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Determination of Riboflavin Binding Protein (Rfbp) in SDS /Native PAGE Electrophoresis

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Abstract

Riboflavin binding protein (Rfbp) was isolated from Hen (*Gallus gallus*) and Peacock (*Pavo cristatus*) egg-white, egg-yolk. The protein was purified in two steps, DEAE-Sephadex A-50 ion exchange chromatography. The protein was eluted with phosphate buffer pH 7.3 containing 0.5 M sodium chloride. The final purification of protein was achieved on sephadex G-100. Collected Protein fractions were determined by the method of Lowry. Absorbance was measured at 280 nm and 455 nm using UV-visible spectrophotometer. The purity of the protein was judged on cylindrical and slab gel electrophoresis, SDS (Sodium do-doceyl sulphate)-PAGE (Poly acrylamide gel electrophoresis) 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 kilodaltons.Interestingly, hen egg-white Rfbp and peacock egg- white, yolk Rfbp had the same molecular weight as revealed by the SDS polyacrylamide gel electrophoresis.

Keywords: Rfbp; Peacock-hen; Egg white-yolk; SDS PAGE/Native

Introduction

Water-soluble, yellow 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 (vitamin-G;) which we know today as vitamin B_2 [1]. All animals are incapable of synthesizing the isoalloxazine skeleton of Rf and require this vitamin in the range of 1-10 mg/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]. 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%;) [4]. Conversely, mammalian and avian serum contains Rf as the predominant flavin with less FAD and only traces of FMN [5,6]. In most instances the flavins are associated with specific

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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 Vitamin B12 binding protein, and Thiamin binding protein 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 the chicken egg white. Rfbp from peacock (Pavo cristatus) egg-white was first isolated and characterized [11-17].

The essential role of Rfbp has been demonstrated from a study of the homozygous recessive mutant (rd rd) of domestic fowl. Developing embryos having this genetic constitution die at around 13 days of incubation, from riboflavin deficiency. A normal hatch was achieved only by a direct injection of riboflavin into such eggs. Subsequently, it was shown that the homozygous recessive (rd rd) hens were unable to synthesize riboflavin binding protein [18].

Present investigation of this study is to purify riboflavin binding protein from Hen (*Gallus gallus*) and peacock (*Pavo cristatus*) and 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, Peacock (*Pavo cristatus*) eggs were obtained from Vana Vignana Kendram, Warangal. 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 Serum albumin, acrylamide, N, N, N1, N1-Tetramethylethylene- diamine, N, N1-methylene-bis-acrylamide, and SDS were procured from Loba Chemical Industrial Company, Bombay, India. All other reagents used were of analytical grade.

Isolation and purification of hen egg-white Riboflavin Binding Protein (Rfbp)

Riboflavin Binding Protein (Rfbp) from hen egg-white was isolated following the methods of Rhodes et.al, (1959), Farrell et.al, (1969) and Hamazume et.al., (1984) with a few modifications [19-21].

Hen egg white was collected and homogenized with an equal volume of 0.1 M sodium acetate buffer pH 4.5. The homogenate was centrifuged at 15000 x g, for 30 minutes at 40°C. The precipitate was discarded. To the clear supernatant DEAE-Sephadex previously equilibrated with 0.1M sodium acetate buffer pH 4.5 was added. The mixture was stirred for 12 hours at 40°C and then suction filtered. The filtrate was discarded. 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. The eluted protein fraction was dialyzed against distilled water.

Fresh DEAE-Sephadex 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 0.1M sodium acetate buffer, pH 4.5 containing 0.5M sodium chloride. Fractions were collected and absorbance measured at 280 nm, 455 nm using UV-visible recording spectrophotometer. Values were expressed as total absorbance at 280 nm and 455 nm per each fraction.

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. The protein was dissolved in 1 ml of the above phosphate buffer, and loaded on the column and eluted with the starting buffer. Fractions were collected. Protein in each fraction was determined by the method of Lowry et.al., (1951) using bovine serum albumin as standard [5]. Absorbance was measured at 280 nm and 455 nm using UV-visible recording spectrophotometer. Values were expressed as total absorbance at 280 nm and 455 nm per fraction. The same steps were followed for purification of protein from Hen (Gallus gallus)

egg-yolk and Peacock (Pavo cristatus) egg-white, yolk.

