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Isolation and Structural Characterisation of Riboflavin Binding Protein from the Egg of Ostrich (*Struthio camelus*) using Maldi – Tof – Ms

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ABSTRACT

Riboflavin Binding Protein (RBP) is isolated and purified for the first time from a single egg of Ostrich (Struthio camelus). The 238 amino acid sequence of this protein is determined using 2DE and Matrix Assisted Laser Desorption Ionization Time of Flight (MALDI-TOF) Peptide Mass Mapping (PMM). The amino acid sequence of the riboflavin binding protein showed 95.8% sequence homology with the RBP isolated from the egg of Emu (Dromaius novaehollandiae), belonging to the same Ratite family. Ostrich egg RBP protein sequence showed homology to a lesser extent with the other avian species. Further, the protein modification sites such as glycosylation, N-myrystoylation sites were identified using Scan Prosite tool.

Keywords: Ostrich, Riboflavin-binding protein, Primary structure, Protein modification sites

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INTRODUCTION

Struthio camelus (Ostrich) is a large flightless bird belonging to the ratite family and the only living member of the genus Struthio that is native to Africa. It is the largest of avian species laying the largest eggs. Ostrich eggs are in a great demand because of its lower cholesterol, and a higher unsaturated/saturated fatty acid ratio, and has the similar chemical and nutritive characteristic compared to chicken eggs [1]. Many countries across the world carry out farming of Ostrich for its eggs and meat, which is low in cholesterol and same levels of protein in comparison to chicken eggs.

Riboflavin binding protein (RBP) or Riboflavin Carrier Protein (RCP) is a reproductive protein, which is evolutionarily conserved and hence becomes functionally important during reproduction of avians and mammals. The RBP which is found in hen egg white has 0.09% concentration [2] and consists of 219 amino acid residues which are a monomeric phosphoglycoprotein [3]. RBP is an estrogen inducible protein, which occupies an important position in riboflavin metabolism and is essential for foetal survival [4]. The RBP of various other species confirmed its presence in the circulation and various functions such as placental transport [5].

Many studies carried out earlier have isolated the Riboflavin Binding Protein (RBP) from eggs of birds such as Ostrich [6], Parrot [7], Eagle, Coot [8], Emu [9], Peacock [10], Pigeon [11], Goose [12], Japanese Quail [13], Duck [14] and reptiles such as Indian Python, Painted Turtle [15], in the plasma of pregnant mammals [16]. However, the primary structure of RBP from a few avian species like Hen [3] and Emu [9] were reported earlier. The increased elevated levels of serum RBP helps in early detection of breast cancer [4].

In the present study for the first time, the amino acid sequence along with phosphorylation and glycosylation clusters of RBP from *S. camelus* egg was elucidated using MALDI-TOF, Proteomics and Bioinformatics tools.

We even described the similarities and the structural and phylogenetic differences between the avian and different other species. The sequence homology of Ostrich RBP with that of the Folate Binding Protein and Retinol Binding Protein of different species were observed.

MATERIALS AND METHODS

The Riboflavin Binding Protein was isolated and purified from *S. camelus* egg white and yolk using DEAE-Sepharose and Sephadex G100 [6]. SDS-PAGE slab gels were carried out according to the method of Laemmli [17]

Spectral studies

The absorption spectrum of free riboflavin was recorded using UV-visible recording spectrophotometer (Lambda 25 Perkin Elmer). The riboflavin solution containing 20 mg of riboflavin was dissolved in 500 ml of distilled water. One ml of this standard solution was diluted to 5 ml with 0.05 M sodium phosphate buffer pH 7.4. The absorption spectra of the purified RBP (Sephadex G-100 fraction) were recorded by diluting the proteins with suitable buffers. Twenty fractions (3 ml each) were collected and the absorbance of each protein fraction was measured at 280 nm and 455 nm using UV Visible recording Spectrophotometer (Lambda 25 Perkin Elmer). The peak fractions were dialyzed against distilled water [18].

2-Dimensional Electrophoresis (2DE) was performed on the purified samples which were obtained from the egg white and yolk of Ostrich. Further, MALDI-TOF-MS was performed using egg white of *S. camelus.*

MALDI-TOF-MS

Gel pieces of 1.5 mm diameter were excised manually from 1 mm thick gels and washed for 30 min at room temperature under vigorous shaking with 400 µl of 10 mM ammonium bicarbonate solution containing 50% (v/v) acetonitrile. After removing the supernatant, gel pieces were dried for 15 min in a vacuum concentrator. The rehydrated gel pieces were incubated in 150 µl reduction solution (10 mM DTT, 100 mm ammonium bicarbonate) for 30 min at 56°C. The reduction solution was then discarded and 100 µl alkylation solutions (50 mM iodoacetamide, 100 mm ammonium bicarbonate) were added for 30 min in the dark room temperature. For digestion, 5 µl trypsin solutions (Sequencing grade modified trypsin, Promega, Madison and 10 ng/µl in 5 mM ammonium bicarbonate / 5% acetonitrile) were added to each sample. After incubation for 5 h at 37-38°C, the reaction was stopped by adding 1 µl of 1% TFA. For better extraction of peptides, the samples were stored overnight at 5°C. Without further purification, 1 µl of supernatant was mixed with 2 µl of matrix solution (5 mg a-cyano-4-hydroxycinnamic acid in 40% (v/v) acetone, 50% (v/v) acetonitrile, 9.9% (v/v) water and 0.1% (w/v) TFA in water). From this mix, 1 µl was deposited onto the MALDI target. Tryptic peptides were analysed with a MALDI – TOF Mass Spectrometer (Bruker – Daltonics, Germany) in positive mode. Background ions from trypsin autolysis and contamination by keratins were removed from mass lists. Protein identification was performed by searching for Rattus proteins in the latest version of the NCBI nr database using the Mascot search engine. The following parameters were applied: Monoisotopic mass accuracy, peptide mass tolerance (0.1 Da); peptide charge state (1+); missed cleavages, 1; allowed variable modifications, oxidation (Met) and fixed modification, carbamidomethyl (C). Fragmentation of selected peptides was measured using the PSD mode.

