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ISOLATION AND PURIFICATION OF RIBOFLAVIN BINDING PROTIEN (RfBP) FROM THE EGG WHITE OF EMU (*Dromaius novaehollandiae*)

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ABSTRACT: Riboflavin-binding protein (RfBP) was isolated and purified from Emu (*Dromaius novaehollandiae*) egg white by DEAE Sephadex A-50 ion exchange chromatography followed by gel filtration chromatography on Sephadex G-100. Further the RfBP was immunologically characterized and compared with the hen (*Gallus gallus domesticus)* egg white RfBP. The purity of the protein was judged by SDS-PAGE technique. Comparison of the mobility of the purified proteins with the standard molecular weight marker proteins revealed that the Emu RfBP had a molecular weight close to 43 kDa and it was approximately 10 kDa larger than the hen egg white RfBP.

Key words: Riboflavin binding protein (RfBP), Emu egg white, purification, UV spectra, SDS-PAGE, antibodies.

Abbreviations: RfBP-Riboflavin Binding Protein; DEAE-Diethyl Amino Ethyl; Kd- kilodalton; SDS-Sodium Dodecyl Sulfate; PAGE-Poly Acrylamide Gel Electrophoresis

INTRODUCTION

Vitamin binding proteins are a group of soluble proteins present in the blood and other body fluids to ensure optimal bioavailability of the fat and water soluble vitamins for growth, metabolism and reproduction of vertebrates. Binding proteins for water soluble vitamins such as riboflavin [1, 2], vitamin B_{12} [3, 4] and thiamin [5, 6] were demonstrated in the sera, egg white and yolk of the egg laying hens.

Riboflavin binding protein (RfBP) is the most abundant egg white vitamin binding protein. It is a phosphoglycoprotein containing a single polypeptide chain of 219 amino acids and a molecular mass of 29.2 kDa. RfBP binds riboflavin tightly (KD=1.3 mm) in a 1:1 ratio. RfBP was isolated for the first time from chicken egg white [1]. This was followed by isolation from chicken egg yolk [2] and from the plasma of egg-laying hen [7].

The indispensability of this protein was demonstrated from a study on the homozygous recessive mutant (rd rd) of the domestic fowl [8], where a gene mutation leading to the absence of a functional RfBP resulted in the death of the developing embryos around the13th day of incubation. A normal hatch was achieved only by a direct injection of riboflavin into such eggs. RfBP was also shown to be an estrogen inducible protein. Further, the discovery, that immunoneutralization of riboflavin binding protein resulted in the abrupt termination of pregnancy in animals clearly established the paramount functional significance of RfBP in fetal growth and development [9].

In the present study RfBP was purified from a single egg of Emu (*Dromaius novaehollandiae)* using a modification of previously developed methods for the purification of hen egg white RfBP. Further the flavoprotein was immunologically characterized and compared with hen (*Gallus gallus domesticus*) egg white RfBP. The emu, an endemic bird of Australia, is a member of the ratite family. In India emu farming is increasingly being taken up as an alternative form of livestock due to its wide commercial potential.

MATERIAL AND METHODS

Fresh hen (*Gallus gallus domesticus*) eggs were obtained from the poultry farm, Madikonda, Warangal. Fresh Emu (*Dromaius novaehollandiae)* egg was obtained from Al-Hassan Ratite farm, Karimnagar. DEAE Sephadex A-50 used in the present study was obtained from Pharmacia Fine chemicals, Uppasala, Sweden. Sephadex G-100, G-75 and Freund's Complete Adjuvant were obtained from Sigma Aldrich Chemical Company, St. Louis, USA. Bovine Serum Albumin, Acrylamide, N, N^1-, melthylene–bis acrylamide, N, N, N1, N1–Tetra methyl ethylene diamine and SDS were procured from Sd Fine Chem. Limited, Mumbai. The Silver staining kit was procured from Bangalore Genei, Bangalore. All other reagents used were of analytical grade. The rabbits used in the present study were obtained from the University College of Pharmaceutical Sciences, Kakatiya University, Warangal, Andhra Pradesh.

