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Comparison of riboflavin binding protein (Rfbp) purified and isolated from egg-yolk, egg-white of Peacock (*Pavo cristatus*)

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ABSTRACT

Riboflavin binding protein (RfBP) was isolated from the peacock (*Pavo cristatus)* egg-yolk, Egg-white. Rfbp was purified on DEAE-Sephadex A-50 followed by Sephadex G-100 ion exchange chromatography. The holoprotein with the bound riboflavin showed an absorption maximum at 455nm.The high intensity of colored peak fraction protein content was estimated.The purity of the protein was judged by cylindrical and slab - gels, SDS-PAGE technique. This protein showed a single band on SDS gels. RfBP of Peacock egg-yolk, egg-white also migrated as a single band during electrophoresis pattern on SDS-PAGE. Comparison of the mobilities of the molecular weight makers with the mobility of peacock egg-yolk RfBP suggested that the RfBP had a molecular weight of approximately 29,000 kD.Interestingly purified peacock egg-yolk RfBP and purified peacock egg-white RfBP had approximately the same molecular weight as revealed by their electrophoresis mobilities on SDS-PAGE.

KEYWORDS

Peacock;
Egg yolk-egg white;
Purification and isolation;
Rfbp comparison.

INTRODUCTION

All animals are incapable of synthesizing the isoalloxazine skeleton of Rf and require this vitamin in the range of 1-10 μg/g diet[1]. All flavins are 10-substituted derivatives of the isoalloxazine tricyclic ring system which is synthesized via a complex pathway from GTP[2]. 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 analysed FAD predominants (ca. 75% of the total tissue flavin), followed by FMN (ca. 22%) and Rf (Ca. 2%;[3]. Conversely, mammalian[4] and avian[5] serum contains Rf as the predominant flavin with less FAD and only traces of FMN. In most instances the 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 the serum of the vertebrates[6-9]. The binding protein for

water soluble vitamins[10,11] Vitamin B_{12}[12,13] and Thiamin[14,15] have been demonstrated in the blood serum, egg-white and egg-yolk of the laying hens. The essential role of Riboflavin binding protein (RfBP) has been demonstrated in the homozygous recessive mutant (rd rd) domesting fowl[16] and in heterozygous leg horn hen[17, 18]. The Riboflavin binding protein (Rfbp) from peacock egg-white was purified and characterized[19].

As the aim of the present study was to prove the Riboflavin binding protein purification, isolation from Peacock (*Pavo cristatus*)Egg-yolk and Egg-white to compare the mobility of the proteins based on the slab and cylindrical gel electrophoresis.

EXPERIMENTAL

Materials

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 was obtained from Sigma-Aldrich Chemical Company, St. Louis, USA. Bovine Serum albumin, acrylamide, N, N, N^1, N^1-Tetramethylethylenediamine, N, N^1-methylene-bis-acrylamide, and SDS were procure from Loba Chemical Industrial Company, Bombay, India. All other reagents used were of analytical grade.

Methods

Isolation and purification of Peacock egg-yolk (Rfbp)

Peacock egg-yolk RfBP was purified in two steps (Batch adsorption to DEAE-Sephadex and Gel filtration column chromatography on Sephadex G-100) following the methods[20,21].

Preparation of peacock egg-yolk

Prior to adsorption onto DEAE-Sephadex, the egg-yolk was collected carefully. Pooled egg-yolk was homogenized with four volumes of 0.1 M sodium acetate buffer pH 5.0. The crude yolk suspension was centrifuged at 10000× g for 20 minutes at 0^0C. The precipitated mucilaginous material was discarded. The supernatant was passed through the cheesecloth to remove the floating material. The clear yellow supernatant was used directly for batch adsorption onto DEAE-Sephadex.

Batch adsorption to DEAE sephadexA-50

To, clear peacock egg-yolk supernatant, DEAE-Sephadex, (previously equilibrated with 0.1 M sodium acetate buffer pH 5.0) was added. The mixture was stirred overnight at 4^0C and then suction filtered. The DEAE-Sephadex was washed with 2 liters of 0.1 M sodium acetate buffer pH 5.0 and re-suspended in 2 liters of buffer, allowed to settle at 4^0C and the supernatant was decanted. The DEAE-Sephadex with bound protein was washed extensively with the same buffer. The Riboflavin Binding Protein bound to the DEAE-Sephadex was eluted with 0.1 M sodium acetate buffer pH 5.0 containing 1 M NaCl by suction filtration. The protein fraction was dialyzed against distilled water.

Fresh DEAE-Sephadex previously equilibrated with 0.1 M sodium acetate buffer pH 5.0 was packed into the column and then the partially purified RfBP was loaded on the column. The column was washed with excess of buffer. RfBP was eluted from the column with 0.1 M sodium acetate buffer pH 5.0 containing 1 M NaCl. Thirty four fractions were collected and absorbances were measured at 280 nm and 455 nm using UV visible recording spectrophotometer (Perkin Elmer). Values were expressed as total absorbance at 280 nm and 455 nm per fraction. The peak fractions having high absorbance both at 280 nm and 455 nm were pooled and dialyzed against distilled water. Further purification was achieved by gel filtration on Sephadex G-100.

