"PURIFICATION, PHYSICOCHEMICAL AND FUNCTIONAL CHARACTERISATION OF GLOSSINA MORSITANS CENTRALIS FIBRINOLYSINS."

BY

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DECLARATION

I, Wilson Ogore Endege, hereby declare that this thesis is my original work and has not been presented for a degree in any other University.

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Abbreviation.	Full name/meaning.
$Alpha_1$-PI	$Alpha_1$-protease inhibitor.
Bz	Benzoyl.
Cbz	Carbobenzoxy.
Chromozym-PL	Tosyl-Gly-Pro-Lys-p-nitroanilide.
Chromozym-PK	Benzoyl-Pro-Phe-Arg-p-nitroanilide.
Chromozym-TH	Tosyl-Gly-Pro-Arg-p-nitroanilide.
CPM	Counts per minute.
Chromozym-TRY	Carbobenzoxy-Val-Gly-Arg-p-nitroanilide.
DFP	Diisopropylfluorophosphate.
DMF	Dimethylformamide.
DMS	Dimethylsuberimidate.
DTT	Dithiothreitol.
EDTA	Ethylenediaminetetetra-acetic acid.
EtOH	Ethanol.
hr	Hour or hours.
HCHO	Formaldehyde.
HOAC	Acetic acid.
HPLC	High Performance Liquid Chromatography.
IEF	Isoelectric focusing.
kDa	Kilo daltons.
Ki	Enzyme inhibitor constant.
Km	Michaelis-Menten Constant.
mg	Milligram.
MGE	Midgut extract.

min	Minutes.
ml	Millilitre.
mM	Millimolar.
mu	Milli units of enzyme.
M	Molar.
Mr	Relative molecular mass.
ng	Nanograms.
nm	Nanometers.
O.D.	Optical density.
PAGE	Polyacrylamide gel electrophoresis.
PBS	Phosphate buffered saline, pH 7.4.
PCMB	Para-choloromercuric benzoate.
pI	Isoelectric point.
PMSF	Phenylmethanesulfonylfluoride.
Temed	N,N,N',N'-tetramethylethylenediamine.
STBI	Soy bean trypsin inhibitor.
SDS	Sodium dodecyl sulphate.
TLCK	N-p-tosyl-L-Lysine chloromethylketone.
TPCK	N-tosyl-phenylalanine chloromethylketone.
Tris	Tris (hydroxy-methyl) aminomethane.
u	Units.
ug	Microgram.
ul	Microlitre.
uM	Micromolar.
rpm	Revolutions per minute.
Vmax	Maximum velocity.

SUMMARY.

Tsetse flies are blood-sucking insects. The ability to keep their blood meal in an anticoagulated state by means of anticoagulant(s), fibrinogenase(s), fibrinolysin(s) or plasminogen activator-like activities is essential to their survival. In this investigation tsetse midgut fibrinolytic activities have been characterised.

Five proteases that hydrolyse Tosyl-Gly-Pro-Arg-p-nitroanilide (chromozym-TH) at different rates, were identified in tsetse midgut extracts and four of them purified to apparent homogeneity using anion-exchange chromatography on DE-52 cellulose followed by isoelectric focusing.

Assays using radio-labelled fibrinogen and fibrin were developed. These showed that two of the proteases with highest fibrinogenolytic activity (P3 and P4) had exo- and endo-peptidase-like activities respectively. The two enzymes had pIs of 6.0 and 5.3 on isoelectric focusing and Mr of 26 and 24.5 kDa on sodium dodecyl sulphate (SDS) denaturing gels, respectively. They appeared electrophoretically homogeneous as judged by SDS polyacrylamide gel electrophoresis (PAGE). The two enzymes seem to digest fibrinogen and fibrin at different sites but within the same domains as does bovine plasmin, a classical fibrinolysin. Tsetse fibrinolysin P3 initially generates very low molecular mass products (range 14-20 kDa), but after extended incubation times it starts generating a breakdown product of apparent Mr 94 kDa. Fibrinolysin P4 also produced the 94 kDa fragment and

another fragment of 42 kDa, as well as larger intermediate breakdown products of approximate Mr 160and 240 kDa.

The two tsetse fibrinolysins have a pH optimum around 8.0. Inhibition studies showed that these two tsetse fibrinolysins were serine proteases since diisopropyl-fluorophosphate (DFP) completely abolished their hydrolytic activity towards Tosyl-Gly-Pro-Arg-pNA. These enzymes had trypsin-like specificity since TLCK, a chloromethyl ketone inhibitor with trypsin-like enzyme-specificity, inhibited 75% of their activity towards this tripeptide substrate. This compared with only 25% inhibition of these enzymes by TPCK, an inhibitor of chymotrypsin-like proteases. Their trypsin-like specificity was also suggested by trypsin specific chromogenic p-nitroanilide substrates, which these enzymes hydrolysed rapidly, and additionally by the inhibition of the enzymes by aprotinin.

A thermolability study showed a biphasic curve with gradual loss of activity upto 50^{o}C after which there was a precipitious loss of activity with further increase in temperature. An antibody against fibrinolysin P4 was raised in rabbits. This antibody reacted with fibrinolysins P3 and P4 thus suggesting that the two share some common antigenic determinants. Fab fragments made from the purified immune IgG did not inhibit <u>in vitro</u> hydrolysis of the Tosyl-Gly-Pro-Arg-PNA thus suggesting that these antibodies were not directed to the active sites of the enzymes.

CHAPTER 1

INTRODUCTION.

1.1 CLOTTING IN BLOOD PLASMA: AN OVERVIEW.

The clotting of plasma in vertebrate blood centers around the fibrinogen molecule, a large protein (Mr 340 kDa) with a disulphide-bridged structure comprising of three different constituent chains, namely, alpha, beta and gamma $(\alpha\beta\gamma)_2$ as shown by Doolittle (1980).

The formation of the blood clot requires the specific cleavage of fibrinogen into fibrin by thrombin. This process involves the release of two peptides, namely fibrinopeptide A and fibrinopeptide B. Events preceeding the cleavage of fibrinogen and generation of fibrin monomer or polymer clots are controlled by a cascade of enzymes which lead to the generation of highly specific proteases. All of these enzymes occur in plasma as inactive precusors, zymogens. When coagulation is initiated, these precusor proteins are converted to active enzymes in a series of concerted reactions.

The model for blood coagulation has evolved from a simple linear sequence of activation of zymogens, into a much more complicated pathway in which protein-protein and protein-lipid-Ca^{2+} interactions occur among proteinases, protein substrates, and enzyme cofactors. At present fibrin clot formation is known to be initiated by two pathways known as the intrinsic and extrinsic pathways of blood coagulation (Davie, 1986). These two pathways are initiated *in vivo* by vascular damage. The precise mechanism for the triggering of these reactions *in vivo*, however, is not known (Davie, 1986).

However, various stages have been defined in both pathways. At each stage in the presence of lipid and cofactors, the zymogen activation rates are amplified several thousand fold over that occuring with the zymogen and proteinase alone.

Under normal physiological conditions, blood coagulation through the intrinsic pathway can be initiated by contact with negatively charged surfaces such as those of collagen and vascular basement membranes (Niewiarowski et al., 1965; Cochrane et al., 1972). The first phase of this pathway involves the allosteric (non-enzymatic) activation of factors XII by binding to one of these initiators, whereas subsequent activation steps involve cleavage of zymogens by limited proteolysis (Kingdon et al., 1964; Soulier et al., 1958; Ratnoff et al., 1961). The initial reaction is greatly accelerated by the participation of kallikrein, an enzyme that generates kinin from kininogens (Cochrane et al., 1972; Weiss et al., 1974). In the presence of factor XIIa, prekallikrein is converted into kallikrein, which is itself capable of activating factor XII and so forming a loop-back activation system. In the next reaction, factor XIIa converts factor XI to an active enzyme.

The allosterically activated factor XII can activate more factor XII by limited proteolysis as well as factor XI in the next phase of the coagulation pathway. The activated factor XI (XIa) then converts factor IX to factor IXa (Waaler, 1959; Ratnoff and Davie, 1962; Cattan and Denson, 1964; Fujikawa et al., 1974). This reaction, which requires calcium ions

occurs in two steps. In the first step of the activation reaction, factor IX is cleaved by hydrolysis of an internal peptide bond giving rise to a two-chain molecule which has no enzymatic activity and is held together by disulphide bonds. In the second step of the activation reaction, factor XIa cleaves a glycopeptide from the amino-terminal end of the heavy chain to generate the active enzyme (IXa).

In the next series of reactions, factor IXa interacts with factor VIII in the presence of calcium and phospholipid to form a complex. It is this complex which converts factor X into factor Xa (Hougie et al., 1967; Chuang et al., 1972). Under physiological conditions, the platelets provide the phospholipid needed for this reaction. In these reactions, factor VIIIa appears to function as a regulatory protein and factor IXa as the catalyst.

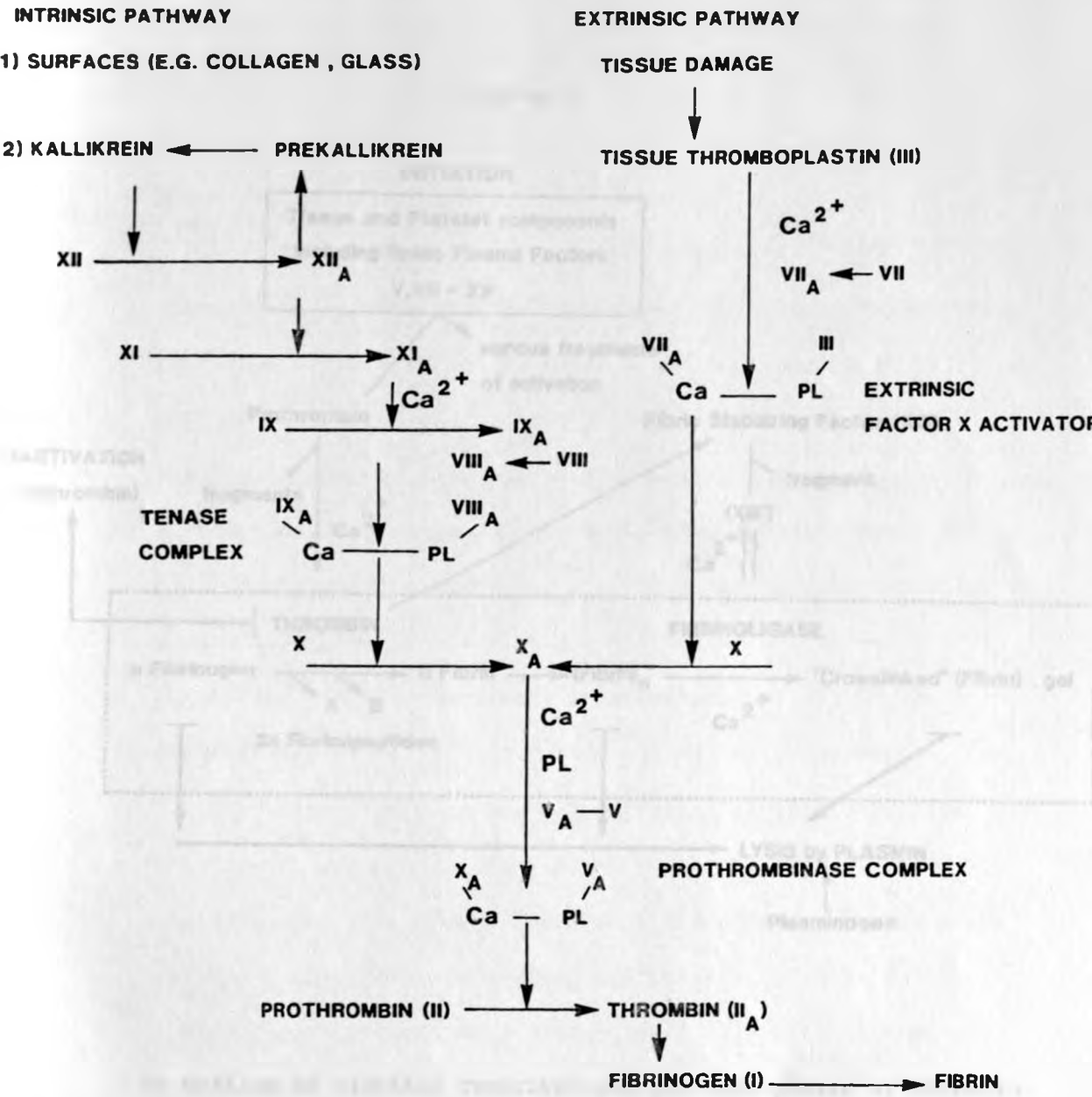
The next reaction of the coagulation pathway involves the formation of the prothrombinase complex consisting of factor Xa, Va, phospholipid and Ca^{2+}. The phospholipid, is supplied by platelets. Factor Xa is the enzyme in this complex and factor Va is a regulatory protein. The activity of factor Va is greatly enhanced by its interaction with thrombin. Thus the role of factor Va in blood coagulation appears to be very similar to that of factor VIIIa. The activation of prothrombin to thrombin is a two-step reaction which initially involves the cleavage and loss of a polypeptide of Mr 33 kDa, followed by a second cleavage which generates a disulphide-linked active thrombin molecule.

