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Purification and characterization of Riboflavin carrier protein from hen egg - yolk and pigeon egg - yolk

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Riboflavin carrier protein (RCP) has been purified from both pigeon and hen egg- yolk by ion exchange chromatography and gel filtration techniques. Purity of pigeon egg- yolk and hen egg-yolk RCPs are determined by SDS-PAGE. Their structures are compared using Circular Dichroism (cd) Spectra and Fluorescence Spectra. For Pigeon egg-yolk RCP amino acid composition was analyzed. In the present study it is observed that the two proteins have similar secondary structure but they differ in their tertiary structures. The amino acid composition of pigeon egg-yolk RCP has close similarity with hen egg-yolk RCP. This study suggests the existence of some significant structural difference between RCPs from pigeon and hen.

Key words : Riboflavin Carrier Protein; Protein purification and Characterization.

INTRODUCTION

VITAMINS are essential for growth, development and metabolism of an individual. It has been shown in recent studies[2,7,14] that transportation of these vitamins to the growing embryo is due to specific carrier proteins. The importance of riboflavin carrier protein in the transport of riboflavin into the egg is particularly clear. A strain of chickens incapable of concentrating riboflavin in the egg can only be maintained by injection of riboflavin into their riboflavin-deficient eggs[16]. The defect in these chickens is the lack of a functional riboflavin carrier[15].

In the present study RCP was purified for the first time from the pegon egg-yolk and compared with hen egg-yolk RCP, with a view to develop this avian model for studying the regulation of RCP production under pathophysiologcal conditions.

MATERIALS AND METHODS

Commercially available hen (Gallus gallus) eggs were used and pigeon (Columba livia) eggs were obtained from a local source. RCP from hen egg yolk was isolated following the methods [3,8] with a few modifications as described below. Pigeon egg-yolk was homogenized with four volumes of 0.1M sodium acetate buffer, pH 4.5; the precipitated protein was removed by centrifugation. 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 ^{0}C and then suction filtered. The DEAE sephadex with bound protein was washed with excess of 0.1M sodium acetate buffer pH 4.5. Bound proteins were eluted with same buffer containing 0.5M sodium chloride by suction filtration. The eluted protein fraction was dialyzed against water.

Fresh DEAE sephadex, previously equilibrated with 0.1M sodium acetate buffer pH 4.5 was packed into the column and then the partially purified RCP was loaded onto the column. The RCP was eluted from the column with the same buffer containing 0.5M sodium chloride, Fractions were collected and absorbance was measured at 280nm and 455nm using uv visible recording spectrophotometer. Values were expressed as total absorbance at 280nm and 455nm per each fraction. The peak fractions are pooled and dialyzed against distilled water and lyophilized.

Further purification of pigeon egg-yolk RCP was achieved by gel filtration column chromatography using sephadex G-100. The protein was loaded on the column and eluted with the 0.025M phosphate buffer pH 7.3 containing 0.5M sodium chloride. Protein in each fraction was determined by the method[6]. Absorbance was measured at 280nm and 455nm using uv visible recording spectrophotometer. Values were expressed as total absorbance at 280nm and 455nm per each fraction. The peak fractions are pooled and dialyzed against distilled water and lyophilized. The purity of the protein was checked by SDS-PAGE. Hen egg-yolk RCP was also purified to apparent homogeneity in two steps: batch absorption to DEAE sephadex and Gel filtration column chromatography on sephadex G-100.

SDS-PAGE:

SDS-Page was carried out according to the method of Leammli[5] using Tris-Glycene buffer containing SDS. 7.5 % gels were used.

SPECTRAL STUDIES

a) UV Spectra:

The absorption spectrum was recorded using UV-visible spectrophotometer (UV 160A, SHIMADZU). The absorption spectrum of the purified RCP preparations were recorded by diluting the proteins with 0.05M Tris-HCl buffer, pH 7.5 or directly from the eluates of the column after diluting the solutions suitably.

b) CD Spectra:

CD Spectra were recorded at 20^{0}C in a cd machine Jovine 750 Dichrograph. Far uv (190 to 260nm) and near uv (260 to 320nm) spectra were recorded. The protein concentration for far uv cd was 0.2 mg/ml and the cell path length was 0.02 cm. The protein concentration for near uv cd was 1 mg/ml and the cell path length of 1 cm were used.

c)Fluorescence Spectra:

Fluorescence spectra were recorded at 20^{0}C in 4D 10 Hitachi Spectrofluorimeter with excitation at 280nm and 295nm. The protein concentration was 1 mg/ml.

Amino Acid Composition:

Amino acid composition of pigeon egg-yolk RCP was analyzed in Beckman HPLC amino acid analyzer. The amino acid contents were calculated with respect to the standard amino acid mixture.

