December 2009 Volume 3 Issue 4

Trade Science Inc.

BioTechnology
An Indian Journal

FULL PAPER

BTAIJ, 3(4), 2009 [251-256]

Immunological characterization of flying and flightless birds based on Riboflavin binding protein (RfBP)

G.Rajender[1]*, G.Benarjee[1], M.S.K.Prasad[2], B.Laxma Reddy[1]

[1]Department of Zoology, Kakatiya University, Warangal-506009

[2]Department of Bio-chemistry, kakatiya University, Warangal-506009

E-mail : gdrajender@yahoo.com; dr.grajender@gmail.com

Received: 7th June, 2009 ; Accepted: 17th June, 2009

ABSTRACT

Flying bird Peacock *(Pavo cristatus),* Hen *(Gallus gallus)* and flightless bird Emu (Dromius) egg Riboflavin binding protein (RfBP) was isolated from these birds. 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. Emu Rfbp contains more non specific protein and contaminants. To avoid this non specific protein column purification repeatedly column filteration.The protein content was estimated with Lowry method. The purity of the proteins was judged by cylindrical and slab–gels, Electrophoresis techniques. The final purified proteins showed a single band on electrophoresis 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 in flying birds. In flightless birds there was no cross reactivity. This study proved that, these two birds (flying and flightless birds) are immunologically relative and phylogentically different from each other.

KEYWORDS

Emu Peacock, Hen;
Rfbp isolation;
Antiserum;
Immunological aspects.

INTRODUCTION

Riboflavin Binding Protein (RfBP) or Riboflavin Carrier Protein (RCP) was first isolated the chicken egg white[1]. It is a phosphor-glycoprotein. The faint yellow color of normal egg white is due to riboflavin bound to a specific protein. The essential role of RfBP has been demonstrated from a study of the homozygous recessive mutant (rd rd) of domestic fowl[2].

Binding proteins for water-soluble vitamins such as Riboflavin binding proteins[3,4], Vitamin B_{12} binding protein[5,6], and Thiamin binding protein[6,7] have been demonstrated in the sera and egg white and yolks of the egg laying hens. All animals are incapable of synthesizing the isoalloxazine skeleton of Rf and require this vitamin in the range of 1-10 μg/g diet[8]. Rhodes[1] first reported the presence of a flavoprotien in egg white which bound riboflavin more avidly than its coenzyme, but devoid of enzymatic action. Further, Farrell,[9,10] isolated RfBP from egg white of heterozygous Leghorn hens, raised antibodies in rabbits and found them immunologically active functionally inactive protein in recessive mutants. These results indicated that these mutants lacked riboflavin binding capacity due to a mutation in the gene

coding for the carrier protein.

Egg white RfBP is a phospho-glycoprotein having a molecular weight of 29,200 containing 219 amino acid residues[11]. The isolation of RfBP from egg yolk was first published[4] and improved methods were subsequently reported[12,13]. The Riboflavin binding protein from peacock (*Pavo cristatus*) egg-white was first isolated[14].

Riboflavin-binding protein (RfBP) mediates the deposition of riboflavin during the formation of egg in birds. Hens of a strain of Single-Comb White Leghorn chickens, which are genetically unable to produce RfBP, lay eggs containing insufficient riboflavin to sustain embryogenesis beyond 13 or 14 days of incubation. Embryos in these eggs grow normally until the day of death, and their heart rate is normal within an hour of death. The effects of riboflavin-deficiency first appear after 10^{th} day of incubation when embryos become severally hypoglycemic and begin to accumulate intermediates of fatty acid oxidation. Although the activities of flavin-dependent enzymes are reduced generally, the 80% reduction in the activity of medium-chain acyl-CoA dehydrogenase further suggests that the major metabolic consequence of riboflavin deficiency is a severe impairment of fatty acid oxidation. The riboflavin-deficient strain provides numerous insights into the metabolism of normal hens and chicken embryos and may be a useful model for sudden death syndromes in humans[15].

EXPERIMENTAL

Materials

Emu (*Dromius*) eggs were obtained from turkey form karimnagar; Peacock (*Pavo cristatus*) eggs were obtained from Vana Vignana Kendram, Warangal. Fresh hen (*Gallus gallus*) eggs were obtained from the poultry farm. DEAE-Sephadex A-50 used in the present study was obtained from Pharmacia Fine Chemicals, Uppasala, Sweden. Sephadex G-100 and Freund's Complete adjuant was procured 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, SDS were procured from Loba Chemical Industrial Company, Bombay, India.

Methods

Isolation and purification of emu egg-white riboflavin binding protein

Emu egg whites were collected and homogenized with an equal volume of 0.1 M sodium acetate buffer pH 4.5. Cheese cloth was used to remove non specific proteins. The homogenate was centrifuged at 15000 x g, for 10 minutes. 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 4^0C 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 water.