In contrast to the instrinsic pathway, the addition of tissue extracts induces clot formation as a result of activation of the extrinsic or tissue factor pathway of blood coagulation, (Figure 1). This involves the activation of factor X by factor VIIa, which occurs as a part of a complex with tissue factor, a lipoprotein present in many tissues (Nermerson and Esnouf, 1973). A number of enzymes have been implicated in the initiation of the extrinsic pathway including factor XIIa, kallikrein, and factor Xa formed by intrinsic pathway activation.

Factor X is thus activated by either pathway and provides a convergence point for early events in the coagulation cascades. Having been formed, factor Xa then activates prothrombin to thrombin by the mechanism which has already been described. Once thrombin is formed, it plays multiple roles in coagulation: (1) It converts fibrinogen to fibrin, (2) converts factor XIII to XIIIa (Figure 2) and (3) activates factor V and factor VIII to Va and VIIIa, respectively.

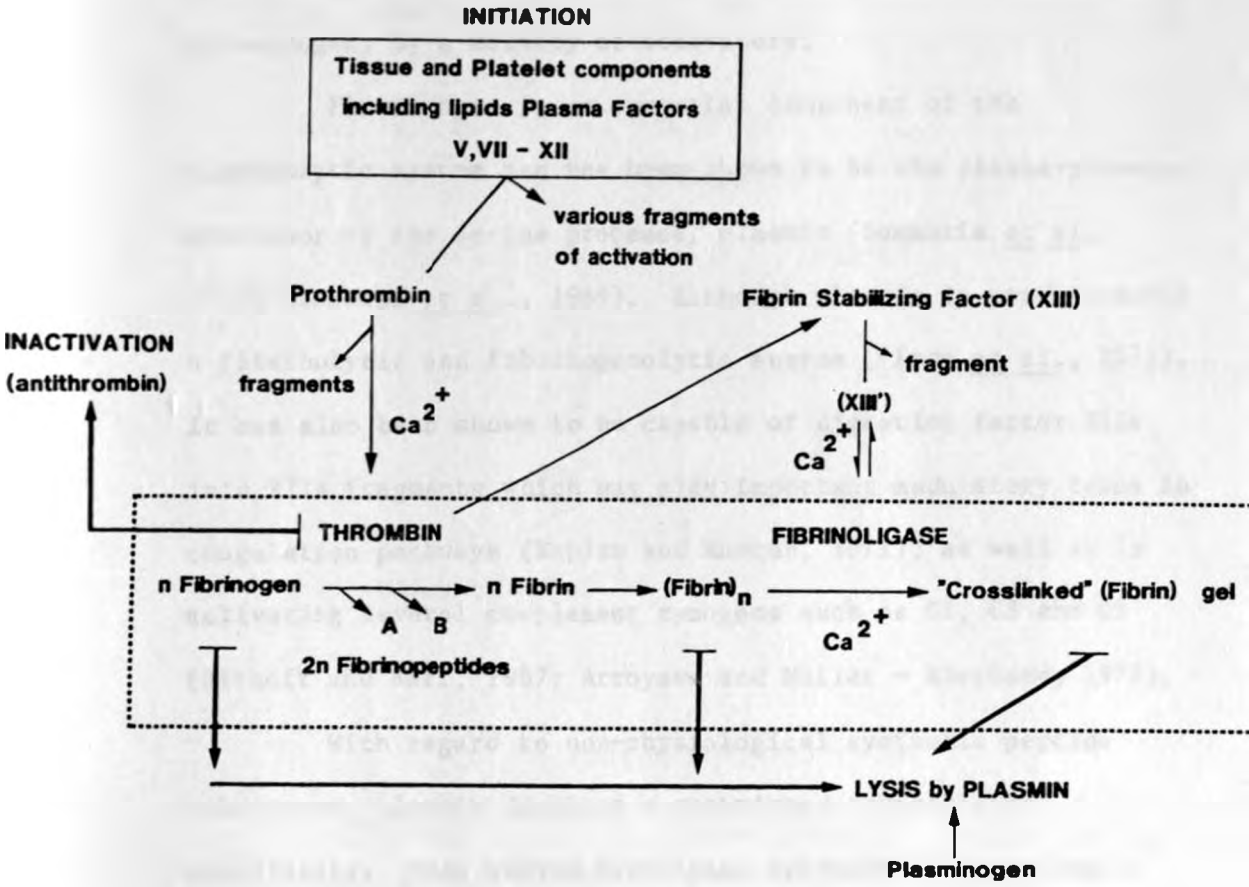
The stabilization of the fibrin clot occurs as a result of the action of the cross-linking enzyme, factor XIIIa, which has been termed the fibrin stabilising factor. This cross-linking reaction involves the formation of an iso-peptide bond between the epsilon amino group of lysine and the gamma glutamic carboxyl group residue. This reaction leads to the formation of a "firm clot". Calcium ions act as cofactors for factor XIIIa, but they are not essential for the reaction to occur.

Figure 1.



The intrinsic and extrinsic pathways of the blood coagulation cascades. Ca and PL refer to calcium ions and phspholipids respectively.

Figure 2.



An outline of clotting reactions and the role played by thrombin in activating and inactivating various factors of the coagulation and lysis pathways. Heavy arrows outline the "shut off" mechanisms (Extracted from Methods in enzymology volume 54, p 32)

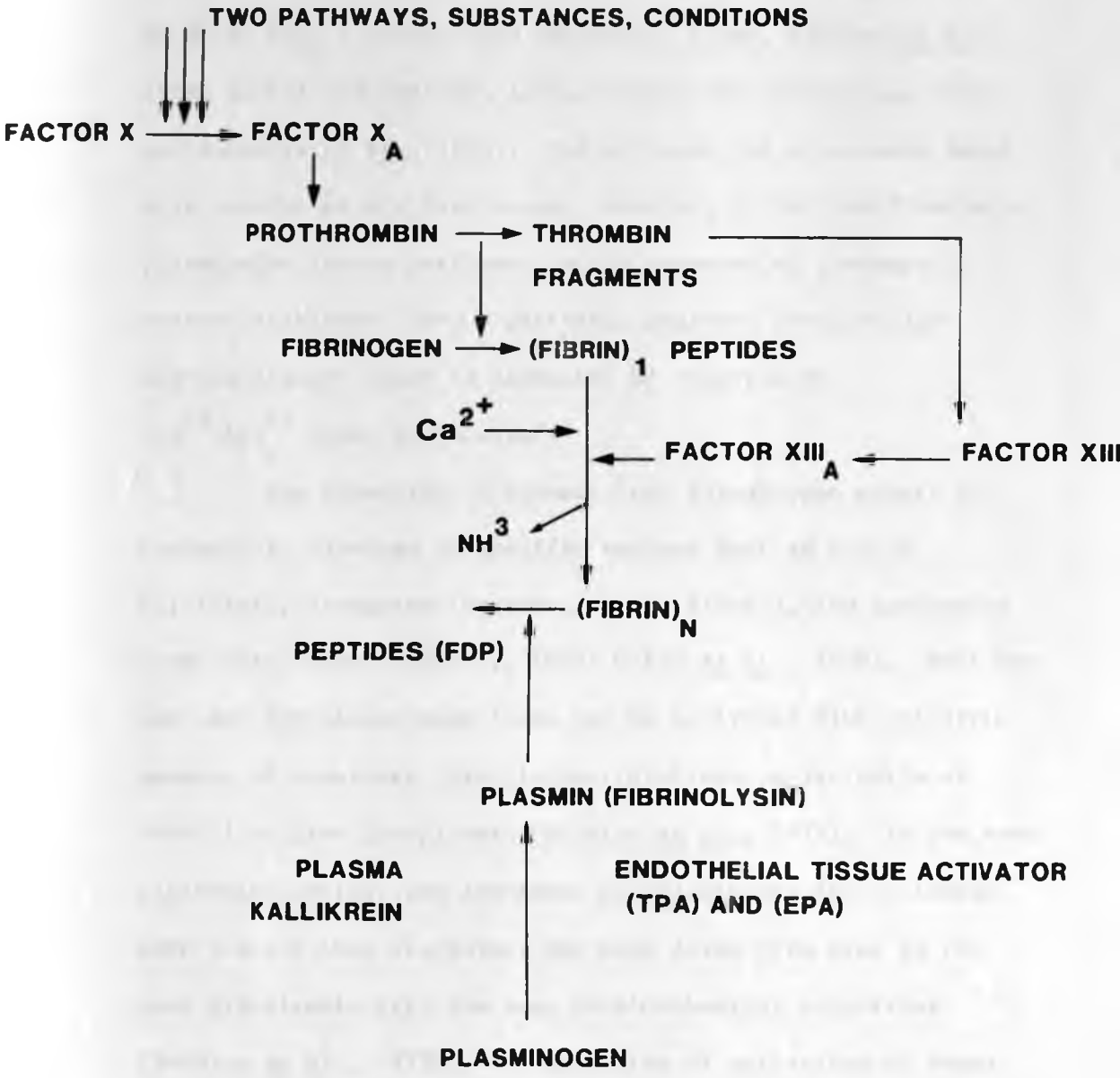
1.2 CLOT LYSIS.

The formation and degradation of fibrin is illustrated in figure 3. The factor XIIIa stablised fibrin clot is not permanent, but can be eliminated by proteolytic degradation processes. Plasmin, a serine protease, is the major enzyme involved in clot dissolution. It is formed from its precursor, plasminogen, by a variety of activators.

Plasminogen is an essential component of the fibrinolytic system and has been shown to be the plasma-protein precursor of the serine protease, plasmin (Summaria et al., 1967; Groskopf et al., 1969). Although plasmin is predominantly a fibrinolytic and fibrinogenolytic enzyme (Pizzo et al., 1972), it has also been shown to be capable of digesting factor XIIa into XIIa fragments which may play important modulatory roles in coagulation pathways (Kaplan and Austen, 1971), as well as in activating several complement zymogens such as C1, C3 and C5 (Ratnoff and Naff, 1967; Arroyave and Muller - Eberhard, 1973).

With regard to non-physiological synthetic peptide substrates, plasmin displays a restricted trypsin-like specificity. This enzyme hydrolyses proteins and peptides at lysyl-and arginyl-bonds (Wallen and Iwanaga, 1968). Various investigators working with plasmin were able to show that the enzyme possesses both esterolytic and amidolytic activity towards basic amino acid esters (Weinstein and Doolittle, 1972) and basic amino acid amides (Christensen and Mullertz, 1974) with a preference for lysyl-residues. In this respect it resembles the other hydrolases of the clotting cascade which

Figure 3.



Formation and degradation of fibrin.

also have trypsin-like specificity (Lottenberg et al., 1981) but which have different subsite specificities.

