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ISOLATION AND PURIFECATION OF RIBOFLAVIN BINDING PROTEIN FROM EAGLE EGG-YOLK (*AQUILA HASTATE*)

Madhukar Rao Kudle, B.Satheesh Kumar and M.S.K Prasad

Department of Biochemistry, Kakatiya University, Warangal, India-506009

Corresponding author: madhukarbiochem@gmail.com

ABSTRACT : Riboflavin binding protein was purified from the egg yolk of *Aquila hastate* (Eagle). The protein was purified using DEAE Sepharose ion exchange chromatography followed by gel filtration on Sephadex G-100. The purity of the protein was judged by cylindrical and slab SDS-PAGE techniques. Comparison of the mobility of RfBP with that of the standard molecular weight marker protein revealed that the RfBP had a molecular weight close to 29 Kd. Interestingly the RfBPs from hen egg yolk and eagle egg yolk had the same molecular weights as revealed by the SDS PAGE.

Keywords: Aquila hastate, DEAE –Sepharose, Electrophoretic characterization, Riboflavin binding protein (RfBP).

INTRODUCTION

Riboflavin binding protein (RfBP) is involved in the deposition of Riboflavin, the essential vitamin in the hen egg yolk and white compartments to meet the needs of the prospective embryos for normal development and growth. Such specific binding or carrier proteins have also been identified in oviparous species for biotin, vitamin B12, folic acid retinol and vitamin D[1] . Among the various vitamin binding proteins hitherto studied hen RfBP from egg white is the best characterized because simple procedures are involved in the isolation of this protein. The paramount functional significance of RfBP in avian reproduction is exemplified by the finding with a mutant strain of hen lacking a functional RfBP gene which results in a failure of riboflavin transport deposition and non hatching of eggs [2]. Similarly, it was discovered that passive immunoneutralization of RfBP terminates pregnancy in mice and rats, while active immunization of monkeys with RfBP prevents establishment of pregnancy [3]. Both the egg yolk and egg white RfBP are produced from a single, estrogen inducible gene, expressed in the liver and the oviduct respectively of egg laying birds. Even though the large amount of lipid make yolk RfBP purification difficult, isolation of hen egg yolk RfBP was reported by Ostrowis *et al* (1962)[4] and Hamazume *et al* (1984)[5]. These methods were slightly modified in the isolation of RfBP from pigeon and peacock egg yolk [6,7]. In the present study, RfBP was purified for the first time from Indian Eagle egg yolk for a comparative study in terms of gross molecular characteristics such as molecular weight and immunological properties, with hen egg yolk RfBP to understand the progressive changes that might have manifested during evolution.

MATERIAL AND METHODS

A. hastate eggs were procured from Old-city, Hyderabad, Andhra Pradesh. The whites and yolk were separated and used immediately or stored at -12^{0}C. DEAE- Sepharose and Sephadex G-100 were obtained from Sigma Aldrich Chemical Company. St. Louis, USA. Bovine Serum albumin, acrylamide, N, N, N^1, N^1- Tetramethylethylene- diamine, N, N^1-methylene-bis-acrylamide and SDS were procured from Loba Chemical, Bombay, India.

Isolation and purification of Eagle egg yolk riboflavin binding protein (RfBP)

RfBP from *Eagle* egg-yolk was isolated following the methods previously reported [8,9,10,11] with a few modifications. *Eagle* egg yolk was collected (10eggs, 250 ml) 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. Fresh DEAE-Sepharose previously equilibrated with 0.1M sodium acetate buffer pH 4.5 was packed into the column and then the partially purified RfBP was loaded onto the column. Riboflavin binding protein was eluted from the column with 0.1 M sodium acetate buffer, pH 4.5 containing 0.5 M sodium chloride. Fractions were collected and absorbances were measured at 280 nm and 455nm. Further purification of eagle yolk RfBP was 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 0.02 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 Lowry *et al*., (1951)[12].

SDS-PAGE: Cylindrical Gel:

The gels were prepared by mixing 2 ml distilled water 8 ml running buffer, 4 ml acrylamide bisacrylamide solution, 20 TEMED AND 2 ml ammonium persulphate solution. The samples were dissolved in 50 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 staining overnight with distaining solutions, and stored in distilled water till they were photographed.

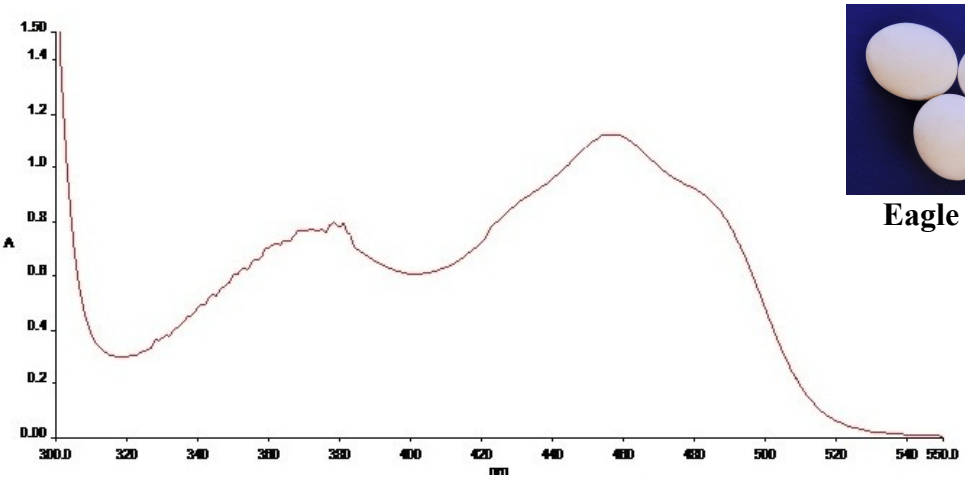
SDS-PAGE: Slab Gel:

The gel 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 2min. The *A. hastate* egg yolk samples (20µl) were loaded into the slots. The remaining 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 15mA for 30 min, after which the current was raised to 30mA. Current supply was terminated when the tracking dye reached the end of the gel. The plates were removed from the chambers, 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.

RESULTS AND DISCUSSION

In the present study it was found that better purification could be achieved by using two successive ion exchange binding steps using DEAE Sepharose. Complete purification of Eagle egg-yolk RfBP was accomplished by gel filtration on Sephadex G-100. The absorption spectrum of the riboflavin apoprotein complex (Fig: 1) indicates that this flavoprotein had absorption maxima at 370 nm and 456 nm. The spectral data were in full agreement with that reported earlier for hen egg white RfBP by Choi and Mc. Cormic (1980)[13].The purity of the isolated protein was judged by SDS-PAGE (Fig: 2). A major band corresponding to RfBP along with a few minor bands was seen in DEAE-Sepharose column eluted fractions (Fig: 3). Complete purification was achieved by gel-filtration on Sephadex G-100, as a single band free from other contaminating proteins could be seen (Fig: 2).

Comparison of the mobility of Eagle egg-yolk RfBP with that of the standard marker proteins revealed that the Eagle egg-yolk RfBP had a molecular weight close to 29Kd. Further, the Eagle egg-yolk RfBP appeared to be having the same molecular weight of hen egg-white RfBPs as these two RfBPs had the same electrophoretic mobilities on SDS-Cylindrical gels (Fig: 3). This data suggests that, even though, the two avian species (Hen and Eagle) phylogenetically distinct, their RfBPs could be structurally similar, mostly unaltered during the evolution.



Eagle eggs

Fig – 1: Absorption spectrum of *A. hastate* egg -yolk RfBP (Sephadex G-100 Fraction)

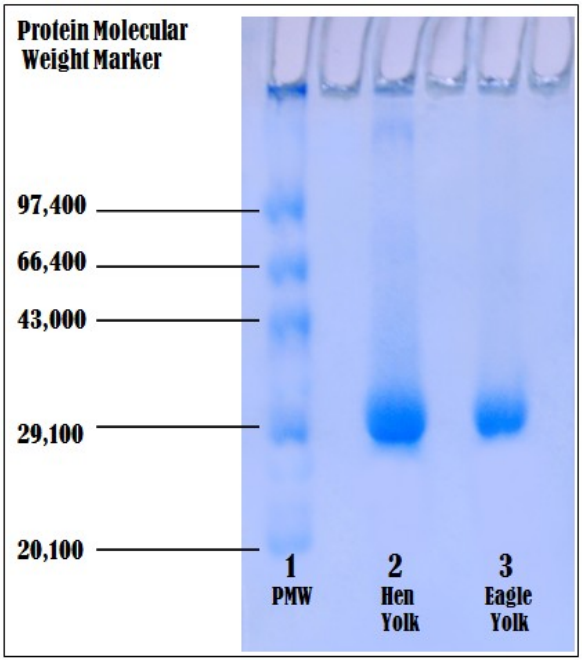


Fig-2: SDS-PAGE Pattern of eagle egg-yolk RfBP

1. Protein Molecular Weight Markers (20,000 to 97,400 Da)
2. Hen egg-yolk RfBP Sephadex G-100 fraction.
3. Eagle egg-yolk RfBP Sephadex G-100 fraction.

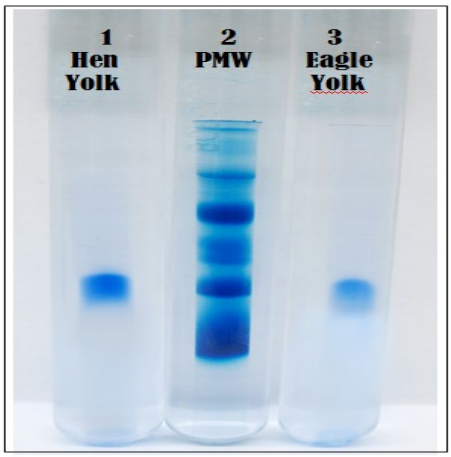


Fig- 3: Cylindrical Gel Electrophoretic pattern of the Eagle egg-yolk RfBP (SDS-PAGE)

1. Hen egg-yolk RfBP Sephadex G-100 fraction.
2. Protein Molecular Weight Markers (20,000 to 97,400 Da)
3. Eagle egg-yolk RfBP Sephadex G-100 fraction.

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