Sodium Dodecyl Sulphate (SDS-Polyacrile amide gel electrophoresis-PAGE)

SDS-PAGE was carried out according to the method of Leammli (1979) using sodium phosphate buffer containing SDS [21]. The following solutions were made:

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.

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

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

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

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, 20ml bromophenol blue was added.

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.

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 ml TEMED and 2 ml of ammonium persulphate solution. The samples were dissolved in 70 □l of sample buffer and heated in a boiling water bath for 2 minutes. 50 ml of the sample was loaded onto the gel tubes. The electrophoresis was carried out at 2-5 mA/tube until the dye reached the end of the tube. The gels were removed and stained overnight with the staining solution and destained by repeatedly washing with destaining solution.

Slab gels

Slab SDS-PAGE was carried out according to the method of Leammli (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 ml TEMED and 4 ml ammonium per sulphate solution.
The prepared gel solution was poured into glass plates (14 cm × 14 cm) separated by 1mm thick spacer. The Hen, Peacock egg-white yolk samples were dissolved in 50 ml sample buffer and kept in a boiling water bath for 2 minutes. Samples (20 ml) were loaded into the slots. The remaining gap was filled with the electrode buffer. The glass plates were fixed to the electrophoresis apparatus without disturbing the samples. The upper and lower electrode chambers were filled with electrode buffer. The electrode chambers were connected to the power supply. Initially electrophoresis was carried out at 15 mA for 30 minutes after which the current was raised up to 30 mA. Current supply was terminated when the tracking dye reached the end of the gel. 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:

Tris Glycine Electrode buffer pH 8.5: 0.6 g of Tris and 2.8g of Glycine was dissolved in 1 lit of distilled H2O.The pH was adjusted to 8.3.

Tris HCl buffer pH 8.8 (sol. A): 36.6 g of Tris was dissolved in 50 ml of distilled H2O and 48 ml of 1N HCl and 0.46 ml of TEMED added. The volume was made upto 100 ml with distilled water.

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

degassed water.

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

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.

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 Peacock, Hen egg-white, yolk sample was dissolved in 50 ml of Tris HCl buffer containing 40% sucrose and 20 ml of bromophenol blue solution. 50 ml of the sample was loaded on the gels. Electrophoresis was carried out at 2 mA-5 mA / tube until the dye reached the end of the tube. The gels were stained overnight with the staining solution and destained by repeatedly washing with destaining solution as described earlier.

Results

Partial purification of hen egg-white Rfbp using DEAE-Sephadex

Hen egg-whites were collected and homogenized with an equal volume of 0.1 M sodium acetate buffer pH 4.5.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 40C. 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 filtration.

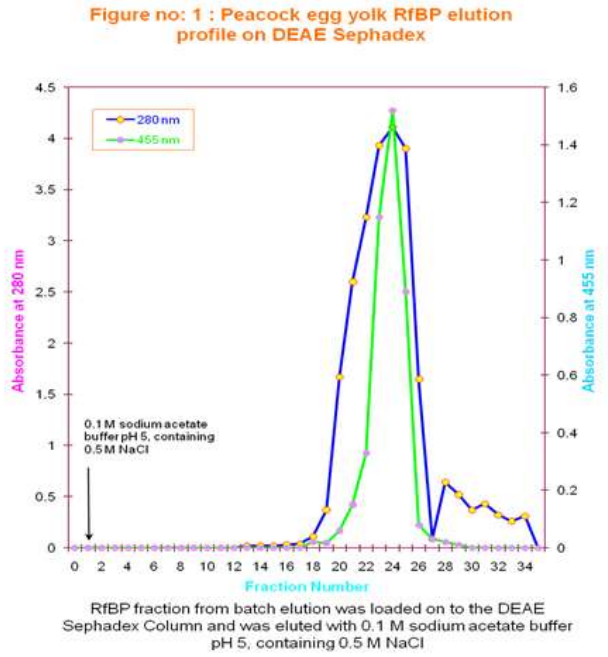

FIG.1. **Peacock egg-yolk elution profile on DEAE-Sephadex.**

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. Fractions were collected. Protein concentration in each fraction was estimated by measuring absorbance at 280 nm. The absorbance at 455 is due to riboflavin bound to the protein. Peak fraction was dialyzed and lyophilized.

