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Study of purification of hemoglobin in *Fasciola gigantica* (Trematoda:Fasciolida)

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Abstract- The hemoglobin of *Fasciola gigantica* a trematode parasite in the bile ductules of the water buffalo *Bubalus bubalis* was isolated and purified by ammonium sulfate fractionation and gel filtration on Sephadex G-75, and 5.7 fold purification was obtained. Gel filtration revealed two fractions designated as FI and FII. Low A412/A280 ratio for the fraction I, suggest it to be a protein impurity while the fraction II represents the true hemoglobin of the trematode. The trematode hemoglobin shows the characteristic absorption maxima at 410, 540 and 576nm. The molecular masses of *Fasciola gigantica* hemoglobins are estimated to be ca 17kDa by both gel filtration and SDS- PAGE, suggesting the monomeric nature of this protein. *Fasciola gigantica* has an n-terminal sequence of the stained, excised and obtained by electroblotting of the SDS-PAGE of the purified hemoglobin in tris-tricine, buffer, pH 8.2. The properties of *Fasciola gigantica* hemoglobin are in conformity with findings on other trematodes.

Keywords : hemoglobin, trematode, *Bubalus bubalis*, etc.

INTRODUCTION

Different molecular devices which serve as carrier in animal respiration are referred to as respiratory pigments.Hemoglobin as an oxygen carrying protein that was first introduced by Hunefeld[1] in 1840. Max-Peruz in 1959 determined the molecular structure of the hemoglobin (Hb) by the X- ray crystallography. For his outstanding work he shared with John Kendrew the Nobel Prize in 1962. Hemoglobin is encountered in all five kingdoms of organisms. Apart from invertebrate, Hemoglobin occurs widely but sporadically in the phyla platyhelminthes to chordata and found in some 33% of presently known classes.

The phylum Platyhelminthes, has around 25000 species and represents one of their major basal animals groups and divided among the four classes: Turbellaria, Monogenea, Trematoda and Cestoda Hemoglobin refers to all classes of O_2 binding heme protein ranging in size from single one domain globins to the most complex ones, & Myoglobins (Mbs) generally denote single, one domain Hbs occurring in muscle, nerve/glial cells and other tissues as well as those found in unicellular organism. Because trematodes are acoelomate, their Mbs may represent most primitive globin among multicellular organisms.Single one domain globins are denoted as: LegHbs (in leguminous

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plant), Neuroglobin (in nerve tissue) & Cyanoglobin (in cyanobacteria).

Major forms of the platyhelminthes'Hbs appear always to be monomeric. Disulfide bonded dimer formation reported may be time dependent as observe with *Isoparorchis hepselobagri's* Hbs Except for the work by Moens and co-workers on trematode there is a paucity of informationon the structure and properties of platyleminthes Hbs. The primary structure of the monomeric Mbs from *Paramphistomum epiclitum* and *Isoparorchis hypselobagri* has been determined. In both the parasite, Mbs exist in at least four isoforms and have Tyr residues in the B10 and E7 position. Spectrophotometric properties of several other digenetic trematodes have been studied[2,3]. Haque *et al.*[4] studied the biochemical properties of the Hbs of trematode *Gastrothylax crumenifer* and *Paramphistomum epliclitum.* They observed that *Gastrothylax* hemoglobin consisted of about 30000 M^r and 18000M^r by the gel filtration. SDS-electrophoresis shows Hbs of both the parasites to be single chain of ca 15000. The molecular weight of *Paramphistomum* hemoglobin by gel filtration was ca 16000. The native molecules of monomeric with Mr 16000-17000 and pI 6.5-7.5, in each species, at least four different globin isoforms occur. Primary structure was determined at the protein level. The globins contain 147 amino acid residues.

The high O_2 affinity favour O_2 transfer from the host, although it appears unfavour for unloading O_2. *Fasciola hepatica* has an intracellular Hb that is located preferentially around the vitellaria and utrine coils and is highly antigenic. In present study attempts have been made to study the biomedical properties of the hemoglobin of the trematode *Fasciola gigantica* in bile ductules of host water buffalo.

