INDO AMERICAN JOURNAL OF PHARMACEUTICAL RESEARCH

SCREENING OF RIBOFLAVIN BINDING PROTEIN (RFBP) FROM AVIAN EGGS TO SCREEN THEIR ANTI-CANCER ACTIVITY

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ARTICLE INFO

Article history
Received 14/12/2016
Available online
31/01/2017

Keywords
Avian Eggs,
RFBP,
SDS-PAGE,
Anticancer Activity.

ABSTRACT

Riboflavin binding protein (Rfbp) was purified from different avian eggs including Pea cock (*Pavo cristatus*) Egg- white and Hen (*Gallus gallus*) egg- white Riboflavin binding protein (RfBP) was isolated. The Rfbp was purified in two steps; DEAE-SephadexA-50 ion exchange chromatography. The final purification of proteins (Rfbp) was achieved on SephadexG100.The protein content was estimated with Lowry method. The purity of the proteins was judged by two methods like cylindrical and slab-gels, SDS- PAGE techniques. These proteins showed a single band on SDS gels and the molecular weight was 29 Kilo Daltons. Antiserum was raised against these Rfbp is in rabbit. These proteins are emulsified in Freund is complete adjuvant and injected subcutaneously at weekly intervals for 4 weeks in to the rabbit at multi places. The rabbit anti serum was collected through the ear vein, 7 days after the booster injection. This serum was analyzed in- vitro method with HeLacervical cancer cell- lines. MTT [(3- (4, 5- dimethyl thiazol-2yl)-2, 5-diphenyl tetrazolium bromide] measures the metabolic activity of the viable cells. The viable cell counting with trypan blue dye exclusion method. There was more than 85 percent of cell death was observed. The anti cancer activity of Riboflavin binding proteins (Rfbp) purified from egg-white of Pea cock (*Pavo cristatus*) 87.7% and Hen (*Gallusgallus*) egg-white 87.5, egg-yolk 86.2% and Emu egg white (*Struthio camelus*) 85.0 contains anti cancer activity on HeLacelllines.

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*Please cite this article in press as **P. Koteshwar Rao** et al. Screening of Riboflavin Binding Protein (RFBP) from Avian Eggs to Screen their Anti-Cancer Activity. Indo American Journal of Pharmaceutical Research.2017:7(01).*

INTRODUCTION

Water–soluble yellow colored pigments is now know to be identical to riboflavin (RF), were first isolated first milk, eggs, and various animal tissues. These materials were initially named in relation to their origin were eventually recognized to be a single compound, Vitamin –G; [1], which we know today as vitamin B_2. All the animals are incapable of synthesizing the isoalloxazine skeletion of RF and require this vitamin in the range of 1-10u/g diet [2]. The specific binding proteins for fat soluble vitamins such as vitamin A and vitamin D are identified in normal serum in all vertebrates [3, 4, 5]. Riboflavin Binding Protein (RfBP) or Riboflavin Carrier Protein (RCP) was first isolated the chicken egg white [6]. Thiamin binding protein, [7, 8, 9] have been demonstrated in the sera and egg white and yolk of the egg laying hens. It is a phosphor-glycoprotein. The faint yellow color of normal egg white is due to riboflavin bound to a specific protein. In [10], encountered a mutant strain of single comb white Leghorn chicken that laid eggs lacking of typical yellow colour of egg white, whose embryos died between 10 and 14 days of incubation. The essential role of RfBPs has been demonstrated from a study of the homozygous recessive mutant (rd rd) of domestic fowl. Developing embryos having this genetic constitution die at around 13 days of incubation, from riboflavin deficiency. A normal hatch was achieved only by a direct injection of riboflavin into such eggs. Subsequently, it was shown that the homozygous recessive (rd rd) hens were unable to synthesize riboflavin binding protein; [11]. It is one of the investigation in our lab purification and characterization of various avian eggs and its characterization, [12, 13, 14, 15].

