Ilorin Journal of Science
Volume 11, Number 2, 2024, pp. 139 – 161 (Printed in Nigeria)
ISSN: 2408 – 4840 © 2024 Faculty of Physical Sciences, University of Ilorin
https://doi.org/10.54908/2024.11.02.004

ILORIN
JOURNAL OF SCEINCE

ILJS-24- 011

Isolation and Characterization of Haemocyanin from *Sudanonautes africanus* (Milne Edward, 1869)

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Abstract

Sudanonautes africanus (Milne-Edward, 1869) Potamonautidae is a freshwater crab, native to Nigeria and the West Africa. There are only few documentations revealing the properties of its haemocyanin. The objective of this study was to isolate, purify and characterize the haemocyanin of Sudanonautes africanus. Haemolymphs from 10 live crabs were obtained via carapace puncture; pooled together, and fractionated into plasma and haemocytes. A 50% saturated ammonium sulphate ($(NH_4)_2SO_4$) solution was added to the plasma to separate it into albumin and globulin fractions. The absorption spectra were determined and λ_{max} was found to be 295 nm; globulin was found to contain more haemocyanin compared to albumin. Gel filtration was used to extract the haemocyanin from the globulin fraction. The eluted globulin fractions with the highest absorbance at 295 nm were pooled together and concentrated via ultra-filtration. The determination of its purity was carried out using Sodium Dodecyl Sulphate Polyacrylamide Gel Electrophoresis (SDS–PAGE). Two bands of haemocyanin were obtained with molecular masses of 46 kDal and 92 kDal respectively. Copper content of the haemocyanin was 0.0383%, while the calculated minimum molecular weight was 829.5 kDal.

Keywords: *Sudanonautes africanus*; Haemolymphs; Haemocyanin; Protein

1. Introduction

A species of African freshwater crabs is the *Sudanonautes africanus* (Milne Edwards, 1869), a Potamonautidae. They live on land, in burrows and under what can cover them well. *Sudanonautes africanus* are active at night (Cumberlidge, 1999), and are vegetarian scavengers. They can run very fast, have well-developed eyes to see from a distance and very responsive to their environment. These species of crabs are found mainly in Nigeria, and Central Africa. *S. africanus* adaptation to freshwater are made possible by their lecithotrophic eggs, direct development (without larvae), and brood care (Cumberlidge, 1999). They are edible and provide a means of livelihood to people who hunt them for sale.

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Evidences show that invertebrates, which lack adaptive mechanisms, have defence systems that respond to pathogen-associated molecular patterns (PAMPs) on the surface of potential pathogens. The methods employed include melanisation, haemolymph coagulation, cell agglutination, active oxygen formation, anti-microbial action, and phagocytic action (Medzhitov, 1997; Salawu *et al*., 2016).

Haemocyanin (Hc), a copper-bound protein, is present in the haemolymph of molluscs and arthropods as high-molecular-weight oligomers. In crustaceans, this di-oxygen-carrier protein is synthesized in the mid-gut gland and transported at high concentrations to haemolymph, comprising 90-95% of the total plasma proteins. The basic structure of all arthropod Hc oligomers is hexamer. In decapods, however, the hexamer (6- mer; 1 × 6) and the dodecamer (12-mer; 2 × 6) forms are common. Crustacean Hc 6-mer proteins can cluster to form aggregates up to 48 (8 × 6) subunits, resulting in molecular masses ranging from 450 to 3900 kDal. Although this blue protein is mostly known as a dioxygen transporter, Hc is a multi-copper protein that can display a remarkable range of functions, including transporting ecdysone, functioning as an osmolyte and storing protein, and acting as a precursor of antimicrobial peptides (Coates and Nairn, 2014, Lorean *et al*., 2022).

Furthermore, Hc can display phenoloxidase (PO) activity when exposed to chemical reagents (Salawu *et al*., 2011), such as percolate and SDS and endogenous defence molecules, such as clotting factors and antibacterial peptides. This has economic consequences because many crustaceans are consumed by humans, and post-mortem melanosis is associated with spoilage, which makes the food product unacceptable to consumers and reduces the value of important commercial shrimp, lobster, and crab fisheries, although the discoloration is not a health hazard. Invertebrate animals, which lack adaptive immune systems, have developed other methods of biological host defence, so called innate immunity, that respond to common antigens on the cell surfaces of potential pathogens. During the past two decades, the molecular structures and functions of various defence components that participated in innate immune systems have been validated in Arthropoda, such as, insects, the horseshoe crab, freshwater crayfish, and the protochordate ascidian. The haemocyanin found in *Concholepas concholepas* blood has immunotherapeutic effects against bladder and prostate cancer (Coates and Nairn, 2014, Lorean *et al*., 2022). In a research conducted in 2006, mice were primed with *C. concholepas* before implantation of bladder tumour (MBT-2) cells. Mice treated with *C. concholepas* showed a significant anti-tumour effect. The effects included prolonged survival, decreased tumour growth and incidence, and lack of toxic effects (Atala 2006). In previous studies, researchers found that haemocyanin, like haemoglobin and many other proteins, is a multi-subunit molecule (Markl and Decker, 1992; Decker and Jaenicke, 2004). For example, the peacock mantis shrimp (*Odontodactylus scyllarus*) has four haemocyanin subunits (Scherbaum *et al*., 2010).

A study investigated the extent to which LPS could potentially impact on coagulation and PO activities in *S. africanus*. This is the first report of its kind on LPS-induced immune responses in *S. africanus* (Salawu and Oloyede, 2011). There is scarce information on the isolation and

characterization of haemocyanin of *Sudanonautes africanus* from Nigeria. The aim of the study was to isolate and characterise the *S. africanus* haemocyanin.

2. Materials and Methods

Animals and reagents

This work was done in the Department of Biochemistry, University of Ilorin, Nigeria, with the approval of the Departmental Postgraduate Committee, and the Faculty of Life Sciences Ethical Review Committee. Ten live, healthy, *S. africanus* crabs, and two healthy Albino rabbits (*Oryctolagus cuniculus*) were purchased from Obo market at Unity, Ilorin and at Sanusi Farms, Gaa-Akanbi, Ilorin, Kwara state respectively. The crab was identified at the University of Ilorin, Department of Zoology, and a specimen deposited. A Voucher Number 002/ZLY was issued dated 10/04/2010.

Crabs were made to acclimatize for 24 hours with free access to water and food in a facility that provided a natural cycle of light/dark hours before their haemolymph were collected. Crab health was determined over the entire study by repeated evaluation of their innate active, aggressive, and evasive natures. Healthy crabs were agile, ready to protect themselves with their claws and try to evade being handled. All the crabs, exhibited these characteristics at purchase, during acclimatization, and before their haemolymph was collected. As the studies here were all performed *in vitro*, and conditions were uniform for all crabs; sample-to-sample variability in haemolymph was deemed not to be a potential problem. All chemicals and reagents used were products of Sigma Aldrich, UK.

Extraction of haemolymphs from *S. africanus*

The crabs were washed thoroughly with distilled water before haemolymphs were extracted to prevent contamination. The haemolymph was obtained through dorsal-ventral carapace puncture, using a sharp screwdriver and allowed to flow into a clean bowl (Salawu and Oloyede, 2011; Salawu *et al*., 2016). Haemolymph was collected using syringes which was transferred into a universal sterile tube with ice packs placed by the tubes to preserve the samples, before storing at 4°C. A total of 115 mL haemolymph was collected from ten crabs. Volumes of 0.1 M Phosphate-Buffered Saline (PBS), pH 7.4 corresponding to the amounts of haemolymph of the crabs was introduced as an anticoagulant.

Fractionation of haemolymph into plasma and haemocytes

The total amount of haemolymph (115 mL) mixed with PBS obtained was sequentially filled into 15 mL centrifuge tubes and centrifuged at 4,000 rpm for 20 minutes. The resulting supernatant, after centrifugation, was plasma (100 mL), while pellets were washed with 1 mL PBS and centrifuged again at 4000 rpm for 10 minutes. The supernatant was discarded, and pellets harvested and stored in 1 mL PBS as haemocytes.

