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Purification and Biochemical Characterization of Serine Proteases from *Plasmodium berghei* NK-65

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Abstract
Serine proteases of human malarial parasites are thought to play key roles in essential pathways of merozoites release, invasion and host hemoglobin degradation during the intraerythrocytic stages of their life cycle. Therefore we have purified and characterized Plasmodium berghei serine protease, to determine if this enzyme can be used as potential drug target and its inhibitors as potential anti-malarial drug. The P.berghei serine protease has been purified by a combination of ion-exchange and Gel chromatography with purification level of the enzyme was about 28-fold with a total recovery of 14.4%. Its properties were examined in order to define a role in the hemoglobin degradation process. The purified enzyme migrated as a doublet band on native SDS/PAGE with a molecular mass of about 28-30kDa. The enzyme has an optimal pH of 7.5 and exhibits its highest activity at 60°c. The enzyme is inhibited by PMSF and Trypsin but not by other inhibitors including EDTA and IAA, supporting it designation as serine protease. Lineweaver burk double reciprocal plot with different substrate concentration and enzyme shows activity with a K_M of 60.33mM and 42.5mM and a V_{max} of 0.914U/mg and 0.215U/mg.

***Key words**: Serine protease, hemoglobin, malaria, Plasmodium berghei.*

Introduction

Malaria places an increasing burden on global public health resources. In the face of growing resistance of the malaria parasite to available antimalarial drugs, due to a very high degree of antigenic variation. There is a need for new drugs and the identification of new chemotherapeutic targets. The malarial parasite has a complex life cycle which includes a number of obligate intracellular stages. Clinical malaria results from cyclic asexual replication of the blood-stage parasite in circulating erythrocytes of the human host.

During the erythrocytic stage of its life cycle, the malaria parasite degrades hemoglobin as a major source of amino acids for protein synthesis. The malaria parasite thus produces/possesses a large number of proteases. These proteases play crucial roles in the life cycles of malaria parasites (Rosenthal 2001, Klemba and Golberg, 2002). They appear to be required for a number of important functions in erythrocytic parasites including hemoglobin hydrolysis, erythrocyte rupture erythrocyte invasion, and processing of precursor proteins. These proteases are thus believed to be promising targets for antimalarial chemotherapy.

In Plasmodium species, serine proteases are known to be involved in the invasion, antigenic processing and the digestion of hemoglobin and the unleashing of merozoites. Serine protease plays an essential part in initiation of hemoglobin degradation before the other proteolytic activities and that function efficiently in food vacuoles has been defined as plasmepsin 4 and two plasmepsins have been identified in Plasmodium falciparum. Two importance serine proteases plasmepsim I and II have been identified in hemoglobin degradation by P.falciparum and the rodent parasite Plasmodium berghei and inhibitors apparently specific to plasmepsin are capable of inhibiting the growth of P.falciparum.

The advent of the structure and biochemical strategies of an enzymes is necessary for facilitate the development of a potent specific inhibitor for potential application as an antimalaria drug. In order to determine whether recombinant forms of plasmepsins are appropriate for use in systematic investigations into inhibitor drug design and development, it was necessary to isolate and characterized and established the naturally occurring properties of the enzyme in respect to activity and specific activity to reflect their recombinant forms. The purification and biochemical characterization and inhibition of hemoglobin catabolism catalyzed by serine proteases in P.berghei can offers an attractive target for chemoltherapeutic serine protease of P.berghei have now been purified and

characterized shown that they share properties similar to those of P.falciparum and may play an essential role in the development of new drugs or drug targets.

Materials and Methods

Malaria Parasite

The malarial parasite Plasmodium berghei (NK-65) strain was obtained from faculty of Pharmaceutical Science Ahmadu Bello University (A.B.U), Zaria-Nigeria.

Albino Mice

They were obtained from faculty of pharmaceutical sciences, Ahmadu Bello University, Zaria-Nigeria.

