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Sanjay Kumar
Research Scholar, Department of Zoology, Patna University, Patna, Bihar, India

Corresponding Author:
Sanjay Kumar
Research Scholar, Department of Zoology, Patna University, Patna, Bihar, India

Study the biochemical properties of the haemoglobins of the nematode ascaris

Sanjay Kumar

Abstract
The exact function of nematode haemoglobin is yet to be elucidated. However, before studying the physiological aspects it is imperative to know that biochemical properties of these unique oxygen birding proteins of the nematodes. In the present study attempts have been made to study the biochemical parameters of the haemoglobins of the Nematode Ascaris such from the small intestine of pigs. The purification of the perienteric fluid haemoglobins and body wall haemoglobins of the Namatode.

Keywords: Haemoglobins and Nematode Ascaris

Introduction
The nematode, *Ascaris lumbricoides*, an intestinal parasite of man, has two distinct haemoglobins, one in the body wall and the other in the perienteric fluid. Davenport (1949) in a notable study partially purified these haemoglobins and demonstrated that they have remarkably high affinities for oxygen resulting from extremely slow rates of dissociation of oxygen. The separations of two haemoglobins have also been reported by Treibs *et al.* (1950) [2]. The perienteric fluid haemoglobin does not give up its oxygen in the living animals, which raises the question: What function does it serve? Smith and Lee (1963) [5] have offered compelling evidence that the perienteric fluid haemoglobin is a store to provide a continuous supply of haemoglobin for incorporation into eggs. Hamada *et al.* (1963) [4] and Smith *et al.* (1963) [5] have reported independently the partial purification of the two *Ascaris* haemoglobins and some of the properties of these preparations. The exact function of the nematode haemoglobin is yet to be elucidated. However, before studying the physiological aspects it is imperative to know that biochemical properties of these unique oxygen binding proteins of the nematodes.
The vertebrate's haemoglobin and myoglobin have been comparatively well studied. However the precise physiochemical properties and physiological roles of haemoglobin in nematodes have remained far less investigated [1–11]. For the understanding of the relationship between the structure and function of nematode haemoglobins, a comparative study of widely different nematode haemoglobins is desirable. Such a study will elucidate the correlation of structural features of nematode haemoglobins with properties peculiar to each. The biological significance of these pigments in anaerobic and aerobic nematodes would help in better understanding of the intrinsic physiological relationship between the host and the parasite nematodes.

Materials and Methods
Ascaris were obtained from the small intestine of the pig from the local slaughter houses. The worms were brought to the laboratory in the normal saline so as to keep them in physiologically active condition. In the laboratory the worms were washed properly. Hb from the perienteric fluid of *Ascaris* was collected by making a longitudinal cuticular incision along the length of the body.
The perienteric fluid was collected in a clean 100 ml beaker. This was centrifuged at 2000 rpm for 15 minutes; the supernatant fluid was separated out and was immediately converted into carbon monoxyhaemoglobin. For body wall Hb the worms were dissected so as to remove all internal organs and the cuticle was washed thoroughly and was cut into pieces.

These pieces were placed in a glass homogenizer along with suitable quantity of tris-glycine buffer (pH 7.4).

It was then homogenized thoroughly and centrifuged at 2000 rpm to remove the suspended coarse debris. The supernatant fluid was re-centrifuged before conversion into carbon monooxyhaemoglobin. In order to increase the concentration of the Hb, the supernatant fluid was dialyzed in a dialyzing bag against sucrose solution. Carbon monoxide was prepared by the dehydration of formic acid by concentrated sulphuric acid. Formic acid was added drop wise to the sulphuric acid in a system with only one outlet.

Fractional precipitation with ammonium sulfate {$(NH_4)_2 SO_4$}.

