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Antiserum raised against riboflavin binding protein purified from peacock (*Pavo cristatus*), Hen (*Gallus gallus*) and observed cytotoxic activity on *in-vitro* cell cultures (HeLa cell lines)

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ABSTRACT

Riboflavin binding protein (Rfbp) was purified from Peacock (*Pavo cristatus*) Egg- white, egg-yolk and Hen (*Gallus gallus)* egg-white and egg-yolk. Riboflavin binding protein (RfBP) was isolated first time in India from peacock eggs (*Pavo cristatus).* The Rfbp was purified in two steps, DEAE-Sephadex A-50 ion exchange chromatography. The final purification of proteins (Rfbp) was achieved on Sephadex G-100.The protein content was estimated with Lowry method. The purity of the proteins was judged by cylindrical and slab-gels, SDS-PAGE techniques. These proteins showed a single band on SDS gels and the molecular weight was 29 Kilodaltons. Antiserum was raised against these Rfbp's in rabbit. These proteins are emulsified in Freund's complete adjuvant and injected subcutaneously at weekly intervals for 4 weeks into the rabbit at multiple sites. The rabbit antiserum was collected through the ear vein, 7 days after the final injection. This serum was analyzed *in-vitro* method with HeLa cervical cancer cell-ines.MTT[(3-(4,5-dimethylthiazol-2yl)-2,5-diphenyltetrazoliumbromide] 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 anticancer activity of Riboflavin binding proteins (Rfbp) purified from egg-white of Peacock (*Pavo cristatus)*94.6 %,egg-yolk87.7 and Hen(*Gallus gallus)*egg-white87.5,egg-yolk 86.2%contains anticancer activity on HeLa cell lines.

KEYWORDS

Peacock;
Hen;
Egg white and yolk;
Rfbp;
Antiserum;
HeLa cell-lines.

INTRODUCTION

Water-soluble, yellow colored fluorescent pigments, now known to be identical or related to riboflavin (Rf), were first isolated form milk, eggs, and various animal tissues. These materials were initially named in relation to their origin were eventually recognized to be a single compound[1] which we know today as vitamin B_2. The specific binding proteins for fat soluble vitamins such as vitamin A and vitamin D are identified in normal serum in all vertebrates[2-5]

Binding proteins for water-soluble vitamins such as

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Riboflavin binding proteins[6,7], Vitamin B_{12} binding protein[8,9], and Thiamin binding protein[10,11] have been demonstrated in the sera and egg white and yolks of the egg laying hens. Riboflavin (7,8-dimethyl-10-(1'-D-ribityl isoalloxazine) in mammals is found predominantly in urine and milk it is also occurs in the eggs of reptiles and birds. All animals are incapable of synthesizing the isoalloxazine skeleton of Rf and require this vitamin in the range of 1-10 μg/g diet[12]. All flavins are 10-substituted derivatives of the isoalloxazine tricycle ring system which is synthesized via a complex pathway from GTP[13]. The two co enzymatic derivatives of Rf, flavin mononucleotide (FMN; Rf 5′-phosphate) and flavin adenine dinucleotide (FAD; Rf 5′-adenine diphosphate) function as prosthetic groups in several mitochondrial oxidation-reduction enzymes. In most vertebrate tissues analyzed FAD predominant (ca. 75% of the total tissue flavin), followed by FMN (ca. 22%) and Rf (Ca. 2%)[14]. Conversely, mammalian[15] and avian[16] serum contains Rf as the predominant flavin with less FAD and only traces of FMN. In most instances the flavins are associated with specific proteins which serve a transport or sequestration function. In the case of Rf the complex is found in the blood or eggs, or in the case of FMN and FAD, as tightly bound prosthetic groups of oxidation-reduction enzymes.

Riboflavin Binding Protein (Rfbp) or Riboflavin Carrier Protein (RCP) was first isolated the chicken egg white[6]. Egg white RfBP is a phospho-glycoprotein having a molecular weight of 29,200 containing 219 amino acid residues[17]. The isolation of RfBP from egg yolk was first published[7] and improved methods were subsequently reported[18,19]. The Riboflavin binding protein from peacock (*Pavo cristatus*) egg-white was first isolated[20].

As the aim of the present study was to observe anticancer activity of riboflavin binding protein purified from Peacock (Pavo cristatus) Egg-white, egg-yolk and Hen (Gallus gallus) egg- white, egg-yolk.