Isolation and purification of emu egg–white riboflavin binding protein:

The Emu egg white RfBP was isolated following the methods of Hamazume et al. [10] and Murthy et al. [11] with a few significant modifications as described below:

Egg white from a single egg (232 ml) was separated and homogenized with three volumes of 0.1 M sodium acetate buffer pH 4.6 at 4^0C. To eliminate the abundant contaminating proteins, 2g of DEAE Sephadex previously equilibrated with 0.1M sodium acetate buffer, pH 4.6 containing 0.15M sodium chloride was added to the crude homogenate. The DEAE Sephadex with bound proteins was washed extensively with 0.1 M sodium acetate buffer pH 4.6 containing 0.15M sodium chloride and the RfBP was eluted with the same buffer containing 1M sodium chloride by suction filtration. The eluted protein was dialyzed against 0.1 M sodium acetate buffer, pH 4.6.

The crude protein was again subjected to ion-exchange column chromatography on DEAE-Sephadex. Fresh DEAE Sephadex (4g) previously equilibrated with 0.1 M sodium acetate buffer, pH 4.6 containing 0.15M sodium chloride was packed into the column and the partially purified RfBP was loaded onto the column. The column was washed with excess of the same buffer. RfBP was eluted from the column with 0.1M sodium acetate buffer, pH 4.6 containing 1M sodium chloride. Fractions were collected and absorbance was measured at 280nm and 455 nm using UV–Visible recording spectrophotometer (SL-164, ELICO). Values were expressed as total absorbance at 280nm and 455nm per fraction. The amount of protein in each fraction was estimated using the method of Lowry et al., (1951) [12]. The peak fraction having high absorbance at both 280nm and 455 nm was dialyzed against distilled water overnight.

Further purification was achieved by gel filtration chromatography on Sephadex G-100. The partially purified Emu egg white RfBP was loaded onto a Sephadex G-100 (6g) column previously equilibrated with 0.05M sodium phosphate buffer, pH 7.4 containing 0.5M sodium chloride. The protein was eluted with the same buffer. The absorbances were measured at 280nm & 455nm using UV–Visible recording Spectrophotometer (SL-164, ELICO). The purity of the protein was checked by polyacrylamide gel electrophoresis.

Isolation and purification of hen egg–white riboflavin binding protein:

Riboflavin binding protein from hen egg white (from20 eggs, 600ml) was isolated and purified in two steps using DEAE Sephadex and Gel filtration column chromatography on Sephadex G-75, following the methods of Rhodes et al., (1959) [1], Farrell et al., (1969) [13] and Hamazume et al., (1984) [10] with a few modifications.

Spectral Studies:

The absorption spectra of partially purified and purified RfBP preparations were recorded using UV-Visible recording spectrophotometer (SL-164, ELICO).

Analytical polyacrylamide gel electrophoresis:

Electrophoresis on analytical PAGE (7.5%) was carried out according to the method of Leammli (1979) [14]. SDS–PAGE was performed on slab gels and cylindrical gels using 0.1Msodium phosphate buffer, pH 8.0, containing SDS. Native PAGE on slab and cylindrical gels were also carried out using Tris –Glycine buffer, pH 8.3. After electrophoresis, the gels were subjected to silver staining and Coomassie brilliant blue staining.