About 10g of fresh frozen *Ascaris* body wall was homogenized in 3-5 volumes of ice-cold 0.05M phosphate buffer, pH 7.4, containing 1mM phenylmethylsulfonyl fluoride (PMSF) an inhibitor of cystein proteases. The PMSF was made soluble by dissolving adequate amount of isopropanol before adding to the homogenate and 5mM ethylenediamine tetraacetic acid (EDTA) in an ice-cold mortar and pestle. The homogenate was centrifuged at 15000 g for 20 min 4^0C to remove the tissue debris. The clear supernatant was made 40% saturated with ammonium sulfate and centrifuged again. The supernatant was then brought to 70% saturation with ammonium sulfate and centrifuged; the resulting supernatant was finally saturated to 95% with ammonium sulfate to precipitate the haemoglobin containing fraction. The entire process of fractionation is called 'salting out'.

The precipitate was dialyzed twice against 2000 volumes of 0.05 phosphate buffer, pH 7.4 for 18 hours at 4^0C and concentrated against sucrose crystal. The process of removal of salt through dialysis is called 'salting in'.

Proteins with surface patches that are easily dehydrated precipitate at lower ammonium sulfate concentrations than those with a tightly bound salvation shell. Once the concentration at which the target protein precipitates is known, fractional precipitation with ammonium sulfate can be used as an early step in a purification protocol. A pilot experiment should be performed using a small volume (10-20 ml) of the protein solution to ascertain the range of ammonium sulfate concentrations over which the target protein precipitates.

When an increasing amount of $(NH_4)_2 SO_4$ added to protein solution, the salt dissolve and resulting ions interacts with freely available water molecules, as more and more $(NH_4)_2 SO_4$ is added, these free available water molecule become scarce, so water molecules are preferentially pulled off from the hydrophobic patches on the protein surface. When the protein is dissolved in water the hydrophobic patches are forced into contact with surrounding water molecules which again dissolved salt into ions. The water molecule associated with the charged and polar groups on the surface of the proteins are bound by electrostatic interaction and are far less easily give up. As the $(NH_4)_2 SO_4$ concentration increases, the hydrophobic surface on the protein are progressively exposed and protein comes together to form insoluble protein aggregate via hydrophobic interactions; i.e. protein– protein interaction.

Obviously those proteins with the largest number of hydrophobic patches aggregates first, where as those with no hydrophobic surface probably will not precipitate even at high conc· of $(NH_4)_2 SO_4$. In crude mixture, co-aggregation is obviously extensive; like molecules do not necessarily all stick together. The solubility of the protein depends upon the ionic strength of a solution.

$I = \frac{1}{2} \Sigma Ci\ Zi$

I = ionic strength

Ci = conc. of the *i*th ion of electrolytes and

Zi = charge of electrolytes of solution.

Gel filtration chromatography

This technique was employed to further purify the partially purified nematode haemoglobins and to estimate their molecular weights. Gel filtration is advantageous in the sense that it does not require highly purified proteins for the estimation of molecular weights and other hydrodynamic properties such as Stake's radius, friction coefficient and partition coefficient (K_{av}). The mechanism involved in gel filtration is that it separates a mixture of molecules on the basis of small difference in their size. With the proper experimental design, a protein can be separated from other protein species differing in molecular weight by a factor of two or slightly less practical simplicity, excellent recovery, free choice of elution condition and straight forward interpretation of results make fractionation by gel filtration an invaluable part of any purification scheme.

It is used to fractionate mixture of gases, liquid or dissolved solid. When the pigment are being separated, forming zones of different colour, the process can easible be followed visually and then each zone is collected as a separate fraction.

Packing of the Column

The dialysed and concentrated solution of trematode haemoglobin were purified over Sephadex-100 (superfine, dry bed diameter 40-120µm, fractionation range- 4000 to 150,000, which is supplied as dry powder whitish in color. Sephadex is bead like structure formed of crossed linked dextran with epichlorohydrin. Large number of hydroxyl group renders the gel extremely hydrophilic that is why Sephadex swells readily in water and in electrolyte solution. Degree of swelling of Sephadex is substantially independent of the present of salt and detergents. For the gel filtration Sephadex is allowed to swell in the proportion of 1gm/ 15 to 20 ml distilled water on boiling steam water bath for about 6 to 8 hours continuously. At certain interval gel is gently stirred to avoid any air trap. Slurry is not kept in fridge but at normal temperature. Fine particles were removed by decantation at this stage. The suspension was also degassed under vacuum. The temperature of the gel suspension should be brought down to room temperature before packing the column.

