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IMMUNOLOGICAL CHARACTERIZATION AND COMPARISON BETWEEN EGG YOLK & WHITE RIBOFLAVIN BINDING PROTEINS OF LARGEST FLIGHTLESS BIRD OSTRICH AND THE FLYING BIRD HEN

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ABSTRACT

Ostrich (Struthiocamelus), the largest flightless bird's and the flying bird (Gallus gallus) hen's egg Riboflavin Binding Protein (RBP) was isolated and purified from their eggs. First, the purification of RBP was undertaken in two steps, using DEAE–Sepharose ion exchange chromatography. Second, the purified (RBP) sample was obtained using gel filtration on Sephadex G-100. The holoprotein had an absorption spectrum characteristic of Flavoproteins. The purity of the RBP, was confirmed by SDS slab-gel electrophoresis, where the isolated RBP migrated as a single band. The mobility of the RBP with that of standard mol.wt proteins marker suggested that the mol.wt was 54 kda (Ostrich) and 29 kda (Hen). Against these RBP's antiserum was raised in Rabbits and this antiserum showed immunological cross reactivity – precipitin lines between the two compounds i.e flightless bird and flying bird RBPs. Hence the study proves that, the largest flightless bird (Ostrich) and flying bird (Hen) are immunologically relatives and phylogenetically different from each other.

KEYWORDS: Riboflavin binding protein (RBP), DEAE-Sepharose, Sephadex G-100, Molecular weight, Immunodiffusion analysis, antiserum.

INTRODUCTION

Vitamins are the important organic compounds which are required in small quantities and hence necessary to maintain normal metabolism and health of the body. The Vitamin Binding

Proteins are a group of soluble proteins which are present in both blood and other body fluids, to ensure optimal bio availability of the fat and water soluble vitamins for growth, metabolism and vertebrate reproduction. The adequate amount of depositions of vitamins and other micro nutrients in the development of the prosthetic embryo involves the participation of specific carrier proteins for each nutrients, Biotin Binding Protein[1&2], Vitamin B_{12}[3], Riboflavin[4&5], Retinol[6] and Cholecalciferol.[7]

Riboflavin (Vitamin B_2) is an important component of B-complex group of vitamins. Riboflavin Carrier Proteins (RCP) or (RBP) was isolated for the first time in the egg white of Hen[4] and its essential role has been demonstrated from a study of the homozygous recessive mutant (rdrd) of domestic fowl[8], hen egg yolk by[9], and later by Miller etal[10] and Murthy etal.[11] All animals are incapable of synthesizing the isoalloxozineskeleton of Riboflavin and hence require this vitamin in the range of 1-10 µg/g diet.[12] Rhodes was the first to report the presence of flavoprotein more avidly bound to riboflavin than its co-enzyme, but devoid of enzymatic action.[4] Further, egg white of heterozygous Leghorn hens RBP was isolated and antibodies were raised in rabbits and found they are immunologically active and functionally inactive protein in recessive mutants. These recessive mutants lack riboflavin binding capacity due to gene mutation coding for the carrier protein and this resulted in the death of the developing embryos.[13&14] Further, the discovery demonstrated that immuno neutralization of RBP could terminate pregnancies in rats and monkeys. Hence, suggesting that RBP a novel, first generation vaccine for regulating the fertility in both the sexes.[15,16&17]
In the present study, RBP was isolated, purified and characterized from a single egg yolk and white of an Ostrich(*Struthiocamelus*) and further the flavoprotein was immunologically compared with Hen (*Gallus gallus*) egg yolk and white RBP.

MATERIALS AND METHODS

Ostrich (*Struthiocamelus*) egg was procured from the private farm at Bengaluru, Karnataka and fresh Hen (*Gallus gallus*) eggs were procured from Poultry Farm PeddaPendyala, Warangal.

