PURIFICATION AND IMMUNOLOGICAL CHARACTERIZATION OF RIBOFLAVIN BINDING PROTEIN FROM EAGLE (Aquila hastate) AND DOMESTIC FOWL (Gallus gallus): A COMPARATIVE STUDY

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ABSTRACT

Riboflavin binding protein (RfBP) was isolated from the eggs of Aquila hastate and Gallus gallus. The protein was purified in two steps, DEAE-Sepharose ion exchange chromatography and gel filtration on Sephadex G-100. The holoprotein had an absorption spectrum characteristic of flavoproteins. The purity of the protein was judged by SDS-PAGE technique. A single band on the slab and cylindrical gels revealed that the protein was pure. Comparison of the mobility of RfBP with that of the standard molecular weight marker proteins suggested that RfBP from the egg white and yolk of Aquila hastate had a molecular weight close to 29 Kd. Ouchterlony double diffusion analysis revealed that the antibodies raised against eagle egg RfBPs failed to cross react with RfBPs isolated from hen eggs, suggesting immunological variation between the RfBPs from these two birds.

Keywords: Riboflavin binding protein (RfBP), DEAE-Sepharose, Electrophoretic characterization, Molecular weight, Immunodiffusion analysis.

INTRODUCTION

Riboflavin is unique among the water soluble vitamins in that egg, milk and dairy products make the greatest contribution to its intake in Indian diets. Meat and fish are also good sources of riboflavin and certain fruits and vegetables especially dark-green vegetables contain reasonably high concentrations. Animals are incapable of synthesizing the isalloxazine skeleton of riboflavin. Hence this vitamin is required in the range of 2 to 10µg/g in the diet [1]. Biochemical signs of depletion arise within a few days of dietary deprivation. Poor riboflavin status in western countries also seem to be of most concern for the elderly and adolescents. Riboflavin deficiency has been implicated as a risk factor for cancer.

Recent studies have established that vitamin binding proteins form a special group of soluble proteins present in eggs and other body fluids which ensure optimal bioavailability of the vitamins during growth and development. The indispensability of RfBP was demonstrated in a study on the homozygous recessive mutant (rd-rd) of domestic fowl wherein, a gene mutation leading to the absence of RfBP resulted in the death of the developing embryos [2]. Further the discovery that immunoneutralization of RfBP in animals such as rats and monkeys resulted in the abrupt termination of pregnancy clearly established the functional significance of RfBP [3].

The present investigation was carried out to isolate and characterize RfBP from the eggs of two different birds. Hen (Gallus gallus) and Eagle (Aquila hastate). For a comparative study Aquila hastate belongs to rait family Accipitridae, an eagle may resemble a vulture in build and flight characteristics but has a fully feathered (often crested) head and strong feet equipped with great curved talons. The Indian eagle is about 60 cm in length and has a wingspan of 1.50 m. It is broad-headed, with the widest mouth of all eagles.

RfBP was first isolated from the hen egg white and recently from Emu (Dromaius novaehollandiae) egg-white [4]. These methods were slightly modified for the isolation of egg white and yolk RfBPs from Aquila hastate.

MATERIALS AND METHODS

Aquila hastate eggs were procured from Old-city, Hyderabad, Andhra Pradesh, and Hen eggs were procured from local Hunter road, Hanamkonda. The white and yolks were separated and used immediately or stored at -12°C. DEAE-Sepharose and Sephadex G-100 were obtained from Sigma Aldrich Chemical Company, St. Louis, USA. Bovine Serum albumin, acrylamide, N, N, N'-Tetramethylethylenediamine, N, N'-methylene-bis-acrylamide and SDS were procured from Loba Chemical, Bombay, India.

Isolation and purification of eagle egg and hen egg white & yolk riboflavin binding protein (RfBP): RfBP from eagle egg white and yolk was isolated following the methods previously reported [5] with a few modifications. Eagle egg white or yolk was collected and homogenized with an equal volume of 0.1 M sodium acetate buffer pH 4.5. To the clear supernatant DEAE-Sepharose previously equilibrated with 0.1 M sodium acetate buffer pH 4.5 was added. The DEAE-Sepharose with bound protein was washed with an excess of 0.1 M sodium acetate buffer pH 4.5. Bound proteins were eluted with the same buffer containing 0.5 M sodium chloride by suction filtration. The same steps were followed for purification of RfBP from Hen egg yolk RfBP was loaded onto the column previously equilibrated with 0.1M sodium acetate buffer pH 4.5 containing 0.5 M sodium chloride. Fractions were collected and absorbances were measured at 280 nm and 455 nm. Further purification of eagle egg white and yolk RfBPs were achieved by gel filtration column chromatography using Sephadex G-100. The almost pure eagle egg yolk RfBP was loaded onto the column previously equilibrated with 0.2 M phosphate buffer pH 7.3 containing 0.5 M sodium chloride and eluted with the same buffer. Fractions were collected and the protein in each fraction was determined by the method of [6]. The same steps were followed for purification of RfBP from Hen egg yolk RfBP was loaded onto the column previously equilibrated with 0.2 M phosphate buffer pH 7.3 containing 0.5 M sodium chloride and eluted with the same buffer. Fractions were collected and the protein in each fraction was determined by the method of [6]. The same steps were followed for purification of RfBP from Hen egg white and yolk.