RESULTS AND DISCUSSION

The SDS-Page pattern of purified RfBPs from hen and pigeon egg-yolk are shown in Fig.1. The pigeon egg-yolk RCPs could be

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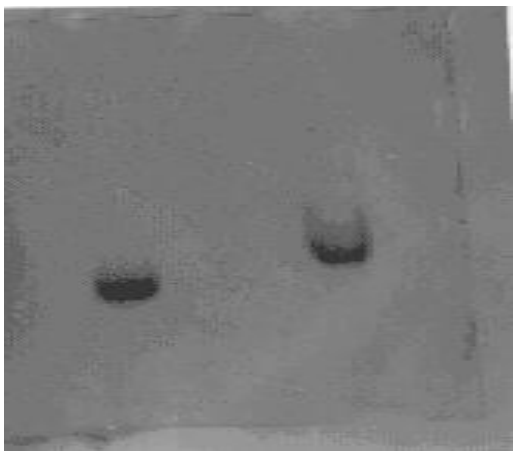
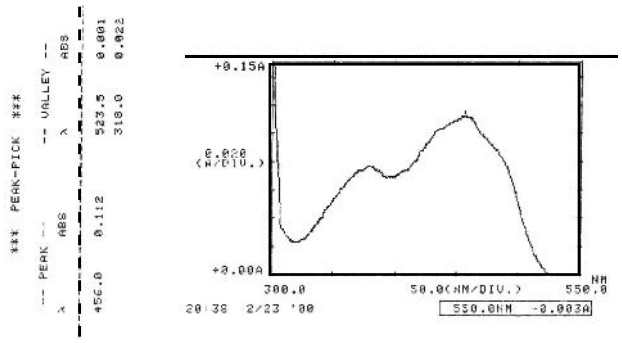
isolated in pure form as revealed by the gel electrophoretic analysis. This was established by judging the purity of RfBPs using SDS-PAGE. The slab gel pattern clearly showed that the purified RCPs could move as one single band establishing thereby the homogeneity of both hen and pigeon egg-yolk RfBPs. Further the molecular weights of pigeon egg-yolk and hen egg-yolk RCPs appears to be nearly same size (Fig. 1).

Fig.1: SDS-PAGE pattern of purified Hen and pigeon egg-yolk RCPs



i) Purified hen egg-yolk RCP ii) Purified pigeon egg-yolk RCP: The near uv absorption spectrum of the riboflavin-apoprotein complex indicated that the protein had a absorption maximum at 274.3nm and a shoulder at about 290nm. This result is in full agreement with the data published[4,9]. Further the visible absorption spectra revealed that the RCPs had absorption maxima at 370nm and 456nm, characteristic of riboflavin-apoprotein forms (holoproteins). The free riboflavin showed absorption maxima at 374 and 445nm. Binding of riboflavin to the protein resulted in the shift of the absorption peak at 445nm to 457nm and shoulders appeared at about 435 and 480nm. At the same time, the absorption at 375nm showed remarkable hypochromism without a shift of band position. The spectral changes observed were characterized by a red shift of the 450nm band. The appearance of the shoulders in the 450nm band suggests that the flavin environment becomes less polar relative to the flavin in water. The fact that the 370nm band of the flavin did not shift when the flavin combined with the protein indicated the concomitant involvement of a hydrophilic or polar interaction. Exactly similar spectral data were reported earlier for hen egg -yolk RCP[1, 11]. The purified pigeon egg - yolk RCP uv absorption spectra is shown in Fig. 2.

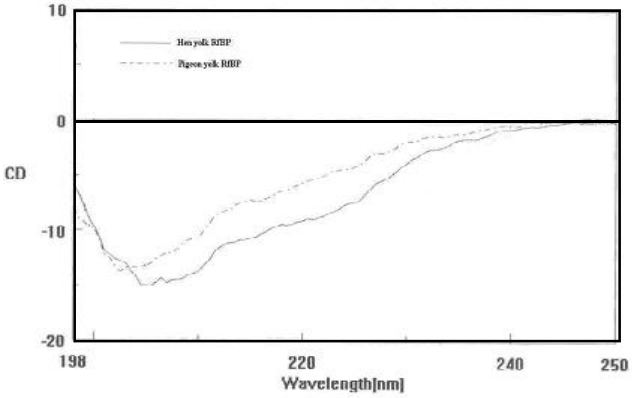
Fig. 2 : The absorption spectrum of purified pigeon egg - yolk RCP



The far u.v.c.d. of hen and pigeon egg yolk RCPs (Fig.3), show a shoulder at 210nm and pigeon yolk RCP has a sharp minimum at 204 whereas hen egg yolk RCP has at 206nm. Hen egg yolk RCP has more negative ellipticity from 205–240nm, but the c.d. profile is similar. The relative content of a, b and random coil is the same.