MATERIALS

Hen (*Gallus gallus*) eggs were obtained from the poultry farm. Emu eggs (*Struthio camelus*) were obtained from the emu form, Karimnagar. DEAE-Sephadex A-50 used in the present study was obtained from Pharmacia Fine Chemicals, Uppasala, Sweden. Sephadex G-100 was procured from Sigma-Aldrich Chemical Company, St. Louis, USA. MTT (3-(4, 5-dimethylthiazol-2yl) - 2, 5-diphenyltetrazolium bromide) from Himedia, Mumbai, India. Fetal bovine RPMI-1640 media serum (Himedia, Mumbai, India). Dimethylsulphoxide (DMSO) (Merck India Ltd, Mumbai, India). HeLa- cervical cancer cell-lines Dabur research foundation, Ghaziabad, U.P.

METHODOLOGY

(3.1). Isolation and purification of emu egg-white riboflavin binding protein

(3.2). Preparation of peacock egg-white

Emu Egg-whites were collected carefully from two eggs. Pooled egg-whites approximately were homogenized with an equal volume of 0.1M sodium acetate buffer pH 5.0. The crude white suspension was centrifuged at 15000 x g for 30 minutes at 4^0C. The precipitate was discarded. The clear yellow supernatant approximately (850 ml) was used directly for batch adsorption onto DEAE-Sephadex.

(3.3). Batch adsorption to deae-sephadex a-50

To 250ml of Emu egg-white solution DEAE-Sephadex (previously equilibrated with 0.1 M sodium acetate buffer pH 5.0) was added. The mixture was stirred overnight at 4^0C and then suction filtered. The DEAE-Sephadex was washed with 2 liters of 0.1 sodium acetate buffer pH 5.0. The Riboflavin Binding Protein was eluted with 0.1 M sodium acetate buffer pH 5.0 containing 0.5 M NaCl by suction filtration. The protein was dialyzed against distilled water,

Fresh DEAE-Sephadex previously equilibrated with 0.1 M sodium acetate buffer pH 5.0 was packed into the column and then the partially purified RfBP was loaded on the column. RfBP was eluted from the column with 0.1 M sodium acetate buffer pH 5.0 containing 0.5 M NaCl. Twenty fractions) were collected. Further purification was achieved by gel filtration on Sephadex G-100.

(3.4). Gel filtration on sephadex g-100

The partially purified peacock egg-white RfBP was dissolved in 1.2 ml of phosphate buffer and was loaded on the Sephadex G-100 (4 gm) column previously equilibrated with 0.05 Phosphate buffer pH 7.4 containing 0.5M NaCl. The protein was eluted with the same buffer. Twenty fractions (5 ml) were collected. Protein in each fraction was determined by the method, [16]. The peak fraction were pooled and dialyzed against distilled water and lyophilized. The purity of the protein was checked by the analytical polyacrylamide gel electrophoresis. [17].

4. Isolation and purification of emu egg-yolk rfbp

Emu egg-yolk RfBP was purified in two steps (Batch adsorption to DEAE-Sephadex and Gel filtration column chromatography on Sephadex G-100).

(4.1). Preparation of emu egg-yolk

Prior to adsorption onto DEAE-Sephadex, the egg-yolks were collected carefully from 2 eggs. Pooled egg-yolks (225 ml) were homogenized with four volumes of 0.1 M sodium acetate buffer pH 5.0. The crude yolk suspension was centrifuged at 10000 x g for 20 minutes at 4^0C. The precipitated mucilaginous material was discarded. The clear yellow supernatant was used directly for batch adsorption onto DEAE-Sephadex.

(4.2). Batch adsorption to deae-sephadex a-50

To 900ml of clear Emu egg-yolk supernatant, DEAE-Sephadex, (previously equilibrated with 0.1 M sodium acetate buffer pH 5.0) was added. The mixture was stirred overnight at 4^0C and then suction filtered. The DEAE-Sephadex was washed with 2 liters of 0.1 M sodium acetate buffer pH 5.0 and re-suspended in 2 liters of buffer, allowed to settle at 4^0C and the supernatant was decanted. The DEAE-Sephadex with bound protein was washed extensively with the same buffer. The Riboflavin Binding Protein bound to the DEAE-Sephadex was eluted with 0.1 M sodium acetate buffer pH 5.0 containing 1 M NaCl by suction filtration. The protein fraction was dialyzed against distilled water.

