Print ISSN: 0974-2115
www.jchps.com Journal of Chemical and Pharmaceutical Sciences

Purification and Characterization of Riboflavin Carrier Protein from Guinea Pig Serum and Cytotoxic Activity of Its Antisera against Hela Cancer Cells

Karunakar Rao Kudle[1], Madhukar Rao Kudle[2], Ramchander Merugu[3], M.P. Pratap Rudra[1]*
[1]Department of Biochemistry, Osmania University, Hyderabad, Telangana State, India
[2]Department of Biochemistry, Kakatiya University, Warangal, Telangana State, India
[3]Department of Biochemistry, Mahatma Gandhi University, Nalgonda, Telangana State, India
***Corresponding author: E-Mail: mpprataprudra@gmail.com, +919640833031**

ABSTRACT

In the present work, Riboflavin Carrier protein was purified from Guinea Pig Serum to investigate the cytotoxic activities of antibodies raised against it. The treated cells with different dose of antisera from Guinea pig serum Riboflavin carrier protein antisera showed 14.391 % cytotoxic activity on HeLa cancer cells and standard drug used was Doxorubicin. HeLa cancer cell lines showed IC_{50} values of 33.31 ± 0.7 for Guinea pig serum Riboflavin carrier protein (RCP) antisera.

KEY WORDS: Riboflavin carrier protein (RCP) antisera, Guinea Pig Serum, HeLa cancer cells.

1. INTRODUCTION

Vitamin binding proteins ensure deposition of adequate amount of the vitamins in the sera of higher animals which prompted us to isolate these proteins. Riboflavin binding protein plays an important role in fetal growth and development. Serum RfBP is synthesized in the liver and complexes with riboflavin in the plasma to form the holoprotein. Muniyappa and Adiga (1980), have reported high-affinity RfBP in pregnant rat serum. Bindu (2010), and Rajender (2010), have reported Folic acid Binding Protein (FBP) antisera, Riboflavin Binding protein (RfBP) antisera *in vitro* cytotoxic activity on some cancer cells lines. Karunakar and Rudra (2015) have reported Cytotoxicity activity of rabbit antisera of RfBP against HeLa cells, MCF-7 and A549 cancer cell lines this provides a base for our interest to produce and test for cytotoxic activity of antiserum against Riboflavin carrier protein. Hence, in present investigations on Riboflavin carrier protein (RCP) antisera at different concentrations were carried out to test the cytotoxic activity against HeLa cells, MCF-7 and A549 cancer cells. No studies were conducted using Guinea pig serum ever before with a specific target towards the cytotoxic activity.

2. MATERIAL AND METHODS

Isolation and Purification of Guinea Pig -Serum RCP: The method for isolation and purification of Guinea pig serum RCP involved mainly three stages: Batch processing of partially purified RCP from Guinea pig serum using DEAE-sepharose. Isolation and partial purification of RCP was done from the Guinea pig serum using DEAE-Sepharose Ion exchange column chromatography. Guinea pig serum was collected from NIN-Hyderabad. Fresh serum 80ml was homogenized with two volumes of 0.1M sodium acetate buffer, pH 4.5 containing 0.15 M sodium chloride. The homogenate was centrifuged and the clear yellow supernatant was mixed with DEAE-Sepharose, previously equilibrated with 0.1M sodium acetate buffer, pH 4.5 and stirred overnight at 4°C. The DEAE-Sepharose was later washed extensively with the buffer to remove the non specific protein. The bound protein were eluted with 0.1M sodium acetate buffer, pH 4.5 containing 0.5 M NaCl. This partially purified RCP was loaded onto a fresh DEAE-Sepharose column, previously equilibrated with 0.1M sodium acetate buffer, pH 4.5. The column was washed and finally the bound RCP was eluted with the same buffer containing 0.5M NaCl. Fractions from the column were collected and the absorbance was measured at 280 nm and 455 nm for protein and bound riboflavin respectively. The purity of the protein was judged by SDS-PAGE. The minor contaminating protein present at this stage could be eliminated by employing gel-filtration column chromatography using Sephadex G-100. This step resulted in the RCP preparation that was homogenous on SDS-PAGE and Ouchterlony double diffusion analysis.