Bioinformatics analysis

The sequence obtained from MALDI-TOF-MS was saved in FASTA format and used for identification of protein modification sites and phylogenetic analysis.

Identification of protein modification sites

S. camelus RBP sequence was used as input in PROSITE, which is a database of protein domains, families and functional sites. The option for "Exclude motifs with a high probability of occurrence from the Scan" was deselected for our analysis.

Phylogenetic analysis

We have retrieved RBP sequences of other species, milk folate binding protein sequence of Bos Taurus, retinol binding protein sequences of Gallus gallus and Homo sapiens from NCBI protein database. The sequences were aligned using ClustalW2 with the default alignment options of Gonnet protein weight Matrix, Penalty of 10 and gap extension Penalty of 0.1. For the comparison of RBP sequences among the various species, the phylogenetic tree was constructed using Mega Version5 Software [19]. The Phylogenetic tree was constructed using Neighbour-joining method [20] with Jones-Taylor-Thornton (JTT) amino acid substitution model [21] and 1000 Boot strap replications.

RESULTS AND DISCUSSION

SDS-PAGE

The Isolated protein RBP was initially separated by SDS-PAGE along with the standard protein marker; the data revealed the isolated RBP had a molecular weight close to 53 KDa (Figure 1) which is higher by approximately 10 KDa when compared to EMU, and 25 KDa higher when compared to Hen egg white RBP. The higher molecular weight of Ostrich and Emu RBPs could be due to the greater extent of glycosylation [6,9].

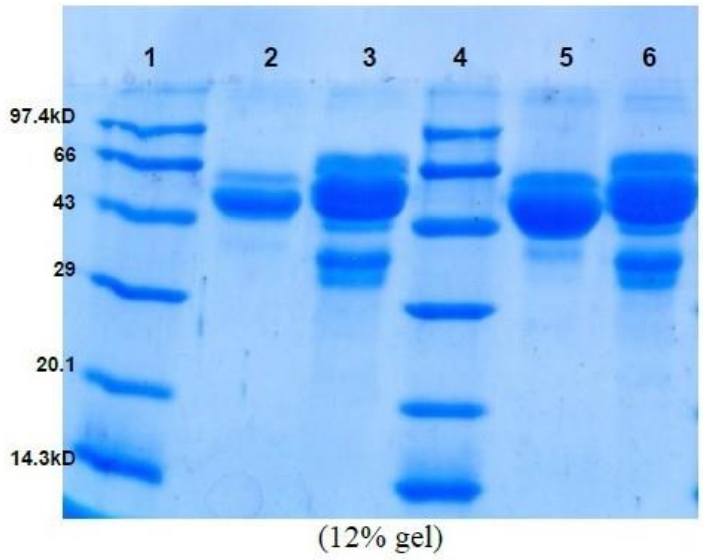

Figure 1: SDS-PAGE

Note: 1. Marker; 2. W (5 µg) sample; 3. Y (5 µg) sample; 4. Marker; 5. W (7 µg) sample; 6. Y (7 µg) sample (All Ostrich egg white (W) and yellow (Y) samples were partially purified using DEAE Sepharose)

Spectral studies

The absorption spectrum of the Ostrich egg yolk RBP was shown in Figure 2a. Binding of Riboflavin to the protein (holoprotein) resulted in the absorption peaks at 372.8 and 454 nm and also the shoulders were appeared. The absorption spectrum of the Ostrich egg white RBP was shown in Figure 2b. Binding of Riboflavin to the protein resulted in the absorption peaks at 374 nm and 457 nm and shoulders appeared. Similar absorption spectrum was reported earlier for hen egg white RBP [2].

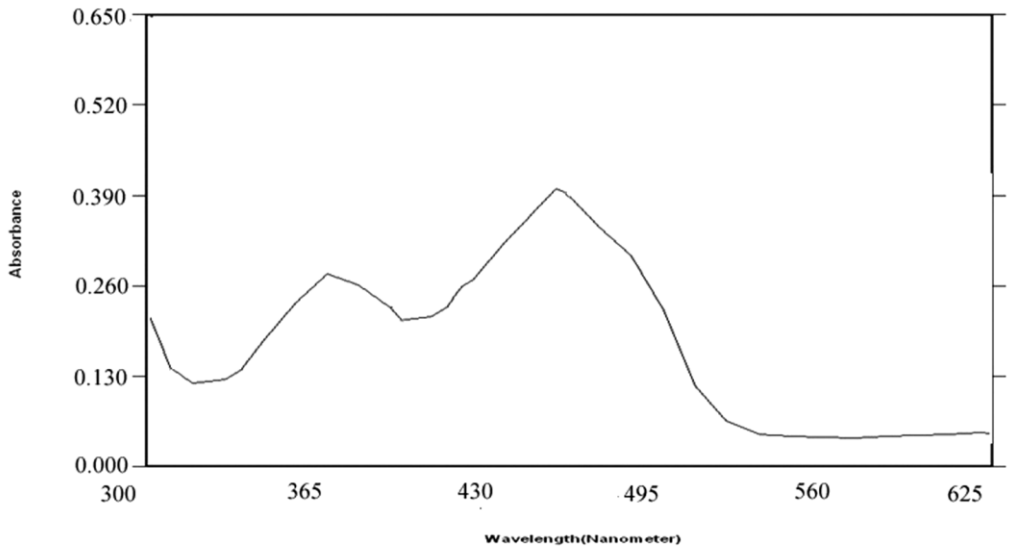

Figure 2a: Absorption spectrum of ostrich egg yolk riboflavin binding protein (Sephadex G -100)