Extraction of globulin and albumin from plasma by precipitation

Plasma was effectively separated into albumin and globulin by the addition of 50% saturated solution of ammonium sulphate ($(NH4)_2SO_4$). To obtain this solution in 20 mL, 7.6 g of ammonium sulphate was used. For 80 mL of plasma, 30.4 g of the salt was used to precipitate globulin out of solution with albumin remaining.

The globulin precipitate was obtained by filtration using Whatman Filter Paper. The total amount of globulin recovered was 8.5 mL. The globulin precipitate was re-dissolved in phosphate buffer saline, and de-salted by ultracentrifugation.

Determination of protein concentrations of plasma, globulin and albumin using Biuret method

The determination of protein concentrations was done according to the method of Gornall *et al*. (1949); (Cansu and Boran, 2015). The blank was prepared by pipetting 0.5 mL of plasma into a test tube containing 4.5 mL of biuret reagent. 0.5 mL of plasma aliquot was mixed with 4.5 mL of PBS in a test tube and pipetted into a cuvette as a blank to be read by the spectrophotometer. It was done in duplicates. The procedure was carried out for both globulin and albumin. The absorbance of the plasma, globulin and albumin were read at 550 nm. Plasma and globulin were too turbid; hence, dilution was made with distilled water using a dilution factor of 10.

Determination of absorbance spectrum for plasma, globulin, and albumin at wavelengths from 280 nm and 800 nm

A 3 mL of diluted plasma (1 mL of plasma + 9 mL of distilled water) was read to obtain the absorption spectrum of the sample, read from 280 to 800 nm. The wavelength was increased by 5nm each time, and the absorbance read and recorded. The same procedure was used for assaying both the globulin and albumin. A plot of absorbance (y-axis) against wavelength (x-axis) was prepared, to obtain the absorption spectrum for the plasma, globulin, and albumin.

Determination of calibration curve for protein standard (egg albumin) using biuret solution

Egg albumin was dissolved in 50 mL of NaCl solution and mixed thoroughly. A final concentration of 20 mg/ml protein was prepared in duplicates. An 0.5 mL aliquot of each concentration was pipetted into a test tube and followed by the addition of 4.5 mL of biuret solution. It was incubated for 20 minutes at room temperature. Its absorbance was then read at 550 nm. A plot of absorbance against concentration was made to obtain the calibration curve for protein concentration.

Isolation of haemocyanin fractions from globulin using Gel filtration

A 50 mL glass column was thoroughly washed with detergent, rinsed with distilled water and stopped with cotton wool. Silica gel of 60-120 mesh was soaked for about 15 min in phosphate buffered saline to form a slurry which was washed twice and allowed to settle. The column was packed with silica gel to the 50 mL mark. This was done carefully to prevent cracks and bubble formation. 425 µL of the globulin fraction was loaded into the column. The column was equilibrated to discharge at a slow rate of not more than 2 mL/min. during separation, as the level of PBS used for elution decreased, more PBS was added intermittently to ensure continuous separation and also to avoid the drying up of the gel packed within the column.

The concentration of pooled Haemocyanin fractions using Centricon® tubes by centrifuging

A 3 mL aliquot of each pooled fractions (HC1, HC2, HC3, HC4, and HC5) were loaded into ten centricon tubes, 3,000 Dal Molecular weight cut off, product of Sartorium Stedim Biotech GmbH, Goettingen, Germany. Two centricon tubes were used for the pooled eluates and spun at a speed of 4,000 rpm for 40 minutes. Proteins of molecular masses lower 3,000 dal were eliminated in the process. The filtrate was discarded, and the concentrate was collected. The total concentrate recovered from each of the sample was 1 mL. The haemocyanin was stored in the centricon tube and preserved for further analysis. After concentration, the protein content of each of the concentrate was determined using biuret method (Gornal *et al*., 1949; Cansu and Boran, 1915).

Determination of protein content of concentrated isolate using Biuret method

At the end of gel filtration, the fractions under each characteristic peak were pooled together. The fractions were designated HC1, HC2, HC3, HC4 and HC5. These fractions were further concentrated using centricon tubes and centrifuged before the protein concentrations of haemocyanin were determined. Firstly, the blank was prepared by the addition of 100 uL of PBS to 900 uL of Biuret reagent. 900 uL of biuret reagent was added to 5 different test tubes already containing 100 ul of the each of the Haemocyanin (HC1, HC2, HC3, HC4 and HC5). This was done in duplicates. The absorbance was then read at 550nm. The average absorbance was read off the protein calibration curve to determine the concentrations of HC1 to HC5.

Quantification of metal ion content in the isolates using Atomic Absorption Spectroscopy

Quantitative analysis was carried for the presence of some metals like, Fe^{2+}, Ca^{2+}, Mg^{2+}, Zn^{2+}, Cu^{2+}, and Na^{+} on the concentrates using Atomic Absorption Spectroscopy, (Buck Scientific 231, UK). A 300 µL aliquot of each of the concentrate was used for this analysis. This was carried out according to the methods of Hill *et al*., 2005.

Determination of the purity of isolates using Sodium Dodecyl Sulphate Polyacrylamide Gel Electrophoresis (SDS-PAGE)

SDS-PAGE was carried out on the concentrate to purify the protein further and determine whether this particular protein occurs in iso-forms. 200 uL of each of the concentrate was used for this analysis. It was carried out according to the methods Singh *et al*., 1991.

Determination of the amino acid composition of the isolates

The amino acid composition of the isolates was carried out with an Applied Biosystems PTH Amino Acid Analyzer (MODEL120A) using the methods of AOAC, 2006. The determination of tryptophan was done using the method described by Maria *et al*., 2004. In this method, hydrolysis was facilitated using sodium hydroxide (NaOH) instead of barium hydroxide to prevent precipitation. The determination of tryptophan in proteins and peptides was problematic, because it chemically decomposes during acid hydrolysis. 6N HCL destroys tryptophan during hydrolysis. For this reason, the concentration of tryptophan was determined separately. The tryptophan in the known sample was hydrolysed with 4.2 M Sodium hydroxide (Maria *et al*, 2004). The known sample was dried to constant weight, defatted, hydrolysed, evaporated in a rotary evaporator and loaded into the Applied Bio systems PTH Amino Acid Analyzer.

Defatting sample

A known weight of the dried sample was weighed into extraction thimble and the fat was extracted with chloroform/methanol (2:1 mixture) using Soxhlet extraction apparatus as described by AOAC (2006) the extraction lasted for 15hrs.

Nitrogen determination

A small amount (200mg) of ground sample was weighed, wrapped in Watman filter paper (No.1) and put in the Kjeldhal digestion flask. Concentrated sulphuric acid (10ml) was added. Catalyst mixture (0.5g) containing sodium sulphate (Na_2SO_4), copper sulphate ($CuSO_4$) and selenium oxide ($Se0_2$) in the ration of 10:5:1 was added into the flask to facilitate digestion. Four pieces of anti-bumping granules were added.

The flask was then put in Kjeldhal digestion apparatus for 3 hours until the liquid turned light green. The digested sample was cooled and diluted with distilled water to 100ml in standard volumetric flask. Aliquot (10ml) of the diluted solution with 10ml of 45% sodium hydroxide was put into the Markham distillation apparatus and distilled into 10ml of 2% boric acid containing 4 drops of bromocresol green/methyl red indicator until about 70ml of distillate was collected.

The distillate was then titrated with standardize 0.01N hydrochloric acid to grey coloured end point, the percentage nitrogen in the original sample was calculated using the formula:

$$\text{Percentage Nitrogen} = \frac{(a - b) \times 0.01 \times 14 \times V \times 100}{W \times C}$$

where:

a	=	Titre value of the digested sample
b	=	Titre value of blank sample
v	=	Volume after dilution (100ml)
w	=	Weight of dried sample (mg)
c	=	Aliquot of the sample used (10ml)
14	=	Nitrogen constant in mg

Hydrolysis of the sample

A known weight of the defatted sample was weighed into glass ampoule. 10ml of 4.2M NaOH was added and oxygen was expelled by passing nitrogen into the ampoule. The glass ampoule was then sealed with Bunsen burner flame and put in an oven pre-set at 105^0C ± 5^0C for 4 hours. The ampoule was allowed to cool before broken open at the tip and the content was filtered to remove the humins. The filtrate was neutralized to pH 7.00 and evaporated to dryness at 40^0C under vacuum in a rotary evaporator. The residue was dissolved with 5ml of borate buffer (pH 8.0) and store in plastic specimen bottles, which were kept in the freezer.