Fluorescence Spectra: The Fluorescence spectra were recorded at 20^0C in 4D10 Hitachi Spectro-fluorimeter with excitation at 280 nm and 295 nm. The protein concentration was 1 mg/ml.

Production of Antiserum to Riboflavin Carrier Protein: Antibodies against Spotted Owlet egg and Hen egg white and yolk Riboflavin carrier protein (RCP) were produced adopting the method of Prasad and Adiga (1979). 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 rabbits 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 0.05M sodium phosphate buffer pH 7.8, 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 of specific antibodies.

MTT ASSAY:

Cell culture: HeLa (cervical cancer) cell lines.
Cell number for subculture: one million cells for flask (30 ml capacity).
Cell loading into plate: 5000 cells per well (96-well plate).
Protein (Rabbit Antiserum antibodies of RCP) solution: 2, 4, 6, 8 and 10µl.
Standard Drug: Doxorubicin (2 µl =10 µM). Concentration of protein (10 µl = 0.0284 µg).

MTT (3-(4, 5-dimethylthiozol-2yl)-2, 5-diphenylterazolium bromide) measures the metabolic activity of the viable cells. The assay is non-radioactive and be performed entirely in a microtiterplate (MTP). It is suitable for measuring cell proliferation, cell viability or cytotoxicity. The reaction between MTT and mitochondrial dehydrogenate was observed in 96-well micro titer plate, and then incubating them with MTT solution for approximately 4 hours. The adherent cells were trypsinized according to protocol and were resuspended in fresh medium after centrifugation. Cell suspension was mixed thoroughly by pippetting several times to get uniform single cell suspension. Different dilution of drug solution were made in media with final 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 (in duplicate) in media was added. Rabbit Antiserum antibodies of RCP were made in media with different concentrations. The plate was then incubated at 37^0C for 72hours 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. 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 absorbance was recorded on the ELISA reader at 562nm wavelength. The absorbances of the test were compared with that of Rabbit serum control to get the % inhibition.

3. RESULTS

All ready reported Riboflavin carrier protein from pregnant Rat serum, Cow serum, Pregnancy sera of Bonnet monkeys (*Macaca radiata*) below data given in the (Table.1)

Table.1. Molecular weight of Riboflavin carrier Protein (RCP) from Different source & Animal Serum

S. No	Vitamin binding protein	Different Source Animal Serum	Mol.Wt (kDa)	Authors
Riboflavin (B2)	Riboflavin binding protein (RfBP)/Riboflavin Carrier protein (RCP)	Pregnant-rat serum	90000±5000Da	(Muniyappa,1980)
		Cow	37,000Da	(Merillet, 1979)
		Pregnancy sera of bonnet monkeys (Macaca radiata)	Molecular weight (approx. 36,000)kDa	Visweswariah, Ad iga, (1987)

The peak fraction (no: 6,7,8,9,10,11) from the DEAE-Sepharose column was dissolved in15 ml of phosphate buffer and loaded on a Sephadex G-100 column (2×40cm) pre-equilibrated with 0.05M sodium phosphate buffer, pH 7.4 containing 0.5M sodium chloride. The protein was eluted with the same buffer. Fractions of 0.5 ml each were collected. The fractions were monitored for absorbance at 280 nm and 455 nm.

The elution profile was given in Figure.1. The fraction no (4, 5, 6, 7, 8, 9, 10, 11 and 12) with the highest absorbance were dialyzed against distilled water. The protein content in all fractions was also estimated by the method of Lowry (1951), (Figure.2). Polyacrylamide gel electrophoresis at pH 8.3 of the DEAE Sepharose fraction revealed the presence of minor contaminating proteins suggesting partial purification of RCP.