Isolation and Purification of Egg White and Yolk RBP

The yolks and whites were separated and used immediately or stored at -12°c. In the present study DEAE-Sepharose, Sephadex G-100, Freund's complete adjuant were obtained from Sigma Aldrich Chemical Company, St Louis, USA; Bovine Serum albumin, N,N,N^1,N^1 – Tetra Methyl ethylene – diamine, N,N – Methylene – bis – acrylamide and SDS were

procured from Loba Chemical, Mumbai, India. Riboflavin Binding Protein from Ostrich egg white and yolk was isolated using DEAE Sepharose and further purified by Sephadex G-100 following the methods previously reported.[18&19] Followed by spectral studies, where fractions were collected and absorbances were measured at 280 nm and 455 nm using UV visible recording Spectro photometer (Lambda 25 Perkin Elmer). The peak fractions were dialyzed against dist. water[20] which was reported earlier.[19] SDS-PAGE slab gels were carried out according to the method of Leammli.[21] The same steps were processed to purify RBP from Hen (*Gallus gallus*) egg yolk & white.

Production of antiserum to Riboflavin Binding Protein (RBP)

Antibodies against Ostrich (*Struthiocamelus*) egg white and Hen (*Gallus gallus*) egg yolk RBPs were produced adopting the method of Prasad and Adiga.[22] Briefly, the obtained protein was emulsified with an equal proportion of Freund's complete adjuant (Sigma Aldrich) and injected subcutaneously at weekly intervals for 4 weeks into Rabbits at various sites. The rabbits were then bled through the ear veins 7 days after the completion of the booster dose. The presence of the antibodies in the serum was tested using Ouchterlony double diffusion analysis.[23] Ouchterlony double diffusion analysis (1958) was carried out as follows: Agarose plates (1.2 %) were prepared in 0.05 M Sodium Phosphate buffer pH 7.8 containing 0.9% Nacl. The antiserum was placed in the central well and the protein dissolved in the serum buffer were placed in the adjacent wells. The appearance of precipitin lines indicated the presence of specific antibodies.

RESULT AND DISCUSSION

In the present study crude egg white and yolk fractions from an Ostrich and Hen were prepared. The crude Ostrich and Hen egg white and yolk were processed as described under materials and methods. The yellow supernatent, obtained after centrifugation was used for batch adsorption onto DEAE Sepharose. The protein bound gel was washed extensively with 0.1 m sod acetate buffer, pH 4.5 on a Buchner funnel and eluted with the same buffer containing 0.5 M Sodium Chloride, by suction filtration process. The eluted protein was dialyzed against 0.1 M sod acetate buffer, pH 4.5and loaded on a fresh DEAE Sepharose, column (2 ×26cm) and bound RBP was eluted as described earlier. Twenty fractions (5ml) were collected andthe peak fractions which were obtained were pooled, dialyzed and lyophilized.

Further purification was achieved by gel filtration on Selphadex G-100. The RBP fractions obtained from DEAE – Sepharose equilibrated with 0.05 M Phosphate buffer pH 7.4containing 0.5 M Nacl. The column was immediately eluted with same buffer. The purified RBP containing peak fractions obtained was used to record the absorption spectrum using UV-visible recording spectro photometer. The absorption spectrum of the Ostrich egg yolk RBP, to the protein (holoprotein) resulted in the absorption peaks at 372.8 nm and 454 nm were reported earlier.[19] The holoprotein in Hen egg white RBP showed absorption maxima at 375 nm and 458 nm in agreement with the data reported[21] and 374 nm & 457 nm.[4]