Loading of the hydrolysate into tsm analyser

The amount loaded was 60 microliters. This was dispended into the cartridge of the analyser. The period of an analysis lasted for 45 minutes.

Method of calculating amino acid values

An integrator attached to the analyzer calculates the peak area proportional to the concentration of each of the amino acids.

Determination of Agglutination and specificity of the concentrate

This following protocol was described by Bing *et al*. (1967) reported by Kuku *et al.* (2009), but was modified using 40% formaldehyde in place of glutaraldehyde.

Fixation of Erythrocytes with Glutaraldehyde

Red blood cells of two rabbits were fixed with formaldehyde. The blood was collected in heparinized bottles and centrifuged at 3000 rpm for 15 minutes at room temperature. The supernatant (Plasma) was removed, and the pellet (Red blood cells) were collected and washed three times with PBS (PH 7.2). 40% Formaldehyde was diluted to 1% (v/v) with PBS and allowed to cool in the refrigerator at 4°C ^{0}C. The chilled formaldehyde-PBS solution was used to dilute the red blood cells to 2% (v/v). The suspension of the cells and formaldehyde was incubated for 1 hour at 4°C with occasional mixing. The fixed cells were collected by centrifugation at 3000 rpm for 15 mins and washed five times with PBS containing 0.02 % (w/v) sodium azide to a final concentration of 4% (v/v) and stored below 0^oC until needed.

Haemagglutination Assay

100 µL of PBS was delivered sequentially into the wells arranged in rows of 12, in a U-shaped micro-titre plate. 100 µL of the extract was added into the first well to obtain 1:2 dilutions. A serial dilution of the extract was carried out by transferring 100 uL of the diluted sample in a particular well into the next well, containing 100 µl of PBS. 50 uL aliquots of the 2 % erythrocyte suspension was dispensed respectively into each well, and the wells were left for about 1 hour to allow the agglutination of red blood cells to occur after which the agglutination titres were noted. The titres were taken as the reciprocal of the highest dilution of the extracts exhibiting visible haemagglutination; these were taken as one haemagglutinating unit.

3. Results

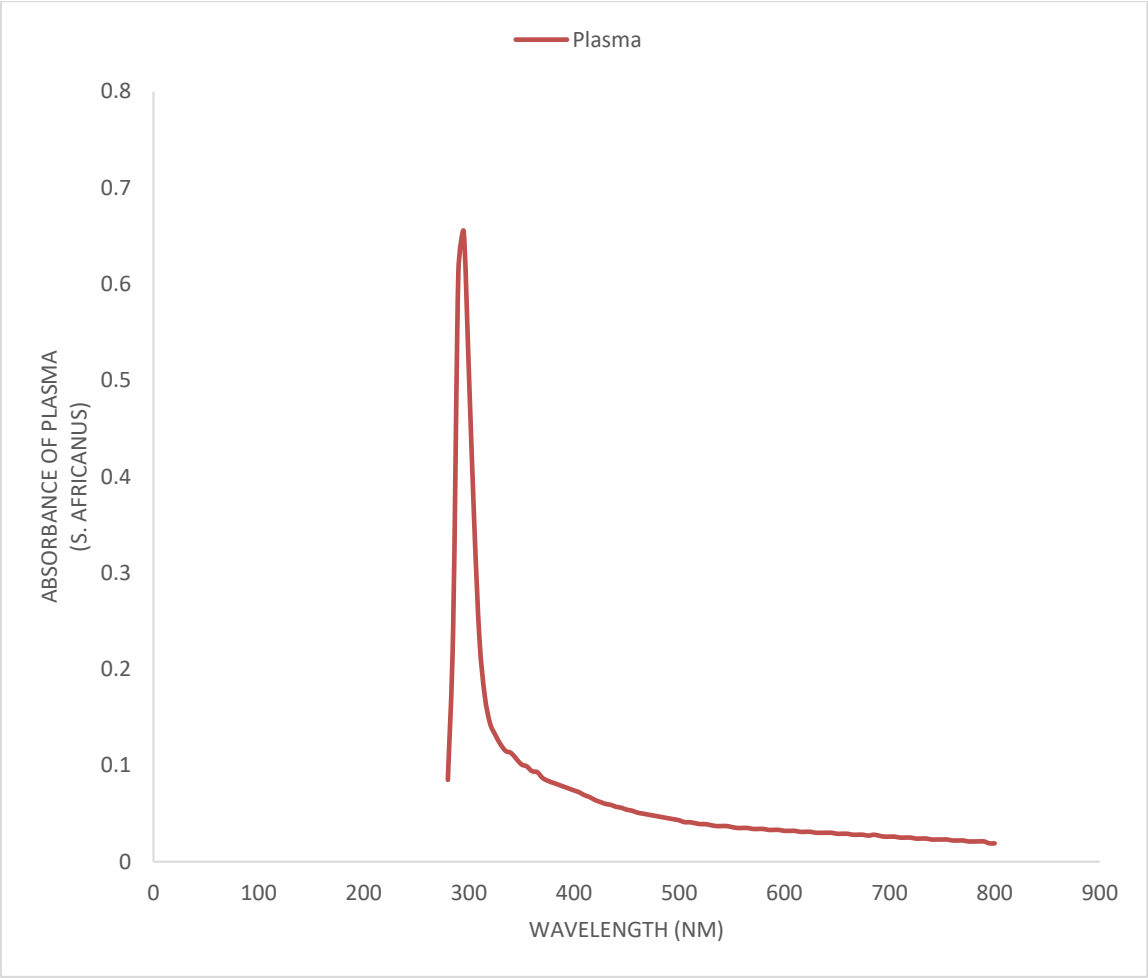

Figure 1: Absorption spectrum of *Sudanonautes africanus* plasma

Figure 1 shows the absorption spectrum of *S. africanus* plasma, scanned from 280 nm to 800 nm at intervals of 5 nm (Figure 1).