The far u.v.c.d. of hen and pigeon egg yolk RCPs (Fig.3),

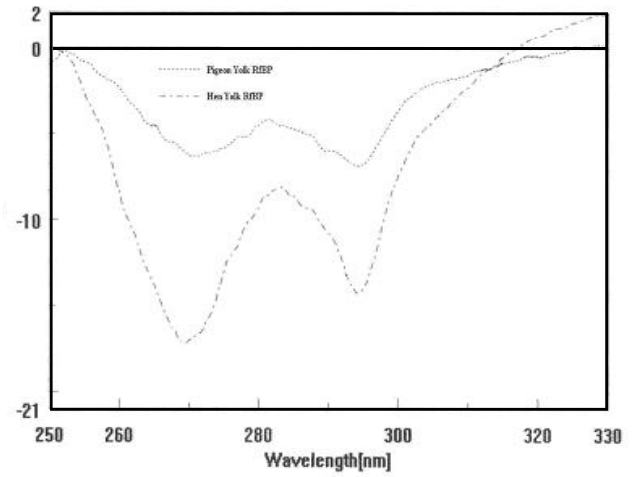
Fig. 3: Far u.v.c.d spectra of RCPs from pigeon and hen egg yolk



show a shoulder at 210nm and pigeon yolk RCP has a sharp minimum at 204 whereas hen egg yolk RCP has at 206nm. Hen egg yolk RCP has more negative ellipticity from 205–240nm, but the c.d. profile is similar. The relative content of a, b and random coil is the same.

Near u.v.c.d. spectra of pigeon and hen yolk RCPs (Fig. 4)

Fig. 4: Near u.v.c.d. spectra of RCPs from pigeon and hen egg -yolk



show a sharp minimum at 270 and 295nm and peaks at 280nm. The near u.v.c.d. profile of both the proteins is similar. For the pigeon egg yolk RCP negative sign is less than that of hen egg yolk RCP. The extent of tertiary structure is less in the pigeon egg yolk RCP. From these spectral studies it appears that the hen proteins are not significantly different in terms of secondary structure. However, the tertiary structure has displayed significant alterations[13]

a)The excitation wavelength was 280nm b)The excitation wavelength was 295nm

The fluorescence spectrum of hen egg-yolk RCP has emission maximum at 340nm. Pigeon egg-yolk RCP has a maximum at 350nm (Fig. 5a and 5b), showing a characteristic of an altered tertiary structure with the tryptophan side chains in the pigeon proteins some what altered extent of exposure to the solvent[12].

The amino acid composition of pigeon egg-yolk RCP is shown in Table-I. It was observed that the amino acid composition

Fig. 5a :

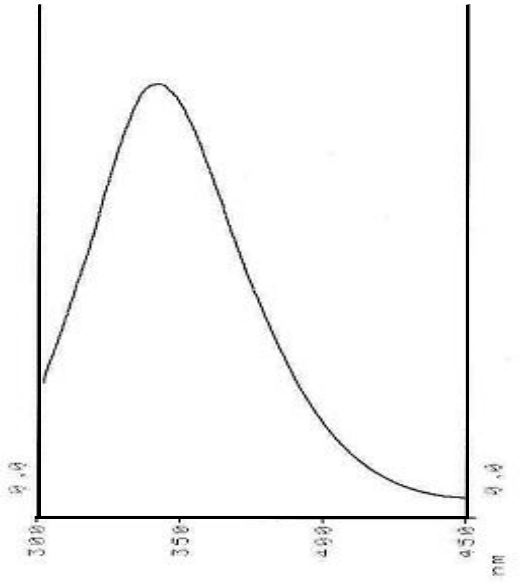
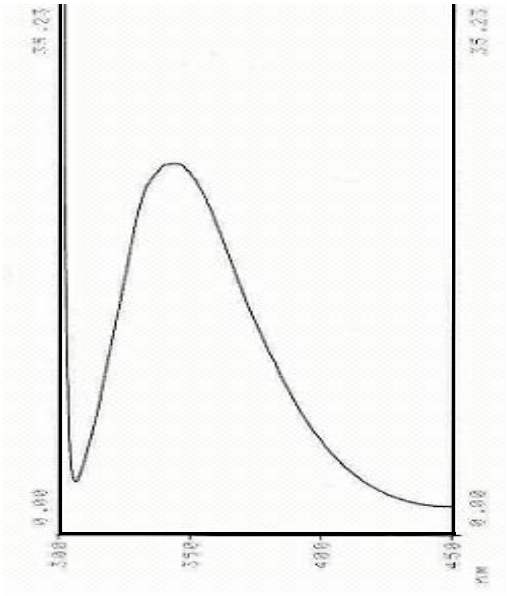


Fig. 5b:



Fluorescence spectrum of RCP from pigeon egg white

of pigeon egg-yolk RCP has close similarity with hen egg-yolk RCP.

TABLE I : Amino Acid content of purified pigeon egg-yolk RCP

Amino acid	μg AA/100μg protein
Aspartic acid	8.90
Threonine	4.94
Serine	7.43
Glutamic acid	9.16
Proline	4.00
Glycine	2.50
Alanine	4.22
½ Cystine	Nd
Valine	4.26
Methionine	Nd
Isoleucine	3.90
Leucine	6.04
Tyrosine	3.42
Phenylalanine	5.04
Lysine	3.98
Histidine	3.02
Arginine	6.86

The cd spectra and fluorescence spectra show that the pigeon and hen egg-yolk RCPs have similar secondary structures but differ in their tertiary structures. This study suggests the existence of some significant structural differences between the RCPs from pigeon and hen.

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