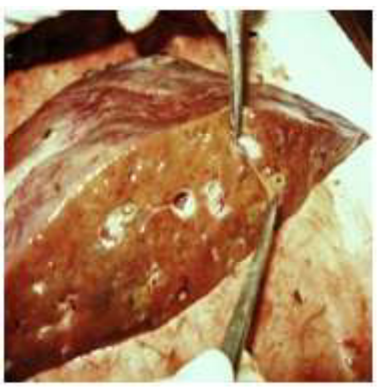

Fig.1 *F. gigantica* pulling out from liver

Fig. 2 *F.gigantica* kept in Tyrode Saline in petridish

MATERIALS AND METHODS

The liver infected of the host with *Fasciola gigantica* (Fig. 1) were collected (Fig. 2) from the local abattoir in Patna. In the laboratory trematode were collected from respective tissue washed and incubated for an hour in normal saline containing 8mM glucose. About 100g of fresh frozen trematode were homogenize in 3-5 volume of ice cold 0.05M phosphate buffer, pH 7.4, containing 1mM phenyl methyl sulfonyl fluoride (PMSF) an inhibitor of cystein proteases.The homogenate was centrifuged at 15000g for 20 min at 4^0C. Ammonium sulfate fractionation of the supernatant was carried out at 40%, 70%, and 95% saturation to precipitate the hemoglobin containing fraction.The precipitate was dialyzed twice against 2000 volume of 0.05M phosphate buffers, pH 7.4 for 18 hour at 4^0C and concentrated against sucrose.

The Hb fraction was applied to chromatographic column (2.5 X 60.0 cm) of Sephadex G-100. Elution was carried out at the rate 75ml/ hr in 0.05M phosphate buffer and 5ml fraction were collected. The absorbance of elute was monitored at 280nm and 412nm. The peak fraction was pooled, concentrated, dialyzed and lyophilized. The absorption maxima of the pooled Hb fraction obtained after gel filtration was done by scanning through 400 to 600nm.

Standard molecular weight used to calibrate the Myoglobin (17kDa), Ovalbumin (43kDa), Bovine serum albumin (BSA) (67kDa). Heme content of the Hb was determined by the method of Darawshe *et al.*[5] Sodium dodecyl sulphate (SDS-PAGE) was performed in a 10-15% acrylamide gradient slab gel (7X10 cm). The gel was stained for protein with Coomassie Brilliant Blue (R-250) and for hemoglobin with Benzidine Reagent.

Peptide mass finger printing Matrix assisted liquid desorption- Time of Flight (MALDI-TOF) was carried out on Hbs isolated from PAGE. The peptide mass data were searched against NCBInr database with the mascot search engine (Matrix Science Ltd., UK. With *Fasciola hepatica* as species searched).

RESULTS AND DISCUSSION

Ammonium sulfate precipitation resulted in 3.02 folds purification of *F. gigantica* hemoglobins and this value is based on A412 / A280 ratio monitored at each stage, as a parameter for the degree of purification. The spectrum of the crude homogenate after 95 % ammonium sulfate precipitation is shown in Fig. 3, where it can be seen that beside hemoglobins there are other protein impurity.

The Fig. 4 depicts basic elution profile of *Fasciola gigantica* hemoglobin in the column of Sephadex G-100. Two sharp peaks designated as E1 and E2 have an absorbance both at 412 nm and 280 nm, whose elution volume corresponds to molecular masses of ca 60kDa and 19kDa respectively. However, the low A412/ A280 ration for peak E_1 suggests it to be a protein impurity, while this ratio for peak E_2 is indicative of the presence of heme. Peak E2 thus represents the hemoglobin fraction of trematode. There is an additional peak also, which absorbed at 280 nm only.

Fasciola gigantica hemoglobin tends to turn brownish, from its original bright red colour, during its passage through the Sephadex G-75 column, a phenomenon attributed to methemoglobin formation as a result of oxidation of iron from ferrous to ferric state[6]. However, the spectral analysis of the E2 peak fraction shows absorbance at 440, 540 and 576nm, corresponding to an oxyhemoglobin spectrum. The ratio of absorbance at 576 to 540nm is 1.06, indication that the sample contains little methemoglobin.