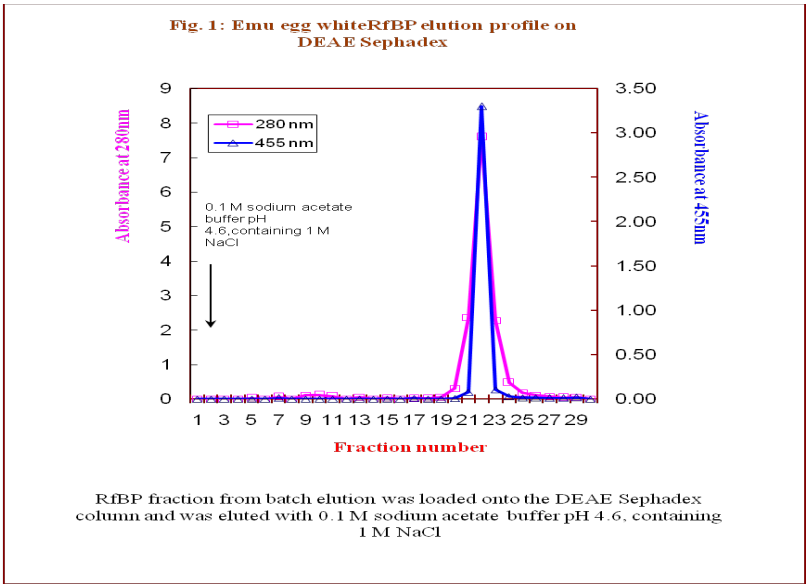
Production of antibodies against riboflavin binding protein:

Antibodies against emu and hen egg white RfBPs were produced adopting the method of Prasad and Adiga (1979) [15]. 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 rabbits at multiple sites. The rabbits were then bled through the ear veins 7 days after the completion of the booster dose. The presence of antibodies in the serum was tested using Ouchterlony double diffusion analysis.

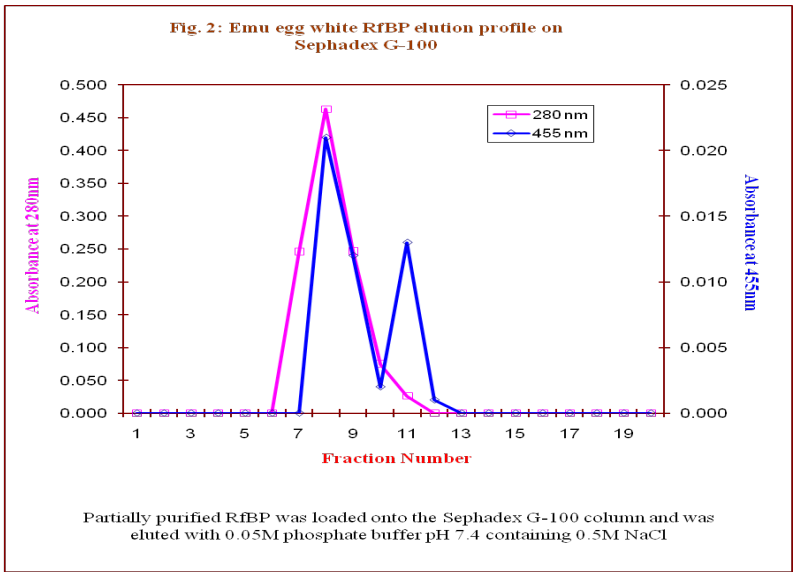
Ouchterlony double diffusion analysis was carried out as follows: Agarose plates (1.2%) were prepared in 0.05M sodium phosphate buffer pH 7.8, containing 0.9% NaCl. The antiserum was placed in the central well and the proteins dissolved in the same buffer were placed in the adjacent wells. The appearance of precipitin lines indicated the presence of specific antibodies.

RESULTS AND DISCUSSION

In the present study crude egg white fractions from emu and hen egg white were prepared. The crude emu egg white preparation was processed as described under Methods. The yellow supernatant (960ml), obtained after centrifugation was used for batch adsorption onto DEAE Sephadex. The protein bound gel was washed extensively with 0.1 M sodium acetate buffer, pH 4.6 on a Buchner funnel and eluted with the same buffer containing 1M sodium chloride, by suction filtration. The eluted protein (16ml) was dialyzed against 0.1 M sodium acetate buffer, pH 4.6 and loaded on a fresh DEAE Sephadex column (2×30cm) and bound RfBP was eluted as described earlier. Thirty fractions of 3ml each were collected (Fig: 1). The fractions also has high absorbance at 280nm. This fraction with highest absorbance at both 280nm & 455nm were pooled and used for further purification.



Final purification was achieved by gel filtration on Sephadex G-100. The elution profiles were given in Fig: 2. The fractions with highest absorbance at 280nm & 455nm were dialyzed against distilled water and the purity was checked by polyacrylamide gel electrophoresis.