Fresh DEAE-Sephadex previously equilibrated with 0.1 M sodium acetate buffer pH 5.0 was packed into the column (2 x 26 cm) and then the partially purified RfBP was loaded on the column. The column was washed with excess of buffer. RfBP was eluted from the column with 0.1 M sodium acetate buffer pH 5.0 containing 1 M NaCl. Thirty four fractions (5 ml) were collected. The peak fractions having high absorbance both at 280 nm and 455 nm were pooled and dialyzed against distilled water. Further purification was achieved by gel filtration on Sephadex G-100.

(4.3). Gel filtration on sephadex g-100

The partially purified Emu egg-yolk RfBP was dissolved in 1 ml of phosphate buffer and was loaded on the Sephadex G-100 (5gm) column previously equilibrated with 0.05 M phosphate buffer pH 7.4 containing 0.5 M NaCl. The protein was eluted with the same buffer twenty nine (5 ml) were collected. The peak fractions were pooled and dialyzed against distilled water and lyophilized. The purity of the protein was checked by the analytical polyacrylamide gel electrophoresis (Fig.3).

Fig.3. Emu eggs and Hen eggs.

(4.4). Mtt- assay

MTT, it is suitable for measuring cell proliferation, cell viability or cytotoxicity. MTT [(3-(4, 5-dimethylthiazol-2yl)-2, 5-diphenyltetrazoliumbromi- de] measures the metabolic activity of the viable cells. Procedure involves culturing the cells in a 96-well microtiterplate, and then incubating them with MTT solution for approximately 2 hours. The reaction between MTT and 'mitochondrial dehydrogenase' produces water-insoluble formazan salt. During incubation period, viable cells convert MTT to a water-insoluble formazan dye. The formazan dye in the MTP is solubilized and quantified with an ELISA plate reader. The absorbance directly correlates with the cell number.

RESULTS

MTT, it is suitable for measuring cell proliferation, cell viability or cytotoxicity. MTT [(3-(4, 5-dimethylthiazol-2yl)-2, 5-diphenyltetrazoliumbromi- de] measures the metabolic activity of the viable cells. Procedure involves culturing the cells in a 96-well microtiterplate, and then incubating them with MTT solution for approximately 2 hours. The reaction between MTT and 'mitochondrial dehydrogenase' produces water-insoluble formazan salt. During incubation period, viable cells convert MTT to a water-insoluble formazan dye. The formazan dye in the MTP is solubilized and quantified with an ELISA plate reader. The absorbance directly correlates with the cell number.

DMSO concentration in the well to be less than 1%.100µl of cell suspension was transferred aseptically to each well of a 96 well plate and to it 100µl of 1% media/ drug solution .The plate was then incubated at 37^0C for 72 hours in CO_2 incubator. After 72 hours of incubation, 20µl of MTT was added to each well and the plate was wrapped in aluminum foil to prevent the oxidation of the dye. The plate was again incubated for 2 hours.80µl of lysis buffer was added to each well and the plate was placed on a shaker for overnight. The absorbances were recorded on the ELISA reader at 562nm wavelength. The absorbances of the test were compared with that of DMSO control to get the %inhibition.

Viable cell assay by dye exclusion method

In this assay white transparent cells are viable cells and blue cells are dead cells. This method is particularly recommended for assay in suspension cultures. Trypan blue is one of dye recommended for use in dye exclusion procedures for viable cell counting. This method is based on the principle that live (viable) cells actively pump out the dye by efflux mechanism, whereas dead (non-viable) cells do not.