Chemicals

Sodium chloride, Tris-HCl, fibrin, Tris-Glycine, Sodium dodeocyl sulphate, commassie brilliant blue, Ammonium persulphate, Acrylamide, Bis-acrylamide, mercaptoethanol N,N,N^1,N^1 tetradiethylenediamine (TEMED), Sephadex-G-75, DEAE-Sephadex-A-50, Distilled water, Fibrinogen, Ethylene diamine tetra acetic acid (EDTA), trichloroacetic acid (TCA) phenylmethylsulfonylfluoride (PMSF), Soyabeans Trypsm all chemicals are of analytical grades.

Methodology

Infection and Parasitemia Development.

Albino mice were infected intraperitoneally with the blood of miced been infected with P.berghei, parasitized RBC (10^6) (Peter and Anatoli: 1998). Parasitemia development was followed by microscopic Giemsa-stained thin blood smears. At peak parasitemia, mice were sacrifice by cardiac plexus puncture and blood was collected into heparinized syringe.

Parasite Preparation

To obtain free parasites, infected erythrocytes were washed with ice-cold PBS, lysed with 0.1% saponin in ice-washed three times with ice-cold PBS (Raphael et al, 2000). To obtain parasite extracts free parasites were suspended in extraction buffer 20mM Tris-Hcl (PH 7.2), 1mM EDTA, 0.1mM PMSF and 1OμM pepstatin, subjected to freeze, thaw cycles, centrifugation at 3000 rpm for 30min and the supernatant was collected.

Ion-Exchange Chromatography

The sample of the malaria parasite extract was passed a column of DEAE-sephadex A-50 equilibrated with 50mM Tris-HCl, pH 7.2. The column was eluted with a step gradient of NaCl from 0.1 to 0.5M in 0.1M increments the samples were eluted at a flow rate of 0.06ml/mins . The fractions were assayed for enzyme activity.

Protease Activity

Proteolytic activity was tested using fibrin as substrate and following the method described by Kunitz (1947) modified by Van Der Watt and Joubert (1971).

50μL of the partially purified enzyme was incubated with 500μL 200mM Tris-HCl buffer pH 7.2, 100μL 1% fibrin in 200mM Tris-HCl buffer pH 7.2 for 30min at 37^0c. The blank was also prepared in a similar manner with the exception of the partially purified enzyme. 200μL of 1M Trichloroacetic acid (TCA) was then added for protein precipitation and to stop the reaction. After removal of precipitated protein by centrifugation (3000rpm for 30min at room temperature) the also absorbance of the supernatant at 280nm was determined.

Inhibitory effect of the proteolytic activity of the partially purified enzyme with high activity was tested with 50mM each of EDTA, PMSF, Soyabeans Trypsin and IAA. This is to determine the fraction with the enzyme of interest for further purification.

Gel-Filtration (Sephadex-G-75)

Gel-filtration of the fraction with the enzyme of interest was run using sephadex-G-75. The fraction was applied to equilibrate with Tris-HCl buffer, pH 7.2. The elution was done with 50mM Tris-HCl buffer, pH 7.2; at a flow rate of 0.02ml/min. 50 fractions were collected of 5ml per fraction. It was then stored at 4^0c for other biochemical analysis

Biochemcial Characterisation

Inhibitory Studies

Inhibitory studies of the enzyme activity were done using various types of inhibitors such as EDTA, IAA, PMSF and Trypsin

Effect of Temperature On Enzyme Activity
The effect of temperature on the rate of hydrolysis of fibrin by the enzyme was studied at 10^o, 25 – 40^oc 50^o, 60^o, 70^o and 80^o as described by Stauffer (1969) for Optimum temperature.

Effect of pH On Enzyme Activity
The effect of pH on the rate of hydrolysis of fibrin by the enzyme was studied at pH range of 4.0 – 8.0 the various buffers used for the pH optimal activity were acetate buffer, (4.0 – 5.5) phosphate buffer (6.0-7.5) and Herpes buffer at 8.0. This was described by vidal etal (1972), Joubat and vander Walt (1975) with slight modifications.