Column Calibration

A calibrated column of Sephadex provides a simple and well established way of determining the molecular weights of proteins in their native state during purification. Gel filtration provides a means of determining the molecular weight or size (Stoke's radius) of native or denatured globular proteins under a wide variety of conditions of pH, ionic strength and temperature etc. Thus gel filtration eliminates the need to set up separate experiments for purification and molecular weight analysis.

The steric approach has been extended in various ways. Porath (1963) [6] derived a theoretical relationship between distribution coefficient kDa and stoke's radius assuming that

the pores in Sephadex area conical. In another treatment Squire (1964) considered pores and crevices as well as cones. An interesting approach by Laurent and Killander 1964 was the assumption that the gel network is composed of rigid roads, randomly arranged. Good correction was found between K_{av} and molecular radius with this model. All of these models have been successfully applied to predict the elution behavior of solutes. However, as has been pointed out by Ackers (1967), none of them may be accurate in a structural sense. Results are equally in accordance with a formulation, where the fraction of the stationary phase available to molecules of different radii are defined by Gaussian probability curve and no assumption is made about the geometric shape of the pores. From a practical stand point, for molecular weight determination, it is still more common to construct a calibration curve and perform estimations graphically.

The Sephadex G-100 (2.5X 60) column was calibrated by determining the partition coefficient K_{av} for the following proteins of known molecular weights according to Schachman (1963): Bovine Serum Albumin (67 kDa) Ovalbumin (43 kDa), and myoglobin (17 kDa). The partition coefficient (K_{av}) was calculated from the following equation;

$K_{av} = (V_e-V_0) / (V_t-V_0)$

Where V_e is the elution volume,

V_0 is the void volume (i.e., elution volume of Blue Dextran (MW- 20, 000, 00), and

V_t is the total volume of the gel bed (Andrews, 1964).

An advantage of calculating K_{av} instead of using elution volume to plot calibration curves is that K_{av} allows one to compare different media. For a well packed, high performance column if the K_{av} values of two molecules differ by 0.2 or more, baseline separation can be achieved.

Consider a buffer composition change to avoid the adsorption effect. If a peak elutes with a volume larger than the total column volume, the peak is not separated according to a gel filtration mechanism alone. Adsorption has occurred and one should consider changing the buffer conditions. For purification purposes, it is best to choose a gel with a selectivity range where the Mr of the molecule of interest is located near the middle of the linear range of the curve (e.g. K_{av} around 0.5). A selectivity curve is usually fairly straight over the range K_{av}= 0.1 to K_{av}= 0.7. The steeper the linear portion of this curve, the greater the difference in elution volume for two molecules of different sizes.

Void Volume

Blue Dextran (Himedia, MW 20, 00,000) 2mg/ml was applied in a total volume of 3ml in 0.05 M phosphate buffer of pH 7.4. Eluation was carried our and110 ml of eluent was collected separately and thereafter collection was done in test tube as 5 ml fraction. So the total void volume calculated up to the peak height as would be 130ml-1.5 ml (half of the sample size i.e. 3/2 ml) =128.5 ml

Total Volume (V_t) $= \pi r^2 h$

$= 3.14 \text{ X } (1.3)^2 \text{ X } 60$

$= 3.14 \text{ X } 1.69 \text{ X } 60$

$V_t = 318.4$

Partition Coefficient (K_{av}) $= V_e - V_o / V_t - V_o$

Table 1: Elution profile of blue dextran to determine the void volume

Sl. No. of tubes	Abs at 412 nm
1	0.034
2	0.043
3	0.054
4	0.067
5	0.067
6	0.048
7	0.044
8	0.04
9	0.031
10	0.025
11	0.018