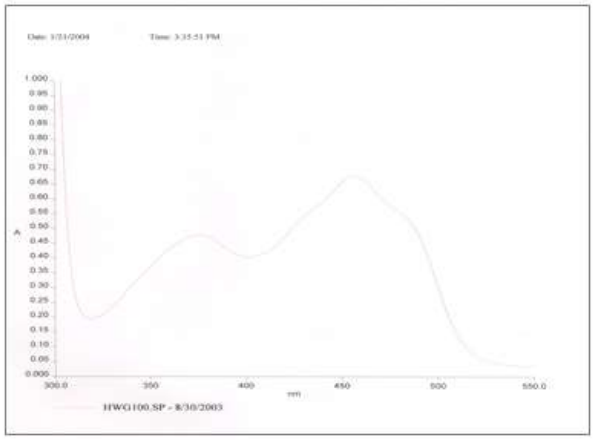

FIG.2. **Absorption spectrum of Hen egg white Riboflavin binding protein (Sephadex G-100 fraction).**

Gel filtration column chromatography

Further purification was achieved by gel filtration on Sephadex G-100. The Rfbp fraction obtained from DEAE-Sephadex was applied onto the Sephadex G-100 column equilibrated with 0.025 M phosphate buffer, pH 7.3 containing 0.5 M NaCl. The following same steps were taken for purification of Rfbp from hen and peacock egg-yolk (FIG.1.), Egg-white. The column was eluted with the same buffer, fractions were collected and the absorbance was recorded at both 280 nm and 455 nm. The fraction having high absorbance both at 280 nm and 455 nm were pooled dialyzed against distilled water and lyophilized.

Spectral studies

UV Absorption Spectra

The absorption spectra of riboflavin Sephadex G-100 fraction from hen egg-white was in FIG.2. The absorption maximum for the flavoprotein was in the spectral range of 456 nm for free riboflavin. The isolated flavoprotein absorption maxima at 376 nm and 457 nm.

SDS Polyacrylamide gel electrophoresis (SDS-PAGE)

Electrophoresis on analytical polyacrylamide gels (7.5%) were 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 3,4,5. As could be seen from Rfbp could be purified to a greater extent by employing the ion-exchange chromatography on DEAE-Sephadex, as one 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 only one 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 with that the Rfbp had a molecular weight close to 29,000 kilodaltons.Interestingly, hen egg-white Rfbp and peacock egg-white, yolk Rfbp had the same molecular weight as revealed by the SDS polyacrylamide gel electrophoresis.

Discussion

In the present study the holoprotein complex from hen egg white was initially isolated using batch adsorption onto DEAE-Sephadex, followed by column chromatography again on DEAE-Sephadex. It was found that a better purification could be achieved using two successive ion-exchange-binding steps. Nearly homogenous preparation of the Rfbp was obtained at this stage of purification, which was revealed by SDS-gel electrophoresis. However, final purification was accomplished using gel-filtration on Sephadex G-100. Adopting this two-stage purification method, Rfbp could be purified to homogeneity.

These techniques were improved during the large-scale purification of Rfbp from peacock egg-white, yolk. Partial purification of the Rfbp could be accomplished by batch adsorption of peacock egg-white homogenate to DEAE-Sephadex followed by a column elution. The bound protein could be eluted as one major sharp peak with 0.5M NaCl. Dialysis of this fraction against distilled water followed by centrifugation resulted in a clear yellow supernatant. Gel electrophoresis of the DEAE-Sephadex fraction revealed the presence of only one major protein band, which had mobility similar to that of the purified Rfbp, and additional minor protein bands. Rfbp could be enriched by many-fold and freed from contaminating proteins to a large extent. Further purification was accomplished using Sephadex G-100 column chromatography. Thus in the present study the peacock egg-white Rfbp could be purified to clear homogeneity as judged by the SDS-Page. Further, the purified peacock egg-white Rfbp also 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.