Thermostability of the Enzyme
The thermal stability activity was investigated by measuring the residual activity of the enzyme after 20mins of incubation at different temperature prior to substrate addition.

Line Weaver Burk
100µL of the enzyme solution was incubated into several concentration of the fibrin substrate i.e 5mg/ml, 10mg/ml 15mg/ml, 17mg/ml and 20mg/ml at 37^oc for 1 hours and the activity was determined

SDS-Polyacrylamide Gel Electrophoresis
Sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) of the partially purified enzyme was performed as described by webster and Pringel 1972.

About 100µL of the enzyme samples along with other protein were treated with 50µL of treatment buffer stacking buffer. One drop of mercaptoethamol and one drop bromophenol blue and was incubated in a boiling water bath 100^oc for 30minutes after which 3 drops of glycerol was added to each sample.

The molecular weight of the purified serine protease protein was determined by SDS-PAGE according to the method of Laemmli. 0.1% gelatin was copolymerized with acrylamide.

After electrophoresis, the gels were washed in 2.5% Triton-X-100 (three washes for 30min each) to remove SDS. The gels were then incubated in glycine buffer (0.1M glycine, 0.002M $cacl_2$, PH 7.0) at 37^oc for 72 hours to allow the digestion by the proteases before the gels were stained with commassie Brilliant Blue.

Results
Three fraction of Plasmodium berghei with serine protease activity were isolated in a two-step purification procedures.First, the parasite was separated by DAE-Sephadex A-50 (ion-exchange chromatography) into five fractions (figure 1); then the fraction with highest specific activity (F3) was chromatography on Sephadex G-75 resulting in three fractions with serine protease activity(figure2).Table 1 present the recovery of protein and activity of all fractions obtained from two-step purification procedure. F3, resulting from first step purification accounted for 6.0% of the parasite protein and the isoenzymes FIII, IV and V accounted for 99%, 99%, and 101% of the protein bounded to column. The isoenzymes resulting from the Gel-chromatography migrated as a single band of in SDS-PAGE (figure 3). These three fractions were the most interesting since they had serine protease activity and protein.

The optimal pH for F IV is 7.0, FIII and FV isoenzyme was 7.5 (figure 4). The optimal temperature for the FIII and FV isoenzyme was 60^oc and F IV is 50^oc (figure 5) FIV showed a thermal stability at a 40^oc, while FIII and FV showed thermal stability at 50^oc for 20minuts (figure 6)

The inhibition of protease activity- the effect of the protease inhibitors EDTA, IAA, PMSE and Trypsin on the activity showed that PMSF and Trypsin seriously inhibit the activity indicating that is a serine protease where as other inhibitor EDTA and IAA for metalloprotease and cysteine protease had no effect on the enzyme activity (figure 7)

The michealis menten constant (K_M) for the isoenzymes are 60.33mM and 42.5mM and a v_{max} of 0.914U/mg and 0.215U/. (Figure 8)

Table 1: Summary of the purification of serine protease enzyme from malarial parasite Plasmodium berghei. NK65

Step DEAE-Sephadex A50	Total protein (Mg)	Activity (nmol/min)	Specific Activity (n mol/min)	Purity	Yield (%)
Crude	0.077	0.053	0.688	1	100
F 1	0.040	0.139	3.475	5.10	51
F 2	0.024	0.074	3.083	4.50	31
F 3	0.005	0.046	9.200	13.40	6.0
F 4	0.028	0.158	5.643	7.30	36
F 5	0.007	0.037	5.286	7.70	9
TOTAL	0.181	0.07			
Sephadex G-75					
Fraction F3xxx	0.186	0.015	0.081	1	100
FI	0.282	0.046	0.163	2.01	152
FII	0.210	0.028	0.133	1.60	113
FIII	0.184	0.069	0.376	4.64	99
FIV	0.184	0.068	0.370	4.56	99
FV	0.188	0.068	0.362	4.47	101
Total	1.234	0.294			