DEAE-Sephadex previously equilibrated with 0.1M sodium acetate buffer pH 4.5 was packed into the column. Partially purified RfBP was loaded onto the column. The column was washed with excess buffer. Riboflavin binding protein was eluted from the column with 0.1M sodium acetate buffer, pH 4.5 containing 0.5M sodium chloride. Twenty fractions, were collected and absorbance was measured at 280 nm, 455nm using UV-visible recording spectrophotometer. Values were expressed as total absorbance at 280 nm and 455 nm per each fraction.

Further purification of Emu 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 Lowery[16] method of using bovine serum albumin as standard. Absorbance was 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 fraction were pooled and dialyzed against distilled water and lyophilized. SDS-PAGE was carried out according to the method of Leammli[17] using sodium phosphate buffer containing SDS.The same steps were pro-

cessed to purify Emu egg-yolk, Peacock egg-white, egg-white and Hen egg-white, egg-yolk.

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. 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 (Figure 2).

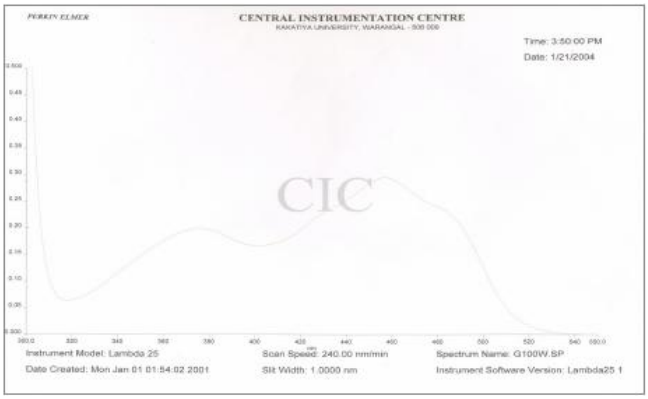


Figure 2 : Absorption spectrum of Emu egg-white riboflavin binding protein (Sephadex G-100 Fraction)

Sodium dodecyl sulphate (SDS-PAGE)

SDS-PAGE was carried out according to the method of Leammli[17] using sodium phosphate buffer containing SDS

Production of antiserum to riboflavin binding protein (RfBP)

Antibodies against RfBPs were produced adopting the method of Prasad and Adige,[18]. Briefly, the protein was emulsified with an equal volume of Freund's complete adjuvant 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.

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

RESULTS & DISCUSSION

Partial purification of emu egg-white RfBP using DEAE-sephadex

Emu egg-whites were collected from 30 eggs and homogenized with an equal volume of 0.1 M sodium acetate buffer pH 4.5. The homogenate was processed as described under Material and Methods. To the crude yellow supernatant (1800 ml), DEAE-Sephadex, previously equilibrated with 0.1 M sodium acetate buffer pH 4.5, was added and stirred overnight at 4^0C. The DEAE-Sephadex was washed extensively with 0.1 M sodium acetate buffer, pH 4.5 as described under Materials and Methods. The bound proteins were eluted with same buffer containing 0.5 M NaCl by 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 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.

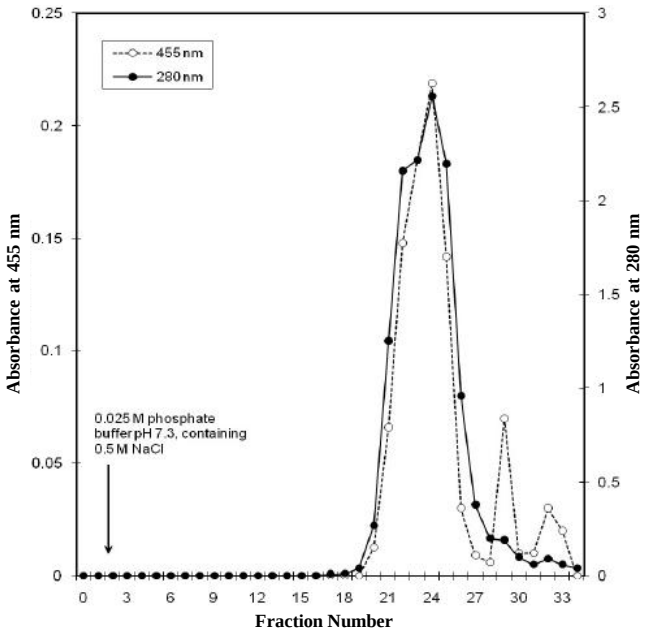
Further purification was achieved by gel filtration on Sephadex G-100. The RfBP fraction obtained from DEAE-Sephadex equilibrated with 0.025 M phosphate buffer, pH 7.3 containing 0.5 M NaCl. Collected fractions and the absorbances were recorded at both 280 nm and 455 nm. The fraction having high absorbance both at 280 nm and 455 nm were pooled, dialysed against distilled water and lyophillised. 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.