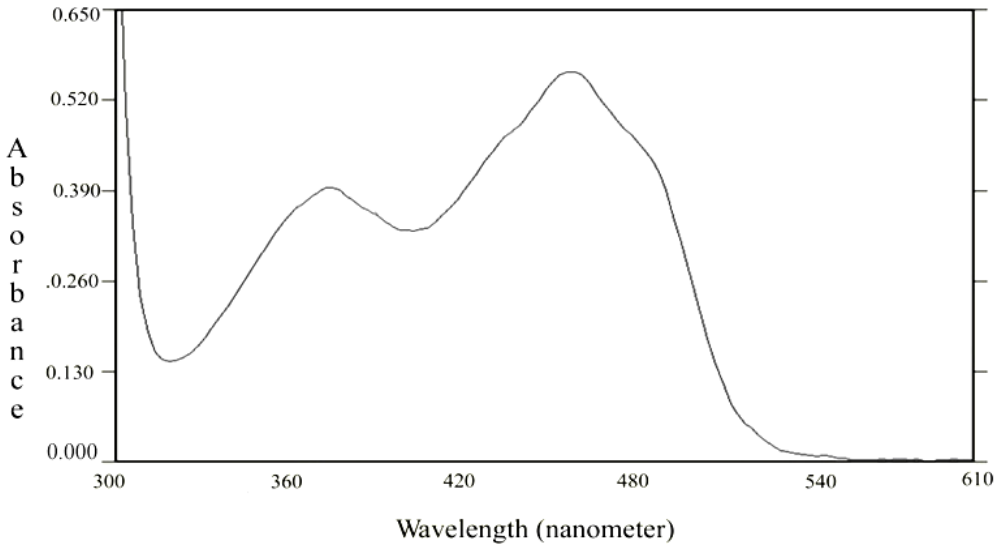

Figure 2b: Absorption spectrum of ostrich egg white riboflavin binding protein (Sephadex G -100)

2DE and MALDI-TOF-MS analysis

The major RBP band was isolated from the Coomassie Brilliant Blue-stained gel, as shown in Figures 3a and 3b, and digested with Trypsin and analysed by MALDI-TOF-MS. High quality peptide mass fingerprinting (PMF) and peptide sequence tag (PST) quality parameters were obtained. The MALDI-TOF graph and database results are shown in Figure 4 and Table 1. A total of 49 peaks were used as a query in MASCOT search, which looks into NCBI database. The applied parameters were: Monoisotopic mass accuracy, peptide mass tolerance (0.1 Da), peptide charge state (1+), maximum missed cleavages (1), allowed fixed modification was` Carbamidomethyl (C) and allowed variable modifications were Oxidation (M) and Propionamide (C). Fragmentation of selected peptides was measured using the PSD mode. This led to the identification of the unknown protein as Riboflavin-binding protein. The sequence of this protein is shown in the following Figure 5.