Gel filtration on Sephadex G-100

The partially purified peacock egg-yolk RfBP was dissolved in 1 ml of phosphate buffer and was loaded on the Sephadex G-100 column previously equilibrated with 0.05 M phosphate buffer pH 7.4 containing 0.5 M NaCl. The protein was eluted with the same buffer fractions were collected and protein was estimated in each fraction by the method of Lowry[22]. The absorbances of each fraction was measured at 280 nm and 455 nm using UV visible recording spectrophotometer (Perkin Elmer). The peak fractions were pooled and dialyzed against distilled water and lyophilized. The purity of the protein was checked by the analytical polyacry-

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lamide gel electrophoresis.

Spectral studies

UV spectra

The Riboflavin solution contains 20.0 mg of riboflavin (LOBA) dissolved in 500 ml of distilled water and 1 ml of concentrated HCl. This standard solution was diluted with 0.05 M Tris-HCl buffer pH 7.5 and absorption spectrum was recorded using UV-visible spectrophotometer (Perkin Elmer). The absorption spectrum of the partially purified RfBP preparations were also recorded by diluting the proteins with 0.05 M Tris-HCl buffer pH 7.5 or directly from the eluates of the column after diluting the solutions suitably.

Sodium dodecyl sulphate (SDS-PAGE)

SDS-PAGE was carried out according to the method of Leammli[23] using sodium phosphate buffer containing SDS.

Protein estimation

Protein was estimated by the method of Lowry[22]. A suitable aliquot from each sample was used for estimation. To 0.1 ml of the sample, 0.9ml distilled water and 5 ml freshly prepared solution (50 ml of 2% Na_2CO_3 in 0.1 N NaOH was mixed with 1 ml of 0.5% $CuSO_4$ in sodium potassium tartarate) were added. After 10 minutes at room temperature 0.5 ml of diluted Folin-Ciocalteau reagent was added and mixed well. The intensity of the color was read at 620 nm against a reagent blank. A standard plot was prepared using bovine serum albumin (BSA) as the standard. Following the same basic steps were followed to isolate and purification of peacock egg-white protein.

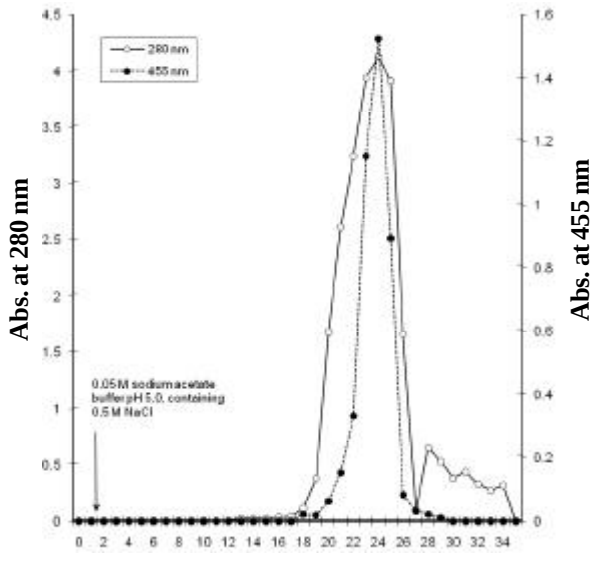
RESULTS AND DISCUSSION

Peacock egg-yolk was collected. The crude egg-yolk solution was prepared as described. To the processed fraction of crude peacock egg-yolk supernatant DEAE-Sephadex previously equilibrated with 0.1 M sodium acetate buffer, pH 5.0 was added. The vast majority of the proteins were unbound to the an ion-exchanger and could be washed off with excess of 0.1 M sodium acetate buffer, pH 5.0. Bound protein was eluted with the above buffer containing 0.1 M NaCl.

The elutant was dialyzed and loaded onto a DEAE-Sephadex column. After washing the column the bound protein was eluted with 0.05M phosphate buffer, pH 5.0, containing 0.5 M NaCl. Fractions were collected. Protein concentrations in the elutes were estimated by measuring absorbance at A_{280} nm with UV-visible recording spectrophotometer (Perkin-Elmer). The elution profile was given in figure 1. Fractions eluted from column were also assayed for protein bound Riboflavin by measuring the absorbance at 455 nm. The peak fraction was yellow in colour with the highest absorbance at 280 nm was dialyzed against distilled water. The protein content in all fractions was also estimated (Figure 2) by the method of Lowry et.al.[22]. Polyacrylamide gel electrophoresis at pH 8.3 of the DEAE-Sephadex eluted protein fraction revealed the presence of contaminating proteins, suggesting partial purification of RfBP at this stage.