The 5.7 fold purification of the hemoglobin obtained as a result of ammonium sulfate fractionations and gel filtration shows the SDS-PAGE pattern, under reducing condition, of the purified hemoglobin of *Fasciola gigantica*and the previously described hemoglobins of *Gastrothylax crumenifer* HbII and *Bubalus bubalis*, lanes a & c respectively[5]; which are here as marker proteins. As can be seen a single polypeptide chain is obtained for *E. explanatum* whose mobility is similar to that of the marker hemoglobin of *G. crumenifer* and *B. bubalis*; having molecular masses of ca 17 kda. This shows that the hemoglobin of *E. explanatum* constitute a ca 17 kDa chain, its apparent molecular mass estimated by gel filtration is slightly higher. These findings thus suggest that the hemoglobin of *E. explanatum* is chemically homogenous and like the hemoglobins of *G. crumenifer*, *P. epiclitum* and *Isoparorchis hypselobagri*, it is also monomeric[4,7]. The hemogobin of *E. explanatum* appears to have a high oxygen affinity with P_{50} value of 16 mmHg at the physiological pH of 7.4; and the Hill coefficient value is calculated to be 1.0.

Fasciola gigantica hemoglobin has an N-terminally blocked residue as it does not provide N- terminal sequence of the stained excised band obtained by electro blotting of the purified hemoglobin after SDS-PAGE in tris-tricine buffer, pH 8.2. The results of sodium dodecyl sulfate polyacrylamide gel electrophoresis under reducing conditions are shown in Fig 5. Hemoglobin of *Fasciola gigantica* resolved into two bands of 17 kDa and 34 kDa. The 17 kDa band is the major hemoglobin. The appearance of 34 kDa band points out to the possibility of dimerisation of the two polypeptides of 17 kDa to 34kDa. Thus 17kDa band cut and run further analyzed on MALDI TOF mass spectrophotometry.

MALDI-TOF mass spectrometry generates Protein identification by in-gel digestion and peptide mass finger printing (PMF) is almost a routine method in MS laboratories. The protein sample is digested with a specific enzyme, e.g., trypsin, which cuts (at c-terminus of arginine and lysine) in a sequence-specific manner to produce a defined set of peptides. The peptides are extracted deposited onto the MALDI target plate. The peptide mixture is then analyzed by MS to produce a rather complex spectrum from which the molecular weights of all of the proteolytic fragments can be read. This spectrum, with its molecular weight information, is called a peptide map or peptide mass fingerprint. This PMF can be used to search protein databases. Many scores are converted to expectation values or *E*-values, which is an estimate of the number random matches of similar score expected in the target database. If the protein already exists on a database, then the peptide map is often sufficient to confirm the protein[8].

Accurate and rapid species identification of pests and parasites is extremely important for initiating species-specific treatment procedures.[9] The most complete peptide maps were obtained when the proteins were reduced and alkylated on the membrane prior to enzymatic digestion followed by separation of the resulting mixture by high performance liquid chromatography prior to mass spectrometric analysis. Such peptide maps cover up to 98% of the sequence and consequently may allow complete characterization of post-translational modifications in proteins for which the amino acid sequence is known[10]. The fastest and most sensitive procedure to obtain peptide maps sufficient for protein identification was direct analysis of the extracted peptide mixture by matrix-assisted laser desorption ionization (MALDI) mass spectrometry. The use of external and internal calibration of MALDI spectra for database searches is evaluated as well as the possibility of including a post-calibration routine within the search program.[11] The PMFs were analysed through search engine/database Swiss-Prot (Fig.7) and peptide sequences were matched *Fasciola gigantica* to the close associated species