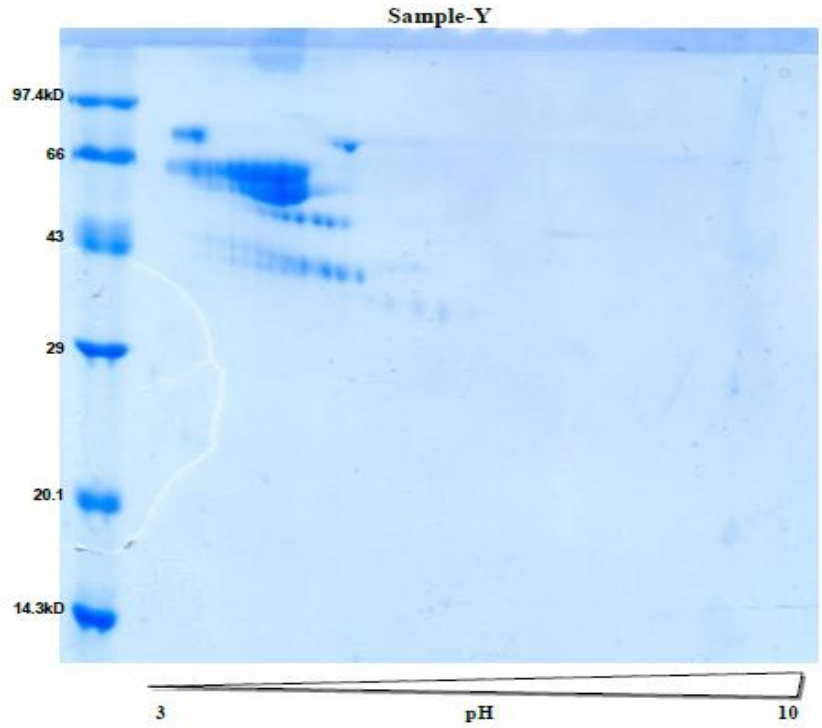

Figure 3a: 12% SDS PAGE (second dimension) 50 µg of proteins is loaded into IEF

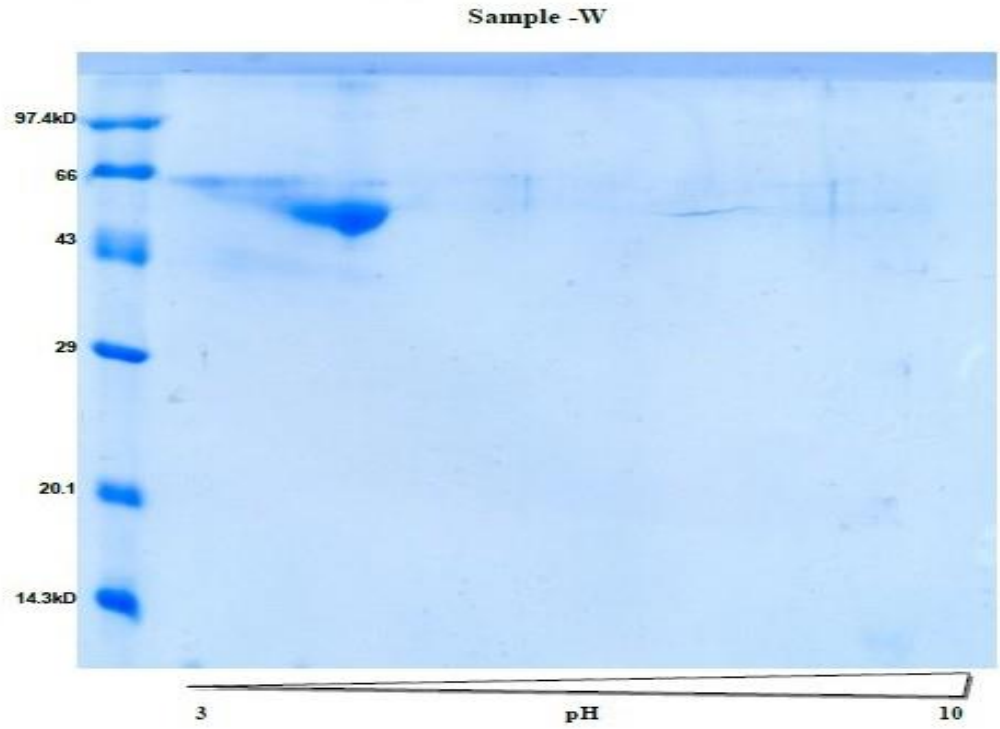

Figure 3b: 12% SDS PAGE (second dimension) 50 µg of proteins is loaded into IEF

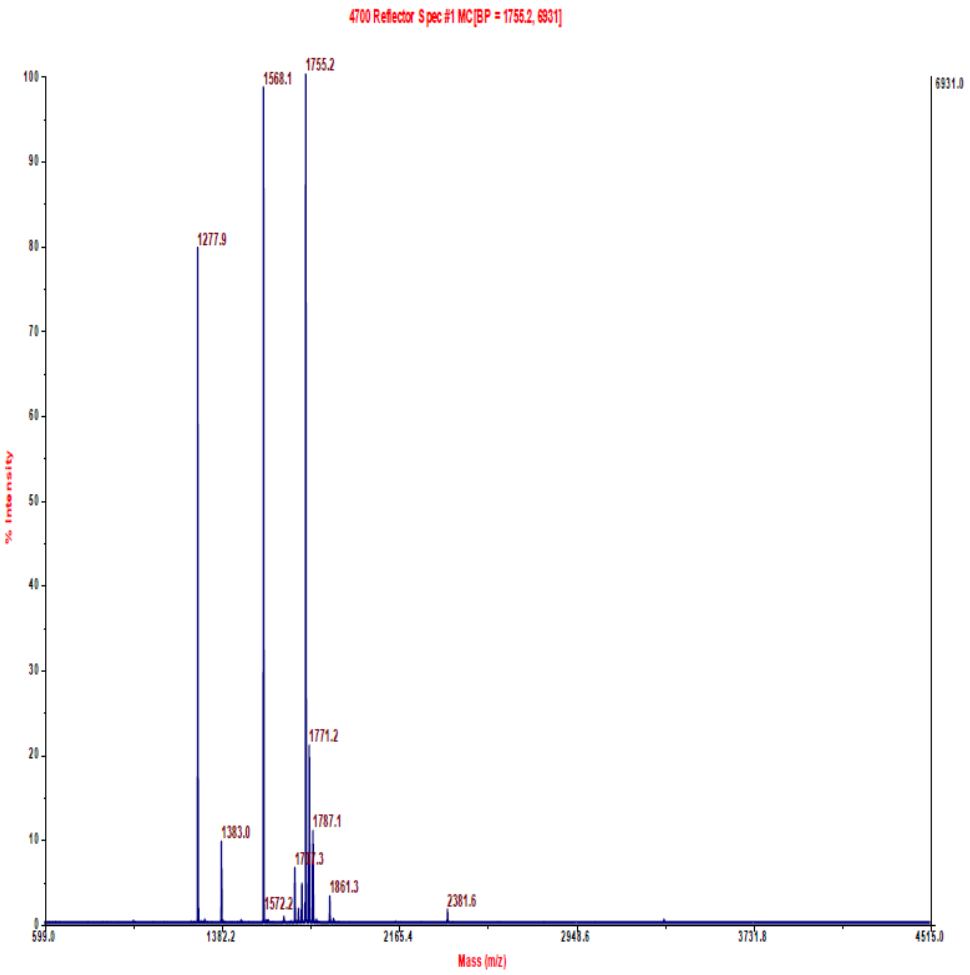

Figure 4: MALDI-MS-TOF analysis of sample 'W'

Search parameters

Table 1: Search parameters

Type of search	Peptide Mass Fingerprint
Enzyme	Trypsin
Fixed modifications	Carbamidomethyl **(C)**
Variable modifications	Oxidation **(M),** Propionamide **(C)**
Mass values	Monoisotopic
Protein Mass	Unrestricted
Peptide Mass Tolerance	0.5 Da
Peptide Charge State	1+
Max Missed Cleavages	1

>Riboflavin-binding protein *(Struthio camelus)*

MLRFAVTLFAVITSSTCKKYSCLEGETHKLKPSPEPNMQECTLYSGSSCCYANFTEQLAH
SPVIKINKSY
WNRCGQLSKSCEDFTKKIECFYRCSPHAAYWIRPNYTAAIRSVPLCQSFCDDWYEACD
DSICVHNWLTD
WEWDESGVNHCKNKCIPYSEMYVNGTDMCQSMWGESFKVSESSCLCLQMNKKDMM
AIKYLLSESSEESSS
VSSSEERACQKKLLKFEKLKEEEGGETR

Figure 5: RBP sequence of *Struthio camelus* in FASTA format

Bioinformatics analysis

Disulphide bridges

The results obtained on Disulphide bond analysis of *S. camelus* (Ostrich) RBP is shown in Figure 6. In Ostrich RBP, all 18 disulphide bonds were conserved and found at exactly same sites as observed in the case of Emu and Hen RBPs.