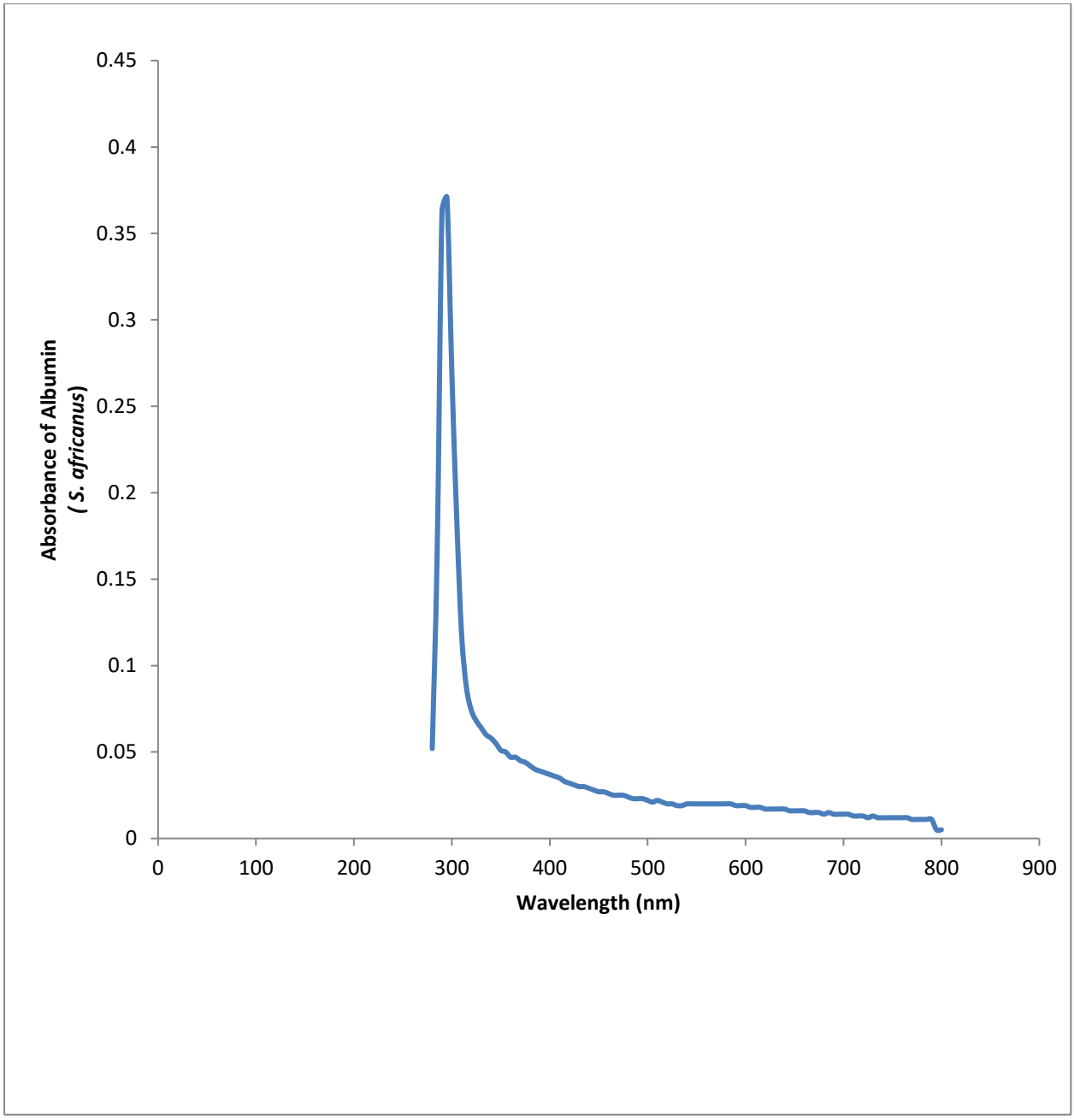

Figure 2: Absorption spectrum of *Sudanonautes africanus* Albumin

Figure 2 shows the plot of the absorbance spectrum of albumin scanned from 280 nm to 800 nm. The λ_{max} was also 295 nm. From the graph, it could be inferred that the concentration of haemocyanin was low in the albumin due to its reduced absorbance at 0.005.

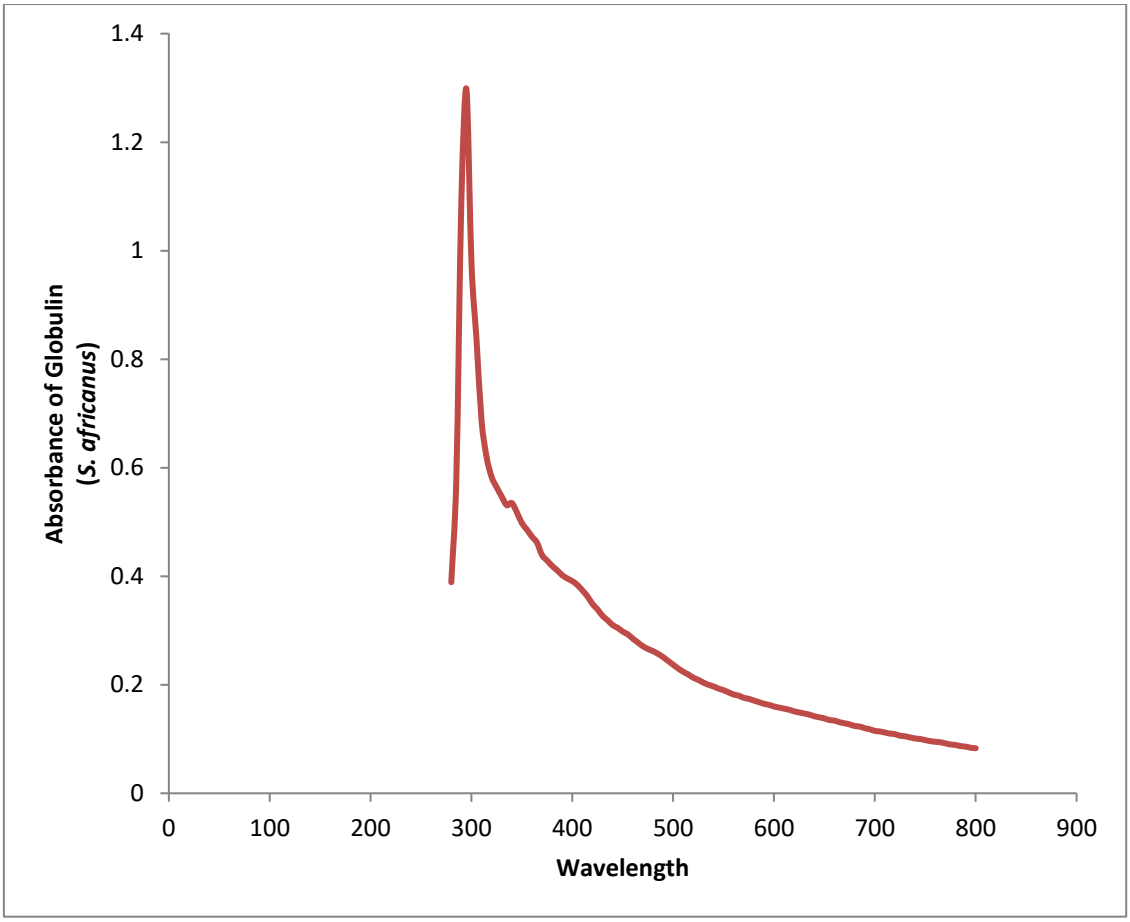

Figure 3: Absorption spectrum of *S. africanus* Globulin

Figure 3 shows the absorption spectrum of globulin having similar trend with those of plasma and albumin from 280 nm to 800 nm. The λ max was also 295 nm. The fraction of globulin extract has haemocyanin in a very concentrated form compared to the albumin fraction. It means that haemocyanin was extracted into the globulin fraction. The lowest absorbance value obtained for globulin was 0.083 compared to albumin, having 0.005 nm. The least absorbance of plasma gave 0.019, which is just a little higher than albumin. This underscores the need for the globulin protein consideration in the haemocyanin extraction.

Table 1: Total protein concentration in Albumin and Globulin

Parameters	**Total Vol. (mL) collected**	**Protein Concentration (mg/mL)**	**Total Protein Concentration (mg/mL) = Determined conc. x dilution factor**
Globulin	13.5	47.036	470.36
Albumin	91.5	18.572	18.572