Native plasminogen has been shown by various investigators to consist of a single-chain protein molecule of Mr 90-94 kDa, with multiple isolectric forms, (Barlow et al., 1969; Rickli and Otavsky, 1973; Violand and Castellino, 1976; and Summaria et al., 1976). The molecule has a glutamic amino acid residue at its N-terminus. However, if the purification of plasminogen is not performed in the presence of proteolytic enzyme inhibitors, then a partially degraded form, called Lys-plasminogen which is generated by cleavage at Lys^{76}-Lys^{77} bond, is obtained.

The formation of plasmin from plasminogen occurs by proteolytic cleavage by specific enzymes such as plasma kallikrein, urokinase (produced by the kidney), and activators from other tissues (Astrup, 1956; White et al., 1966). Both the Glu- and Lys-plasminogen forms can be activated with catalytic amounts of urokinase (plasminogen: urokinase molar ratio of 1000:1) to give Lys-plasmin (Robbins et al., 1973). In the same activation system, the Lys-forms of plasminogen are activated more rapidly than Glu-forms, but both forms give rise to the same Lys-plasmin with the same physicochemical properties (Robbins et al., 1975). The mechanism of activation of human Glu-plasminogen to plasmin by urokinase has been shown to involve two specific bond cleavages (Robbins et al., 1975). An arginyl-valine peptide bond within the carboxyl-terminal portion of the zymogen is cleaved by urokinase to give a two-chain plasmin molecule containing a Glu-heavy (A) chain and a

Val-light (b) chain. The second cleavage involves a lysine-lysine peptide bond in the amino-terminal portion of the Glu-heavy (A) chain. This is cleaved by plasmin itself to give rise to a Lys-heavy (A) chain and other peptides.

There are also non-protease plasminogen activators such as streptokinase and staphylokinase which are extracellular proteins produced by Streptococcus hemolyticus and Staphylococcus aureus, respectively. Unlike urokinase or tissue activator, streptokinase has been shown (Zybler et al., 1959; Kline and Fishman, 1972) to activate plasminogen without the involvement of limited proteolysis but simply by forming a strongly associated one-to-one molar complex with the zymogen. Fibrinolytic activity is generated within the plasminogen moeity of this complex (Reddy and Markus, 1972). Furthermore, in the absence of specific inhibitors, the plasminogen-streptokinase complex can rapidly convert into a plasmin-streptokinase complex which in turn effectively activates plasminogen to plasmin, a reaction not catalysed by plasmin itself.

Knowledge about staphylokinase is more limited, although it has been suggested (see Jackson et al., for a Review, 1981) that, the activation of human plasminogen by staphylokinase occurs in a similar manner to that described for streptokinase.

1.3 PLASMA INHIBITORS OF THE FIBRINOLYTIC ENZYME SYSTEM.

Having described the mechanisms that lead to the production of plasmin, it is necessary to address the role played by plasma inhibitors of the fibrinolytic system which exert their effects of regulation in two ways, namely, inhibition of plasminogen activation and neutralization of any plasmin formed.

Both fast and slow acting plasmin inhibitors have been described (Schultze et al., 1963; Garnot, 1967; Wiman and Collen, 1977). Five of the six known plasma protease inhibitors, namely, alpha$_2$- macroglobulin, alpha$_1$-protease inhibitor, inter-alpha-trypsin inhibitor, antithrombin III and C1 inactivator have been shown to possess different degrees of antiplasmin activity (see review by Ogston, 1977).

Alpha$_2$-macroglobulin has been established to be the most potent inhibitor of the proteolytic activity of plasmin (Schultze et al., 1963; Garnot, 1967; Harpel, 1975). For example, Harpel (1975) studied the competitive binding of ^{125}I-labelled plasmin to mixtures of alpha$_2$-macroglobulin, C1 inactivator and alpha$_1$-protease inhibitor using sucrose density ultracentrifugation and SDS-PAGE gels. He showed that, over 80% of the bound plasmin was complexed to the alpha$_2$-macroglobulin. The complexing of alpha$_2$-macroglobulin with plasmin, occurs in a 1:1 ratio and is accompanied by proteolytic modifications within the subunit structure of the inhibitor and the trapping of the enzyme within an alpha$_2$ macroglobulin cage-like structure (Harpel, 1973). Being trapped within the alpha$_2$-macroglobulin molecule, the proteinase molecule is

unable to digest large protein substrates or to be inhibited by protein inhibitors. The changed conformation of the "closed" alpha$_2$-macroglobulin molecule leads to its rapid clearance from the circulation by the reticuloendothelial system along with the trapped proteinase.

Recently a fast-acting plasmin inhibitor in plasma was purified and partially characterised (Wiman and Collen, 1977). This molecule is a single-chain glycoprotein with a molecular mass of about 70 kDa on SDS-PAGE. It forms a very stable complex of Mr 140 kDa with human plasmin which does not dissociate on SDS-PAGE gels. These investigators have shown that this human antiplasmin acts very rapidly by forming a very stable complex with plasmin and at the same time releasing a 14 kDa fragment.

Alpha$_1$-proteinase inhibitor has been shown to inhibit plasmin (Schultze et al., 1963; Rimon et al., 1967) and represents the slow-acting antiplasmin described by Norman (1958). Inhibition of plasmin can also be achieved with antithrombin III the reaction of which is accompanied by the formation of an undissociable complex representing an equimolar stoichiometric combination of the two components (Highsmith and Rosenberg, 1974).

1.4 SNAKE VENOMS: BLOOD COAGULATION AND FIBRINOLYTIC PROTEINASES.

Some snake venoms have been shown to initiate blood coagulation by activating factor X or prothombin, or by exerting direct thrombin-like activity. Other venoms have factors that

function as anticoagulants by inactivation of thromboplastin, Ac-globulin, prothrombin or by cleavage of fibrin or fibrinogen (see Seegers and Ouyang, 1981 for review) thus rendering blood uncoagulable.

Four fibrinolytic hydrolases, with arginine esterase specificities, have recently been purified from the venom of Trimeresurus mucrosquamatas (Sugihara et al., 1986). Two of these enzymes cleave the B-beta chain of fibrinogen before the A-alpha chain while the other two cleave A-alpha and B-beta chain simultaneously. Naja nigricollis (Spitting Cobra) venom contains proteolytic activity which degrades the A-alpha chain of fibrinogen exclusively and also readily degrades the A-alpha polymer of highly crosslinked fibrin, without apparent cleavage of the B-beta chain or the gamma-dimer fibrin (Evans, 1981). This fibrinogenolytic activity was inhibited by EDTA or 1,10-phenanthroline thus suggesting that it was due to a metalloproteinase. All of the fibrinogenases from Crotalus and Trimeresurus mucrosquamatus venoms consit of single polypeptide chains with molecular masses in the range 22.4-26 kDa, whereas proteinase F1 from Naja nigricollis venom consits of a single polypeptide chain of about 58 kDa.

1.5 ANTICOAGULANTS AND FIBRINOLYTIC MOLECULES IN HAEMATOPHAGOUS INSECTS AND OTHER INVERTEBRATES.

Many arthropods belong to the haematophagous group of bloodsucking insects. It is essential for their survival that they keep their ingested blood meal anticoagulated and that they have the capacity to clear any possible fibrin clots that may

form in parts of the alimentary canal. It has been known for sometime that haematophagous arthropods have some kind of anticoagulant, such as plasminogen activator(s) or fibrinolytic molecules within their salivary glands or gut. Indeed an anticoagulant-like activity was demonstrated by Sabbatani in the tick Ixodes ricinus as far back as 1898. Further work that confirmed the presence of this tick salivary gland anticoagulant was later described (Nuttal and Strickland, 1908; Cornwall and Patton, 1914; Jucci, 1927; and Markwardt and Landmann, 1961). Using whole tick extracts from the tick, Ornithodorus moubata, Hellman and Hawkins (1967) were able to demonstrate that these extracts lysed only unheated bovine fibrin containing plasminogen, but not fibrin in which plasminogen was inactivated by heat denaturation. They also showed that these tick extracts did not lyse purified human fibrinogen unless plasminogen was present, thus suggesting that the ticks secreted some endogeneous plasminogen activator molecule(s), which activated plasminogen into plasmin, within the blood meal.

In 1907 Stulhmann suggested that the salivary glands of tsetse flies might contain anticoagulant molecules. In 1928 Lester and Lloyd showed that when the salivary glands were removed from the living tsetse fly, feeding and digestion continued normally for sometime, but eventually the proboscis and the crop became clogged with clots of blood. They attributed the anticoagulin activity within the salivary glands of tsetse to an unknown antikinase.

Little further work was done until 1966 when Hawkins performed some elegant experiments that gave insight into the

origin of the anticoagulant and fibrinolytic activities of the tsetse fly Glossina austeni. Using the fibrin plate-lysis assay (Christensen and Macleod, 1945), she demonstrated that heated bovine fibrin was lysed by midgut extracts. Thus these extracts contained fibrinolytic molecules. She also demostrated fibrinolytic activity in G. austeni salivary glands extracts but subsequent work by Parker and Mant (1979) suggested that the salivary gland extracts she used for experiments were possibly contaminated by material from the gut. Using partially purified tsetse salivary gland anticoagulant from G.m.morsitans Westwood, Parker and Mant (1979), showed that this anticoagulant prolongs the prothrombin, thrombin, and activated partial thromboplastin times of human plasma. They also demonstrated that this anticoagulant molecule acted as an antithrombin and had no specific anticoagulant activity against any other clotting factors. The same investigators also confirmed the presence of fibrinolytic molecule(s) within the guts of unfed tsetse flies and were able to show that these fibrinolytic activities were absent from salivary gland homogenates which had been dissected free of gut extracts. Recently Gooding and Rolseth (1976), have identified, partially purified and characterized six midgut proteases from G. morsitans morsitans. However, they did not relate these proteases to the fibrinolytic activities which are present in midgut of the flies.

Anticoagulant and fibrinolytic activities of salivary glands are not restricted to haematophagous arthropods. Fibrinolytic activity has been found in extracts prepared from the salivary gland of the giant leech, Haementeria ghilianii.

The activity was found entirely in the cytosol fraction and was associated with only one protein band on paper electrophoresis (Budzynski et al., 1981). From the closely related species, Hirudo medicinalis, chemically pure hirudin, an anticoagulant which has antithrombin effects in vivo, was prepared (see Markwardt 1970 for a review).

1.6 PROJECT PROPOSAL.

From the above reports, it is clear that little attention has been directed towards the exact source of the fibrinolytic activities in the tsetse or to their characterisation. The aim of this project was (1) to develop an assay system and establish optimal conditions for assaying the molecules that are associated with this fibrinolytic activity in G. m. centralis; (2) to purify these fibrinolytic molecules; (3) to characterise their enzymatic activities using synthetic p-nitroanilide and protein substrates, various inhibitors, both of peptide and protein origin; and (4) raise antibodies against purified fibrinolytic molecules and determine if they inhibit in vitro activities of these molecules.

CHAPTER 2

MATERIAL AND METHODS.