Table 2: Effect of protease inhibitors on Plasmodium berghei NK 65 serine protease F III, FIV and FIV

Inhibitors	Relative Activity (%)		
	FIII	FIV	FV
EDTA	76.8	72.1	70.6
IAA	98.6	95.6	91.2
Trypsin	17.4	13.2	11.8
PMSF	28.9	26.5	23.5

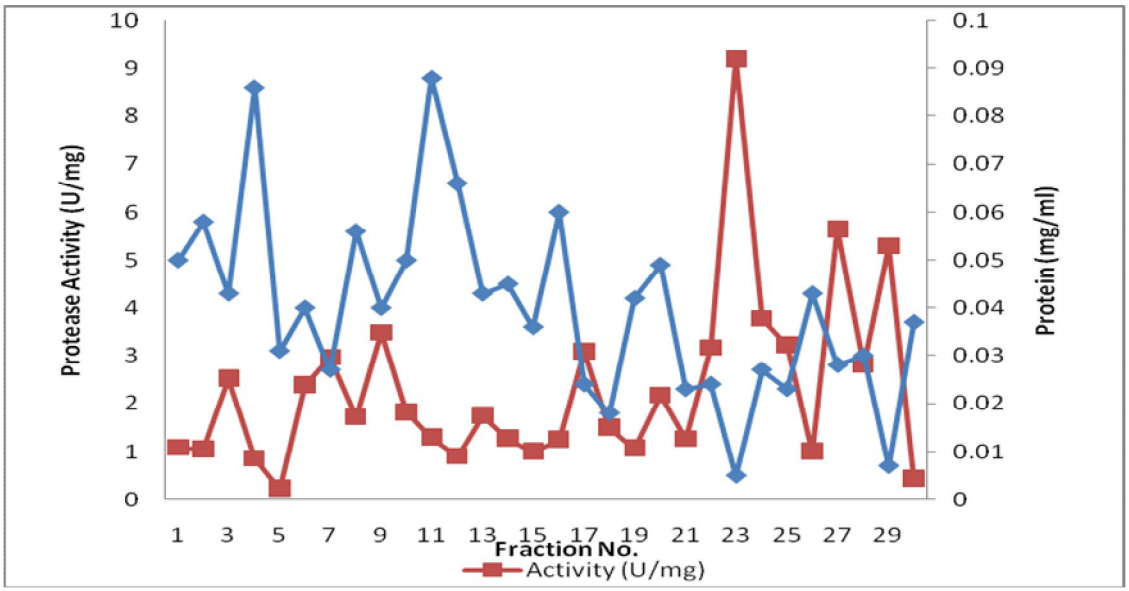


Figure 1. Elution profile for ion- exchange chromatography of *Plasmodium berghei* protease on DEAE-sephadex-A-50 equilibrated with 50mM Tris- HCl buffer, pH 7.2 at a flow rate 5ml/h and step gradient elution with NaCL of 0.1-0.5M