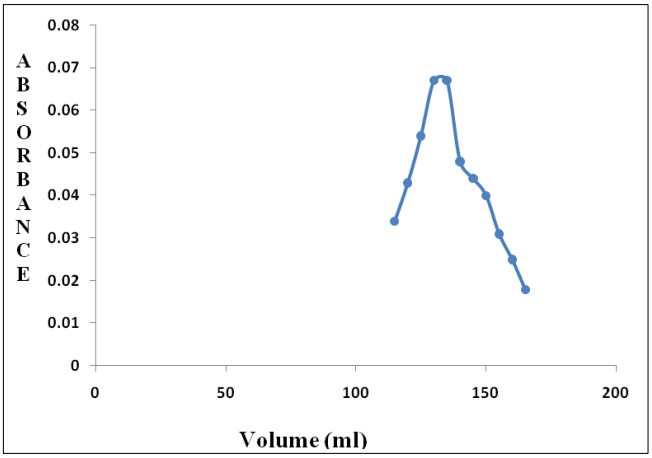

Fig 1: Elution profile blue dextran

Application of sample

The ammonium sulfate precipitated *Ascaris* haemoglobin was dialyzed against the elution buffer and applied on the Sephadex bed, during which the outlet of the column is closed. For the fair application at least two inches of top of the column is kept empty and buffer must be elevated to a substantial height from the gel bed for successful loading. However, the gel bed surface was prevented from drying by not draining the excess of eluent. Sample was layered on the top of gel bed surface. Sample may be applied manually by pipette through the inner wall of the column carefully without any hindrance with a uniform speed touching the eluent buffer. Sample loading can also be performed using peristaltic pump connected to column inlet. The sample stacking on the column wall and bed surface was refilled with the elution buffer. For sample application under the eluent, the sample was drawn into a syringe connected with fine needle; little air was drawn into the needle to prevent mixing of sample with eluent. The column outlet was closed and sample was dispensed slowly along the inner wall of the column a few millimeters above the bed surface.

Results and Discussion

Haemoglobins of both the perienteric fluid as well as the body wall of *Asacarissuum* reveal the presence of more than on fraction and these are designated as Haemoglobin1, 2, 3, and so on as shown in Figs. 1. In the perieteric fluid there are at least seven fractions of haemglobins, out of these four appear as minor bands; two a little darker and one major

and rapidly migrating band. Except the last one, all other bands are confined to the anodal half of the small pore gel. The body wall haemoglobin contains at least five visible fractions. Out of these, four are minor bands and one major fraction which migrates rapidly and is most obvious. Except the major one, all are restricted to the anodal half of the small pore gel. The two major bands in both haemoglobins migrated to an equal distance in both cases. It appears that the haemoproteins of perienteric fluid and body wall have same electrophoretic mobillty and are, therefore, similar in nature and physico-chemical properties. Among the minor fractions haemoglobin, 1, 2 & 3 of both the body wall and the perienteric fluid are similar to each other, as it is evident from their electrophortic mobility. The absorption spectra of Ascaris body wall and perienteric fluid haemoglobins are shown in Fig. 2 & 3. The purification profile of *Ascaris* perienteric fluid haemoglobin has been shown in Table: 2.

Table 2: Summary of purification of haemoglobin from perienteric fluid of Ascaris

Step in procedure	Volume (ml)	Absorbency		Ratio 410mμ/280mμ
		280mμ	410mμ	
Extract	30	46.3	6.7	0.14
First (NH4) SO_4 Fractionation	30	28.3	4.9	0.17
Second (NH4) SO_4 Fractionation	6	28.3	13.2	0.47
Sephadex gel Chromatography	70	1.54	1.15	0.75

Table 3: Readings of elution profile at 280nm & 412 nm of *Ascaris* perienteric fluid haemoglobin after Sephadex G-100 column purification