The absorption spectra of partially purified emu egg white RfBP (DEAE Sephadex fraction) and purified RfBP by gel-filtration were shown in figures 3 & 4 respectively. The protein with bound Riboflavin (holoprotein) showed a typical spectrum similar to that of the flavin-apoprotein complex reported earlier by others. The protein with bound riboflavin showed absorption maxima at 376 and 458nm, characteristic of riboflavin-apoprotein forms. The spectral data thus confirmed the identity of the purified protein [16, 1].

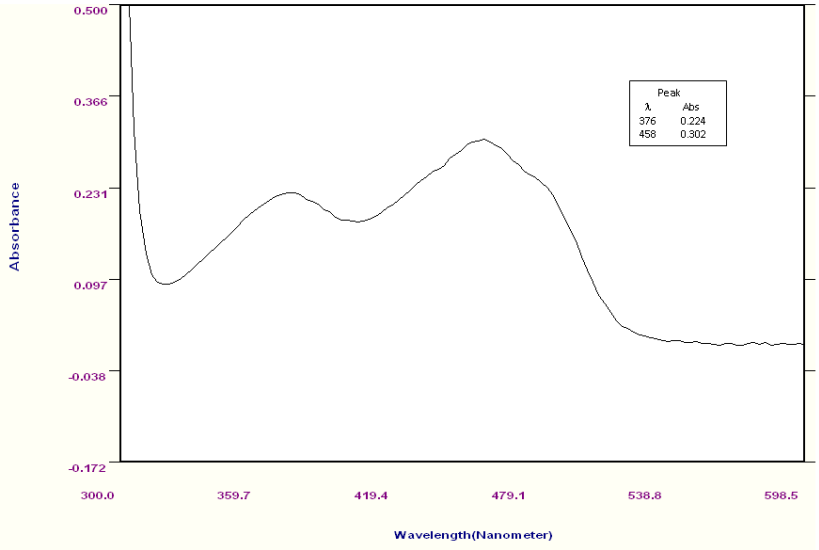


Fig. 3: The Absorption spectrum of Emu Egg White Riboflavin binding protein DEAE Sephadex fraction)

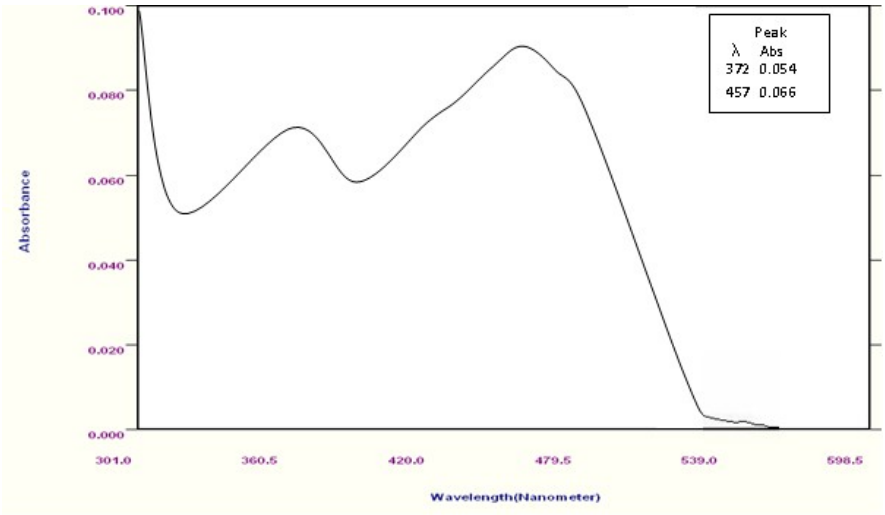
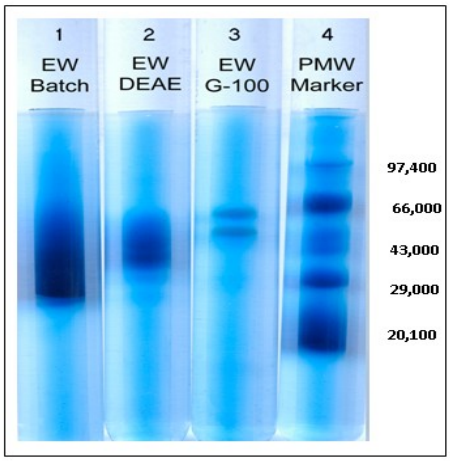