EXPERIMENTAL

Materials and methods

Peacock (*Pavo cristatus*) eggs were obtained from Vana Vignana Kendram, Warangal. Fresh hen (*Gallus gallus*) eggs were obtained from the poultry farm. DEAE-Sephadex A-50 used in the present study was obtained from Pharmacia Fine Chemicals, Uppasala, Sweden. Sephadex G-100 and Freund's Complete adjuant was procured 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, SDS were procured from Loba Chemical Industrial Company, Bombay, India. MTT (3-(4,5-dimethylthiazol-2yl)-2,5-diphenyltetrazolium bromide) (Himedia, Mumbai, India). Fetal bovine RPMI-1640 media (Himedia, Mumbai, India)serum (Himedia, Mumbai, India). Trypan blue (Himedia, Mumbai, India). Dimethyl sulphoxide (DMSO) (Merck India Ltd, Mumbai, India). HeLa- cervical cancer cell-lines Dabur research foundation, Ghaziabad, U.P.

Isolation and purification of hen egg-white riboflavin binding protein

Riboflavin Binding Protein from hen egg-white was isolated following the methods[6,17,21] described below.

Hen egg whites were collected and homogenized with an equal volume of 0.1 M sodium acetate buffer pH 4.5. The homogenate was centrifuged at 12000 × g, for 20 minutes. The precipitate was discarded. To the clear supernatant DEAE-Sephadex previously equilibrated with 0.1M sodium acetate buffer pH 4.5 was added. The mixture was stirred for 12 hours at 4^0C and then suction filtered. The filtrate was discarded. The DEAE-Sephadex with bound protein was washed with excess of 0.1M sodium acetate buffer pH 4.5. Bound proteins were eluted with the same buffer containing 0.5 M sodium chloride (50 ml) by suction filtration. The eluted protein fraction was dialyzed against water.

DEAE-Sephadex previously equilibrated with 0.1M sodium acetate buffer pH 4.5 was packed into the column (2 × 28 cm). Partially purified RfBP was loaded onto the column. The column was washed with excess buffer. Riboflavin binding protein was eluted from the column with 0.1M sodium acetate buffer, pH 4.5 containing 0.5M sodium chloride. Twenty fraction (5 ml each), were collected and absorbance was measured at 280 nm, 455nm using UV-visible recording spectrophotometer (Perkin Elmer). Values were expressed as total absorbance at 280 nm and 455 nm per each fraction.

Further purification of hen egg white RfBP was achieved by gel filtration column chromatography using Sephadex G-100. The column (2 × 36 cm) was equili-

brated with 0.025 M phosphate buffer pH 7.3 containing 0.5 M sodium chloride. The protein was dissolved in 1 ml of the above phosphate buffer, and loaded on the column and eluted with the starting buffer. Fractions (5 ml) were collected. Protein in each fraction was determined by the Lowery[22] method of using bovine serum albumin as standard. Absorbance was measured at 280 nm and 455 nm using UV-visible recording spectrophotometer (Perkin Elmer). Values were expressed as total absorbance at 280 nm and 455 nm per fraction. The peak fraction were pooled and dialyzed against distilled water and lyophilized. SDS-PAGE was carried out according to the method of Leammli[23] using sodium phosphate buffer containing SDS.

Production of antiserum to riboflavin binding protein (RfBP)

By adopting Prasad and adiga[24] antibodies were produced. The Rfbp's were emulsified with equal amount of Freund's complete adjuvant. At weekly intervals 4 weeks injected this emulsified protein to rabbit at multiple sites. The rabbit was bleed after final injection.

The following same steps carried out to purify and raising of antiserum against Peacock (*Pavo cristatus*) egg white, egg-yolk Hen(*Gallus gallus*) egg-white, egg-yolk riboflavin binding protein(Rfbp).

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.

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 × dilution factor.
Total viable cells = Cells/ml × original volume of cell suspension.
Cell viability % = Total viable cells (unstained)/ Total cells.