Total volume of plasma used= 80ml

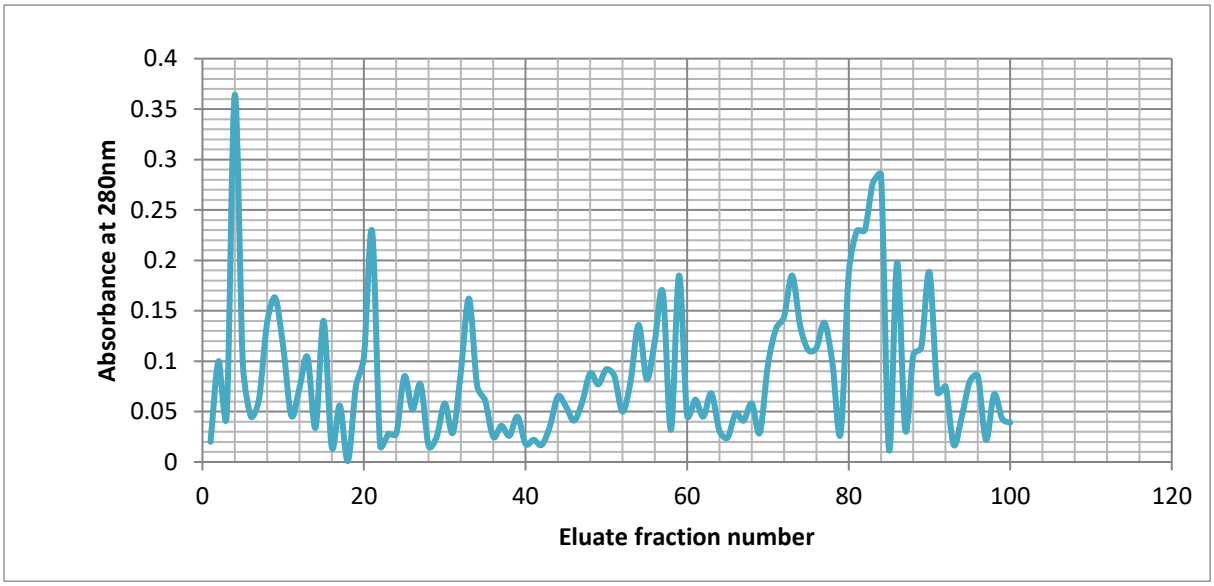

Figure 4: Elution profile of *S. africanus* globulin at 280 nm

Figure 4 shows the absorbance at 280 nm for each eluate against the fraction number on the x-axis. It shows the presence of protein content in each characteristic peak at 280 nm. Absorbance read at this wavelength in the UV-region gives us a hint of the protein concentration of the haemocyanin fractions. This can be seen from the characteristic peak on the graph.

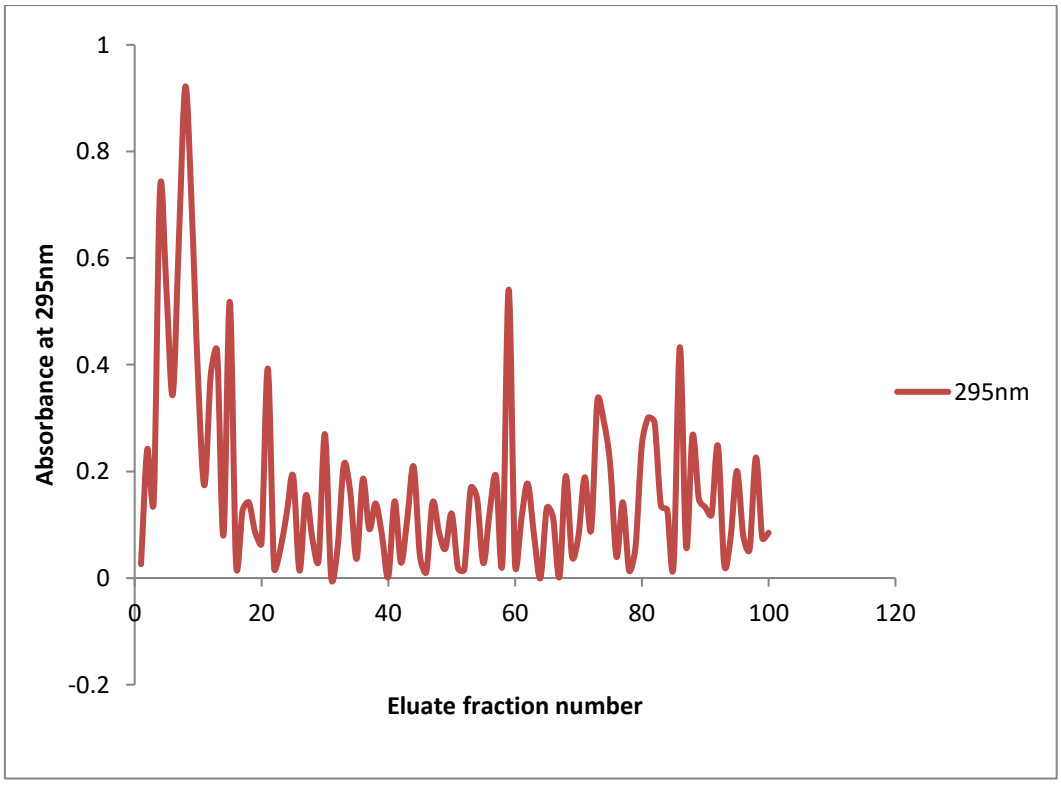

Figure 5: Elution profile of *S. africanus* globulin at 295 nm

Figure 5 shows the plot of absorbance of each eluate against the fraction number on the x-axis measured at 295 nm. From the elution profile, haemocyanin appears to be in more significant

concentrations and of higher molecular weights coming out as the first peak (fractions 1-3) with highest absorbance at 295 nm.

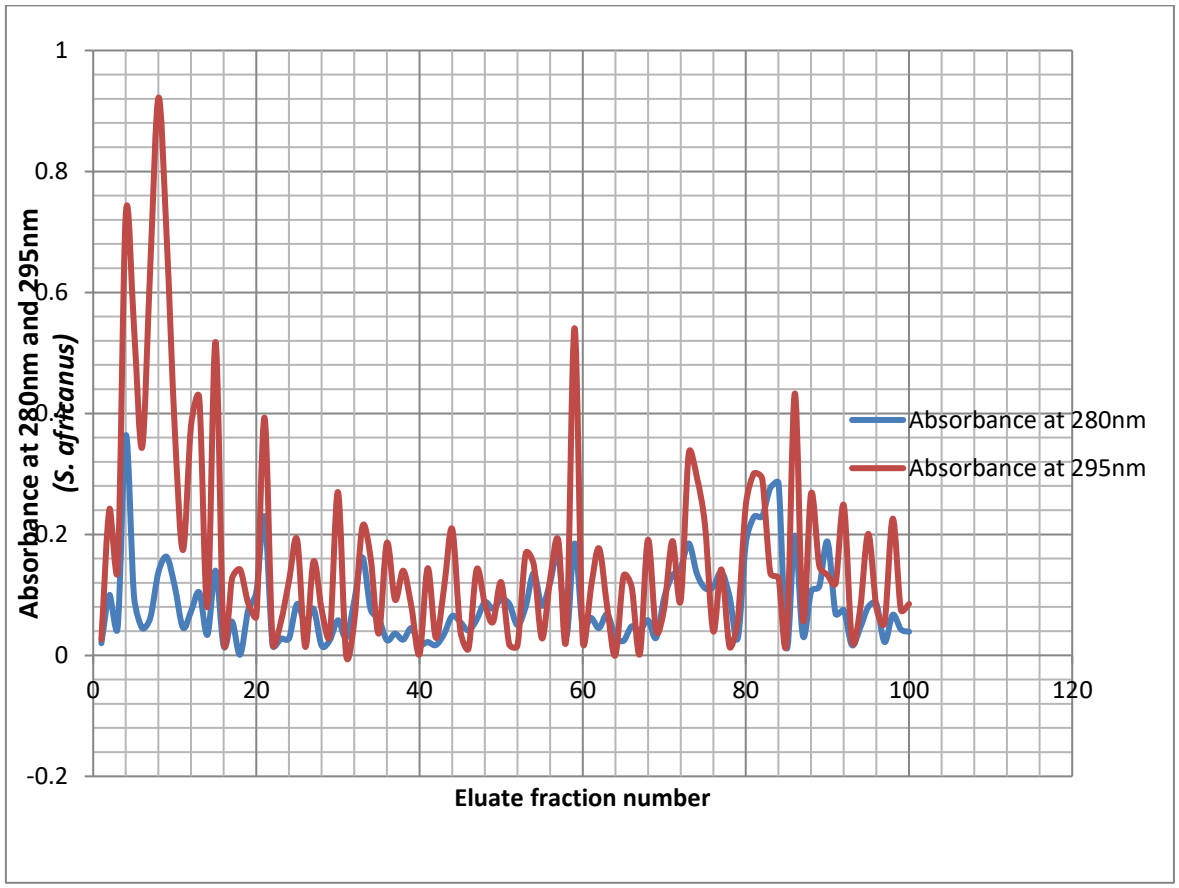

Figure 6: Elution profile for Gel filtration of *S. africanus* haemocyanin on Silica gel

The chromatograms show the protein content of each fraction (Absorbance at 280nm, Orange) and haemocyanin elution monitored at 295nm (Blue).