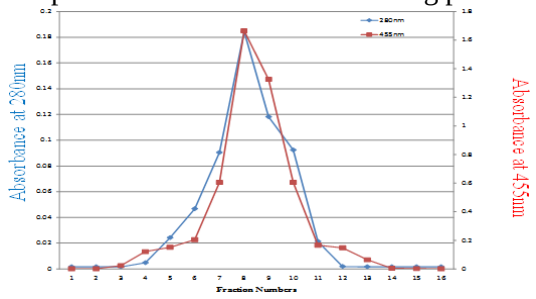

Figure.1. Guinea Pig Serum RCP elution profile on DEAE-Sepharose

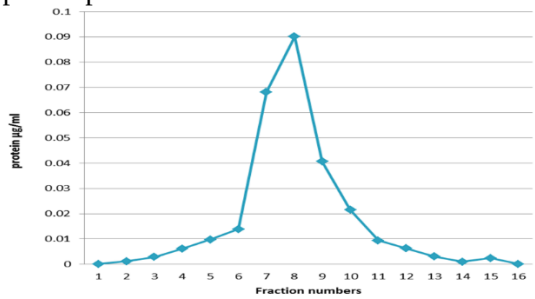

Figure.2. The partially purified RCP was eluted from DEAE-Sepharose Using Phosphate buffer pH 7.3 containing 0.5M NaCl. The protein concentration was estimated by the method of Lowry method.

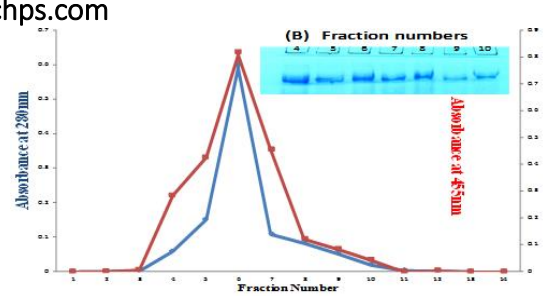

Figure.3. Guinea Pig Serum Riboflavin carrier protein elution profile on Sephadex G-100 and (B) Fraction no (4, 5, 6, 7, 8, 9, 10) SDS-Polyacrylamide Gel Electrophoresis Pattern of Riboflavin carrier protein (RCP) from Guinea Pig Serum.

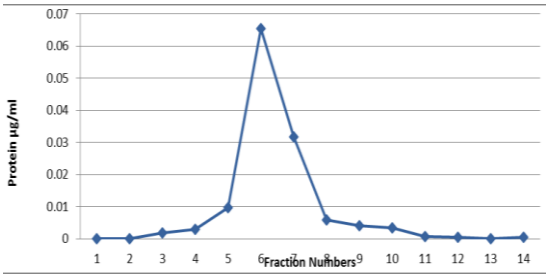

Figure.4. The partially purified Riboflavin carrier protein(RCP) was eluted from Sephadex G-100 Using Phosphate buffer pH 7.3 containing 0.5M NaCl. The protein concentration was estimated by the method of Lowry method.

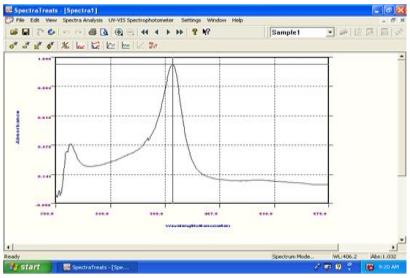

Figure.5. Absorption spectrum of Guinea Pig serum Riboflavin carrier Protein (DEAE-Sepharose Fraction 6, 7, 8 and 9)

The Guinea Pig Serum (50 ml) was processed as described under Materials and Methods. After centrifugation, 45 ml of the pale yellow Guinea Pig Serum solution was used for batch adsorption onto DEAE Sepharose. The non-specific proteins were removed by washing the protein bound DEAE –Sepharose gel with excess of chilled buffer on a Buchner funnel. The bound protein was eluted with 0.1M sodium acetate buffer, pH 4.5 containing 0.15M NaCl by suction filtration total volume (22 ml) collected. The eluted was dialyzed and loaded onto a DEAE-Sepharose column. After washing the column, the bound protein was eluted with 0.1M sodium acetate buffer, pH 4.5, containing 0.5M NaCl. 1ml fractions were collected. The protein concentrations in the eluates were estimated by measuring absorbance at 280 nm using a UV-Visible recording spectrophotometer. Fractions eluted from the column were also assayed for protein bound Riboflavin by measuring the absorbance at 455 nm. The elution profile was presented in (Figure.3). The 14 fractions no (3, 4, 5, 6, 7, 8, 9, 10) with highest absorbance at 280 nm was dialyzed against distilled water. The protein content in all fractions was also estimated by the method of Lowry (1951), (Figure.4). The total protein recovered corresponded to 2.4 mg.