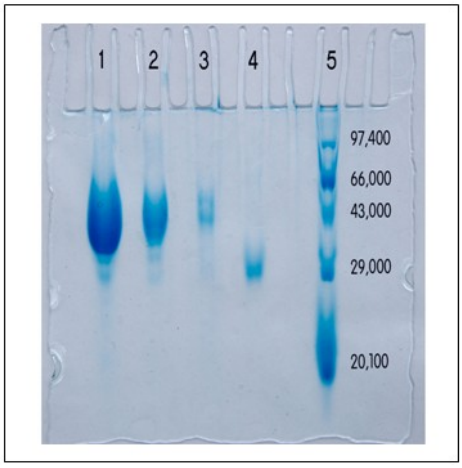
Fig. 4: The Absorption spectrum of purified Emu Egg white Riboflavin binding protein (Sephadex G-100 fraction)

The purity of the isolated protein was judged by native and SDS PAGE (cylindrical and slab gels). Partial purification of emu egg white RfBP could be achieved by using DEAE Sephadex, as this fraction contained a few minor contaminants. However final purification was achieved by gel filtration chromatography (Fig: 5). SDS-PAGE analysis resolved the protein into two bands that could be due to microheterogeniety of this glycoprotein (Fig: 6).A comparison of the electrophoretic mobilities revealed that the emu egg white RfBP had a molecular weight of approximately 43,000Da (Figs: 5&6). In addition comparison of the SDS-PAGE pattern of hen egg white RfBP and emu egg white clearly indicated that the emu RfBP was approximately 10 kDa larger than hen RfBP. Similar pattern was obtained using more sensitive silver staining technique (Figs: 7&8).



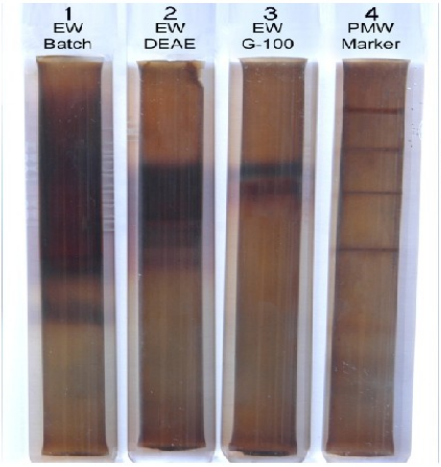
1. Emu egg white crude homogenate
2. Emu egg white DEAE Sephadex elution fraction
3. Emu egg white Sephadex G-100 Elution fraction
4. Protein Molecular Weight Markers (20,100 - 97,400 Da)

Fig 5: Cylindrical Gel Electrophoretic pattern of the Emu egg white RfBP(SDS PAGE)



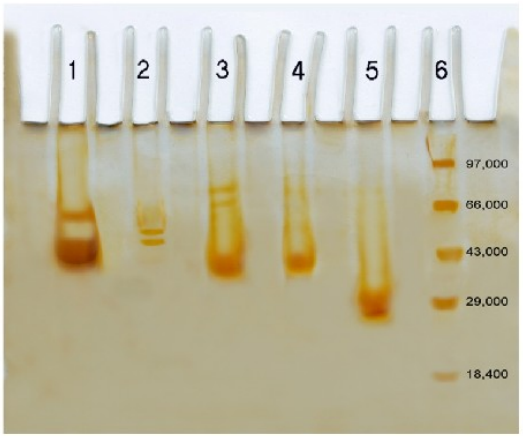
1. Emu egg white crude homogenate
2. Emu egg white DEAE Sephadex elution fraction
3. Emu egg white Sephadex G-100 fraction
4. Hen egg white Sephadex G-75 fraction
5. Protein Molecular Weight Markers (20,100 - 97,400 Da)