2.1 REAGENTS.

All the reagents used in these studies were of analytical grade or better. Alpha$_1$-protease inhibitor, aprotinin, Chromozym-PL, Chromozym-PK, Chromozym-TH, Chromozym-TRY and alpha$_2$-macro globulin were purchased from Boehringer-Mannheim GmbH Biochemica (Sandhofer St., Mannheim, West Germany). Benzamidine, L-cysteine, diisopropyl-flurophosphate (DFP), bovine fibrinogen, dimethylsuberimidate (DMS), dithiothreitol (DTT), 2-mercaptoethanol, iodoacetamide, p-chloromercuribenzoate (PCMB), phenylmethanesulfonyl-fluoride (PMSF), human plasmin, thrombin, tosyllysine chloromethyl ketone (TLCK), tosylphenylalanine chloromethyl ketone (TPCK), were all products of Sigma, Chemical Co. (St. Louis, MO., U.S.A.). Soybean trypsin inhibitor was supplied by Millipore Corporation (Freehold, NJ. U.S.A). Chromatography resins were products of the following companies: CM-52 cellulose and DE-52 cellulose were both obtained from Whatman Ltd. (Maidstone, Kent, U.K.), whereas Sephadex G-25, G-100, G-150, low molecular weight protein markers (SDS-PAGE), protein markers for sizing columns and blue dextran 2000, were all products of Pharmacia Fine Chemicals (Uppsala, Sweden). Ampholines (pH 3.5-10) were obtained from LKB Bromma (Sweden). Acrylamide, bisacrylamide, sodium dodecyl sulphate (SDS) and glycine were purchased from Serva (New York, U.S.A). Freund's complete and incomplete

adjuvants were products of DIFCO laboratories, (Detroit, MI, U.S.A.). All radioisotopes were supplied by Amersham International plc (Buckinghamshire, U.K.). Papain and leupeptin were products of Worthington Biochemical Corporation (Freehold, NJ, U.S.A) and Cambridge Research Biochemicals (Cambridge, U.K.), respectively. The rest of the reagents and chemicals were either products of May and Baker Ltd. (UK), Peninsular Biochemicals, (Belmont, CA., U.S.A.) or as indicated in the text.

2.2 ANIMALS.

Rabbits: Adult half-lop eared New Zealand rabbits supplied by the ILRAD small animal breeding unit, were maintained at the ILRAD tsetse vector laboratory and used in feeding the tsetse flies.

Tsetse flies: Glossina morsitans centralis (G.m. centralis) female flies, which had been fed on rabbits 72 hours earlier, were used in this study. Female flies were chosen because they have a higher activity of midgut proteases (Graf et al., 1986). This appears to be the case because the amount of proteases secreted into the midgut lumen are inducible by feeding and also positively correlate to the size of blood meal ingested. Female flies are known to ingest a lot more blood during feeding than males (Gooding, 1966 and 1975; Brigel and Lea, 1975). Besides the normal nutritional requirements needed by a female fly, part of the blood meal is also used in nourishing the pupa during development.

2.3 ISOLATION OF MIDGUTS AND PREPARATION OF CRUDE TSETSE MIDGUT HOMOGENATES.

Eleven day old G. m. centralis were allowed to ingest a blood meal by feeding on ears of rabbits, after which they were starved for three days. At the end of starvation, they were immobilized by chilling for 30 min at -20°C. The wings were carefully detached from the thorax and the inactive flies were mounted onto a microscope slide for dissection in 0.1 M Tris-HCl buffer, pH 8.5, at 4°C. The abdomens of the flies were opened carefully so as to avoid any possibility of damaging the internal organs. Fat deposits around the midguts were carefully teased away leaving behind a clean midgut. A portion of the gut (Figure 4) stretching from just behind the proventriculus at the foremidgut and before the malpighian tubules, was carefully excised and transferred immediately into chilled 50 mM Tris-HCl, pH 8.5, on ice. Great care was exercised during dissection to avoid any contamination arising from the salivary glands which have been shown to contain an antithrombin-like, blood anticoagulant (Hawkins, 1966; Parker and Mant, 1979).

G.m. centralis crude midgut extract was obtained by a four step procedure. One thousand five hundred midguts were resuspended in 30 ml of 50 mM Tris-HCl, pH 8.5, at 4°C. This suspension was homogenised while on ice using a Dounce ball hand homogenizer (Thomas Scientific, NJ. U.S.A.). The homogenate obtained was then transferred into 5 ml plastic tubes, and passed through three cycles of freezing and thawing using liquid nitrogen (-196°C) and a water bath maintained at

Figure 4. Tsetse (G.m. centralis) digestive system.

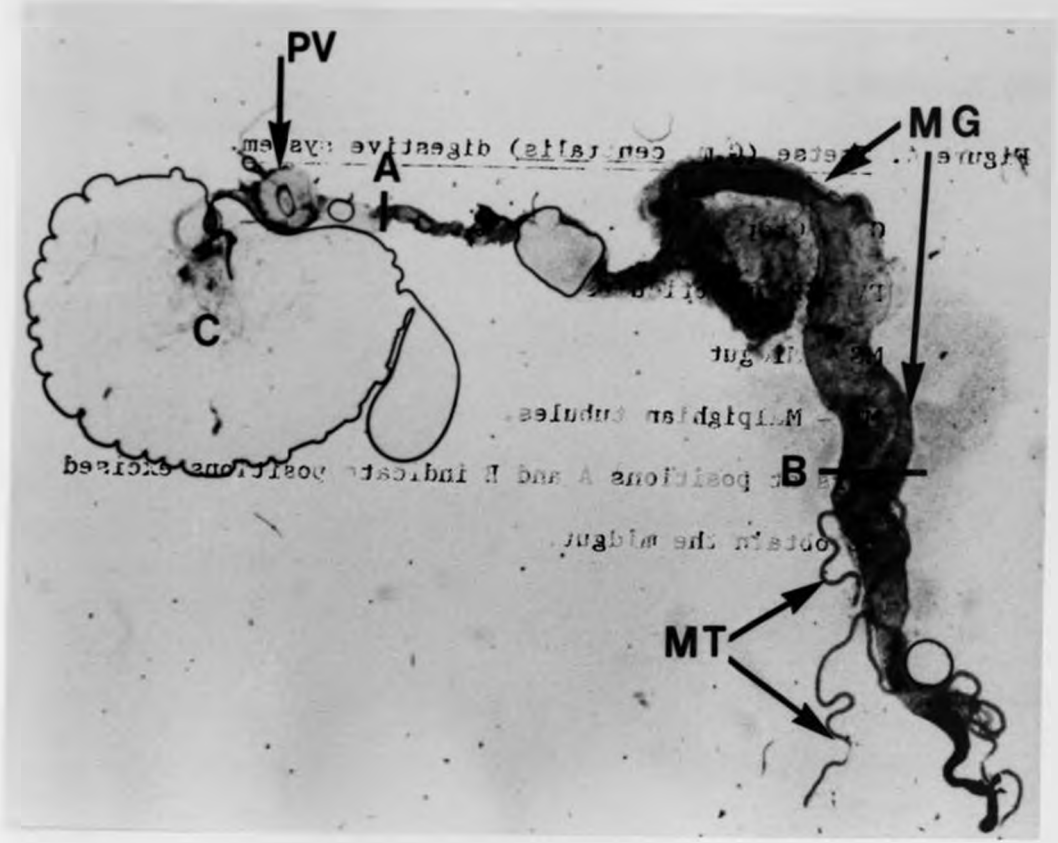
C - Crop

PV - Proventriculus

MG - Midgut

MT - Malpighian tubules.

Bars at positions A and B indicate positions excised to obtain the midgut.


PV
A
MG
C
B
MT

37°C. After freeze-thawing, the homogenate/lysate was subjected to centrifugation at 30,000 rpm for 30 min at 4°C in a Beckman TY65 fixed angle rotor. The supernatant obtained at this stage, which will subsequently be referred to as midgut extract (MGE), was stored at -80°C until needed for further processing.

2.4 ASSAY FOR PROTEASE(S) ACTIVITY IN CRUDE HOMOGENATE AND COLUMN FRACTIONS.

A spectrophotometric assay employing Tosyl-Gly-Pro-Arg-pNA, a chromogenic para-nitroanilide substrate, was used to monitor column fractions. The reaction mixture, in a cuvette, was made up of 2.5 ml 0.1 M Tris-HCl pH 8.0, containing 40 umoles Tosyl-Gly-Pro-Arg-pNA, taken from a stock solution of 1 mM Tosyl-Gly-Pro-Arg-pNA dissolved in 2% dimethylformamide (DMF), and 100 ul of each column fraction to be assayed. The release of para-nitroanilide from this tripeptide substrate was monitored at 405 nm on a Beckmann DU-7 spectrophotometer. The reaction was allowed to proceed for about four minutes after which the change in absorbance per minute (A_{405}) was determined by the inbuilt kinetic programme of the spectrophotometer.

2.5 PROTEIN ESTIMATION.

Most proteins have an absorption spectrum maximum around 280 nm. This was exploited in determining the elution profile of various protein from purification columns. A Beckman DU-7 spectrophotometer was used in all the ultraviolet protein estimation studies.

Protein concentration in crude homogenates and pooled fractions was estimated quantitatively according to the method of Lowry et al. (1951). Commercial bovine serum albumin at a concentration of 1 mg/ml was used in generating a standard curve (Figure 5, see appendix) from which the amount of protein present in test samples could be determined. The assay system utilized folin-phenol reagent diluted in the ratio 1:1 with three times glass distilled water, reagent D prepared by mixing 1% Copper sulphate, 2% sodium potassium tartrate and 2% sodium carbonate solution made up in 0.1 M sodium hydroxide solution in the ratio 1:1:100 (v/v). Protein samples were diluted to 1 ml using distilled water and incubated with 2 ml of reagent D for 30 min at room temperature. Folin-phenol reagent (0.2 ml) was then added and the mixture vortexed thoroughly and left to incubate for a further 30 min at room temperature. At the end of the 30 min period, the optical densities of test and standard samples was read off a Beckman DU-7 spectrophotometer at 700 nm, the wavelength at which the blue colour formed in this reaction absorbs maximally and is least interfered with by other products present within the reaction mixture.

2.6 PREPARATION OF CHROMATOGRAPHY RESINS.

DE-52 cellulose: Four hundred grams of preswollen DE-52 cellulose anion exchanger was gently resuspended in 1.6 litres of 5 mM Tris-HCl, pH 8.0, at 4°C in the cold room. The pH of the suspension was adjusted to 8.0 using concentrated hydrochloric acid. The suspension was then left to settle in the cold. After 2 hours the fine particles were decanted off the settled resin. DE-52 buffer (1.6 litres; 5 mM Tris-HCl, pH 8.0) was added to the slurry, the pH of this was again checked and corrected to 8.0. The process of buffer changes was repeated until the pH of the slurry was constant at 8.0 and, also to a point where no fine particles could be seen in the buffer on the top of the sedimented resin.

CM-52 cellulose: Preswollen CM-52 cellulose cation exchanger was also used. Two hundred grams of the resin was gently resuspended in 800 ml of sodium acetate/acetic acid buffer pH 5.5 at 4°C in the coldroom. The pH of this suspension was adjusted to 5.5 using glacial acetic acid. The suspension was then left to settle in the coldroom for 2 hours and fine particles were aspirated off the settled resin. Two changes of sodium acetate/acetic acid, pH 5.5, buffer (each 800 ml) were used in equilibrating the resin until a constant pH and conductivity were obtained. When this had been achieved a 150 ml bed volume column was set up at 4°C using the equilibrated resin.

Sephadex G-100: Ten grams of Sephadex G-100 beads of particle size 40-120 microns was suspended in 400 ml of 50 mM Tris-HCl/0.2 M NaCl, pH 7.5. This suspension was allowed to equilibrate and swell overnight in a waterbath maintained at 40°C. Fine particles were aspirated off with the aid of a water pump. The resin was resuspended in the buffer in the ratio of 1:3 (v/v) of buffer to resin. A 200 ml bed volume column of 100 cm length and 1.6 cm internal diameter was set up in the cold room using this resin.

The void volume of this column was approximated by applying 1 mg of blue dextran 2000 dissolved in 3 ml of 50 mM Tris-HCl/0.2 M NaCl pH 7.8. The column was run at a flow rate of 18 ml/hr and 2 ml fractions were collected. For further calibration of this column, low molecular weight protein markers were loaded onto the column in two separate lots: 20 mg of bovine serum albumin (Mr 67 kDa) and 5 mg chymotrypsinogen-A (Mr 25 kDa) were dissolved in 3 ml of running buffer and resolved on this column at a flow rate of 18 ml/hr. Eighteen milligrams of ovalbumin (Mr 43 kDa) and 18 mg of ribonuclease-A (Mr 13.7 kDa) were dissolved in 3 ml of buffer and resolved on the column under the same conditions. Pooled fractions (P3 and P4) recovered from isoelectric focusing, as described below, were either frozen immediately for later experiments or dialysed overnight against 3 litres of 0.1 M Tris-HCl/0.2 M NaCl pH 8.0 and 3 ml of each of the two enzymes were loaded independently onto the G-100 column for size determination. The column was run under the same conditons as for the standard protein markers.