Purification of egg-yolk Rfbp using gel-filtration chromatography on sephadex G-100

The fraction from DEAE-Sephadex was dialyzed against distilled water and lyophilized. The partially purified RfBP was dissolved in 1.0 ml of phosphate buffer



RfBP fraction from batch elution was loaded on to the DEAE sephadex column and was eluted with 0.05M sodium acetate buffer pH 5.0 containing 0.5M NaCl

Figure 1 : Peacock egg yolk RfBP elution profile on DEAE sephadex

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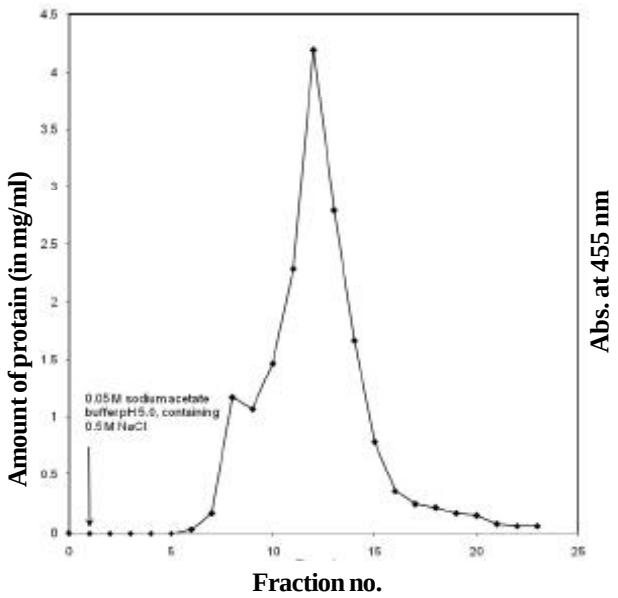

The partially purified RfBP was eluted from selphadex G-100 using 0.05 M phosphate buffer pH 5.0 containing 0.5 M NaCl. The protain concentration was estimated by the method of lowry et al.

Figure 2 : Peacock egg yolk RfBP elution profile on sephadex G-100

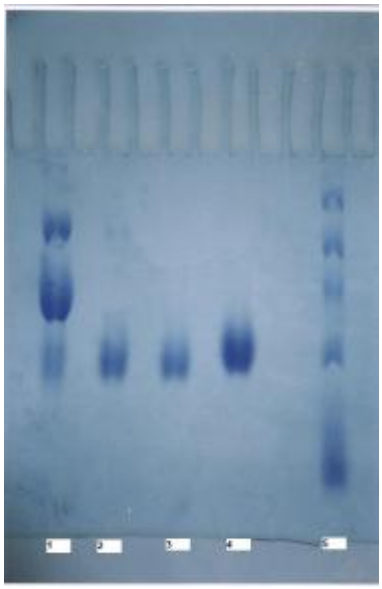

Figure 3: Electrophoretic pattern of Riboflavin binding protein (1. Crude homogenate of peacock egg-whitem, 2. Batch elution of Peacock egg-white, 3. Sephadex G-100 fraction of Peacock egg-white, 4. Sephadex G-100 fraction of Peacock egg-yolk, 5. Protein molecular weight marker)

and loaded on a Sephadex G-100 column (2 × 42 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. Fractions were collected. The fractions were monitored for absorbance at 280 nm and 455 nm. The peak fractions were yellow in colour with highest absorbance at 280 nm were pooled and dialyzed against distilled water. The protein content in the fractions were also estimated by the method of Lowery[22].

Spectral studies

UV absorption spectra

The holoprotein with the bound riboflavin showed an absorption maximum at 455 nm. Similar absorption spectra were reported earlier for the flavoprotein complexes[10,24].

SDS-polyacrylamide gel electrophoresis

The purity of the isolated peacock egg-yolk RfBP was judged using SDS-PAGE technique. Purification of peacock egg-yolk RfBP was accomplished by employing initially DEAE-Sephadex ion exchange chromatography followed by gel-filtration on Sephadex G-100 column chromatography. SDS gel electrophoresis of the RfBP fraction obtained from gel filtration on Sephadex G-100 resulted in a single band on the cylindrical gels suggesting complete purification of RfBP. Comparison of the mobilities of the molecular weight makers with the mobility of peacock egg-yolk RfBP suggested that the RfBP had a molecular weight of approximately 29,000 kD. Interestingly purified peacock egg-yolk RfBP and purified egg-white RfBP had approximately the same molecular weight as revealed by their electrophoretic mobilities on SDS-PAGE.

These techniques were improved during the large-scale purification of RfBP from peacock egg-white. 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. Further purification was accomplished using Sephadex G-100 column chromatography. Thus

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in the present study the peacock egg-white RfBP could be purified to clear homogeneity as judged by the SDS-Page (7.5% gels were used). 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 Peacock egg-white RfBP (Figure 3).

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