RESULTS AND DISCUSSION

The pooled fraction from DEAE-Sephadex was

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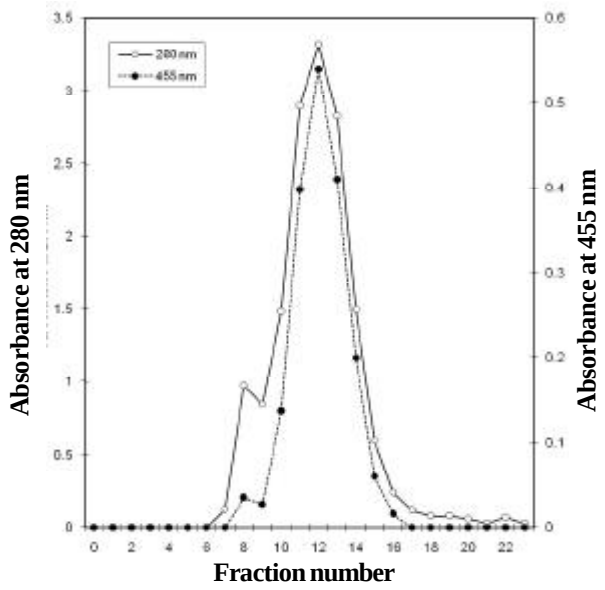

Partially purified RfBP was loaded on to the Sephadex G-100 and was eluted with 0.025M phosphate buffer pH 7.3 containing 0.5 M NaCl

Figure 1: Partially purified Hen egg-white Rfbp elution profile on Sephadex G-100 column.

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 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 (Figure 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 Kilodaltons. 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 of Prasad and Adiga[24].

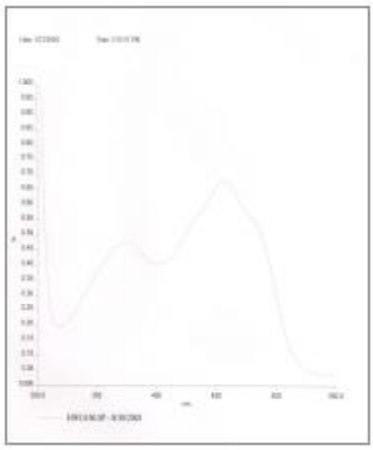

Figure 2: Absorption spectrum of Hen egg-white riboflavin binding protein (Sephadex G-100 fraction)

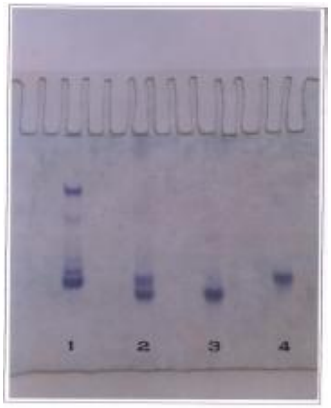

Figure 3: Electrophoresis pattern of riboflavin binding protein on slab-gels: (1) Protein molecular weight marker, (2) Partially purified hen egg-white, (3) Partially purified peacock egg-white (4) Sephadex G-100 fraction of hen egg-white

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^{0}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.

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

TABLE 1

Blank Control		Hen egg white ½ dilution		Hen egg yolk ½ dilution		Peacock egg white ½ dilution		Peacock egg yolk ½ dilution	
10.849	9.249	1.271	1.346	1.315	1.241	0.543	0.472	0.445	0.439
10.849	9.149	1.288	1.007	1.218	1.171	0.479	0.460	0.541	0.458
10.149	9.649	1.187	0.826	1.228	1.071	0.549	0.513	0.386	0.545
9.149	10.749	1.371	1.150	1.341	1.312	0.353	0.488	0.514	0.628
10.149	8.849	1.278	1.306	1.274	1.312	0.512	0.445	0.476	0.513
10.149	9.549	1.123	1.177	1.345	1.019	0.443	0.607	0.532	0.458
9.749	10.949	1.410	1.238	1.249	1.214	0.471	0.765	0.429	0.449
10.449	10.249	1.608	1.219	1.129	1.239	0.629	0.734	0.512	0.529
Average									
10.186	9.786	1.284	1.158	1.262	1.147	0.497	0.560	0.479	0.502
		1.221		1.204		0.528		0.490	
Percentage of inhibition									
		87.5		86.2		94.6		87.7	

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 investigation in our lab and well known fact that antiserum produced against folic acid binding proteins showed significant activity[25] on HBL-100 (Human breast cancer).This provides a base for our interest to produce and test for cytotoxic activity of antiserum against riboflavin binding proteins.

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