Isoelectric focusing: A pH gradient, 3.5-10 was generated using an LKB 110 ml column with a 5 to 50% (w/v) sucrose gradient containing 2.5% (v/v) LKB ampholines. The anode solution consisted of 15 g of sucrose, 12 ml glass distilled water and 4 ml 1 M orthophosphoric acid. The cathode solution was made up of 2.5 ml 1 M NaOH solution and 7.5 ml glass distilled water. All solutions were degassed before pouring into the focusing column. Each gradient chamber contained 53 ml solution. The enzyme was prepared as part of the high sucrose gradient mixture. The column was subjected to a constant voltage of 1600 for a period of at least 19 hours at 4^{0}C.

2.7 ENZYME PURIFICATION.

All steps in the purification of tsetse midgut enzymes were carried out at 4^{o}C unless otherwise stated. Because the proteases were easily detectable using the amidase hydrolytic activity of Tosyl-Gly-Pro-Arg-pNA, this substrate was used throughout the isolation procedures. Column fractions at each step were assayed for activity and enzyme units of activity were expressed as described by Erlanger et al. (1961).

A 300 ml bed volume Pharmacia column, (2.6 X 75 cm) was packed with DE-52 cellulose resin and equilibrated overnight at a flow of 24 ml/hr in 5 mM Tris-HCl buffer pH 8.0. The tsetse MGE supernatant (7.6 ml), dialysed against column equilibrating buffer (5 mM Tris-HCl, pH 8.0), was loaded onto the column using an LKB peristalic pump at the same flow rate as that used for equilibrating the column. The column was washed extensively with equilibrating buffer until the protein

absorbance at 280 nm was almost equal to that of the buffer blank. A salt gradient (0 - 0.15 M NaCl, 400 ml) was used to elute the enzymes. The fractions containing activity against Tosyl-Gly-Pro-Arg-pNA were collected and dialysed in Spectrapor-3 membranes against two 6 litre changes of 5 mM Tris-HCl pH 8.0 and either stored at -80°C or used immediately for further purification by isoeletric focusing.

2.8 ELECTROPHORESIS, STAINING AND FLUOROGRAPHY.

The preparations described in this study were analysed on discontinous polyacrylamide slab gel system according to the method by Laemmli (1970). The acrylamide solutions used for all gels contained acrylamide: bis acrylamide in the ratio of 30: 1. The mixtures were degassed and polymerisation initiated by the addition of 10% ammonium persulphate and N, N, N', N', tetramethylethylenediamine (Bio-Rad, California, USA). A 3% stacking gel was cast on top of the 5-20% acrylamide gradient resolving gel two to three hours before use. Sample wells were obtained by using a comb made of Plexi glass. The electrophoresis buffer was 25 mM Tris-HCl, 192 mM glycine and 0.1% SDS, pH 8.3.

For samples ran under reducing conditions, experimental samples and molecular mass markers were boiled on a waterbath for 5 mins in 92.5 mM Tris-HCl sample buffer, pH 6.8, containing 2.5% SDS, 5% 2-mercaptoethanol or 5% dithiothreitol (DTT), 20% glycerol and 0.02% bromophenol blue.

Table 1. Staining Procedure for the silver-based gel electrophoresis colour development system.

Steps		Solutions	Duration of agitation.
1.	Fix	50% EtOH, 10% HOAC	2 hr or more
2.	Wash	50% EtOH, 10% HOAC	2 hr
		25% EtOH, 10% HOAC	1 hr 2x
		10% EtOH, 0.5% HOAC	1 hr 2x
3.	Equilibrate gel	$AgNO_3$ (1.9 g/l)	2 hr or less
4.	Rinse	H_2O	10-20 sec.
5.	Reduce Silver	$NaBH_4$ (87.5 mg/l)	10 min or les
		HCHO (7.5 ml/l) in 0.75 N NaOH	
6.	Enhance Colour	Na_2CO_3 (7.5 g/l)	1 hr, store

Electrophoresis was done for about 4 hours at a constant current of 30 mA or 40 mA per gel until the bromophenol blue tracking dye just migrated to the bottom of the gel. At the end of electrophoresis, gels were removed from the glass plates. The gels were stained by either Coomassie brilliant blue R-250 or silver stain (Sammons et al., 1981).

Staining: For Coomassie staining, the gels were incubated, with gentle shaking, in a solution containing 25% (v/v) methanol, 10% (v/v) acetic acid and 0.025% (w/v) Coomassie brilliant blue R-250 in distilled water for several hours (4 hours to overnight). Destaining was achieved by shaking the gel gently in several changes of 10% (v/v) acetic acid in distilled water.

The silver staining procedure described here is a modification of a method developed by Sammons et al. (1981). The principle of the method is based upon the complexing of silver with "proteins reactive centres" in polyacrylamide gels. This method is about fifty-fold more sensitive than are techniques employing Coomassie blue. The glass plates used for casting gels were washed thoroughly in detergent, rinsed with water, finally rinsed with 100% ethanol, and air dried. Gloves were worn throughout the preparations. The steps taken following removal of gel from the electrophoretic plates were all carried out at room temperature on a shaker to ensure thorough mixing as summarised in table 1. Extensive washing (step 2) in Pyrex trays was done to remove SDS.

The gel was equilibrated with silver nitrate solution and then rinsed briefly in filtered double-distilled water to remove excess surface silver before placement into a reducing solution made up of sodium borohydride, sodium hydroxide and formaldehyde. The formaldehyde was added to the developing solution mixture immediately before the developing reaction was started. When the bands were sufficiently stained the gel was either equilibrated by two changes of an enhancing solution, made up of sodium carbonate. The sodium carbonate was necessary for optimal colour development. The gel was either stored in 7.5% sodium carbonate solution or dried.

The molecular masses of the protein bands on the gels were estimated by comparing the relative mobilities of the unknown proteins with known protein standards. The protein standards used were: Phosphorylase b (Mr 94 kDa), bovine serum albumin (Mr 67 kDa), ovalbumin (Mr 43 kDa), carbonic anhydrase (Mr 30 kDa), soybean trypsin inhibitor (20 kDa) and lactalbumin (Mr 14.4 kDa).

Fluorography: Fluorography of SDS-polyacrylamide gels containing tritium-labelled proteins was performed by equilibrating the gel for at least 1 hour in 60 ml of a fixing solution containing 10% (v/v) glacial acetic acid and 30% (v/v) methanol. The gel was then incubated in EN^{3}HANCE (New England Nuclear) for 1 hour. Following impregnation with enhancer, cold water was added to the tray containing the gel in order to precipitate the fluorescent material within the gel. The precipitation procedure was carried out for only 30

min. The gel was then dried under vacuum on a slab gel drier for 2 hours. Detection of labelled protein was achieved by exposure of the dried gel to a Fuji X-ray film for 4 days at -80°C. The film was developed as described under section 2.10. Approximately 1.5 x 10^5 cpm were loaded per lane for each of ^{3}H-aprotinin-enzyme complex to be analysed.

2.9 PRODUCTION OF ANTIBODIES, PURIFICATION OF IgG AND PREPARATION OF Fab FRAGMENTS.

Two adult rabbits, ranging in weight from 3.5-5.0 kg, were selected for immunization. Prior to immunizations, 50 ml of pre-immune sera were prepared from blood collected from the peripheral ear vein of each rabbit. Immunizations were performed by injecting various amounts of purified heat-inactivated tsetse fibrinolysin, P4, which had been thoroughly emulsified in complete Freund's (DIFCO) adjuvant in the ratio 1:1 (v/v). The first injections (40 ug), antigen were given at three different sites; intradermally on the neck and intramuscularly on both the gluteal muscles. After three weeks, a booster injections (20 ug) was given to each rabbit several times in the same manner in incomplete Freund's adjuvant and again after a further 14 days a 10 ug booster injection was administered. Rabbits were then bled one week after the second booster.

IgG preparation: Antibodies from immune and normal serum were purified by a combination of ion-exchange chromatography on

DEAE cellulose (DE-52 cellulose) and Sephadex G-150. The IgG was purified to remove proteases, complement proteins and other serum components which would interfere with the clotting assay, fibrin(ogen) lysis assay and western blotting analyses.

Serum was dialysed using Spectrapor-2 dialysis tubing (a Mr cut-off 8-10 kDa) at room temperature against three, one litre changes of 10 mM potassium phosphate buffer pH 7.5 until the conductivity of the sample was equal to that of the fresh equilibrating buffer. The dialysed sample was applied to a column of DE-52 (90 ml DEAE-cellulose resin per 20 ml serum) equilibrated with 10 mM potassium phosphate pH 7.5 (conductivity 1-1.5 mu) at room temperature. The antibody activity, which was largely associated with IgG, appeared in the column effluent. Under these conditions, most of the other serum proteins were retained on the DE-52 cellulose resin.

The pooled IgG fractions were concentrated to 10 mg/ml using PM 30 Amicon membranes. This was then dialysed against 3 litres of 50 mM Tris-HCl, 0.2 M NaCl pH 7.6. The dialysate was filtered through a Sephadex G-150 column previously equilibrated with the dialysis buffer. The IgG fractions recovered from the gel filtration column were concentrated to 10 mg/ml using PM 30 Amicon membrane and then dialysed aganst 3 litres of 0.1 M potassium phosphate buffer pH 7.0.

Preparation of Fab fragments: The method described by Porter (1959) was employed in the preparation of Fab fragments. One hundred milligrams of both normal and immune rabbit IgG (10 mg/ml) obtained from the Sephadex G-150 step were dialysed for

24 hours against 0.1 M potassium phosphate buffer pH 7.0, using Spectrapor-2 membranes. Seventeen milligrams of L-cysteine monohydrate and 7.5 mg ethylenediaminetetra-acetic acid (EDTA) disodium salt, were added to each of the immune IgG and normal rabbit IgG so as to bring the final concentrations of L-cysteine monohydrate to 10 mM and EDTA to 2 mM in each reaction mixture of 10ml. Cleavage of IgG into Fab and Fc fragments was started by adding into each reaction mixture 1.0 mg of papain. The reaction mixture was incubated in a controlled environment incubator shaker (New Brunswick Scientific Co. Inc. New Brunswick, NJ, U.S.A.) for 20 hours at 37^{o}C. At the end of the 20 hour incubation, the digest was put in Spectrapor-3 (Mr cut off 2.8-3.8 kDa) dialysis tubing and dialysed against 0.154 M NaCl for 24 hours with two changes, each consisting of three litres.

A 150 ml CM-52 cellulose column (50 x 2.4 cm) was equilibrated with 0.1 M sodium acetate/acetic acid buffer, pH 5.5, for 12 hours at a flow rate of 60 ml/hour. The IgG digest was also equilibrated against the same buffer by dialysis for 12 hours with 2 changes, each of 5 litres. The digest was then loaded onto the CM-52 cellulose and the column was run at a flow rate of 60 ml/hour using equilibrating buffer while 10 ml fractions were collected. Fab fragments were in the column effluent fractions whereas Fc fragments, which bound onto the resin under these condition, were eluted using a gradient starting from 0.1 M sodium acetate/acetic acid pH 5.5 to 0.9 M sodium acetate/acetic acid, pH 5.5. The gradient was made up

of 500 ml buffer in each chamber. The presence of Fab fragments in the pooled fall through fractions was confirmed by precipitin lines seen on radial immuno-diffussion (Ouchterlony) on 1% agarose petri dishes using sheep anti-rabbit IgG.