Spectral Studies:

UV Absorption Spectra: The absorption spectra of the partially purified Guinea Pig Serum RCP and purified RCP were shown in (Figures.5 and Table.2). The holo protein with the bound Riboflavin showed an absorption maximum at 458nm. Similar absorption spectra were reported earlier for the flavoprotein complexes (Rhodes, 1959; Choi & Cormick, 1980).

Table.2. Absorbance peaks of riboflavin carrier protein (RCP) Protein at 280nm – 600nm

Name of Instrument	Scanning range (nm)	Absorbance O.D Value		Intensity Peak
		Ion Exchange Column Chromatography (DEAE-Sepharose)	406.2nm	1.032
ELICO-	280nm-600nm	Gel Filtration (G-100)	477.7nm	1.095

Florescence Spectra: Guinea Pig Serum Riboflavin carrier protein Fluorescence spectra recorded with excitation at 280 nm and 295 nm were shown in (Figure.6). They had an emission maximum at 340 nm.

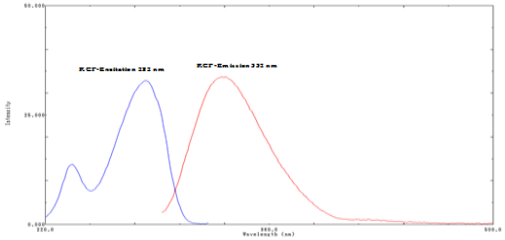

Figure.6. Fluorescence spectrum of Riboflavin Carrier protein (RCP) Excitation and emission

Polyacrylamide gel electrophoresis: The purity of the isolated protein was judged by SDS PAGE (slab gels) and Fraction no (4, 5, 6, 7, 8, 9, 10) the gel pattern was shown in (Figure.7). Partial purification of Guinea Pig Serum RfBP could be achieved by using DEAE Sepharose, as this fraction contained minor contaminants. However gel-filtration chromatography on Sephadex G-100 could yield RCP (or) RfBP fraction that was homogenous on SDS-PAGE (only one major band) suggesting complete purification of RCP (Figures.8). On comparison with standard molecular weight markers, the Guinea Pig Serum RCP had a molecular weight of approx 95,000Da.

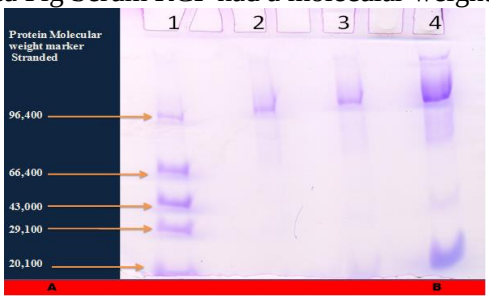

Figure.7. SDS-Polyacrylamide Gel Electrophoresis Pattern of (A) single band of RCP from Guinea pig serum (B) purification steps in DEAE-sepharose, Sephadex G-100, Riboflavin carrier protein (RCP) of Guinea Pig Serum

- Protein Molecular weight standard Markers
- Guinea Pig serum Sephadex G-100 fraction
- Guinea Pig serum DEAE-Sepharose eluted fraction
- Guinea Pig serum crude

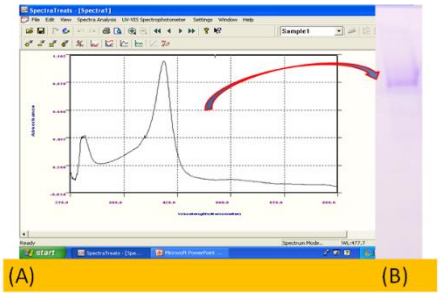

Figure.8. Absorption spectrum of Guinea Pig serum Riboflavin carrier Protein (A) Sephadex G-100 Fraction 6, 7 and (B) SDS PAGE Single band protein

The antisera obtained from the rabbits injected with purified Guinea pig serum RCP gave single precipitin lines respectively, when tested against purified Guinea pig serum RCPs respectively by immunodiffusion. In addition the antiserum raised against purified RCPs showed cross reactivity with purified RCPs from Guinea pig serum (Figure.9).