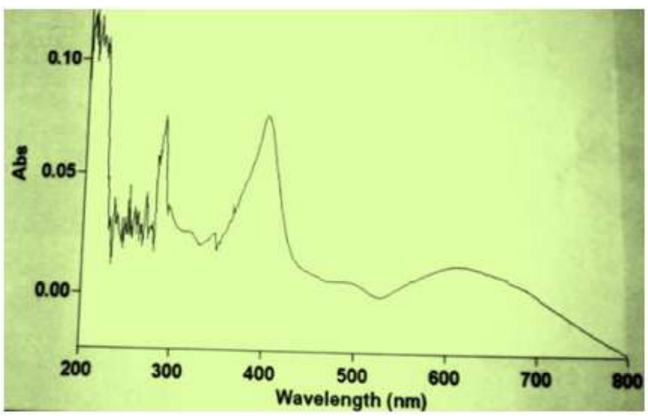

Fig. 3. Spectral profile of the crude hemoglobin preparation of *Fasciola gigantica* after 95% ammonium sulfate precipitation

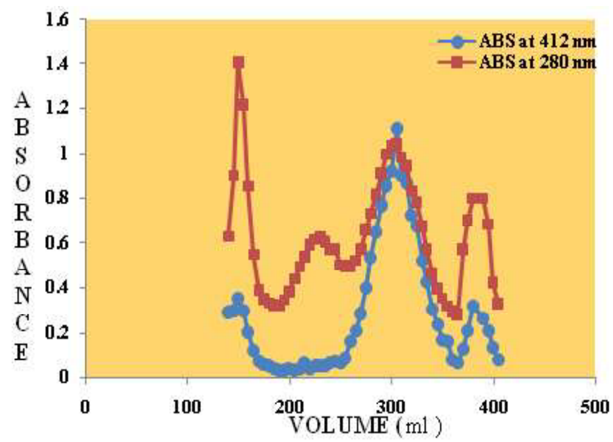

Fig. 4. Elution profile of *Fasciola gigantica* Hbs on Sephadex G-100 column

Fig. 5. Electrophretic pattern of *Fasciola gigantica* (Band 1) Hb after Sephadex G-100 column purfication

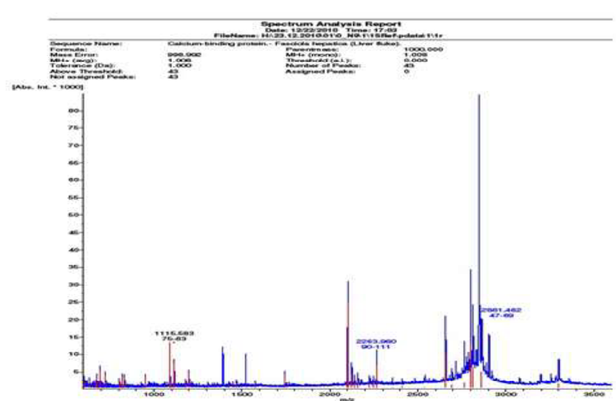

Fig. 6. Maldi spectral analysis report

Sequence data:

Calcium-binding protein.- Fasciola hepatica (Liver fluke).

Intensity Coverage:	9.8 % (18743 cnts)	Sequence Coverage MS:	28.4%
Sequence Coverage MS/MS:	0.0%	pI (isoelectric point):	5.2

```
         10         20         30         40         50         60         70         80         90
MTTAYKLHDV ESLIDWFMEL DKNNDEAVDK KELTAYYKSK GVSDSKINEW MEHFDADSDG KITLMEFCRG LGLRIDEVRV EQKQPPLQR
        100        110        120        130        140        150        160        170        180
GTAPALGTDI EMIATTMSQP RQVEVTEKFK SLVTAHNGKD EEMKDVAHEL KTFLDDTYGR VWQCVILTGS YWMQFSHEPF LSIQFRYGR
        190        200
ICLAWRTPRG
```

Display Parameter:

Sequence Name:	Calcium-binding protein.- Fasciola hepatica (Liver fluke).		
Parentmass:	1000.000	Mass Error:	998.992
MH+ (mono):	1.008	MH+ (avg):	1.008
Threshold (a.i.):	0.000	Tolerance (Da):	1.000
Number of Peaks:	43	Above Threshold:	43
Assigned Peaks:	0	Not assigned Peaks:	43