Figures 4 and 5 which helps in the identification of the protein peaks that are haemocyanin revealed several distinct peaks at 295 nm were noted from the graph above. The peaks were compared with the peaks generated at 280 nm (showing protein content). There was an overlap between these peaks which facilitated the pooling of the elution fractions rich in proteins together (Figure 6). Five different haemocyanin fractions were derived by pooling the elution fractions from five different peaks designated as follows: HC1, HC2, HC3, HC4, HC5.

Table 2: Protein concentration of Haemocyanin concentrates

Haemocyanin	Absorbance @550nm	Conc. (mg/ml)
HC1	0.0215	1.536
HC2	0.0915	6.536
HC3	0.0525	3.75
HC4	0.0105	0.75
HC5	0.004	0.286

From Table 2, HC2 has the highest protein concentration of 6.536 mg/mL when compared to others.

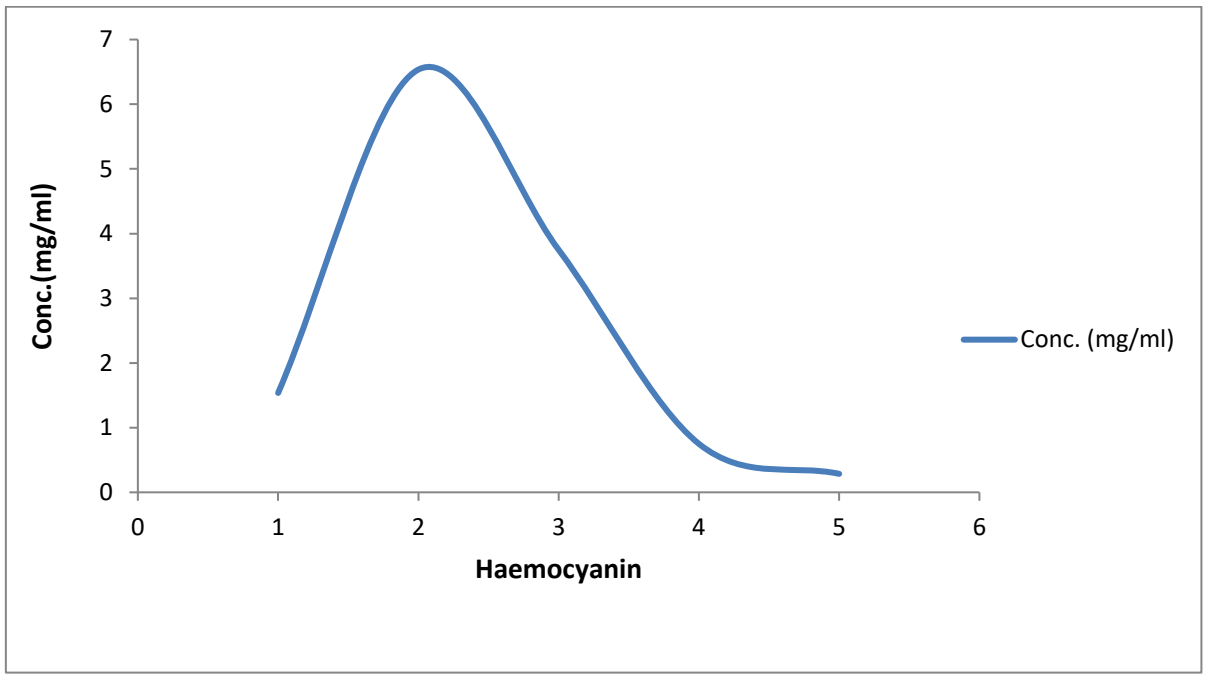

Figure 7: Protein content of *S. africanus* in the haemocyanin eluates

Table 3 shows some of the metal ions present in the haemocyanin concentrates.

Table 3: Result of atomic absorption spectrum indicating the metal ions in the haemocyanin concentrates

Metal(mg/L)	HC1	HC2	HC3	HC4	HC5
Fe	0.00	0.00	0.00	0.00	0.00
Ca	0.00	0.00	0.00	0.00	0.00
Mg	0.00	0.00	0.00	200	0.00
Zn	0.00	0.00	0.00	0.00	0.00
Cu	0.00	0.25	0.00	0.00	0.00
Mn	0.00	0.00	0.00	0.00	0.00

The only metal detected in one of the fractions is Copper, indicating that the only fraction that is haemocyanin is HC2.

From the result below, SDS-PAGE separation and Coomassie blue staining of haemocyanin protein as investigated, showed two major bands of the protein. It shows that haemocyanins are all the same but occur in different sub-units of 9 KDaltons and 18 KDaltons. From Table 3, the calculated Minimum Molecular weight was 829.503 KDalton.

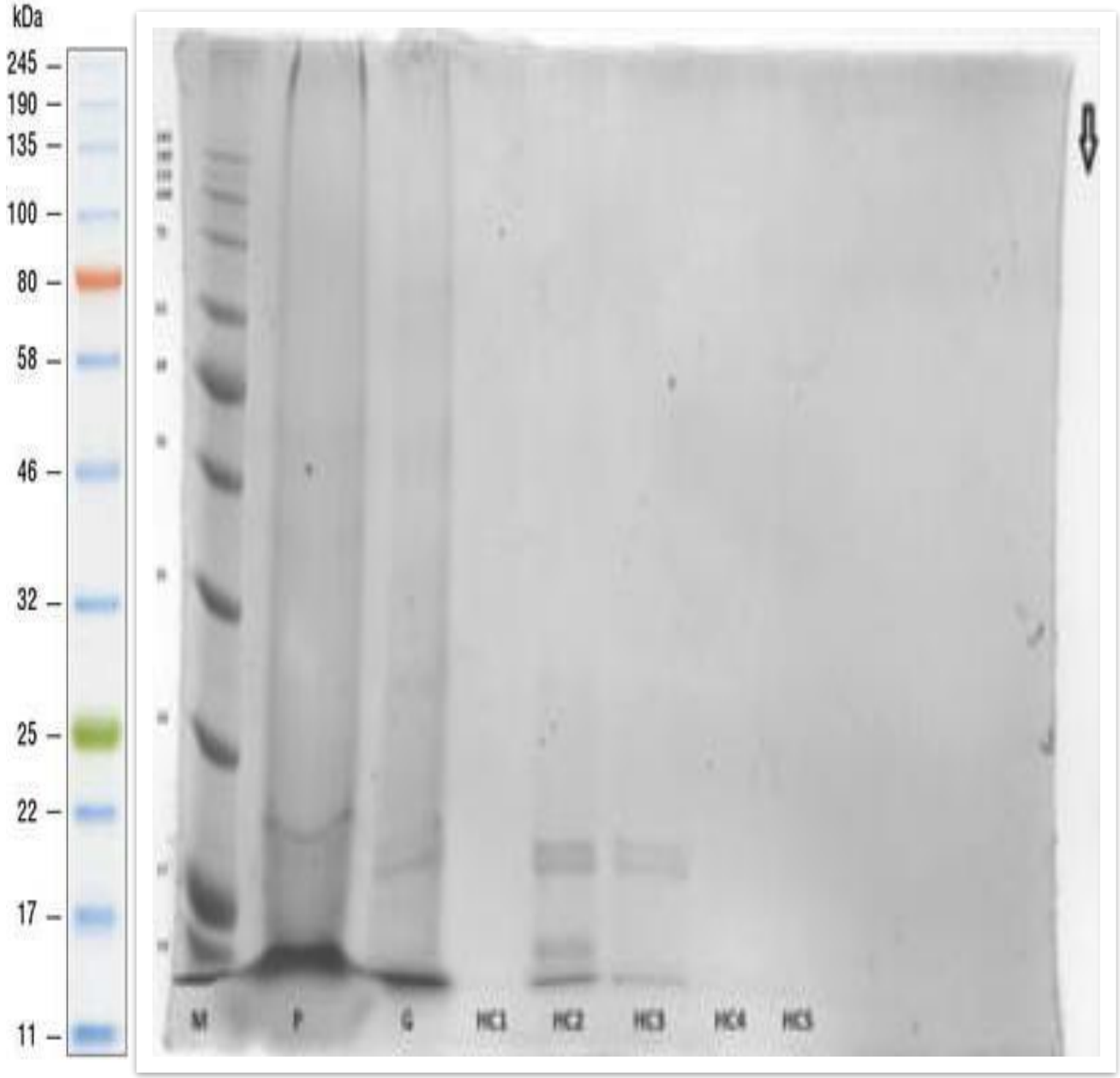

Figure 8: SDS-PAGE of *Sudanonautes africanus* Haemocyanin

Key: M=Marker; P=Plasma=18.5; G= Globulin=18; HC1= 18 kDal; HC2=9-18kDal; HC3= 9-18 kDalHC5= 9-18kDal; HC= Haemocyanin