The contents of the culture flask were transferred into a centrifuge tube aseptically and then centrifuged at 2000 rpm for 2 minutes. Supernatant was discarded and the pellet was resuspended in fresh medium and mixed thoroughly to get a uniform cell suspension. 0.3 ml of the cell suspension was added aseptically to each well in the 96-well plate. Drug solutions are made in medium such that the final concentration of the solvent (DMSO) is less than 1%. 1% DMSO in media served as control. Each well was added with 0.7ml of medium/1% DMSO/ drug solutions. The plate was then incubated at 37^0C for 72 hours in CO_2 incubator. After 72 hours of incubation the plate was taken and the viable cells were counted as follows: 100µl of the cell suspension is taken in a sterile eppendorf tube; to it 100µl of trypan blue is added. Then 10 µl of the dye-cell suspension mixture was transferred onto heamocytometer. A separate count was maintained for viable and non-viable cells. The % inhibition of growth was calculated by comparing the % viability in the well with test compound with that of the control.

Calculations

Cells/ml = Average count per square x dilution factor.
Total viable cells = Cells/ml x original volume of cell suspension.
Cell viability % = Total viable cells (unstained)/ Total cells.

DISCUSSION

The pooled fraction from DEAE-Sephadex was dialyzed against distilled water and lyophilized. Further steps carried out according method. The elution profile of partially purified Hen (*Galluas gallus*) egg-white elution profile Sephadex G-100. (Figure: 1) The absorbance's were measured at 280 nm and 455 nm using UV-visible recording spectrophotometer (Figure no: 2), the purity of the isolated protein was judged by Native-PAGE and SDS-PAGE. Partial purification of peacock egg white RBP could be achieved by using DEAE-Sephadex. This evident by the fact that the eluted protein fraction contained additional protein bands along with RfBP both on negative slab gel and SDS slab gel, Further, electrophoresis using cylindrical gels in the presence of SDS clearly demonstrated that RfBP could be purified to homogeneity after gel filtration on Sephadex G-100. RfBP moved as a single band both on the Slab and Cylindrical gels (Figures: 3). Comparison of the mobility of RfBP with that of the molecular weight marker proteins revealed with that the RfBP had a molecular weight close to 29,000 Kilo Daltons. Interestingly hen egg-white, egg-yolk RfBP and peacock egg- white, egg-yolk RfBP had the same molecular weight as revealed by the SDS polyacrylamide gel electrophoresis. Antibodies against hen egg-white, yolk peacock egg-white; egg-yolk RfBP's were produced in the rabbits adopting the method [18].

Cytotoxicity studies

Maintenance of the cell lines was carried out using RPMI-1640 media, and the sub-cultures of the cell lines were maintained for optimum (<P25) and properly maintained in the deep freezer (-80^{o}C). The cell counts were done using the trypan blue dye exclusion method on Neubaur slide (heamocytometer). The results of cytotoxicity with percentage inhibition of half-diluted antiserum were indicated in (Table-1).

Table 1. Cytotoxicity with percentage of inhibition.

Blank	Control	Hen egg white ½ dilution		Hen egg yolk ½ dilution		Emu egg white ½ dilution		Peacock egg yolk ½ dilution	
10.849	9.249	1.271	1.346	1.315	1.241	1.823	1.972	0.445	0.439
10.849	9.149	1.288	1.007	1.218	1.171	1.979	1.960	0.541	0.458
10.149	9.649	1.187	0.826	1.228	1.071	0.699	0.793	0.386	0.545
9.149	10.749	1.371	1.150	1.341	1.312	1.853	1.888	0.514	0.628
10.149	8.849	1.278	1.306	1.274	1.312	0.712	0.745	0.476	0.513
10.149	9.549	1.123	1.177	1.345	1.019	0.843	0.807	0.532	0.458
9.749	10.949	1.410	1.238	1.249	1.214	1.771	1.765	0.429	0.449
10.449	10.249	1.608	1.219	1.129	1.239	1.829	1.934	0.512	0.529
Average:									
10.186	9.786	1.284	1.158	1.262	1.147	1.440	1.480	0.479	0.502
		1.221		1.204		1.460		0.490	
Percentage of inhibition									
		87.5		86.2		85.0		87.7	