Production of antiserum of Riboflavin Binding Protein

Antibodies against Ostrich (flightless bird) egg white and Hen (flying bird) egg yolk RBP were produced by adopting method of Prasad and Adiga[22] as described earlier in materials and methods. Studies on the immunological aspects of RBPS are very limited and hence detailed analysis is required. RBP is immunologically a multi-determinant antigen consisting of at least six immunodominant regions each eliciting distinct size specific antibodies.[24,25,26 and 27] In the present study, the Ouchterlony immunodiffusion analysis revealed that the antibodies raised against Ostrich egg white RBP could show clear immunological cross reactivity with1. Ostrich egg white (Sephadex G-100 fraction), 2. Ostrich egg yolk (Sephadex G-100 fraction), 3. Hen egg white (Sephadex G-100 fraction) and 4. Hen egg yolk (Sephadex G-100 fraction) as shown in **Fig.1.** Further, antiserum raised against hen egg yolk RBP showed immunological cross reactivity with 1. Hen egg yolk (Sephadex G-100 fraction), 2.Hen egg white (Sephadex G-100 fraction), 3. Ostrich egg white (Sephadex G-100 fraction) and 4. Hen egg yolk (Sephadex G-100 fraction) as shown in **Fig. 2**.

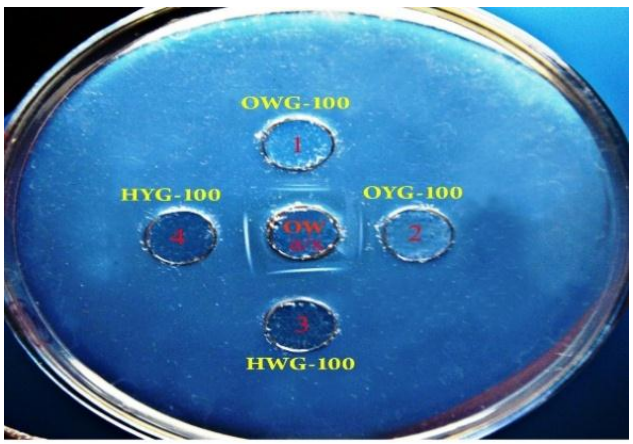

Fig 1: Ouchterlony double diffusion Analysis.

(The central wall contains Ostrich Egg white antiserum)

1. Purified Ostrich egg white Sephadex G-100 fraction.
2. Purified Ostrich egg yolk Sephadex G-100 fraction.
3. Purified Hen egg white Sephadex G-100 fraction.
4. Purified Hen egg yolk Sephadex G-100 fraction.

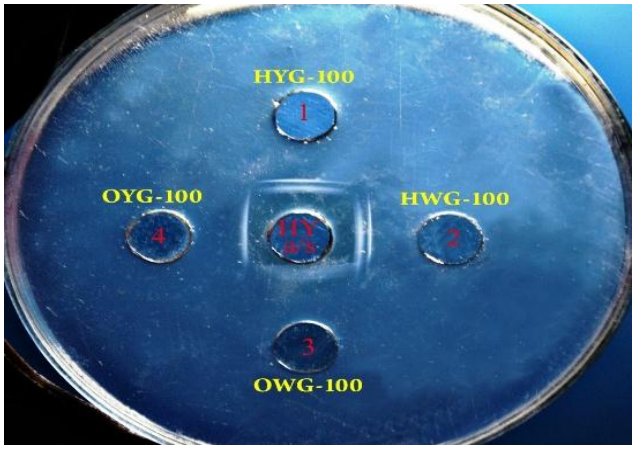

Fig 2: Ouchterlony double diffusion Analysis.

(The central wall contains Hen Egg yolk antiserum)

1. Purified Hen egg yolk Sephadex G-100 fraction.
2. Purified Hen egg white Sephadex G-100 fraction.
3. Purified Ostrich egg white Sephadex G-100 fraction.
4. Purified Ostrich egg yolk Sephadex G-100 fraction.

CONCLUSION

Thus the present study clearly suggested that the antibodies raised against Hen (flying bird) egg yolk RBP and Ostrich (flightless bird) egg white RBP could immunologically cross react with Hen egg white & yolk RBPs and Ostrich egg white & yolk RBPs. Hence the data clearly established that the Ostrich (flightless bird) and Hen (flying bird) are immunologically identical and phylogenetically different from each other and highly conserved during evolution.

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