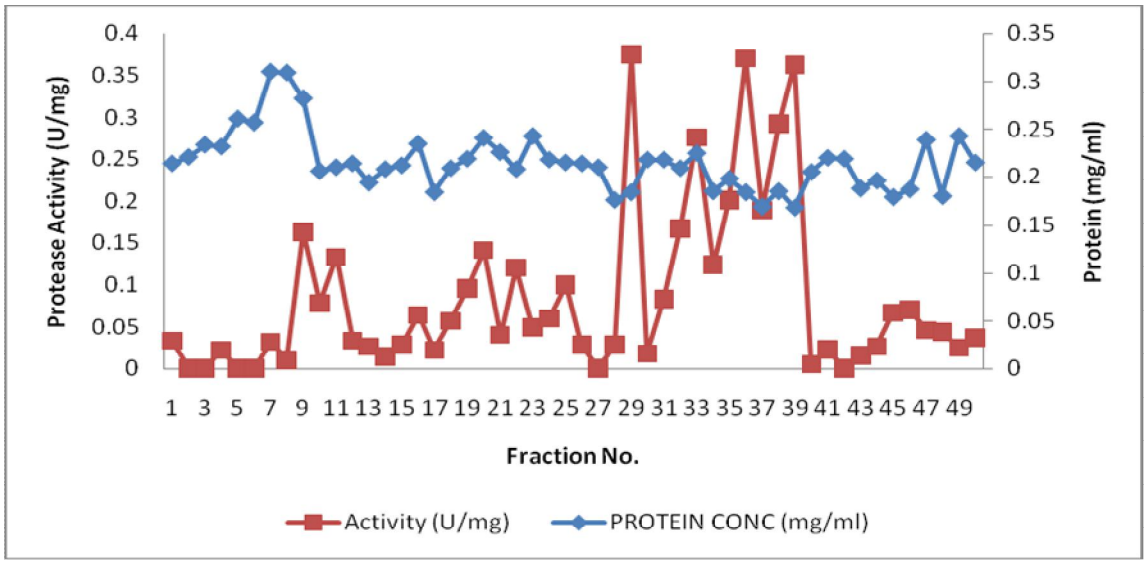
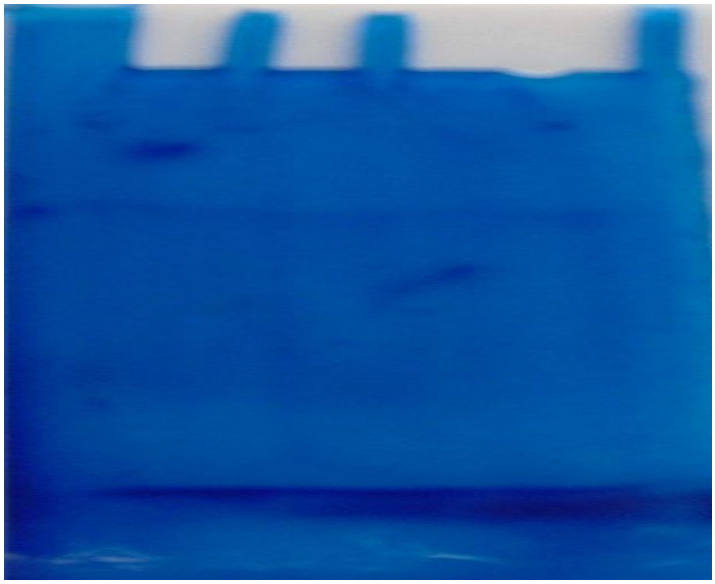


Figure 2. Elution profile for the Gel chromatography of *Plasmodium berghei* protease Sephadex-G-75 from F3 on Sephadex – A -50.



LANE : 1: F-3 2: F-4 3: F-5

Figure 3. SDS- PAGE analysis of the purified malaria serine protease from *plasmodium berghei* NK-65