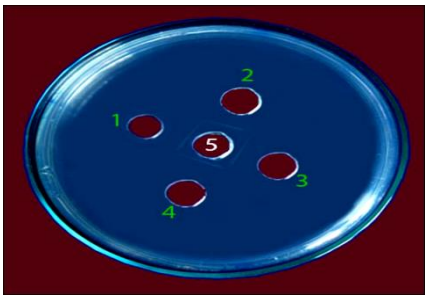

Figure.9. Ouchterlony double diffusion analysis (The central well (5) Contains Guinea pig RCP against antiserum antibodies.

- Purified Guinea pig RCP DEAE Batch Crude
- Purified Guinea pig RCP DEAE Sepharose Fraction
- Purified Guinea pig RCP Sephadex G-100 Fraction
- Purified Guinea pig RCP Sephadex G-100 Fraction
- Guinea pig RCP against antiserum antibodies

Cytotoxicity activity of antisera rabbit of riboflavin carrier protein (rcp) against cancer cells:

Cytotoxicity studies: RPMI-1640 Medium was used for the maintenance of the cell lines. Sub-cultures were maintained in a deep freezer at -80^{0}C. Neubar slide (heamocytometer) was used for cell counting by trypan blue dye exclusion method. Results of the cytotoxicity with percentage inhibition are presented. Rabbit Riboflavin carrier protein (RCP) antibodies (Antisera) of rabbit showed more significant anticancer activity as compared to the positive

control and standard drug i.e. Doxorubicin (Figure.10). Remain procedure according (Karunakar Rao, 2015) was also reported.

Cytotoxicity and Viability (%) of Riboflavin Carrier Protein Antisera from Guinea Pig Serum on Hela Cancer Cells (Human Cervical Cancer Cell Line):

Table.3. *In-vitro* cytotoxicity HeLa cancer cells of RCP antisera Guinea pig serum, Normal Rabbit serum and standard Drug Doxorubicin

S.No	Concentration (µl)	Rabbit serum Absorbance at 570nm	% Viability	Guinea pig serum RCP Antisera absorbance	% of viability	% of cytotoxic activity
1	2	1.62014	100	1.5608	96.337	3.662
2	4	1.59402	100	1.4981	93.982	6.017
3	6	1.32901	100	1.1540	86.635	13.364
4	8	1.1876	100	1.0246	86.274	13.725
5	10	1.0562	100	0.9042	85.608	14.391
6	Doxorubicin	0.8284		----	51.472	48.528
7	Control cells	1.6094		-	-	-

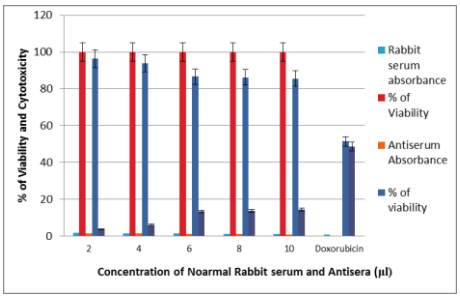

Figure.11. Graphical representation of cytotoxicity and percentage of call viability versus different concentration for HeLa cancer cells Guinea pig serum RCP antisera, Normal Rabbit serum and standard Drug Doxorubicin

Riboflavin carrier protein antisera ic_{50} values of guinea pig on hela cancer cells (human cervical cancer cell lines):

Table.4. Riboflavin Carrier Protein Antisera IC_{50} Values of Guinea Pig on Hela Cancer Cells and standard drug Doxorubicin

S.No	Concentration (µl)	Sources of Antisera RCP	IC $_{50}$ Values
1	10	Guinea pig serum RCP antisera	33.31 ± 0.7
2	2 µl (10 µM)	Doxorubicin	10.30 ± 0.4

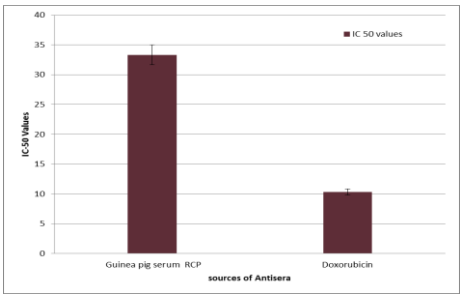

Figure.12. Comparison of IC 50 Values Riboflavin Carrier Protein Antisera of Guinea pig and standard drug Doxorubicin