Polyacrylamide Gel Electrophoresis: SDS-PAGE was carried out according to the method of [7] using sodium phosphate buffer containing SDS. The following were made.
1. Sodium phosphate stock buffer (pH 8.0): To 461ml of 0.2 M sodium hydroxide, 500 ml of 0.2 M sodium dihydrogen phosphate was added and made up to 1 litre with distilled water. To this 5 mM EDTA, and 1g SDS were added.
2. Electrode Buffer: 500 ml of stock buffer was diluted to 1 litre with distilled water.
3. Acrylamide - Bis acrylamide Solution: 30g of acrylamide and 0.8 g of bisacrylamide were dissolved in 10ml degassed water.
4. Ammonium persulphate solution: 150 mg of ammonium persulphate was dissolved in 100 ml degassed water.
5. Sample Buffer: 20 ml of the electrode buffer was degassed and 600 mg of SDS was added to 1 ml of this buffer 30 mg SDS, 500 mg sucrose, 20µl β- mercaptoethanol and 20µl bromophenol blue were added.
6. **Protein staining solution**: Coomassie blue (0.2 g) was dissolved in a solution containing 50 ml methanol, 7 ml of acetic acid and 43 ml water.

7. **Destaining solution**: The gels were destained with the solutions containing 50% methanol and 7% acetic acid.

**SDS-PAGE: Cylindrical Gel**

The gels were prepared by mixing 2 ml distilled water 8 ml running buffer, 4 ml acrylamide-bisacrylamide solution, 20 µl TEMED AND 2 ml ammonium persulphate solution. The samples were dissolved in 50 µl of sample buffer. The samples were heated for 2 min. in a boiling water bath. 20 µl 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 stained overnight with staining solutions, and after destaining the gels were stored in distilled water till they were photographed.

**SDS-PAGE: Slab Gel**

The gels were prepared by mixing 4 ml of distilled water, 16 ml electrode buffer, 8 ml acrylamide-bisacrylamide, 40 µl TEMED and 4 ml ammonium per sulphate. The prepared gel solution was poured into glass plates (14x14 cm) separated by 1 mm thick spacer. The samples were dissolved in 50 µl sample buffer and kept in a boiling water bath for 2 min. The A. hastate and hen egg white and yolk samples (20 µl) were loaded into the slots. The reaming gap was filled with the electrode buffer. The glass plates were fixed to the electrophoresis apparatus without disturbing the samples. The upper and the lower electrode chamber were filled with the electrode buffer. The electrode chambers were connected to the power supply. Initially electrophoresis was carried out at 15 mA for 30 min, after which the current was raised to 30 mA. Current supply was terminated when the tracking dye reached the end of the gel. The plates were removed from the chambers and the gel was removed from the glass moulds by flushing buffer between the plates. The gel was stained immediately at room temperature. Later the gels were destained using the destaining solution. Proteins were also stained by the silver staining method using a silver staining kit (Bangalore Genei Ltd).

**Production of antibodies against riboflavin binding protein**

Antibodies against eagle and hen egg white RfBPs were produced adopting the method of [13]. 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 rabbit 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 0.05 M sodium phosphate buffer pH 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 antibodies.

**RESULTS AND DISCUSSION**

The purified RfBP containing peak fraction obtained from Sephadex G-100 gel filtration column chromatography was used to record the absorption spectrum using UV-Visible recording spectrophotometer. The spectral characteristic of Riboflavin are altered upon binding to the apo-RfBP. These spectral changes are characterized by a red shift of the 450 nm band which accompany the appearance of shoulders and a remarkable hypochromism at the 370 nm band (Figs. 1, 2a & 2b). The holoprotein showed absorption maxima at 375 nm and 458 nm in agreement with the data reported by [10].

Electrophoresis on analytical polyacrylamide gels (7.5%) was conducted at pH 8.3. The purity of the isolated protein was judged by slab and cylindrical SDS-PAGE methods. The electrophoretic pattern obtained was shown in Fig. 3. A major band corresponding to RfBP along with few minor bands were seen in the DEAE-Sepharcose eluted fraction. Complete purification was achieved by gel-filtration chromatography on Sephadex G-100, as a single band free from other minor contaminating proteins was obtained (Fig. 3 & 4a). RfBP moved as a single band on the cylindrical gels also (Fig. 5). Comparison of the mobility of RfBPs with that of the standard molecular weight marker proteins revealed that the RfBPs had a molecular weight close to 29 kilodaltons.