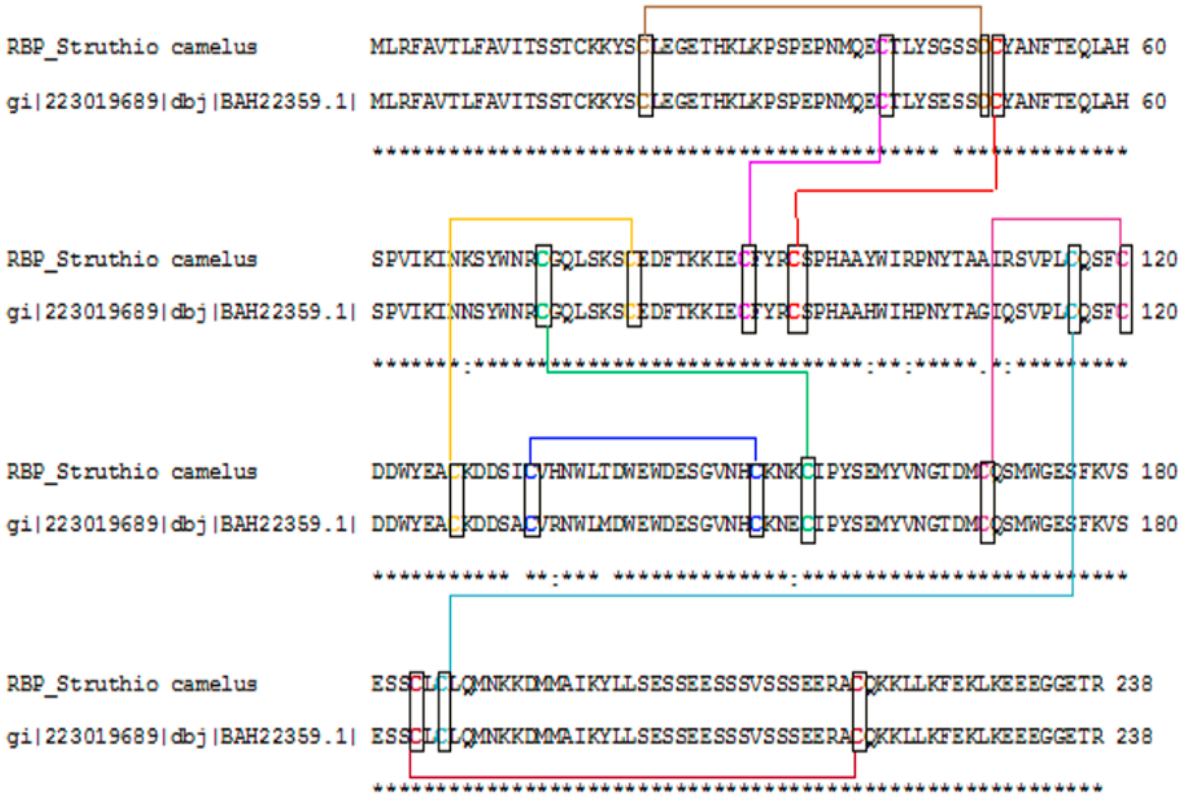

Figure 6: RBP of *Struthio camelus* showing the disulphide bridges

Protein modification sites

From the analysis of *S. camelus* RBP sequence in Prosite, which identified protein modification sites based on homology, the following insights were obtained. The Threonine (Thr) amino acid at positions 16, 27, 85 were predicted to undergo Phosphorylation i.e., they are the sites for Protein Kinase C phosphorylation. The Serine (Ser) residues at positions 21, 80, 118, 139, 212, and 213 were predicted to undergo phosphorylation, shown in Figure 7.

N-myristoylation sites

The predicted sites for N-Myristoylation were: 46 – 51 (GSscCY), 147 – 152 (GVnhCK) and 165 – 170 (GTdmCQ), shown in Figure 8.

Glycosylation sites

The PROSITE predicted sites for glycosylation were: 53 – 56 (NFTE), 67 – 70 (NKSY), 105 – 108 (NYTA) and 164 – 167 (NGTD). Further, our analysis using Prosite also identified Tyrosine Kinase phosphorylation site at positions 86 – 92 (Kki.Ecf.Y). Further cyclic AMP and cyclic GMP dependent phosphorylation sites were observed at 18 – 21(KKYS). The riboflavin binding sites (Tyr – 91, Trp – 173) were found to be conserved, as in Emu [9] and Chicken [22]. The glycosylation site at ASN 88 in Emu, which was reported to be conserved in Turtle, Toad and Frog [9]. The same was also found to be conserved in *S. camelus* RBP in our study as shown in Figure 9.

Interpro predicted domain

The software has identified presence of single functional domain in the RBP sequence of *S. camelus* binding to folate and reduced folic acid derivatives and is required for transport of riboflavin to the developing Oocyte. The main functional part is 22 – 188. The residues from 1 – 22 may serve as a signal peptide. From 188 – 250 may code for polyadenylation signal [9], shown in Figure 10. Predicted protein modification sites, generated by Prosite; Prosite reference [23] (Web Server issue).

Phosphoserine, Phosphothreonine modification sites in *S. camelus* RBP sequence are highlighted below:

MLRFAVTLFAVITSSTCKKYSCLEGETHKLKPSPEPNMQECTLYSGSSCCYANFTEQLAH
SPVIKINKSYWNRCGQLSKSCEDFTKKIECFYRCSPHAAYWIRPNYTAAIRSVPLCQSFC
DDWYEACKDDSICVHNWLTDWEWDESGVNHCKNKCIPYSEMYVNGTDMCQSMWGE
SFKVSESSCLCLQMNKKDMMAIKYLLSESSEESSSVSSSEERACQKKLLKFEKLKEEEGG
ETR

Figure 7: Phosphoserine and phosphothreonine modification sites

N-myristoylation is an acylation process specific to the N-terminal amino acid glycine in proteins [24]. N-Myristoylation sites in *S. camelus* RBP sequence are highlighted below:

MLRFAVTLFAVITSSTCKKYSCLEGETHKLKPSPEPNMQECTLYSGSscCYANFTEQLAH
SPVIKINKSYWNRCGQLSKSCEDFTKKIECFYRCSPHAAYWIRPNYTAAIRSVPLCQSFC
DDWYEACKDDSICVHNWLTDWEWDESGVnhCKNKCIPYSEMYVNGTdmCQSMWGESF
KVSESSCLCLQMNKKDMMAIKYLLSESSEESSSVSSSEERACQKKLLKFEKLKEEEGGET
R

Figure 8: N-myristoylation sites

Glycosylation sites in *S. camelus* RBP sequence are highlighted below:

MLRFAVTLFAVITSSTCKKYSCLEGETHKLKPSPEPNMQECTLYSGSSCCYANFTEQLAH
SPVIKINKSYWNRCGQLSKSCEDFTKKIECFYRCSPHAAYWIRPNYTAAIRSVPLCQSFC
DDWYEACKDDSICVHNWLTDWEWDESGVNHCKNKCIPYSEMYVNGTDMCQSMWGE
SFKVSESSCLCLQMNKKDMMAIKYLLSESSEESSSVSSSEERACQKKLLKFEKLKEEEGG
ETR

Figure 9: Glycosylation sites

Interpro predicted domain for *Struthio camelus* RBP sequence: 22 – 188 Folate receptor-like (IPR018143) [25].