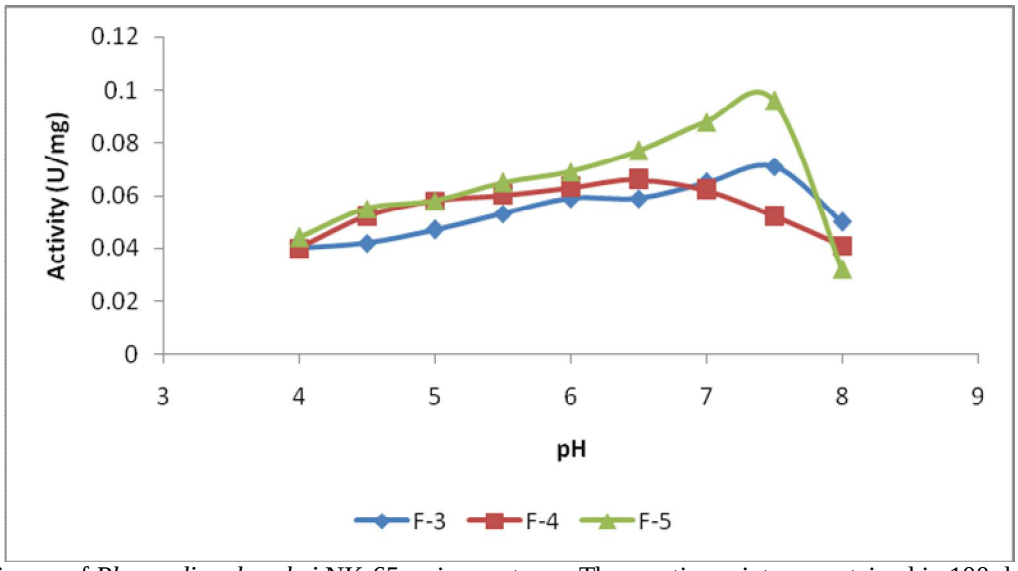


Figure 4. pH optimum of *Plasmodium berghei* NK-65 serine protease .The reaction mixture contained in 100µl of fibrin, suitable amount enzyme and 50mM acetate buffer (pH 4.0-5.5), phosphate buffer (6.0-7.5), and herpes buffer 8.0.

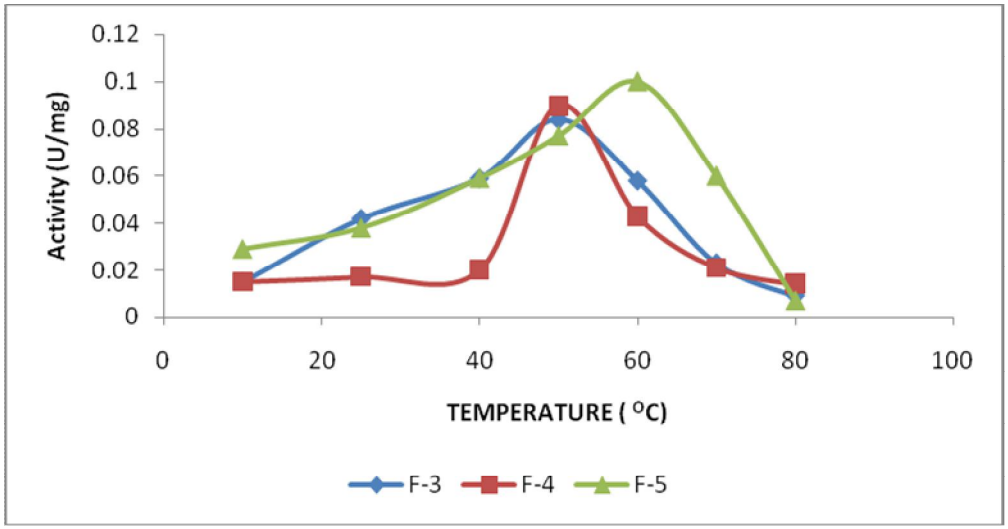


Figure 5. Temperature optimum of *Plasmodium berghei* serine protease FIII, FIV and FV at the same substrate concentration.