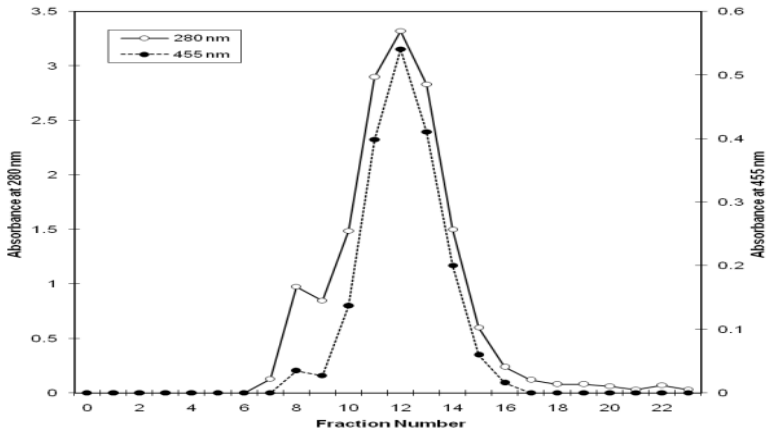
Initially partial purification of RfBP could be accomplished by batch adsorption of peacock egg-yolk homogenate to DEAE-Sephadex followed by a column elution. Gel electrophoresis of this fraction revealed the presence of one major protein band which had mobility comparable to that of the purified RfBP and minor protein band. It was clear that at this stage of purification itself, RfBP could be enriched by many-fold and freed from contaminating proteins to a large extent. Further purification using Sephadex G-100 column chromatography The partially purified and completely purified hen egg-white, egg-yolk and peacock egg- white, egg-yolk RfBP's 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.

The antiserum produced against Riboflavin Binding Protein from Hen egg-white, as well as egg-yolk and Peacock egg-white, egg-yolk have been collected from the rabbit. The cytotoxic activity was carried out using human cervical cancer cell lines (HeLa). A

remarkable reduction in absorbance was observed with antiserum against Hen egg-white, egg-yolk as well as peacock egg-white and egg-yolk, which explain the inhibitory activity of antiserum and forms a good evidence for cytotoxicity activity in initial cytotoxic assays. The further studies for the calculation of the IC-50 values are yet to be undertaken.

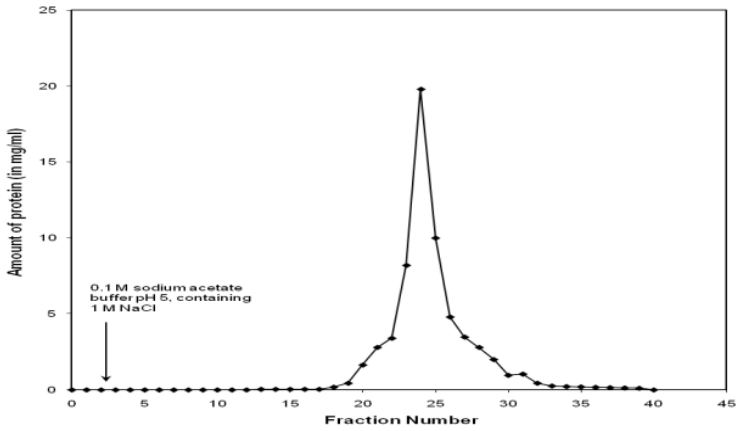
It is one of the investigations in our lab and well known fact that antiserum produced against RfBP of Pea cock egg white folic acid binding proteins showed significant activity, on HBL-100 (Human breast cancer) [19]. This provides a base for our interest to produce and test for cytotoxic activity of antiserum against riboflavin binding proteins. (Fig 1&2).

Fig. I : Partially Purified Emu Egg White Riboflavin Binding protein on Sephadex G-100



Partially purified RfBP was loaded on to the Sephadex G-100 and was eluted with 0.05 M phosphate buffer pH 7.4 containing 0.5 M NaCl

Fig. II : Emu Egg white partially purified Rfbp on DEAE Sephadex



RfBP fraction from batch elution was loaded on to the DEAE Sephadex Column and was eluted with 0.1 M sodium acetate buffer pH 5, containing 1 M NaCl. The protein concentration was estimated by the method of Lowry *et al.*

ACKNOWLEDGEMENTS

Authors are very much thankful to University Grants Commission (UGC), New Delhi, India. For his financial support of this work.

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