Table 4: Amino acid composition of HC2

Amino Acid	Net height (mm)	NH/2 (mm)	Width NH/2 (MM)	Sstd	Concentration: g/100 g protein
Leucine	108	54	1.00	5.91	6.04
Lysine	90	45	1.00	5.37	4.58
Isoleucine	45	22.5	1.00	6.63	2.83
Phenylalanine	20	10	1.00	17.96	3.40
Tryptophan	23.0	11.50	1.00	5.32	1.21
Valine		45.0	1.00	5.92	4.50
Methionine	39	19.5	1.00	5.41	2.00
Proline	17	8.5	1.00	20.56	3.31
Arginine	25	12.5	1.00	17.42	4.12
Tyrosine	8.0	4	1.00	34.84	2.64
Histidine	70	35	1.00	6.47	4.29
Cysteine	10	5.0	1.00	12.26	1.16
Alanine	70	35	1.00	7.68	5.00
Glutamic acid	50	25	1.00	15.33	7.38
Glycine	100	70	1.00	4.81	4.55
Threonine	50	25	1.00	5.62	2.66
Serine	68	34	1.00	5.47	3.52
Aspartic acid	170	65	1.00	6.28	10.12

Table 5: Result of Haemagglutination titre of *Sudanonautes africanus* Plasma, Globulin and Haemocyanin

S/N	Parameters	Haemaggltination titre	Last positive dilution
C	Control	0	0
P	Plasma	128	0.016
G	Globulin	2	0.500
1	HC1	16	0.063
2	HC2	4	0.250
3	HC3	8	0.125
4	HC4	32	0.031
5	HC5	64	0.016

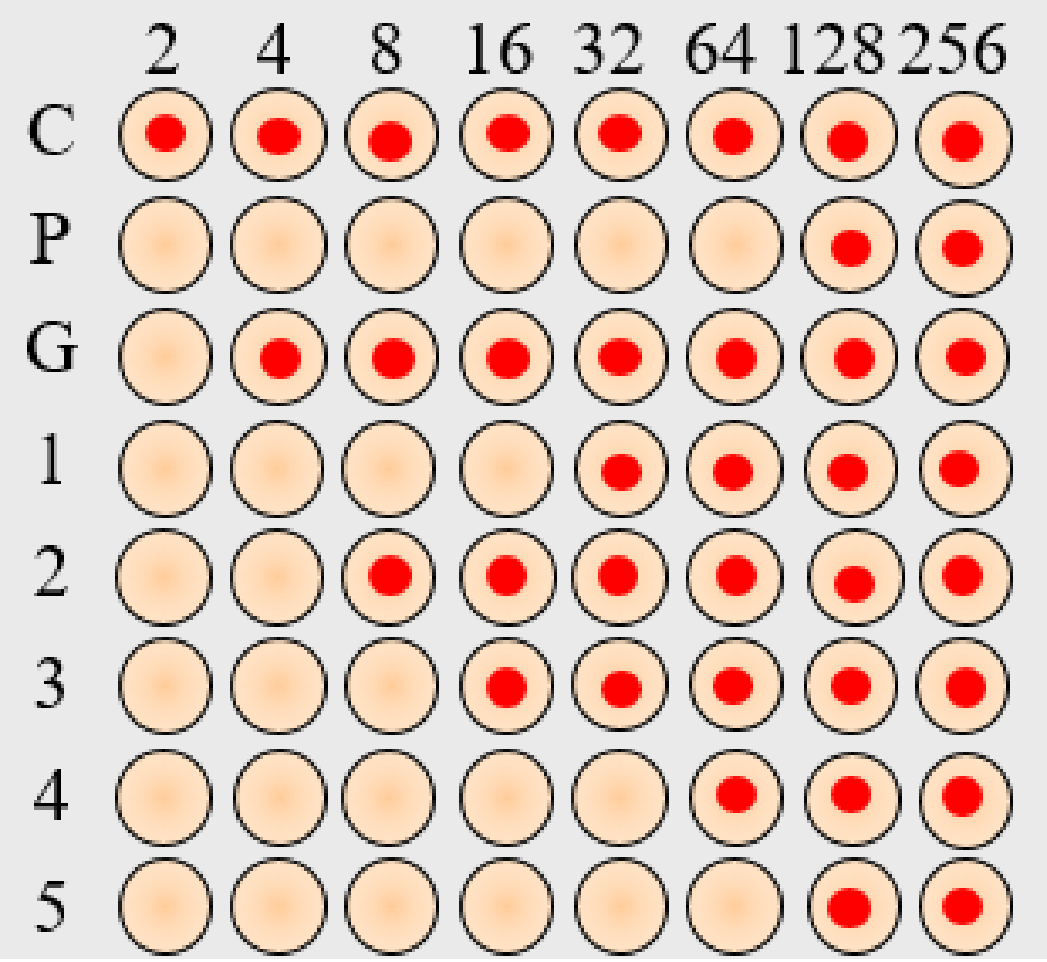

Figure 9: Haemagglutination titre of *S. africanus* Plasma, Globulin and Haemocyanin

4. Discussion

Haemocyanin are proteins that transport oxygen in the body of some invertebrates. These metalloproteins contain two copper atoms that reversibly binds to a single oxygen molecule (O_2). They are second only to haemoglobin in frequency of use as an oxygen transport molecule in animals. Unlike the haemoglobin in red blood cells found in vertebrates, haemocyanin are not bound to blood cells but are instead suspended directly in the haemolymph. Oxygenation causes a colour change between the colourless Cu (I) in its deoxygenated form and the blue Cu (II) in its oxygenated form. (Coates and Nairn, 2014). From the absorption spectrum, the sample has absorbance at λ_{max}, 295 nm, indicating the presence of haemocyanin in the plasma of *Sudanonautes africanus*. From the spectrophotometric analysis, *S. africanus* plasma was shown to have a characteristic maximum absorbance at 295 nm. The λ max of globulin was also obtained to be 295 nm indicating the presence of a significant amount of haemocyanin absorbing at 295 nm. The absorbance at 295 nm indicates the presence of the copper-containing protein, haemocyanin (blue). A comparison between the elution profile data at maximum wavelength (295nm) in Figure 6 (orange) and protein content at 280 nm (blue) also confirmed earlier results shown by the elution profile as there was a corresponding high amount of protein in the peaks noted (Blue).