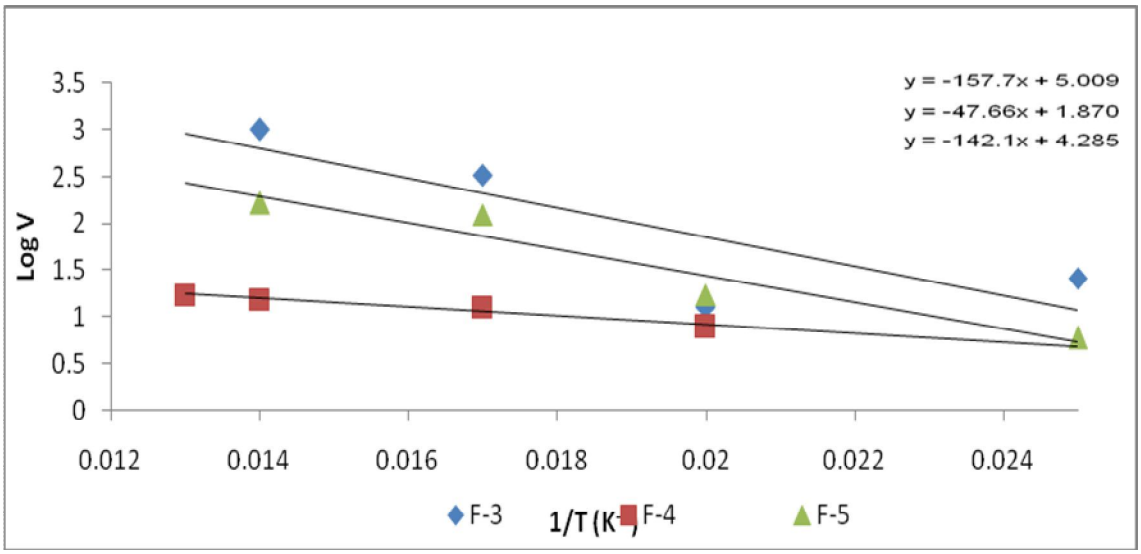


Figure 6. A graph showing the Arrhenius plot for the determination of Activation Energy of Malaria serine protease from *Plasmodium berghei* NK-65.

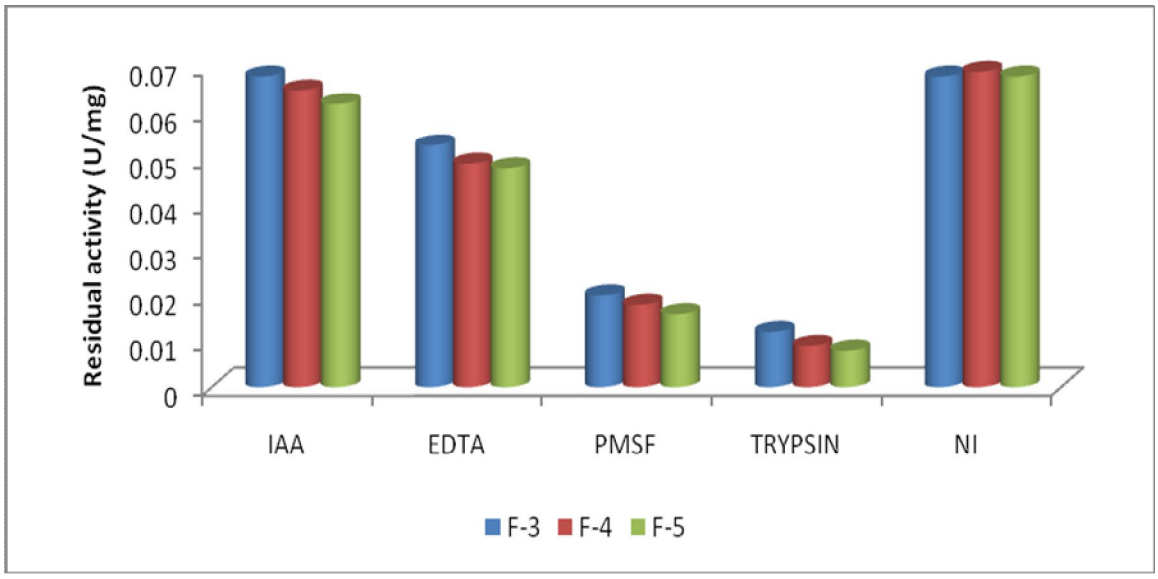


Figure 7. Inhibition of serine protease activity by various inhibitors. The protease activity was tremendously inhibited by PMSF and TRYPSIN indicating it a serine protease as described under the Materials and Methods.