Fig 6: Slab gel pattern of Emu egg white RfBP (SDS PAGE)



1. Emu egg white crude homogenate
2. Emu egg white DEAE Sephadex elution fraction
3. Emu egg white Sephadex G-100 fraction
4. Protein Molecular Weight Markers (18,400 - 97,000 Da)

Fig 7: Cylindrical Gel Electrophoretic pattern of the Emu egg white RfBP - Silver Staining



1. Emu egg white DEAE Sephadex elution fraction
2. Emu egg white Sephadex G-100 fraction
3. Emu egg yolk DEAE Sephadex elution fraction
4. Emu egg yolk Sephadex G-100 fraction
5. Hen egg white Sephadex G-75 fraction6. Protein Molecular Weight Markers (18,400 - 97,000 Da)

Fig 8: SDS Polyacrylamide gel Electrophoretic pattern of Emu and Hen RfBP- Silver Staining

Ouchterlony double diffusion analysis was performed using the antisera raised against emu and hen egg white RfBPs. The antiserum was placed in the central well and the purified and partially purified proteins were placed in the adjacent wells. The antiserum raised against purified emu egg white RfBP could show clear immunological cross reactivity with (i) Purified emu egg white RfBP (Sephadex G-100fraction) (ii) partially purified emu egg white RfBP (DEAE-Sephadex fraction) and (iii) crude emu egg white RfBP. However, the antisera failed to cross react with hen egg-white RfBP (Fig: 9).

1. Purified Emu egg white RfBP (Sephadex G-100 fraction)
2. Partially Purified Emu egg white RfBP (DEAE Sephadex fraction)
3. Crude Emu egg white homogenate
4. Partially purified Hen egg white RfBP (DEAE Sephadex fraction)

Fig 9: Ouchterlony double diffusion analysis of Emu RfBP (Agarose) (The central well contains Emu egg-white RfBP antiserum)

In the present study RfBP was purified from hen as well as Emu eggs and immunological characterization of the flavoproteins were carried out, as no detailed study was undertaken on RfBP from emu eggs. The isolation of RfBP, purification and characterization of the flavoprotein apoprotein system of chicken egg white was first reported by Rhodes et.al. (1959) [1]. 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 Hamazume et al. (1984) [10] isolated the hen egg white and egg yolk RfBPs using DEAE-Sephadex at a pH of 5.5, followed by gel filtration chromatography on Sephadex G-100. 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 by 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 by adopting this two-stage purification method, RfBP could be purified.

Further the Visible absorption spectra revealed that the RfBP had absorption maxima at 376 and 458nm, characteristic of riboflavin-apoprotein forms (holoprotein). The free riboflavin showed absorption maxima at 363 and 446 nm. Binding of riboflavin to the protein (holoprotein) resulted in the shift of the absorption peak at 446 to 458 nm. At the same time the absorption at 366 nm showed remarkable hypochromism without a shift of band position. The spectral changes observed were characterized by a red shift of the 450 nm band. The appearance of the shoulders in the 450 nm band suggests that the flavin environment becomes less polar relative to the flavin in water. The fact that the 370 nm band of the flavin did not shift to any significant extent when the flavin combined with the protein indicated the concomitant involvement of a hydrophilic or polar interaction. Exactly similar spectral data were reported earlier for hen egg white RfBP [1, 16].

SDS gel electrophoresis revealed the presence of two bands with an approximate molecular weight of 43,000Da. In addition comparison of the SDS-PAGE pattern of hen egg white RfBP and emu egg white clearly indicated that the emu RfBPs were approximately 10 kDa larger than hen RfBP. It is to be noted that this range of molecular weights has been reported in other species like turtles and alligators and is unusual in the avian species. It is presumed that this large difference in the molecular weights could be attributed to the differences in the glycosylation patterns [17].