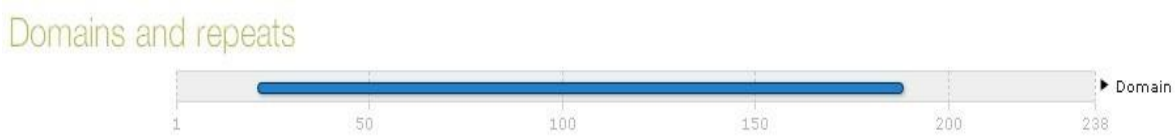

Figure 10: Interpro predicted domain for *Struthio camelus* RBP sequence

Sequence and phylogenetic analysis

Comparison of *S. camelus* RBP with the other species

From the multiple sequence analysis using ClustalW, the RBP sequence of *S. camelus* (Ostrich) and Dromaius novae hollandiae (Emu) were found to be 95.8% identical, indicating a close homology between the RBP sequences of these two species. Gallus gallus (Hen) RBP and *S. camelus* (Ostrich) shared 86.97% homology; on the other hand, Cotornix japonica (Japanese quail) RBP and *S. camelus* shared 81.51% homology. Interestingly, the phylogenetically distinct species belong to sea–born amphibian species Pelodiscus sinensis japonicas (Tortoise) RBP and *S. camelus* shared 73.11% homology. The rest of the species analysed shared equal or less than 50% homology; these identities were also reflected in phylogenetic tree, shown in Figure 12. Hence, the RBP of the avian species are very closely related with the high degree of homology and highly conserved. Further, we also observe the amphibian species; Pelodiscus sinensis japonicas (Chinese Soft Shell Turtle) also shared the higher degree of homology with *S. camelus* RBP. When compared to the other non-avian species this might indicate the certain degree of evolutionary conservation with regard to this protein between avian and amphibian species, as shown in Figure 11 and Table 2.

Comparison of *S. camelus* RBP with milk folate binding protein

The RBP sequence of *S. camelus* and milk Folate binding protein of Bos Taurus (cattle species) shared 20.59% similarity. The low similarity may be attributed to evolutionary diversions leading to difference in the functioning of these proteins, as shown in Figure 13 and Table 3.

Comparison of *S. camelus* RBP with retinol binding protein of different species

The sequence of *S. camelus* RBP shows 8.67% homology with Retinol binding protein of Gallus gallus (Hen) and 9.45% with Homo sapiens. The low similarity may be attributed to evolutionary diversions leading to difference in the functioning of these proteins, as shown in Figure 14 and Table 4.

RBP of struthio camelus – other different species RBPs

CLUSTAL 2.1 multiple sequence alignment

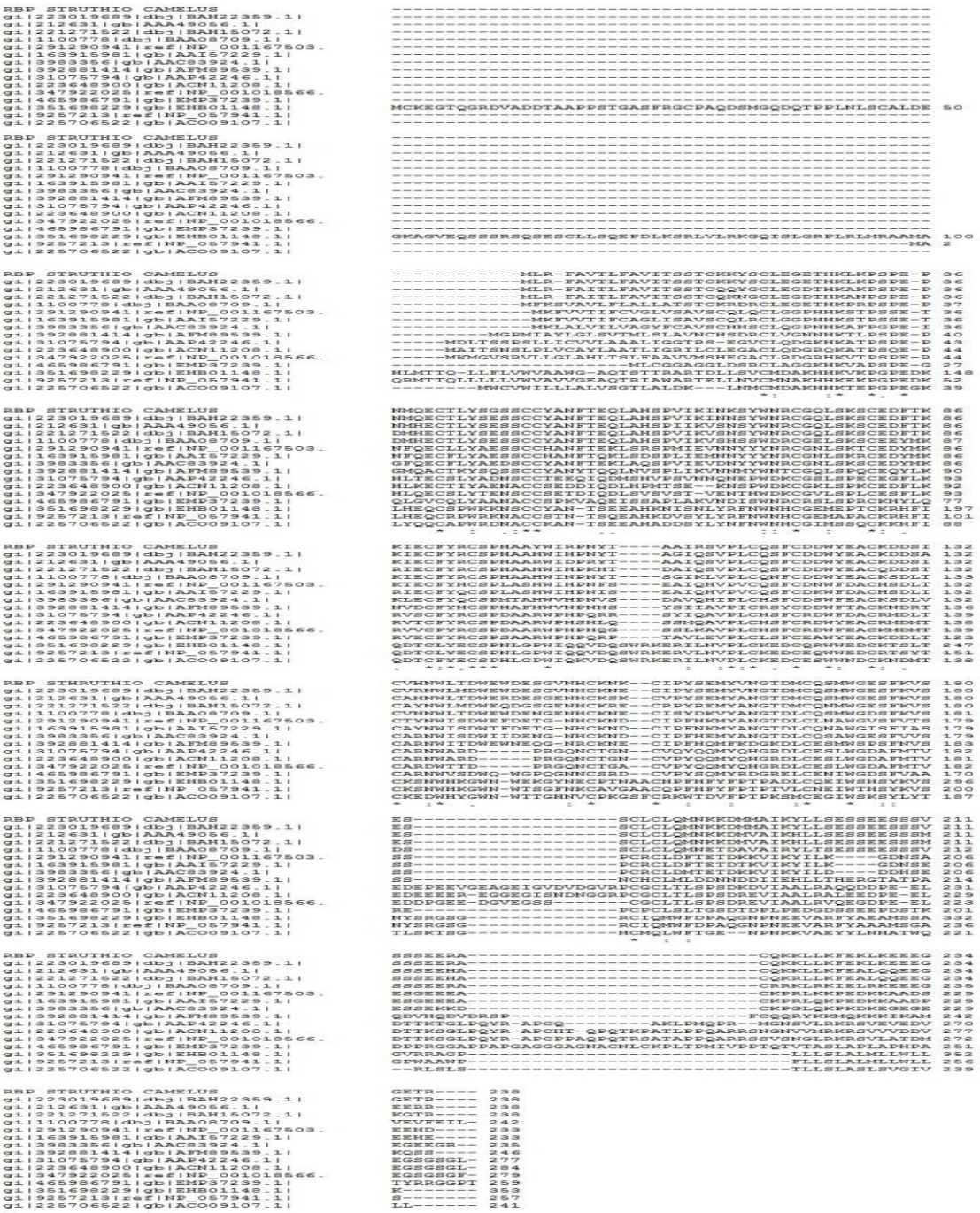

Figure 11: RBP multiple sequence alignment of *Struthio camelus* and other different species