2.10 IMMUNOCHEMICAL METHODS USED FOR DETECTION OF ANTIBODIES.

Ouchterlony's double immunodiffusion: Double immunodiffusion analyses were carried out according to the method described by Ouchterlony (1949). The procedure was performed in precoated petri dishes using 1% (w/v) solidified agarose gels containing 0.04% sodium azide in 0.1 M sodium barbitone, pH 8.0. The agar was poured when still warm and allowed to solidify by cooling. Six peripheral wells and one central well, each 2 mm in diameter, were cut out in the solidified agarose using an LKB well template. Antigen antibody immunoprecipitates were allowed to form in a moist chamber, after which the gels were extensively washed with 0.1 M PBS and then stained with 0.01% Coomassie brilliant blue R-250. Destaining was achieved using 10% acetic acid when necessary. The plates were then dried after destaining. This method was used to identify antibodies in rabbit immune sera and also in locating the Fab fragments eluted from the CM-52 celllulose column (section 2.8), according to the scheme described in the paragraph below.

Twenty five microlitres samples, equivalent to 5 ug of purified antigen (enzyme inactivated by boiling for 10 min), were put in the central well. Twenty five microlitres of serially diluted (i.e. neat, 1/2, 1/4, 1/8, 1/16, 1/32) Fab,

IgG or serum was put in the six peripheral wells. Diffusion in gels was allowed to take place in a humid chamber at room temperature for up to 48 hours. Because of the differences in sizes of antigen and antibody molecules, antibody was allowed to diffuse for approximately 3 hours before the antigen was put in the central well.

After the precipitin lines of antigen-antibody complexes had developed sufficiently, the gels were thoroughly washed with 5 changes of 0.9% saline or 0.1 M PBS to remove the unprecipitated proteins and protein/antibody complexes before staining.

Western Immunoblotting: For western blotting, protein samples were subjected to electrophoresis on a 5 to 20% SDS-polyacrylamide slab gel as already described in section 2.8. Protein transfer to nitrocellulose paper was performed using a Bio-Rad Trans Blot electrophoretic transfer cell apparatus, which was used according to the manufacturer's instructions. Before transfer, sponge/fibre pads and filter paper (Whatmann 3 MM chromatographic paper cut to the same dimensions as the pads), were saturated with transfer buffer (25 mM Tris, 192 mM glycine, pH 8.3, 20% [v/v] methanol). The gel and nitrocellulose membranes (pore size; 0.45 microns) were also equilibrated in the transfer buffer. Methanol was used to improve binding of SDS-proteins onto nitrocellulose filters.

After equilibration, the gel for transfer was placed between two sheets of pre-wetted Whatman 3 MM filter paper, which were in turn sandwiched between supportive porous foam

pads and sponges. Pre-equilibrated nitrocellulose filter paper was then carefully layered onto the gel. Since air bubbles interfere with the transfer of protein molecules by creating local points of high resistance within the nitrocellulose filter matrix, special care was taken to remove air bubbles between the gel and nitrocellulose filter by rolling a glass pipette on top of the membrane gel surface. The sandwich was then placed in the gel holder and submerged into the slot in a Transblot tank containing 4 litres of chilled ($4^{o}C$) transfer buffer. Since the proteins from the SDS-polyacrylamide gels have a net negative charge, they were eluted as anions towards the anode. The sandwich was placed in the Transblot tank in such a manner that the nitrocellulose filter paper faced the anode and the gel faced the cathode. A magnetic stirrer was used to improve heat exchange. Tranfer was performed in the cold at 75 volts (1 amp) for 3 hours. The nitrocellulose filter was then briefly rinsed in 0.1 M Tris-saline before incubation for 1 hour at room temperature in a tray containing 5% (w/v) milk powder in 10 mM Tris, 0.9% NaCl pH 7.8, to saturate the free protein binding sites and also minimize background effects. The transfered proteins were reacted with the complimentary sera or purified antibody diluted in freshly prepared milk powder-Tris-saline containing 0.04% sodium azide. The incubations were performed for a few hours or overnight on a shaker at room temperature. This was followed by three 15 min washes in Tris-saline, pH 7.8, at room temperature. The nitrocellulose filter papers were then washed through two changes of 15 min each in 0.05% NP-40 in

Tris-saline buffer pH at 7.8 to remove any unreacted antibody.

The blot was then incubated in fresh 5% milk powder, 10 mM Tris-0.9% NaCl containing 20 microcuries of ^{125}I-labelled protein - A ($1\text{-}5\times10^{5}$ cpm/ml) for 1 hour at room temperature. The blot was passed through the same washing procedure as described above to remove non-specifically bound and excess labelled probe. After this step the filter was dried between two sheets of Whatman 3 MM filter paper, sealed in a plastic bag and exposed to a Fuji X-ray fast film with an intensifying screen at room temperature. The autoradiogram was developed using Kodak KLX developer and Kodak fixer. Exposure times varied from overnight to 7 days.

2.11 PREPARATION OF TRITIUM-LABELLED APROTININ AND ^{3}H-APROTININ-ENZYME COMPLEX.

Aprotinin labelling: Two millicurie in 2 ml of toluene of N-Succinimidyl (2, 3-^{3}H) propionate was carefully transferred into a 5 ml Wheaton vial on an ice bath at 0^{o}C. Into this was directed a gentle flow of dry nitrogen (dried by blowing it through a trap of anhydrous calcium chloride) to evaporate away the carrier solvent, toluene. The free radioisotope and toluene were adsorbed to charcoal contained in a fine charcoal trap connected in series with the drying apparatus. Dry nitrogen was also used to eliminate any moisture present which would hydrolyse the active sites of the N-succinimidyl (2, 3-^{3}H) propionate.

After evaporating the toluene, the radioisotope was mixed with 2.8 ml of a 1 mg/ml solution of aprotinin in 0.1 M phosphate buffered saline, pH 6.8. The mixture was left to react, with occassional shaking, at room temperature for a period of between 15 to 30 min. At this pH the N-terminal amino acid residues of aprotinin remain unprotonated and are thus able to donate lone pair nitrogen electrons to form an amide bond with the carbonyl group of propionic acid. We thus preferentially labelled the N-terminal end amino acid residues of aprotinin. Labelling at higher pH would have inactivated the inhibitor by binding through the epsilon amino group of lysine which constitutes part of the protease binding domain.

Free radioisotope was separated from tritium-labelled aprotinin (^{3}H-Aprotinin) using a 30 ml bed volume column of Fine Sephadex G-25. The 2.8 ml ^{3}H-aprotinin and free radioisotope mixture was loaded onto the column and eluted at 60 ml/hr using 0.1 M PBS/0.25 M NaCl pH 6.8. Three millilitre fractions were collected and aliquots counted using a Beckman LS 6800 beta liquid scintillation counter. Fractions in the void volume of the column, which had the highest activity, were pooled (6 ml) and dialysed using Spectrapor-3 tubing against 0.1 M PBS pH 6.8 and then against two x 3 litre changes of H_2O to further remove any free tritium counts which might not have been separated from ^{3}H-aprotinin by the Sephadex G-25 step. The final dialysis against distilled water was necessary to remove PBS salt from ^{3}H-aprotinin. The ^{3}H-aprotinin sample was frozen at -80^{o}C and lyophilized using Labconco freeze dryer Model 18. Powdered ^{3}H-aprotinin was then

recontituted in 3.0 ml of 0.1 M Tris-HCl, pH 8.0, a smaller volume which allowed for increased activity of radioisotope per ml. The specific activity of ^{3}H-aprotinin was calculated to be 2.1 x 10^{10} cpm/ug of protein. The ^{3}H-labelled aprotinin was aliquoted into 250 ul vials and stored at -80°C until needed for enzyme-aprotinin complex preparations.

Enzyme inhibitor complex formation: The amidase activity of tsetse proteases was assayed with Tosyl-Gly-Pro-Arg-pNA as substrate using a method similar to that described by Erlanger et al. (1961). One hundred microlitres of each enzyme solution, appropriately diluted to have equal activity against the substrate, was incubated with 25 ug of ^{3}H-aprotinin at 37°C for 30 min to allow for ^{3}H-aprotinin-enzyme complex formation. Purified tsetse midgut proteases, refered to as P1, P2, P3, P4, P5, or total MGE tsetse proteases were used individually in the preparation of ^{3}H-aprotinin-enzyme complex. For each enzyme preparation 77 mu of enzyme activity towards Tosyl-Gly-Pro-Arg-pNA were used to form enzyme-inhibitor complexes. One Tosyl-Gly-Pro-Arg-pNA unit of activity was defined as $\Delta A_{405nm/min}$ X 1000 X 2.8 x $(8800)^{-1}$ at pH 8.0 where 8800 is the extinction coefficient of p-nitroanilide, Erlanger et al. (1961). After the 30 min incubation period, each fraction was assayed for residual protease activity using the same substrate and if any was detected, more ^{3}H-aprotinin was added and the incubation continued. If no residual hydrolytic activity was present, 59 micrograms of solid dimethylsuberimidate (DMS) (aprotinin: DMS

w/w 1:2.4) was added to each ^{3}H-aprotinin-enzyme complex mixture. This was done to ensure formation of stable complexes between ^{3}H-aprotinin-enzyme by cross linking the complex. The ^{3}H-aprotinin-enzyme-DMS mixture was mixed on a roller for one hour and then aliquoted and stored at -80^{o}C until needed.

2.12 LABELLING OF FIBRINOGEN WITH (N-SUCCINIMIDYL 3-[4-HYDROXY-3, 5-^{125}I DIIODOPHENYL] PROPIONATE).

The procedure used in the preparation of ^{125}I-fibrinogen was essentially the same as the one described for ^{3}H-aprotinin preparation under section 2.11 . Labelling by this reagent essentially involves conjugation to protein amino groups via an active ester of the Bolton-Hunter reagent.

Four milligrams of bovine fibrinogen, whose purity had been confirmed on a sizing TSK 250/125 HPLC column, was dissolved in 2 ml of 0.1 M PBS/0.25 M NaCl, pH 6.8. This was mixed with 1.5 mCi of radioisotope and left to incubate on ice for 15-30 min with occassional agitation. At the end of incubation, free radio-isotope was separated from labelled fibrinogen using the method described under section 2.11. The dialysis step of ^{125}I-fibrinogen employed 0.1 M Tris-HCl/0.25 M NaCl, pH 8.0, as buffer, since this was the reaction buffer used in the assays described below. The labelled material (5 x 10^{5} cpm/microgram of protein) was aliquoted into 500 ul portions and stored at -20^{o}C until required.

2.13 ^{125}I-FIBRIN CLOT DIGESTION ASSAYS.

A soft ^{125}I-fibrin clot (i.e. one that had not been cross-linked by factor XIIIa) was obtained by mixing 200-300ul, 2.5 mg/ml unlabelled fibrinogen with 10-32 ul ^{125}I-fibrinogen and 0.17 clotting units of bovine thrombin in desired ratios and incubated at room temperature for 10-15 min. The digestion of the clot was assayed by a single time point assay, a time course assay, and a dose response assay.

Time point assay for ^{125}I-fibrin clot disolution: All purified tsetse midgut proteases obtained by a combination of DE-52 cellulose and isoelectric focusing procedures were used in this assay. An equal number of units (80 mu), of each protease fraction determined by Tosyl-Gly-Pro-Arg-pNA as described under section (2.11), was used per assay. For each protease, the assay was done in quadruplicate. Prior to the formation of the clot, 200 ul of unlabelled bovine fibrinogen (stock 2.5 mg/ml) was mixed with 10 ul of ^{125}I-fibrinogen. The mixture was vortexed and clotting initiated by addition of 0.17 clotting units of bovine thrombin and left to set for 10-15 min. The clot was then dislodged from the sides of the eppendorf tube, and pelleted by centrifugation in an Eppendorf microfuge at 12,000 rpm for five min. The supernatant was discarded into an appropriate radioactive waste collection bin and the pellet was processed through two cycles of washing and centrifugation each of 400 ul of 0.1 M Tris-HCl/0.25 M NaCl pH 8.0 each time. This extensive washing was essential for

removal of any unclotted substrate which would interfere with subsequent enzymatic clot dissolution reactions. After the last wash, the clot was drained for 15 min by inverting the Eppendorf tubes on top of layers of soft adsorbent tissue paper.