Electrophoresis on analytical polyacrylamide gels (7.5%) were conducted at pH 8.3. Chromatography on DEAE-Sephadex, as one major band correspond-

FULL PAPER

ing to RfBP along with a few minor bands were obtained with the DEAE-Sephadex fraction. Complete purification was achieved by Gel-filtration chromatography on Sephadex G-100. A comparison of the electrophoretic mobilities revealed that the hen egg-white had a molecular weight marker approximately 29,000kD.

After centrifugation 440 ml of the 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 0.25 M sodium acetate buffer pH 7.3 containing 0.5 M NaCl filtration by suction. The elutant was dialysed and loaded onto a DEAE-Sephadex column. After washing the column the bound protein was eluted with 0.025 M sodium acetate buffer, pH 7.3 containing 0.5 M NaCl. Fractions were collected.(Figure 1) The peak fractions with the highest absorbance at 280 nm were pooled and dialysed against distilled water. The protein content in all fractions was also estimated. 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.



RfBP fraction from batch elution was loaded onto the DEAE sephadex column and was eluted with 0.025M sodium acetate buffer pH 7.3, containing 0.5 M NaCl

Figure 1 : Emu egg-yolk RfBP elution profile on DEAE-sephadex

Production of antiserum of riboflavin binding protein

Antibodies against hen egg-white, peacock egg-white, peacock egg-yolk RfBPs were produced in the rabbits adopting the method of Prasad and Adiga[18] as described under Materials and Methods. Ouchterlony double diffusion analysis was carried out using Agar/Agarose plates. The antiserum raised against hen egg-white RfBP could cross react with (i) Purified peacock egg-white RfBP, (ii) Purified peacock egg-yolk RfBP, and (iii) & (iv) Purified hen egg-white RfBP, as revealed by the appearance of the cross reacting precipitin lines. Further the antibodies raised against hen egg-white RfBP could cross react with peacock egg-white RfBP, peacock egg yolk RfBP and hen egg-white RfBP. However, the antisera failed to show any cross reactivity with purified emu egg-white RfBP as no cross reacting precipitin line (Figure 3) could be obtained.

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 emu egg-white RfBP (Sephadex G-100 fraction

Figure 3 : Ouchterlony double diffusion analysis

RfBP from the domestic fowl has been investigated most thoroughly. RfBP or RCP is the most abundant egg-white vitamin binding protein. RfBP contains a single polypeptide chain of 219 aminoacids of molecular mass 29.2 kD. RfBP binds riboflavin tightly (K_d = 1.3 mm) in a 1:1 ratio. RfBP undergoes a number of following post-translational modifications. Formation of nine disulphide bonds. Extensive glycolysation and phosphorylation of eight serine side chains. Undergoes limited proteolysis with the cleavage of a 17-residue signal peptide from

the N-terminus and two arginine residues from the C-terminus.

These features coupled with the relatively small size of the protein and exceptional high thermo-stability of RfBP, make it an interesting protein for further studies such as: (1) Isolation and purification from different sources for a comparative study (2) Mode of biosynthesis, post-translational modifications and hormonal induction and (3) The mode of transport of ligand and related functional aspects of RfBP.

In the present study work RfBP was purified from both the hen and peacock eggs for the first time and immunological characterization was carried out, as no detailed study was undertaken earlier on RfBP from peacock eggs.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. RfBP constituted less than 1% of the total proteins in hen egg white, and 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. 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 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 hen egg-white RfBP.

Initially, RfBP from chicken egg-yolk was purified by Ostrowski[19], and later by Hamazume[11]. The concentration of RfBP of the egg-yolk is not much less than that of it in the egg-white, large amounts of lipids and other proteins make purification more difficult. In fact, Ostrwoski[19] extracted the egg-yolk suspension with ether to remove lipids before employing the ammonium sulphate precipitation step. Further, during the purification of hen egg-yolk biotin binding protein, White[15] prepared an egg-yolk acetone dried powder to remove the most of the lipids. On the other hand, Murthy[13] could isolate crude egg-yolk RfBP from egg-yolk homogenates directly by adsorption onto DEAE-Cellulose at pH 7.5. The bound RfBP with the other contaminating proteins were eluted with 0.1 M Tris-HCl buffer, at pH 7.5 containing 1 M NaCl. Gel-exclusion chromatography of this crude RfBP resulted in the purification of RfBP to homogeneity. Later, Hamazume[11] used the anion-exchanger, DEAE-Sephadex and partially purified RfBP from hen egg-yolk by adjusting the pH of the yolk suspension to 5.5. Non-specific proteins were removed by washing the DEAE-Sephadex with 0.1M and 0.15M NaCl before eluting the bound protein with the 0.5M NaCl.