Further the antiserum obtained from the rabbit injected with purified Emu egg white RfBP gave a single precipitin line when tested against purified Emu egg-white RfBP by immunodiffusion. However, the antisera against purified Emu RfBPs failed to show cross reactivity with hen egg-white RfBP, suggesting that these two RfBPs may be immunologically distinct and different from each other. Thus the present study clearly unraveled for the first time the molecular size and immunological differences between hen and emu egg-white RfBPs.

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REFERENCES

1) Rhodes M. B., Bennett N. and Feency R. E., (1959): The flavoprotein-apoprotein system of egg white. J. Biol. Chem. 234: 2054-60.
2) Ostrowski W., Skarzynski B. and Zak Z., (1962): Isolation and properties of flavoprotein from the egg yolk. Biochem. Biophys. Acta. 59: 515-19.

3) Grasbeck R., (1969): The vitamin B12-binding protein in human saliva isolation, characterization and comparison with the unpurified protein. Prog. Hematol. 6: 233.
4) Sonneborn D. W. and Hansen H. J., (1970): Vitamin B12 binders of chicken serum and chicken proventriculus are immunologically similar. Science. 168: 591-592.
5) Naber E. C., Craves W. W., Baumann C. A. and Bird H. R., (1954): The effect of thiamine analogs on embryonic development and growth of the chick. J. Nut. 54: 579-591.
6) Coates M E., (1971): Physiology and Biochemistry of the Domestic Fowl (Bell D J. and Freeman B M. Ed.) Vol. 1: 373-392, Academic Press, London and New York.
7) Murthy U. S. and Adiga P. R., (1978): Estrogen-induced synthesis of riboflavin-binding protein in immature chicks - kinetics and hormonal specificity. Biochim. Biophys. acta. 538: 364-375.
8) Winter W. P., Buss E. G., Clagett C. O. and Boucher R. V., (1967): The nature of the biochemical lesion in avian renal riboflavinuria. I. Effect of genotype on renal riboflavin metabolism. Comp. Biochem. Physiol. 22: 879-906.
9) Adiga P. R. and Murthy C. V. R., (1983): Vitamin carrier protein during embryonic development in birds and mammals; in Molecular Biology of Egg Maturation (Ciba Foundation Symposium 98) pp. 111-136 eds R. Porter and J Whelan (London: Pitman).
10) Hamazume Y., Mega T. and Ikenaka T., (1984): Characterization of hen egg white- and yolk- riboflavin binding proteins and amino acid sequence of egg white-riboflavin binding protein. J. Biochem. 95: 1633-44.
11) Murthy U. S., Srikrishna K. and Adiga P. R., (1979): Simplified method for the purification of riboflavin-binding protein from hen's egg-yolk. Analytical Biochem. 92: 345-350
12) Lowry O. H., Rosen Borough N. J., Far A. L. and Randall R. J., (1951): Protein measurement with the folin phenol reagent. J. Biol. Chem. 193: 265-275.
13) Farrell H. M., Mallette M. F., Buss R G. and Clagett C. O., (1969): The nature of the biochemical lesion in avian renal riboflavinuria: The isolation and characterization of the riboflavin-binding protein from egg albumen. Biochim. Biophys. Acta. 194: 433-442.
14) Leammli U. K., (1970): Cleavage of structural proteins during the assembly of the head of bacteriophage T4. Nature. 227: 680-685.
15) Prasad M. S. K. and Adiga P. R., (1979): Prolactin-testosterone interaction in the male-rat - Modulation of ornithine decarboxylase in various tissues. Ind. J. Biochem. Biophys. *16***:** 354-361
16) Choi J. D. and Mc Cormick D. B., (1980). The interaction of flavins with egg white riboflavin-binding protein. Arch. Biochem. Biophys. 204: 41-51.
17) Maehashi K., Matano M., Uchino M., Yamamoto Y., Takano K. and Watanabe T., (2009). The primary structure of a novel riboflavin-binding protein of emu (*Dromaius novaehollandiae*). Comp. Biochem. Physiol., Part B. *153***:** 95-100.