Clot solubilization was initiated by adding 80 milli-units of enzyme to each tube and then standardizing the reaction volume in each assay mixture to 200 ul by adding an appropriate amount of reaction buffer, 0.1 M Tris-HCl/0.25 M NaCl, pH 8.0. The mixture was vortexed thoroughly before incubation for two hours at room temperature. At the end of the incubation period, the residual (undigested) clot was pelleted by centrifugation at 12000 rpm in an Eppendorf microfuge for 10 min. In order to find out which of the purified tsetse midgut proteases released the highest amount of ^{125}I-fibrinopeptides, 20 ul of supernatant was carefully pipetted out from each assay mixture, without disturbing the pellet, and counted on a Packard Auto-Gamma Spectrometer. The control for this assay was made up of essentially the same reagents except the enzyme was substituted for by adding 20 ul of reaction buffer to each of the control assay mixtures.

Time course assay of ^{125}I-fibrin clot digestion: This assay was set up in the same manner as that described for the single time point assay above. The volumes of the various reagents used in this assay were also kept the same. The major difference was that various periods of incubation (15, 30, 60, 90, 120 and 180 min) were chosen as time points for each of the four tsetse enzymes used. Each time point was tested in

quadruplicate for each of the four tsetse enzymes (P2, P3 and P4 and P5) used. Again, at the end of each time point the incubation mixture was centrifuged in an Eppendorf microfuge at 12000 rpm for 5 min to pellet any undigested residual clot. Twenty microlitres aliquots of the supernatant were counted in quadruplicate for each time point.

Enzyme dose response assay: Two tsetse proteases, fibrinolysin P3 and fibrinolysin P4 were used in this assay. These enzymes were selected because they had been demonstrated, in the last two assays, to solubilize fibrin clots more efficiently for any given period of time, than any of the other tsetse MGE proteases. The experimental set up for this assay was essentially the same as that outlined in the foregoing two sections. The only differences are reflected in the volumes of some reagents used per assay to accommodate the variation of enzyme dose. The assay mixture was made up of 300 ul of 2.5 mg/ml unlabelled bovine fibrinogen, 10 ul of ^{125}I-fibrinogen and to this was added 0.25 units of bovine thrombin. The mixture was vortexed and left to incubate at 37^{o}C for 15 min to allow clot formation. The clot was dislodged from the sides of the incubation vials, subjected to washings and centrifugation as already decribed. Having removed excess buffer from the clot by inverting the Eppendorf tubes over soft adsorbent tissue paper, the fibrin clot digestion reaction was initiated by adding 0, 40, 80, 160 and 320 milliunits of either fibrinolysin P3 or fibrinolysin P4. The reaction was allowed to proceed for 1 hour at 37^{o}C after which each incubation

mixture was centrifuged at 12000 rpm for 10 min. Each experiment was done in triplicate and each test counted in triplicate. The rest of the supernatant was discarded, leaving an undigested pellet of the fibrin clot which was treated as follows: Each pellet was washed twice in 200 ul of 1 N HCl, in order to stop any further clot digestion by the enzymes. After the second washing step, the clot was dissolved in 200 ul of 0.1 M Tris-HCl/0.25 M NaCl pH 8.0, containing 2.5% SDS. This was vortexed vigorously to resuspend the clot and 20 ul of each solution mixture was counted, this last step enabled the determination of counts left behind in the residual fibrin clot after enzyme digestion.

2.14 SDS-PAGE ANALYSIS OF PEPTIDES FROM FIBRINOGEN AND FIBRIN.

An assay of the soluble products generated by ^{125}I-fibrin clot lysis by various tsetse enzymes and plasmin was developed. Three hundred microlitres of a 2.5 mg/ml bovine fibrinogen solution was mixed with 0.17 units of bovine thrombin and left to incubate for 15 min. The resultant fibrin clot was pelleted by centrifugation at 12,000 rpm for 10 min in an Eppendorf microfuge. The pellet was resuspended to an appropriate volume using 0.1 M Tris-HCl/0.25 M NaCl pH 8.0. To initiate the fibrin clot lysis, 160 milliunits of each tsetse protease or plasmin was added to each suspension mixture. To facilitate a higher release of polypeptide products from the fibrin clot by these enzymes, longer incubation times (up to 4 hr) were used. The fibrin clot-lysing reaction was stopped at

the end of each time point by adding 150 ul of 4 mM benzamidine into each incubation mixture for the tsetse proteases and by adding 150 ul of 4 mM leupeptin to the plasmin incubation mixture. Benzamidine was used for stopping the fibrinolysin reactions since it had been shown to be a very efficient inhibitor of these two proteases (Ki ranging from 20-30 uM, see section 3.9). Because benzamidine was not a very efficient inhibitor of plasmin, leupeptin was used since it was found to be a potent inhibitor of this enzyme in pilot studies with chromogenic substrates (unpublished observations). Incubation mixtures were centrifuged at 12,000 rpm for 5 min in an Eppendorf microfuge. The supernatant which contained the lysis products generated from fibrin by the enzymes was concentrated by drying under vaccum in a Speed Vac microfuge concentrator (Savant Instruments, Hicksville, NY, U.S.A.) maintained at 42^{o}C. These samples were reconstituted in 100 ul of distilled water and stored at -80^{o}C until needed for analysis by SDS-PAGE.

An assay, which employed excess unlabelled bovine fibrinogen and only small amounts of ^{125}I-fibrinogen as tracer, was developed to analyse the cleavage products generated by the two purified tsetse fibrinolysins, and to compare these products with those obtained when plasmin, a classical fibrinolytic enzyme, was used to cleave fibrinogen.

Four hundred microlitres of 2.5 mg/ml unlabelled bovine fibrinogen, was used for each test. To this was added 32 ul of ^{125}I-fibrinogen. The fibrinogen digestion reaction was started by adding 160 milliunits of either fibrinolysin P3,

fibrinolysin P4 or plasmin. Each experiment comprised seven time points between 0 and 180 min. At the end of each incubation time, the reaction was terminated by adding 150 ul of 4 mM benzamidine for the tsetse fibrinolysins and 150 ul of 4 mM leupeptin for plasmin. After stopping the reaction all test mixtures, which had a total volume of 700 ul, were frozen immediately at -20°C, and only taken out when they were to be further analysed on SDS-PAGE.

2.15 KINETICS OF HYDROLYSIS OF PARA-NITROANILIDES BY FIBRINOLYSINS.

The 16 p-nitroanilide substrates listed below (see also table 4) were used in studying the amidase hydrolytic specificities of the two purified tsetse fibrinolysins as compared to bovine plasmin. A stock concentration of 4 mM for each substrate was obtained by dissolving the individual substrate in dimethylformamide (DMF) and then diluting this to a concentration of 5% DMF using 0.1 M Tris-HCl buffer pH 8.0. The substrates used in this study were:-

a) Tosyl-L-Gly-L-Pro-L-Arg-pNA (chromozym-TH)

b) Tosyl-L-Gly-L-Pro-L-Lys-pNA (chromozym-PL)

c) Benzoyl-L-Pro-L-Phe-L-Arg-pNA (chromozym-PK)

d) Carbobenzoxy-L-Val-L-Gly-L-Arg-pNA (Chromozym-TRY)

e) N alpha-Carbobenzoxy-L-Arg-pNA

f) N alpha-Carbobenzoxy-L-Tyr-L-Lys-L-Arg-pNA

g) D-Val-L-Leu-L-Lys-pNA

h) N-Acetyl-L-Phe-L-Gly-pNA

i) Carbobenzoxy-L-Arg-L-Arg-pNA

j) L-Arg-pNA

k) Acetyl-L-Arg-pNA

l) Benzoyl-L-Phe-L-Val-L-Arg-pNA

m) Benzoyl-L-Arg-pNA

n) H-D-Ile-L-Phe-L-Lys-pNA

o) Acetyl-L-Lys-pNA.

i) Benzoyl-L-Try-pNA

Each substrate was assayed in quadruplicate. The assay volume was made up of 2.5 ml 0.1 M Tris-HCl buffer pH 8.0 and 25 ul (40 mmoles) of substrate. Hydrolysis was started by adding 10 ul (26 milliunits) of purified enzyme. Substrate hydrolysis was monitored at 405 nm for 3 min. One Tosyl-Gly-Pro-Arg-pNA unit of activity was defined as ΔA_{405} X 1000 x 2.8 x $(8800)^{-1}$ at pH 8.0.

For more detailed analyses, the best substrate, Tosyl-Gly-Pro-Arg-pNA, was subjected to dose-response and Michaelis-Menten analyses. For the dose response study the substrate stock solution (1 mM) was prepared by dissolving 20 mg of Tosyl-Gly-Pro-Arg-pNA in 1.0 ml of DMF and adjusting this to the required concentration using 0.1 M Tris-HCl pH 8.0. Stock enzyme solutions were made in the same buffer at 1.3 ug/ml for fibrinolysin P3 and 1.65 ug/ml for fibrinolysin P4. Two hundred microlitres of substrate was added to 2.3 ml of buffer in a cuvette. The mixture was allowed to equilibrate at 37°C for 5 min in a thermostatic peltier controlled kinetics accessory sample holder. Varying concentrations of enzymes ranging from 1.3 to 10.4 ng for fibrinolysin P3 and from 0.825

to 6.6 ng for fibrinolysin P4 were added, and the reaction was allowed to proceed for 5 min. Each point was tested in triplicate.

For the Michaelis-Menten analysis, substrate stock solutions were prepared by diluting to 2.5 ml an initial stock of 4 mM Tosyl-Gly-Pro-Arg-pNA solution appropriately to final concentrations ranging from 0.375 to 96.0 uM. The kinetics of Tosyl-Gly-Pro-Arg-pNA amidase reactions at 37°C, were determined in triplicate by establishing the rates of hydrolysis for each substrate concentration. Before addition of enzyme, the substrate and buffer mixtures were allowed to equilibrate for 5 min at 37°C. The hydrolysis for each substrate concentration was initiated by adding about 10 ng (10 ul) of either fibrinolysin P3 or P4 and allowing the reaction to proceed for 2 min. The initial rates were obtained using an inbuilt programme which calculates the rates and analyses them on the basis of linear regression.

2.16 INHIBITION OF FIBRINOLYSINS.

A series of protease inhibitors were tested against the two purified tsetse fibrinolysins. The inhibitors included: diisopropylfluorophosphate (DFP), tosyllysine chloromethylketone (TLCK), benzamidine, tosylphenylalanine chloromethylketone (TPCK), phenylmethanesulfonylfluoride (PMSF), p-chloromercuric benzoate (PCMB), iodoacetamide, soybean trypsin inhibitor (SBTI), aprotinin and $alpha_1$-protease inhibitor ($alpha_1$-PI). The protein inhibitors (SBTI, aprotinin and $alpha_1$-PI) were each

prepared at a stock concentration of 2 mg/ml in 0.1 M Tris-HCl buffer pH 8.0, while the rest were prepared at stock concentrations ranging from 2-10 mM by first dissolving the salts in the appproriate amount of DMF and then diluting in 0.1 M Tris-HCl pH 8.0 to the required volume to give a final concentration of 5-10% DMF. The enzymes (26 milliunits) and inhibitors were preincubated at 37°C for 15 min. Each incubation was carried out in 2.5 ml of 0.1 M Tris-HCl pH 8.0. Residual enzyme activity was determined by adding 40 umoles of Tosyl-Gly-Pro-Arg-pNA into the incubation mixture and monitoring the release of p-nitroanilide. Controls for the three enzymes were performed without adding any inhibitor.

A detailed study of the inhibition of fibrinolysins P3 and P4 by benzamidine was performed using three concentrations of stock solutions of inhibitor in the presence of different concentrations of substrate. The reactions were set up and calculated in a similar manner to that described in section 2.15, except that 2.4 ml of substrate and 100 ul of the inhibitor were used.

The mixtures were pre-equilibrated at 37°C for 5 min and the reaction started by adding 10 ng (10 ul) of enzyme and 100 ul inhibitor mixture. Each of the different substrate concentrations were tested against three different final inhibitor concentrations (20, 40, 80 uM).