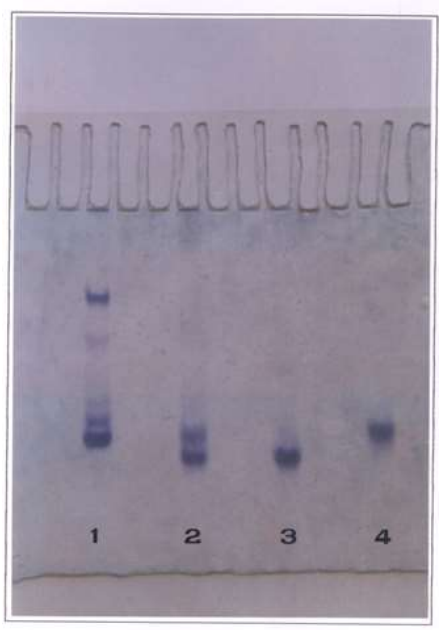

FIG.3. **Native-PAGE gel pattern of Peacock egg-white RfBP; 1): Peacock egg-white crude homogenate; 2): Peacock egg-white DEAE-Sephadex batch eluted fraction; 3): Peacock egg-white DEAE- Sephadex column eluted fraction; 4): Peacock egg-white Sephadex G-100 fraction.**

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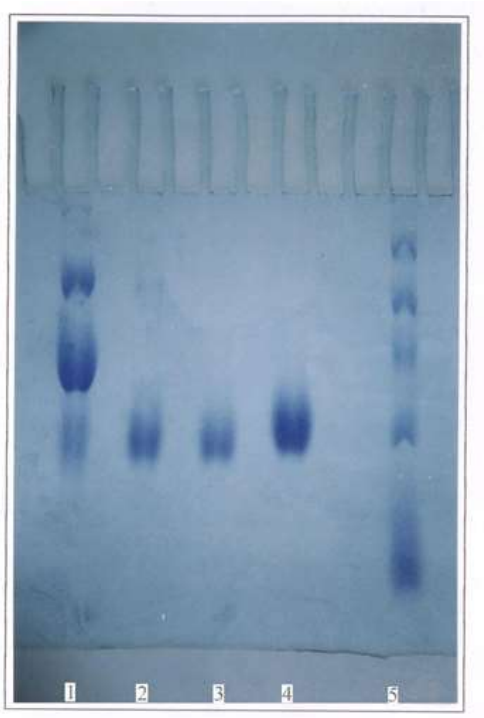

FIG.4. **SDS polyacrylamide gel electrophoresis pattern of Peacock and hen egg white RfBP; 1): Peacock egg-white crude homogenate; 2): Peacock egg-white DEAE-Sephadex eluted fraction; 3): Peacock egg-white DEAE- Sephadex G-100 fraction; 4): Hen egg-white Sephadex G-100 fraction; 5): Protein Molecular Weight Markers (20,000 to 97,400 kD).**

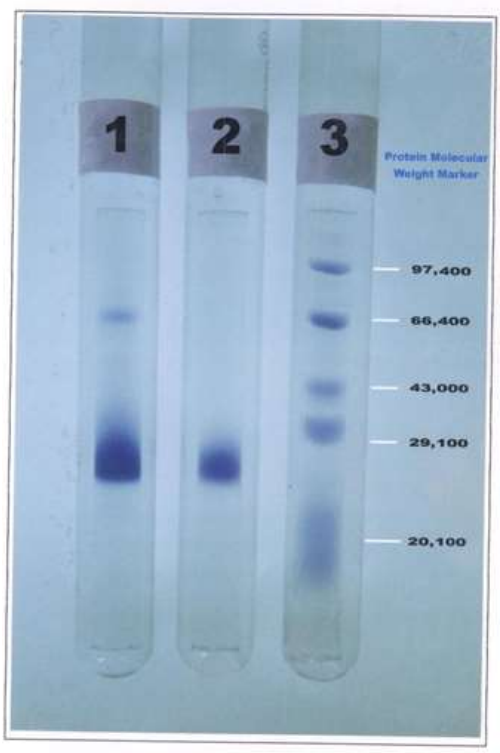

FIG.5. **Cylindrical Gel Electrophoresis Pattern (SDS-PAGE); 1): Peacock egg-white DEAE-Sephadex eluted fraction; 2): Peacock egg-white DEAE- Sephadex G-100 fraction; 3): Protein Molecular Weight Markers (20,000 to 97,400 kD).**

Conclusion

Riboflavin binding protein extracted in two different steps 1.Sephadex A-50 and G-100 methods, and confirmed through SDS Page/Negative gel electrophoresis.This protein was confirmed it's specific molecular weight Marker.

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