Peaklist:

Peak	Mass	Intensity	Peak	Mass	Intensity
1	603.299	1920.136	2	605.238	2348.737
3	606.232	2074.150	4	634.298	2180.463
5	672.216	1232.004	6	675.346	3121.954
7	691.345	5113.758	8	725.280	3922.623
9	804.325	2036.883	10	817.428	3604.104
11	833.400	3138.687	12	950.440	3636.533
13	1091.560	12988.000	14	1091.560	12233.632
15	1094.579	1436.780	16	1115.583	7182.695
17	1181.558	1978.688	18	1198.591	4154.530
19	1200.596	2472.720	20	1307.665	1084.463
21	1425.803	1141.392	22	1468.703	1748.008
23	1576.729	1166.903	24	1743.781	4069.942
25	2097.995	1764.262	26	2100.974	22625.449
27	2100.981	23497.000	28	2122.964	4270.574
29	2138.924	1716.060	30	2157.996	2050.590
31	2223.069	1604.944	32	2245.123	1306.243
33	2263.960	6711.062	34	2353.484	540.039
35	2410.116	834.800	36	2537.464	452.285
37	2656.276	9943.218	38	2692.937	1191.139
39	2763.438	1774.689	40	2797.453	12343.467
41	2810.504	5910.488	42	2861.462	4849.159
43	3299.725	1554.451			

Fig. 7 Maldi spectral analysis report

Matched Sequences:

Calcium-binding protein.- Fasciola hepatica (Liver fluke).

```
MTTAYKLHDVESLIDWFMELDKNNDEAVDKKELTAYYKSKGVSDSKINEWMEHFDADSDGKITLMEFCRGLGLRIDEVRVEQKQPPLQRAGTAPALGTDI
EMIATTMSQPRQVEVTEKFKSLVTAHNGKDEEMKDVAHELKTFLDDTYGRVWQCVILTGSYWMQFSHEPFLSIQFRYGRYICLAWRTPRG
```

Digest Matches
Tolerance = 100.000000 ppm, Trypsin
Score = 33.800000, Rank = 1, Database = MSDB, Accesskey = O46121_FASHE
Modifications: \ML C -H+CH2COOH Carboxymethyl (C), \MO M O Oxidation (M),

```
  Mass Mr      Dev.    Range     P Sequence
* 1114.576   -0.022   75 -  83  1 IDEVRVEQK
* 2262.953   -0.129   90 - 111  0 AGTAPALGTDIEMIATTMSQPR 102: Oxidation (M)  107: Oxidation (M)
* 2860.455    0.241   47 -  69  1 INEWMEHFDADSDGKITLMEFCR 51: Oxidation (M)
```

Fig. 8 Peptide mass finger printing match through Swiss-Prot engine

The data base search has revealed that there exist around 28.4% sequence (Fig.8) match of *Fasciola gigantica* with sequence of previously known *Fasciola hepatica* hemoglobin. Different levels of purification obtained could be due to different protein content in the trematodes.

CONCLUSION

In the present study the hemoglobin characteristics of the trematode *Fasciola gigantica* were studied using spectrophotometry, gel filtration chromatography and MALDI-TOF. The trematode hemoglobin is 17 KD M^r protein, found to occur in dimeric form as well as monomeric forms. It has been reported that the hemoglobin in nematodes serve in oxygen transport.[12] The oxygen affinity of trematode hemoglobin is more than the vertebrate hemoglobin and as such it takes greater time to release the oxygen than the vertebrate counterpart.[12] This could be an adaptation on part of the parasitic forms of nematodes which take refuge inside the host bodies where oxygen is limiting. This may function to store oxygen and release a little as they have no energy intensive function to do as an endoparasite. The absorption intensity of trematode Hb is greater in the beta band region than in alpha band region could be due to this reason. The modification may help the animal to sustain aerobic respiration in spite of anaerobic surrounding. The *F. giganticum* hemoglobin dimer may be in accordance to this adaptation.

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