Note: * conserved in all species, conservation between groups of strongly similar properties. Conservation between groups of weakly similar properties

Sequence identities from ClustalW RBP

SeqA	Name	Length	SeqB	Name	Length	Score
1	RBP *S. camelus*	238	2	RBP *Gallus gallus*	238	86.97

1	RBP *S. camelus*	238	3	RBP *Coturnix japonica*	238	81.51
1	RBP *S. camelus*	238	4	RBP *Dromaius novaehollandiae*	238	95.8
1	RBP *S. camelus*	238	5	RBP *Pelodiscus sinensis japonicus*	242	73.11
1	RBP *S. camelus*	238	6	RBP *Scaphiopus couchii*	235	52.77
1	RBP *S. camelus*	238	7	RBP *Callorhinchus milii*	246	44.96
1	RBP *S. camelus*	238	8	Folate receptor *Xenopus laevis*	233	52.36
1	RBP *S. camelus*	238	9	Folate receptor *Xenopus (silurana) tropicalis*	233	51.93
1	RBP *S. camelus*	238	10	RBP *Chelonia mydas*	259	32.35
1	RBP *S. camelus*	238	11	RBP *Dreochromis niloticus*	277	34.03
1	RBP *S. camelus*	238	12	RBP *Salmo salar*	284	33.19
1	RBP *S. camelus*	238	13	RBP *Danio rerio*	279	33.19
1	RBP *S. camelus*	238	14	Folate receptor *Heterocephalus glaber*	353	24.79
1	RBP *S. camelus*	238	15	Folate receptor *Osmerus mordax*	241	21.01
1	RBP *S. camelus*	238	16	Folate receptor *Homo sapiens*	257	22.27

Table 2: Interpretation of RBP and folate receptor sequence identities from ClustalW of *Struthio camelus* and other different species

Protein Name	**GenBank Accession Number**	**Organism**
RBP	BAK22263	*Struthio camelus*
	AAA49056	*Gallus gallus*
	BAH15072	*Coturnix japonica*
	BAH22359	*Dromaius novaehollandiae*

	BAA08709	*Pelodiscus sinensis japonicus*
	AAC83924	*Scaphiopus couchii*
	AFM89539	*Callorhinchus milii*
	EMP37239	*Chelonia mydas*
	AAP42246	*Oreochromis niloticus*
	ACN11208	*Salmo salar*
	NP_001018566	*Danio rerio*
Folate Receptor	NP_001167503	*Xenopus laevis*
	AAI57229	*Xenopus (Silurana) tropicalis*
	EHB01148	*Heterocephalus glaber*
	ACO09107	*Osmerus mordax*
	NP_057941	*Homo sapiens*

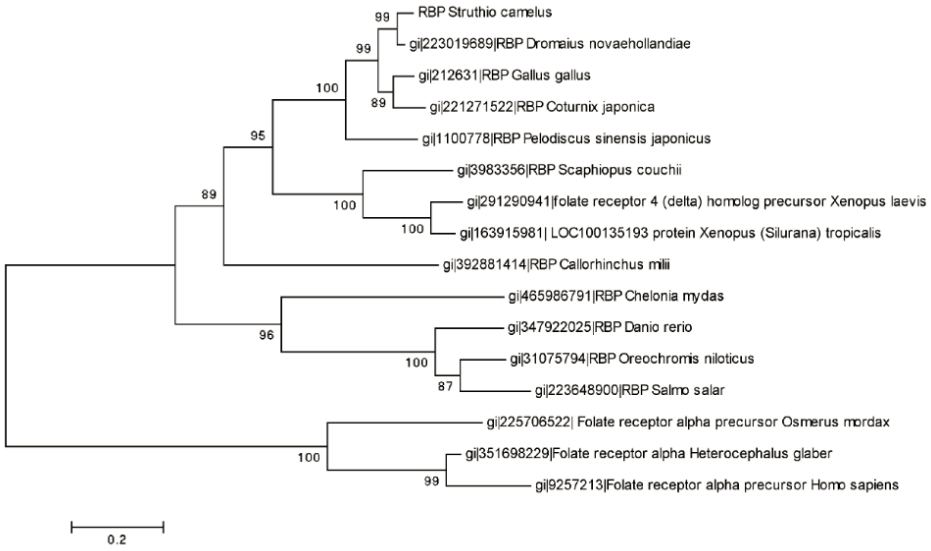

Figure 12: Phylogenetic tree of RBPs based on protein sequences, with human folate binding protein (FBP) as the out group

The Phylogenetic tree was constructed using Neighbour – joining method with Jones-Taylor-Thornton (JTT) amino acid substitution model and 1000 Boot strap replications.