The metal ions Fe^{2+}, Ca^{2+}, and Mn^{+} were not found in any of the concentrates. Mg^{2+} was the only ion detected in HC4 with a concentration of 200 mg/L. Mg^{2+} is required for coagulation reaction to occur. Cu^{2+} was absent in all the concentrates except for HC2 which had a concentration of 0.25 mg/L. Cu^{2+} in haemocyanin of crustaceans is very crucial in oxygen transport. Haemocyanins are proteins applied in immunology as they have been reported to activate dendritic cells and are useful for vaccination against small molecules and in tumor immunotherapy studies (Lorean *et al*., 2022)

Typically, haemocyanins have high molecular weights and multiple subunits. Also, pH, temperature, and ionic concentration modulate the oxygen affinity of the molecule. The subunits of haemocyanin tend to aggregate, whereas the haemocyanin of arthropods, function as multi-subunit aggregates of about 75 kDal, molluscan haemocyanin are built from very much larger polypeptide chains, ranging from 350 to 450 kDal in mass (Coates and Nairn, 2014, Giannaza *et al*., 2021). In this study, the *S. africanus* haemocyanin was low in the plasma albumin. The absorbance of globulin at 295 nm was 0.083 compared to albumin, having 0.005 at same dilution factor. It shows that haemocyanin is a globular protein. For this reason, globulin was considered for isolation of the haemocyanin in this research. The albumin/globulin ratio in this study was obtained at 39:100.

Purification of the isolates was carried out using column chromatography loaded with silica gel, and 0.1 M phosphate-buffered saline. Results indicated the presence of a metalloprotein containing copper. Desalting and concentration of the isolates were carried out in Sartorious™ Centricon tubes via ultrafiltration. Ultrafiltration, when compared to other traditional methods,

is more energy-efficient, has consistent product quality, and doesn't denature proteins as moderate operating conditions were used. The low molecular weight solutes and solvents were driven by centrifugal force through the membrane into the filtrate vial. The retained protein solutes were above and the membrane inside the sample reservoir. As the sample volume diminished, retained solute concentration increased. The elution profile and protein concentration of the peaks were determined spectrophotometrically.

In the spectrophotometric analysis, the haemocyanin isolates showed a peak absorbance at 295 nm, which is typical for oxygenated haemocyanin and also reflects the presence of the copper-oxygen complex. It corresponds with Nobumasa Kitalima's Model (Kitajama *et al*., 1992). The amount of copper determined in HC2 was 0.25 mg/l (Table 4). The recovered haemocyanin concentrate in HC2 is 6536 mg/L. From this value, the percentage of copper composition for HC2 is 0.00383 %. The calculated minimum molecular weight of haemocyanin was approximately 829.5 Kilo Dalton. This shows that one major band of protein migrated with molecular mass ranging between 46-92 kDal, which is corresponding to the molecular mass of haemocyanin of 75 kDal. The number of hexamers found in HC2 was five (5) which correspond to haemocyanin from literature review, which occurs between 4-6 hexamers subunits.

Since cases of cooperative binding in haemocyanin were arranged in protein sub-complexes of 5 units (hexamers), each with oxygen binding sites, the hexamer complex is likewise arranged in chains or clusters with weights exceeding 1500 kDal (Waxman, 1975, Coates and Naim, 2014). Haemocyanin in arthropods can form multiples of hexamers, up to eight hexamers, depending on the species (Giannazza *et al*., 2021). The subunits are usually homogenous and heterogeneous with 2 variant subunit types. Each hexamer complex was arranged together to form more massive dozens of hexamers, since they are not covalently linked together. In one study, cooperative binding was found to be dependent on hexamers being arranged together in the larger complex, suggesting cooperative binding between hexamers. Since arthropods haemocyanin functions as multi-subunits aggregates of promoters of polypeptides of about 75 kDal (Markl, 1986), arthropod haemocyanin are built as multiples of hexamers, with each made up of monomers of about 75 kDal. Their ability to withstand different environmental temperatures are dependent on their survival upon haemocyanin as the oxygen-carrying respiratory protein of the haemolymphs, since both oxygen affinity and cooperativity are optimized for specific temperature range. A change in temperature can lead to a change in haemolymphs pH.

Proteins are among the most abundant biological macromolecules and are incredibly versatile in their function and interaction during the metabolism of proteins, amino acids, enzymes, and co-enzymes. The biological value of protein is reflected upon its amino acids' concentration.

The role played by amino acids in isosmotic intracellular regulation has been illustrated in several investigators (Weber and Van Marrewik, 1972), and it can be an essential source of energy-producing compounds (Van Marrewijk and Ravestein, 1974). In addition, amino acids play important roles in physiological functions such as osmo-regulation and buffer capacity in the tissues of aquatic animals (Sankar and Yogamoorthi, 2012; Huang *et al*., 2023) and some amino acids are involved in neurotransmission (Paulraj and Sridhar, 2001). crustaceans have a

balanced distribution of all essential amino acids required for an adult per day (Huang et al., 2023).

There are 20 amino acids found in the crustacean haemocyanin. Some of these are listed as essential amino acids (EAAS), i.e. arginine, histidine, isoleucine, leucine, lysine, methionine, phenylalanine, threonine, tryptophan and valine because these are not synthesized in the human body (Voet and Voet, 2011). Essential amino acids are required for the maintenance of life, growth, synthesis of vitamins, and reproduction. The non-essential amino acids were alanine, cysteine, proline, aspartic acid, glutamic acid, glutamine, glycine, serine and tyrosine. The non-essential amino acids (NEAAS) play important roles when compared to the essential ones since they help in the regulation of cellular osmotic pressure (Sudhakar, 2009). Literatures on amino acids' composition of crustacean's species are scanty except for few reports.

There is scarcity of information on the isolation and characterisation of haemocyanin from *Sudanonautes africanus*, hence this research. In our findings, the isolated haemocyanin of the crab contains cysteine, tryptophan, methionine, tyrosine, gave the lowest concentrations of 1.16, 1.21, 2.00 and 2.64 respectively while the highest concentrations were seen in aspartic acid (10.12), glutamic acid (7.38), leucine (6.04), alanine (5.00), lysine (4.58), valine (4.50), and histidine (4.29). Furthermore, the recorded results indicate the presence of 7 non- essential amino acids (NEAAS) represented by (alanine, glycine, proline, tyrosine, serine, aspartic acid and glutamic acid, asparagine, cysteine. Arginine and glutamine were non- detectable (Table 5). Cysteine, tryptophan, methionine, tyrosine, gave the lowest concentrations of 1.16, 1.21, 2.00 and 2.64 respectively while the highest concentrations were seen in aspartic acid (10.12), glutamic acid (7.38), leucine (6.04), alanine (5.00), lysine (4.58), valine (4.50), and histidine (4.29).

Haemagglutinins are proteins that possess a specific affinity for certain sugar molecules. Since carbohydrate units exist in most animal cell membranes, the haemagglutinins may attach to these receptor groups. This attachment will occur only if the lectin molecule has at least two active groups. Haemagglutinins are characterized and detected by their action on red blood cell membranes, causing the blood cells to clump together. Haemagglutination is a specific form of agglutination used when antibodies bind to red blood cells, which act as a particulate antigen. Red blood cells are particularly useful targets as they are readily available and agglutination is observable using the naked eye. In this work, rabbit erythrocyte was used to determine the titres of *S. africanus* haemocyanin.

The result shows HC2 have a minimum haemagglutination titre of 4, indicating the presence and relatively low amount of agglutinin capable of causing complete agglutination of the erythrocytes. HC3, HC1 and HC4 have moderate titres while HC5 was the highest with a titre of 64. The globulin is very rich in hemagglutinin compared to that in plasma which was quite low. The heamagglutination of the haemocyanin indicates that it may be behaving as a lectin.

5. Conclusion

The purpose of the research was to isolate and characterise haemocyanin from Sudanonautes africanus. The total protein content of albumin and globulin of *S. africanus* was 18.57 L and 470.36 mg/mL, respectively, with a ratio of 39:100. The λ max of the globulin containing the haemocyanin was also at 295 nm, indicating abundance of haemocyanin in the globulin fraction. The highest protein concentration of haemocyanin isolated from the globulin fractions after concentration via ultrafiltration was 6.536 mg/ml. The haemocyanin isolates from *Sudanonautes africanus* revealed the presence of copper ion. The composition of copper ion was 0.00383% with a calculated minimum molecular weight of approximately 829.5 kDal. The haemocyanin has two major bands of the protein with five (5) hexamers sub-units. The molecular mass ranged between 46 - 92 kDal which corresponds to the molecular mass of arthropod haemocyanin of 75 kDal.

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