on the Slab and Cylindrical gels. Comparison of the mobility of Rfbp with that of the standard molecular weight marker proteins revealed that the Rfbp had a molecular weight close to 29,000 kilodaltons. Interestingly, hen eggwhite Rfbp and Peahen egg- white, yolk Rfbp had the same molecular weight as revealed by the SDS polyacrylamide gel electrophoresis.

Fig.2. SDS-PAGE electrophoresis



 Peahen egg-white Sephadex fraction.
Peahen egg-white Sephadex G-100 fraction 3. Protein molecular weight marker.

Discussion

The purified peahen egg-white Rfbp migrated as a single band during electrophoresis on SDS-PAGE. The molecular weight appeared to be nearly the same as that of hen egg-white Rfbp. This is a novel finding for the purification of riboflavin binding protein in two steps Sephadex A-50 followed G-100 and this protein was characterized by SDS/Native PAGE electrophoresis. This rfbp band showed similar to that of standard protein.

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