RBP Struthio camelus -milk folate BP

CLUSTAL 2.1 multiple sequence alignment

```
RBP STRUTHIO CAMELUS            ---------MLRFAVTLFAVITSSTCKKYS-----CLEGETHKLKPSPE- 35
gi|514825580|ref|NP_001265502.  MAWQMTQLLLLALVAAAWGAQAPRTPRARTDLLNVCMDAKHHKAEPGPED 50
                                         :* :..: :.. :. * :  :     *::.: ** :*.**

RBP STRUTHIO CAMELUS            PNMQECTLYSGSSCCYANFTEQLAHSPVIKINKSYWNRCGQLSKSCEDFT 85
gi|514825580|ref|NP_001265502.  SLHEQCSPWRKNACCSVN-TSIEAHKDISYLYRFNWDHCGKMEPACKRHF 99
                                .  ::*: :  .:** .* *.  **. :  : :  *::**::. :*: .

RBP STRUTHIO CAMELUS            KKIECFYRCSPHAAYWIRPN----YTAAIRSVPLCQSFCDDWYEACKDDS 131
gi|514825580|ref|NP_001265502.  IQDTCLYECSPNLGPWIREVNQRWRKERVLGVPLCKEDCQSWWEDCRTSY 149
                                 :  *:*.***: . ***       .  : .****:. *:.*:* *: .

RBP STRUTHIO CAMELUS            ICVHNWLTDWEWDESGVNHC--KNKCIPYSEMYVNGTDMCQSMWGESFKV 179
gi|514825580|ref|NP_001265502.  TCKSNWHKGWNWT-SGYNQCPVKAACHRFDFYFPTPAALCNEIWSHSYKV 198
                                 *  ** ..*:*  ** *:*  *  *  :.  : . : :*:.:*..*:**

RBP STRUTHIO CAMELUS            SESSCLCLQMNKKDMMAIKYLLSESSEESSSVSSSEERACQKKLLKFEKL 229L
gi|514825580|ref|NP_001265502.  SN-------YSRGSGRCIQMWFDPFQGNPN-----------EEVARFYAE 230
                                *:        .: .  .*:  :.  . :..           ::: :*

RBP STRUTHIO CAMELUS            KEEEGGETR-- 238
gi|514825580|ref|NP_001265502.  NPTSGSTPQGI 241
                                :  .*. .:
```

Figure 13: RBP *Struthio camelus* – milk folate binding protein multiple sequence alignment

Note: * conserved in all species, conservation between groups of strongly similar properties, conservation between groups of weakly similar properties

RBP-MFBP

SeqA	Name	Length	SeqB	Name	Length	Score
1	RBP *S. camelus*	238	2	gi|514825580|ref|NP_001265502.1|	241	20.59

Table 3: Interpretation of RBP-MFBP sequence identities from ClustalW

Protein Name	GenBank Accession Number	Organism
RBP	BAK23263	*Struthio camelus*
Milk Folate Binding Protein	NP_001265502	*Bos taurus*

RBP-retinol BP

CLUSTAL 2.1 multiple sequence alignment

```
gi|45382541|ref|NP_990569.1|      -MAYTWRALLLLALAFLG--SSMAERDCRVSSFKVKENFDKNRYSGTWYA 47
gi|55743122|ref|NP_006735.2|      -MKWVW---ALLLLAALG--SGRAERDCRVSSFRVKENFDKARFSGTWYA 44
RBP STRUTHIO CAMELUS              MLRFAVTLFAVITSSTCKKYSCLEGETHKLKPSPEPNMQECTLYSGSSCC 50
                                   : :.     ::  :     *    .  ::..    :  :   :**:  .

gi|45382541|ref|NP_990569.1|      MAKKDPEGLFLQDNVVAQFTVDENGQMSATAKGRVRLFNNWDVCADMIGS 97
gi|55743122|ref|NP_006735.2|      MAKKDPEGLFLQDNIVAEFSVDETGQMSATAKGRVRLLNNWDVCADMVGT 94
RBP STRUTHIO CAMELUS              YANFTEQLAHSPVIKINKSYWNRCGQLSKSCEDFTKKIECFYRCSPHAAY 100
                                   *:   :  .     : :   :. **:* :.:. .: :: :  *:   .

gi|45382541|ref|NP_990569.1|      FTDTEDPAKFKMKYWG---VASFLQKGNDD-----HWVVDTDYD------ 133
gi|55743122|ref|NP_006735.2|      FTDTEDPAKFKMKYWG---VASFLQKGNDD-----HWIVDTDYD------ 130
RBP STRUTHIO CAMELUS              WIRPNYTAAIRSVPLCQSFCDDWYEACKDDSICVHNWLTDWEWDESGVNH 150
                                   :  .: :* ::          .: :  :**     :*:.* ::*

gi|45382541|ref|NP_990569.1|      --TYALHYSCRELNEDGTCADSYSFVFSRDPK----------------- 163
gi|55743122|ref|NP_006735.2|      --TYAVQYSCRLLNLDGTCADSYSFVFSRDPN----------------- 160
RBP STRUTHIO CAMELUS              CKNKCIPYSEMYVNGIDMCQSMWGESFKVSESSCLCLQMNKKDMMAIKYL 200
                                    . .: **   :*  . * . :.  *. . .

gi|45382541|ref|NP_990569.1|      -GLPPEAQKIVRQRQIDLCLDRKYRVIVHNGFCS-------- 196
gi|55743122|ref|NP_006735.2|      -GLPPEAQKIVRQRQEELCLARQYRLIVHNGYCDGRSERNLL 201
RBP STRUTHIO CAMELUS              LSESSSEESSSVSSSEERACQKKLLKFEKLKEEEGGETR---- 238
                                   . ..* .. * . :   *  :  :.   :   .
```

Figure 14: ClustalW multiple sequence alignment

Note: * conserved in all species, conservation between groups of strongly similar properties, conservation between groups of weakly similar properties

SeqA	Name	Length	SeqB	Name	Length	Score
1	RBP *S. camelus*	238	2	gi|45382541|ref|NP_990569.1|	196	8.67
1	RBP *S. camelus*	238	3	gi|55743122|ref|NP_006735.2|	201	9.45

Table 4: Interpretation of RBP-retinol BP sequence identities from ClustalW

Protein Name	GenBank Accession Number	Organism
		Struthio camelus
Retinol-binding protein 4 precursor	NP_990569	*Gallus gallus*
Retinol-binding protein 4 precursor	NP_006735	*Homo sapiens*

CONCLUSION

From the data present in the study, it could be concluded that the Ostrich egg white and egg yolk RBPs were significantly larger in their molecular mass mainly due to the difference in the extent of glycosylation [9].

Further, the amino acid sequence appeared to be highly conserved (95.8% with Emu and 86.97% with Hen). However, in post translational modification, the protein glycosylation appeared to be significantly altered leading to the absorbed increase in molecular mass; nevertheless, the post translational modification site interestingly remains highly conserved in these species. Thus, the study adds to the evolutionary information with regard to RBP protein, especially avian species.

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