Analysis of carbohydrate composition of hen egg white RfBP revealed that the protein contains 10% hexosamine and 4% neutral sugars with a single sialic acid at the terminus of a highly branched oligosaccharide chain [14]. Hen egg yolk RfBP also contains both hexosamine and hexose as well as multiple sialic acid residues [15]. In the present study, the isolated RfBPs from Hen, Coot and Eagle eggs were analyzed using SDS PAGE and then subjected to glycoprotein specific silver staining. All these glycoproteins (Fig. 6), as revealed by the gel banding profiles, isolated from three birds eggs had the the same electrophoretic mobility with an approximate molecular mass of 29 kDa.

Studies on the immunological aspects of RfBPs are very limited and a detailed analysis is needed. RfBP is immunologically a multideterminant antigen consisting of at least six immunodominant regions each eliciting distinct site specific antibodies [15]. In the present study, the Ouchterlony immunodiffusion analysis revealed that the antibodies raised against eagle egg white and yolk RfBPs could cross react with eagle egg white and yolk RfBPs only and failed to cross react with hen egg white and yolk RfBPs (Figs 7g & 8). Absence of precipitin line formation against the RfBPs from hen eggs could be due to some extent, differences in the antigenicity and antigenic domains. This could be due to less conserved secondary and tertiary structures of these proteins.

The present study clearly showed that RfBP from *Aquila hastate* (flying bird) had an electrophoretic mobility similar to that of hen (non flying bird) egg-white and yolk RfBP having a molecular weight close to that of the hen egg-white and yolk RfBP suggesting that this protein mostly remained unaltered in these species. However, the lack of immunological cross reactivity as judged by Ouchterlony double diffusion analysis indicate some differences during evolutionary conservation of these proteins.

Thus the present study while clearly establishing the similarities in the physicochemical properties between hen egg RfBPs and eagle egg RfBPs the protein might not be totally immunologically related.
Fig. 2a: Absorption spectrum of Eagle egg-white Riboflavin Binding Protein

Fig. 2b: Absorption spectrum of Eagle egg-yolk Riboflavin Binding Protein

Fig. 3: SDS Polyacrylamide Gel Electrophoresis pattern of Eagle egg white and egg yolk RfBPs.

1. Eagle egg white crude
2. Eagle egg white DEAE Sepharose eluted fraction
3. Eagle egg white Sephadex G-100 fraction
4. Eagle egg yolk crude
5. Eagle egg yolk DEAE Sepharose eluted fraction
6. Eagle egg yolk Sephadex G-100 fraction
7. Protein molecular weight marker (20,000 to 97,400 kD)

Fig. 4: SDS Polyacrylamide Gel Electrophoresis pattern of Eagle egg white and Hen egg white RfBPs.

1. Eagle egg white DEAE Sepharose eluted fraction
2. Eagle egg white Sephadex G-100 fraction
3. Hen egg white DEAE Sepharose eluted fraction
4. Hen egg white Sephadex G-100 fraction

Fig. 5: Cylindrical Gel Electrophoresis (SDS Polyacrylamide) pattern of Eagle egg white and Hen egg white RfBPs.

1. Eagle egg white G-100 eluted fraction
2. Eagle egg yolk G-100 eluted fraction
3. Hen egg white G-100 eluted fraction
4. Protein molecular weight marker (20,000 to 97,400 kD)

Fig. 6: SDS Polyacrylamide Gel Electrophoresis pattern of Hen, Coot, Eagle egg white and egg yolk RfBPs’s Silver staining

1. Hen egg white Sephadex G-100 fraction
2. Hen egg yolk Sephadex G-100 fraction
3. Coot egg white Sephadex G-100 fraction
4. Coot egg yolk Sephadex G-100 fraction
5. Eagle egg white Sephadex G-100 fraction
6. Eagle egg yolk Sephadex G-100 fraction
7. Protein molecular weight marker (18,400 to 97,000 kD)
CONCLUSION

The present investigation was clearly showed these are correlated to both electrophoretic mobility and molecular levels of egg and hen, and also physiological properties between hen egg RBP and egg yolk RBP the proteins might not be totally immunologically related. That RBP from *Aquila hastate* (flying bird) had an electrophoretic mobility similar to that of hen (non-flying bird) egg-white and yolk RBP having a molecular weight close to that of the hen egg-white and yolk RBP suggesting that this protein mostly remained unaltered in these species. However, the lack of immunological cross-reactivity as judged by Ouchterlony double diffusion analysis indicate some differences during evolutionary conservation of these proteins.

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