S. No. of tubes	A 280	A412	S. No. of tubes	A 280	A412
1	0.121	0.03	27	0.787	0.063
2	0.155	0.04	28	0.859	0.055
3	0.181	0.04	29	0.911	0.048
4	0.545	0.03	30	0.988	0.033
5	0.731	0.04	31	1.155	0.032
6	0.987	0.04	32	1.265	0.029
7	1.419	0.039	33	1.375	0.03
8	1.418	0.041	34	1.49	0.031
9	1.19	0.042	35	1.512	0.035
10	0.898	0.013	36	1.397	0.039
11	0.563	0.014	37	1.199	0.042
12	0.5	0.014	38	0.971	0.029
13	0.476	0.014	39	0.751	0.093
14	0.428	0.015	40	0.648	0.21
15	0.407	0.016	41	0.648	0.5
16	0.359	0.017	42	0.655	0.739
17	0.311	0.031	43	0.777	0.898
18	0.299	0.043	44	0.999	1.11
19	0.294	0.067	45	1.376	1.222
20	0.299	0.076	46	1.365	1.19
21	0.321	0.081	47	1.242	1.133
22	0.355	0.08	48	1.111	0.943
23	0.399	0.084	49	0.976	0.777
24	0.43	0.079	50	0.572	0.222
25	0.461	0.065	51	0.231	0.198
26	0.598	0.064	52	0.092	0.099

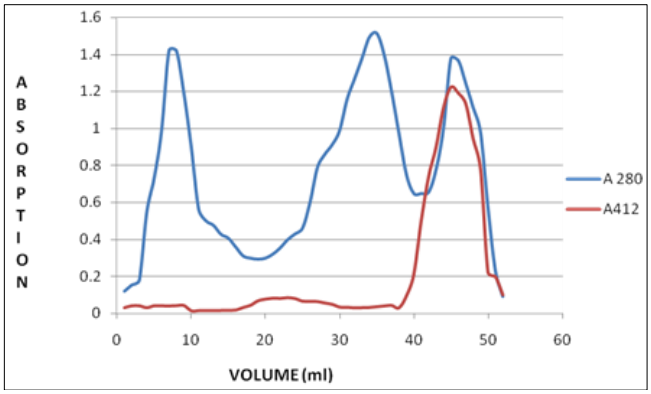

Fig 2: Elution profile of *Ascaris* perienteric fluid haemoglobin through Sephadex G-100 gel filtration chromatography.

Table 4: Readings of elution profile at 280nm & 412 nm of *Ascaris* body wall haemoglobin after Sephadex G-100 column purification

Sl. No. of tubes	A 280	A412	Sl. No. of tubes	A 280	A412
1	0.292	0.2	27	1	0.8
2	0.301	0.21	28	0.8	0.787
3	0.33	0.28	29	0.535	0.701
4	0.35	0.28	30	0.403	0.698
5	0.38	0.29	31	0.387	0.501
6	0.43	0.29	32	0.099	0.486
7	0.5	0.291	33	0.02	0.301
8	0.9	0.298	34	0.01	0.192
9	1.4	0.301	35	0.01	0.101
10	1.8	0.311	36	0.02	0.019
11	**2.4**	0.319	37	0.03	0.018
12	1.9	0.333	38	0.05	0.029
13	1.4	0.361	39	0.1	0.093
14	1	0.377	40	0.328	0.21
15	0.7	0.381	41	0.5	0.5
16	0.1	0.399	42	0.8	0.739
17	0.045	0.408	43	0.899	0.898
18	0.053	0.423	44	0.999	1.11
19	0.053	0.52	45	**1**	**1.734**
20	0.199	0.591	46	0.9	1.11
21	0.343	0.619	47	0.738	0.983
22	0.511	0.783	48	0.549	0.781
23	0.719	0.855	49	0.2	0.543
24	0.9	0.9	50	0.099	0.308
25	**1.11**	**1.309**	51	0.04	0.198
26	1.001	0.969	52	0.01	0.099