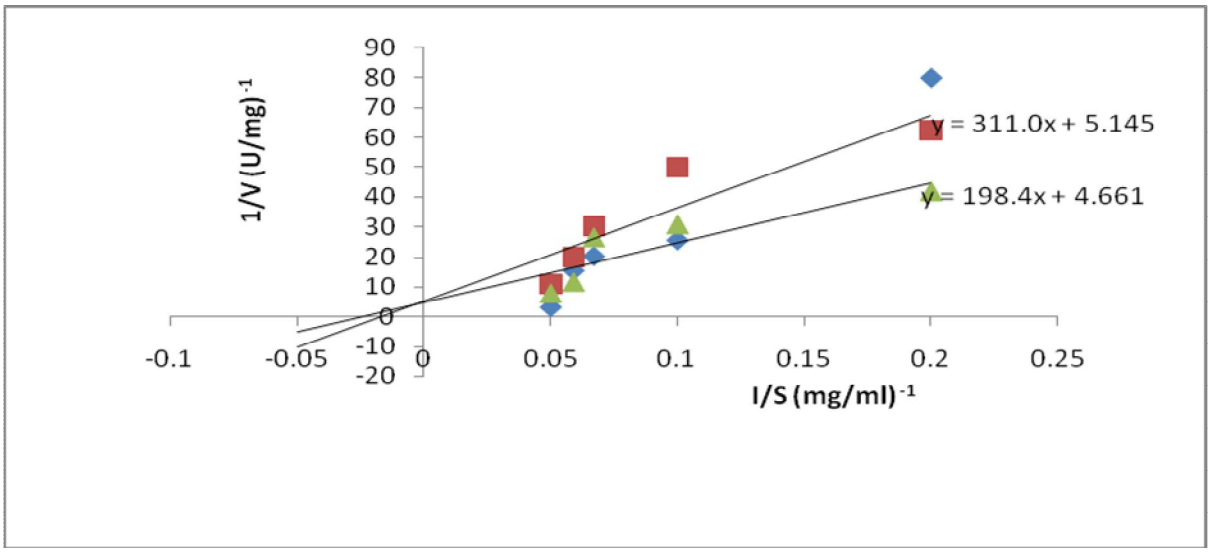


Figure 8. Line-Weaver Burk Plots of Initial Velocity data for the determination of K_M and V_{max} for malaria serine protease from *Plasmodium berghei* NK-65

Discussion

In the list of targets explore for chemotherapeutic development are families of proteases that are vital in the life cycle of protozoa parasites. Hemoglobin proteolysis in the digestive vacuoles of the malaria parasites is an ordered process due to limited capacity of the parasite to synthesized amino acids. In view of the slightly alkaline nature of the other food vacuole, we say that the parasite would utilize hemoglobin degrading proteases with slight alkaline pH optima.

This paper described the characterization of an enzyme that is known to play an essential role in the catabolic pathway of hemoglobin. Detailed of proteases specificity with its natural substrate have been studied and the substrate properties of this serine protease is noteworthy because it is obvious from substrate specificity studies/Kinetics analysis and structural information that plasmepsins show unique binding characteristic for native and recombinant enzymes. The ability of serine proteases to catalyzed the hydrolysis of native hemoglobin is consistent with the observation that purified enzyme preparation were capable of cleaving hemoglobin and utilized as food.

From previous work done by other people on other types of Plasmodium protease was shown that the mode of action chloroquine may be due in part to a diversion of ferriprotoporphyrin IX from its normal processing into the malarial pigment by forming a complex. The antimalarial activity may be as a result of the inhibition of hemoglobin degrading proteases by free ferriprotoporphyrin IX by its complex with chloroquine primaquine etc.This present study provides additional support for this model by demonstrating that the hemoglobin degrading proteases are located in the same place in the food digestive vacuole where malaria pigment is produced.

The results from this experiment have shown that protease are present in P.berghei and are involved in plasmodial development. The presence of serine protease in P.berghei opens new avenues to chemotherapy through the designed of specific inhibitors that may block essential metabolic pathways of hemoglobin degradation by the parasites.

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