The partially purified and completely purified hen egg white, hen egg-yolk, peacock egg white and peacock egg-yolk RfBPs were also characterized by recording the absorption spectra. The near ultraviolet absorption spectrum of the riboflavin aproprotein complex indicated that the protein had an absorption maximum at 274.3 nm and a shoulder at about 290 nm. This result is in full agreement with the data published by Nikhikimi and Kyogoku[20]. Further the visible absorption spectra revealed that the RfBPs had absorption maxima at 370 and 456 nm characteristic of riboflavin-apoprotein forms (holoproteins). The free riboflavin showed absorption maxima at 374 and 445 nm. Binding of riboflavin to the protein (holoprotein) resulted in the shift of the absorption peak at 445 to 457 nm and shoulders appeared at about 435 and 480 nm. At the same time the absorption at 375 nm showed remarkable the antiserum obtained from tested against purified peacock egg-white RfBP by immunodiffusion. Similar single precipitin lines were also obtained by testing this

antiserum against peacock egg-white crude homogenate and partially purified peacock egg white RfBP. These lines showed a reaction of identify with the line obtained by reacting the antiserum against purified RfBP. Immunodiffusion studies were also 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 peacock egg-yolk RfBP as well as and (3) Hen egg-white, egg-yolk RfBP.

Studies using the antiserum raised against purified peacock egg-yolk RfBP showed cross reactivity with the purified RfBPs from hen egg-yolk, hen egg-white and peacock egg-yolk. However, this antiserum failed to cross react with RfBP isolated from emu egg-yolk. As expected, ouchterlony double diffusion analysis using purified peacock egg-yolk RfBP could clearly show immunological cross reactivity with the RfBPs obtained from (i) Peacock egg-white, (ii) Peacock egg-yolk, and (iii) Hen egg-white.

The antiserum produced an immunoprecipitation line when allowed to diffuse against peacock egg-yolk crude homogenate, partially purified peacock egg-yolk RfBP and purified peacock egg-yolk RfBP. On the other hand the antiserum failed to interact with the purified hen egg-yolk RfBP. These results indicate that hen RfBP and peacock RfBP may immunologically distinct and different from each other.

REFERENCES

[1] M.B.Rhodes, N.Bennett, R.E.Feeney; J.Biol. Chem., **234**, 2054 **(1959)**.

[2] W.P.Winter, E.G.Buss, C.O.Claget, R.V.Boucher; Comp.Biochem.Physiol., **22**, 897 **(1967)**.

[3] M.B.Bennett, R.E.Feeney; J.Biol.Chem., **234**, 2054 **(1959)**.

[4] W.Ostrowski.B.Skarzynski, Z.Zak; Biochem. Biophysics.Acta., **59**, 515 **(1962)**.

[5] R.Grasbeck; Prog Hematol., **6**, 233 **(1969)**.

[6] D.W.Sonneborn, H.J.Hansen; Science, **168**, 591 **(1970)**.

[7] M.E.Coates; Academic Press, New York, **1**, 373 **(1971)**.

[8] R.H.Dadd; Pergamum Press, New York, **4**, 313 **(1958)**.

[9] H.M.Farell, M.F.Mallette, E.G.Buss, C.O.Clagett; Biochim.Biophys.Acta., **194**, 433 **(1969)**.

[10] H.M.Farell, E.G.Jr.Buss, C.O.Claggett; Int.J.Biochem., **1**, 168 **(1970)**.

[11] Y.Hamazume, T.Mega, T.Ikenaka; J.Biochem., **95**, 1633 **(1984)**.

[12] D.M.Miller PhD; Dissertation, William marsh Rice University **(1981)**.

[13] U.S.Murthy, K.Sreekrishna, P.R.Adiga; Anal Biochem., **92**, 345 **(1979)**.

[14] G.Rajender, G.Benarjee, M.S.K.Prasad; Current science, **93**, 11 **(2007)**.

[15] H.B.White 3rd, A.H.Merrill Jr; Comp.Biochem. Physiol., **90**, 243 **(1988)**.

[16] O.H.Lowry, N.J.Rosebrough, A.L.Farral, R.J.Randall; J.Biol.Chem., **193**, 265 **(1951)**.

[17] U.K.Leammli; Nature, **227**, 680 **(1979)**.

[18] M.S.K.Prasad, P.R.Adiga; Ind.J.Exp.Biol., **17**, 1166 **(1979)**.

[19] W.Ostrowski, B.Skarzynski, Z.Zak; Biochem. Biophysics.Acta., **59**, 515 **(1962)**.

[20] M.Nishikimi, Y.Kyogoku; J.Biochem., **73**, 1233 **(1973)**.