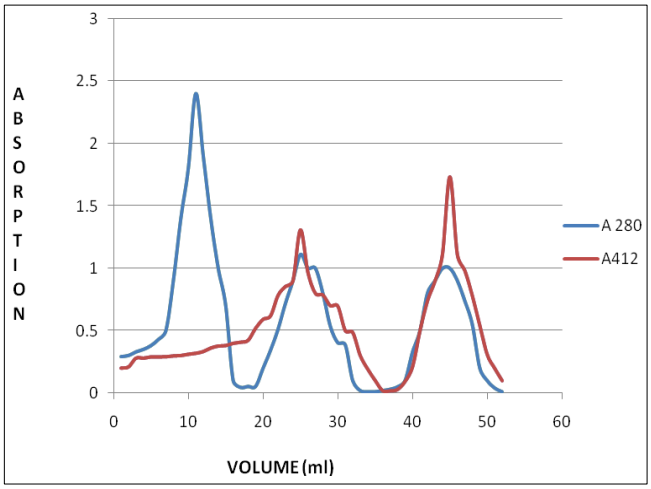

Fig 3: Elution profile of *Ascaris* body wall haemoglobin through Sephadex G-100 gel filtration chromatography

In the present works by employing polyacrylamide disc gel electrophoresis the author have found at least at seven haemoprotein fractions in the perienteric fluid and at least five fractions in the body wall. The presence of multiple haemoglobin fractions in the body wall and perienteric fluid confirm to some extent the reports of other workers that the two haemoglobins are different in their physiochemical properties. Contrary to it, the presence of larger fraction showing equal electrophoretic mobility in both the cases leads to the conclusion that the two haemoglobins resemble each other in many aspects. This also confirms that the body wall haemoglobin is not a single pigment as it was thought earlier but is composed of multi haemoprotein fractions. Wittenberg *et al.* (1965) too has shown that the minor colored fraction displays a spectrum between 500 to 600 mμ very similar to body wall haemogloin.

Conclusion

Spectra of the derivatives of *Ascaris* perienteric fluid haemoglobin have been presented by several workers. However there are appreciable and unexplained discrepancies between the data from different laboratories. The spectra in the visible region of the deoxygenated carbon monoxide and ferrous cyanide forms are similar to those of mammalian haemoglobin. The spectrum of the oxygenated from differs from mammalian haemoglobin in that the extinction at the α–band is much less. The outstanding chemical characteristic of this haemoglobin is the extreme slowness with which it dissociates oxygen. The unusual spectrum of the oxygenated form may reflect the chemical structure responsible for this property.

Polyacrylamide disc gel electrophoresis technique is used to study and differentiate the haemoglobins of body wall and the perienteric fluid of *Ascaris suum*.

References

1. Davenport HE. The haemoglobins of *Nippostrongylus muris* (Yokagawa) and *Strongylus* spp. Proc R Soc Lond B Biol Sci 1949;136:271-280.
2. Treibs A, Mendheim H, Lorenz M. *Naturwissenschaften* 1950;37(1650):378.
3. Lee DL, Smith MH. Haemoglobin of parasitic animals. Expt Parasit 1965;16:392-424
4. Hamada K, Okazaki T, Shukuya R, Kaziro K. Haemoglobin from *Ascaris lumbricoides* III. A haemoglibin from perienteric fluid. J Biochem Tokyo 1963b;53:484-488.
5. Smith MH, Morrison M. Isolation of *Ascaris* haemoglobins. Biochimica et Biophysica Acta 1963;71:364-370.
6. Porath J. Some recently developed fractionation procedures and their application to peptide and protein hormones. Journal of Applied Chemistry 1963;6:233-244.
7. Squire PG. A relationship between the molecular weights of macromolecules and their elution volumes based on a model for Sephadex gel filtration. Archives of Biochemistry and Biophysics 1964;107:471-478.
8. Laurent TC, Killander J. a theory of gel filtration and its experimental verification. Journal of Chromatography 1964;14:317-330.
9. Acker GK. A new calibration procedure for gel filtration columns. Journal of Biological Chemistry 1967;242:3237-3238.