DISCUSSION

The antiserum produced against Riboflavin carrier protein from Guinea pig serum RCP antibodies have been collected from rabbit. The cytotoxic activity was carried out using Human cervical cancer cell line (HeLa). A remarkable reduction in absorbance was observed with antiserum against guinea pig RCP the cytotoxic activity was carried out using Human cervical cancer cell line (HeLa) (Figure.11 & Table.3) and which explains the inhibitory activity of antiserum and forms a good evidence for cytotoxicity activity in initial cytotoxic assay. The IC $_{50}$ values were calculated (Figure. 12 and Table. 4). This provides a base for our interest to produce and test for cytotoxic activity of antiserum against Riboflavin carrier protein. Hence, in present investigations on Riboflavin carrier protein (RCP) antisera at different concentrations were carried out to test the cytotoxic activity against HeLa cells. The

treated cells with different dose (2,4,6,8 and 10) of antisera from Guinea pig serum Riboflavin carrier protein antisera showed 3.662%, 6.017%,13.364%,13.725% and 14.391 % cytotoxic activity on HeLa cancer cells and standard drug used was Doxorubicin 2 µl (10 µM) 10.30 ± 0.4. HeLa cancer cell lines showed IC_{50} values of 33.31 ± 0.7 for Guinea pig serum Riboflavin carrier protein (RCP) antisera. The antiserum against RCP must have binded to Riboflavin carrier protein. Due to this depletion of riboflavin in the cancer cell lines leading to cell growth inhibition must have taken place as RCP could not carry riboflavin. No studies were conducted using Guinea pig serum ever before with a specific target towards the cytotoxic activity. We have come out with the anticancer antibodies produced against Riboflavin carrier Protein's of Guinea pig serum.

4. CONCLUSION

Riboflavin Carrier protein was purified from Guinea Pig Serum and the cytotoxic activities of antibodies raised against it showed good cytotoxic activity on HeLa cancer cells. These studies may pave a way for production of biopharmaceuticals for tumor treatment.

REFERENCES

Alfred Merrill H, John Froehlich A and Donald B. McCormick B, Purification of Riboflavin-binding Proteins from Bovine Plasma and Discovery of a Pregnancy-specific Riboflavin-binding Protein, J. Biol. Chem, 254, 1979, 9362-9364.

Bindu Mary Rajan and Prasad M.S.K, Isolation and purification of riboflavin binding protein (rfbp) from the egg white of Emu (Dromaius novaehollandiae), IJPAES, 2010.

Choi JD, McCormick DB. The interaction of flavins with egg white riboflavin-binding protein, Arch Biochem Biophys, 204 (1), 1980, 41–51.

Karunakar Rao Kudle and M.P, Pratap Rudra, Cytotoxicity activity of rabbit antisera of riboflavin carrier protein (RCP) against HeLa cells, MCF-7 and A549 cancer cell lines, Journal of Chemical and Pharmaceutical Research, 7 (12), 2015, 706-719.

Lowrey OH, Rosebrough N, Farrl AL and Randall RJ, Protein measurement with the Folin phenol reagent, J. Biol. Chem, 1951, 193, 265-275.

Muniyappa K and Adiga P.R, Occurrence and functional importance of a Riboflavin-carrier protein in the pregnant rat, Febs letters, 110 (2), 1980.

Muniyappa K and Adiga P.R, Occurrence and functional importance of a riboflavin-carrier protein in the pregnant rat. Febs letters, 110 (2), 1980.

Prasad M.S.K and Adiga P.R, Prolactin-testosterone interaction in the male-rat - Modulation of ornithine decarboxylase in various tissues, Ind. J. Biochem. Biophys, 16, 1979, 354-361.

Rhodes MB, Bennett N, Feeney R.E, The flavoprotein-apoprotein system of egg white, J Biol Chem, 234 (8), 1959, 2054–2060.

Visweswariah SS, Adiga PR, Purification of a circulatory riboflavin carrier protein from pregnant bonnet monkey (M. radiata), comparison with chicken egg vitamin carrier, Biochim Biophys Acta, 915 (2), 1987, 141–148.