2.17 pH OPTIMA AND THERMAL STABILITY.

The effect of pH (2-12) on the rate of hydrolysis of Tosyl-Gly-Pro-Arg-pNA by tsetse fibrinolysis was determined according to the method of Erlanger *et al.* (1961). The buffers employed are indicated in the results sections.

To determine thermostability of the two tsetse fibrinolysins, the enzyme solutions were maintained in 0.1 M Tris-HCl buffer pH 8.0 for 30 min at different temperatures, ranging from 20 to 80°C, at 10°C intervals, then cooled rapidly in an ice bath for 5 min before the residual activity was assayed using Tosyl-Gly-Pro-Arg-pNA at 37°C. Each point in either of the experiments was assayed in triplicate.

CHAPTER 3

RESULTS.

3. PURIFICATION AND PARTIAL CHARACTERISATION OF TWO TSETSE FIBRINOLYSINS.

3.1 DE-52 FRACTIONATION.

Crude extracts of tsetse midguts, prepared from 1500 flies, were fractionated by chromatography on DE-52 cellulose. The elution profile of hydrolytic activity against Tosyl-Gly-Pro-Arg-pNA is shown in figure 6. At the fraction number indicated by the arrow, the adsorbed substances were eluted using a linear salt gradient from 0 to 150 mM NaCl in the equilibration buffer. The resolved peaks of activity shown in figure 6, which will be subequently refered to as P1, P2, P3, P4 or P5, based on their order of elution, were pooled individually as indicated by horizontal bars over each peak. The salt gradient profile reveals a biphasic elution pattern which might have been the result of having an excess of the initial buffer at the top of the resin before the salt gradient developed. Further purification of the enzymes was carried out using IEF. With this particular technique, recovery of units of enzyme activity dropped by half, a factor most likely due to the exposure of these enzymes to high voltage for long periods (21 hours) at $4^{o}C$ or to loss of activity due to protein precipitation at the pI of each enzyme.

The fibrinolysins P3 and P4 were obtained from 3.9 mg of crude MGE (1500 female G.morsitan centralis flies). A combined yield of 21 ug of purified protein with a combined recovery of about 12.7% of the initial MGE protease activity was obtained. An outline of the purification of the two tsetse fibrinolysins (P3 and P4) is summarised in tables 2 and 3 respectively. The low yield of activity obtained is partly due to the presence of several proteases in the orginal crude MGE that hydrolyse Tosyl-Gly-Pro-Arg-pNA and also due to losses incurred during purification.

Figure 6. Elution profile of crude taeras midgut proteinases on DE-52 cellulose.

The elution profile obtained during DE-52 cellulose chromatographic fractionation of crude taeras Mga. The chromatogram shows absorbance at 280 nm (—●—●—) and enzyme activity measured by hydrolysis of Tosyl- Gly-Pro-Arg-pNA (—●—●—) and replaced as per absorbance at 405 nm.

Figure 6. Elution profile of crude tsetse midgut proteases on DE-52 cellulose.

The elution profile obtained during DE-52 chromatographic fractionation of crude tsetse MGE. The chromatogram shows absorbance at 280 nm (-X-X-X-X-) and enzyme activity measured by hydrolysis of Tosyl- Gly-Pro-Arg-pNA (-●-●-●-) and depicted as net absobance at 405 nm.

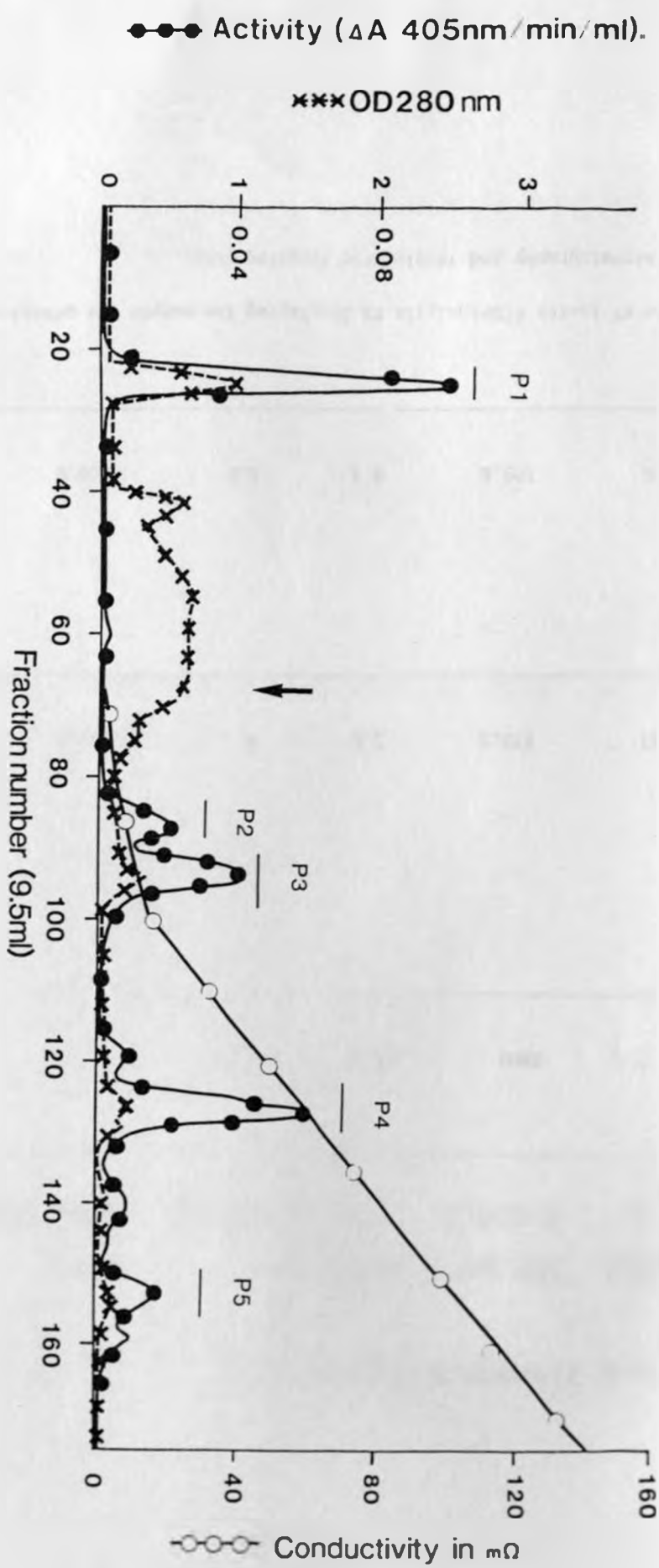

●—●—● Activity (ΔA 405nm/min/ml).
×××OD280 nm
0
1
0.04
2
0.08
3
20
40
60
80
100
120
140
160
Fraction number (9.5ml)
P1
P2
P3
P4
P5
0
40
80
120
160
○—○—○ Conductivity in mΩ

Table 2. Tsetse Fibrinolysin P3 purification scheme.

FRACTION	VOLUME ML	TOTAL UNITS OF ACTIVITY	UNITS/ML	TOTAL PROTEIN (UG)	SPECIFIC ACTIVITY (U/MG)	% RECOVERY	PURIFICATIO FACTOR
CRUDE EXTRACT	7.6	2800	368.4	3900	717.9	100	0
ANION EXCHANGE CROMATOGRAPHY ON DE-52 CELLULOSE	57	313.5	5.5	9	34833.0	11.3	48.5
ISOELECTRIC FOCUSING (pH RANGE 3.5-10)	16	129.6	8.1	8.0	16200.0	4.6	22.6

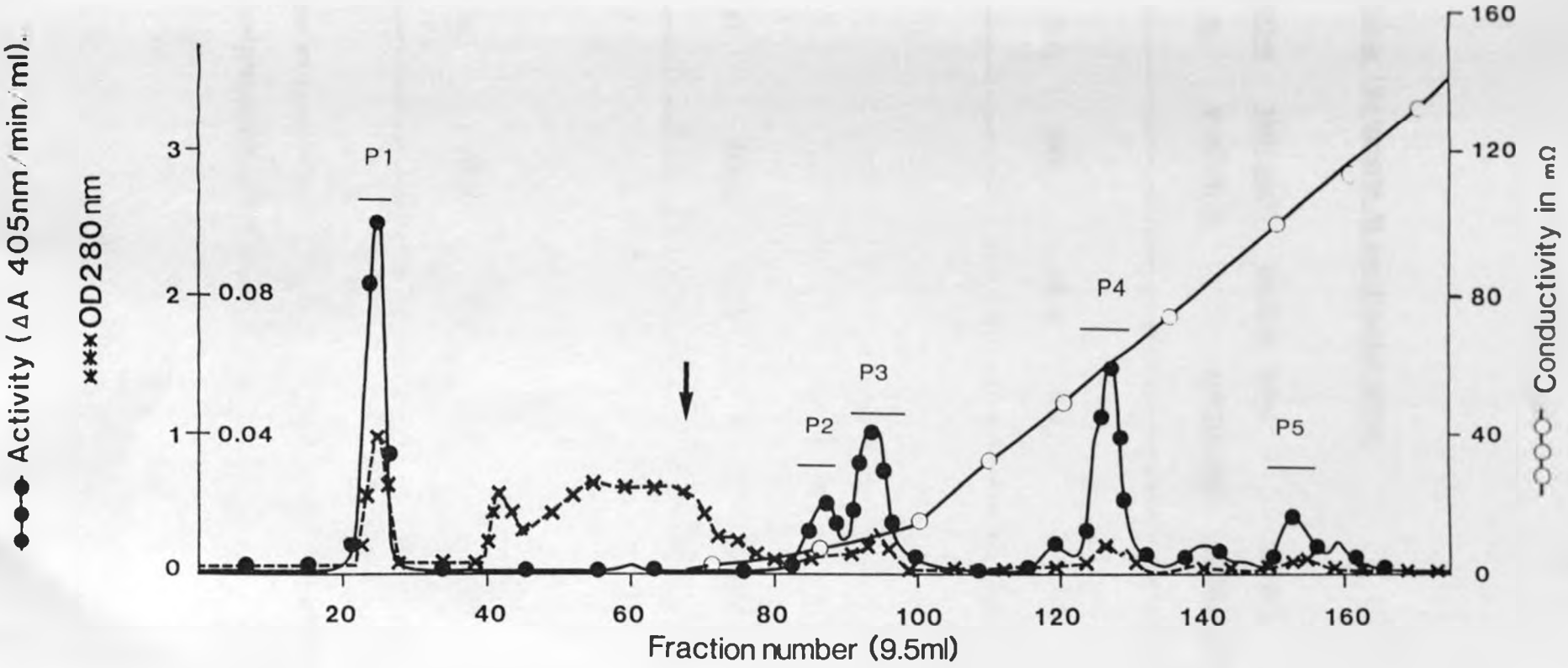
Purification table of tsetse fibrinolysin P3 displaying the enzyme and protein content obtained from DE-52 cellulose chromatography and isoelectric focusing steps.

Table 3. Tsetse fibrinolysin P4 purification scheme.

FRACTION	VOLUME ML	TOTAL UNITS OF ACTIVITY	UNITS/ML	TOTAL PROTEIN (UG)	SPECIFIC ACTIVITY (U/MG)	% RECOVERY	PURIFICAT FACTOR
CRUDE EXTRACT	7.6	2800	368.4	3900	717.9	100	0
ANION EXCHANGE CROMATOGRAPHY ON DE52 CELLULOSE	110	517	4.7	13	39769.2	18.5	55.4
ISOELECTRIC FOCUSING (pH RANGE 3.5-10)	48	225.6	4.7	13	17353.0	8.1	24.2

Purification table of tsetse fibrinolysin P4 displaying the enzyme and protein content obtained from tl

DE-52 cellulose chromatography and isolectric focusing steps.


Activity (ΔA 405nm/min/ml).
***OD280 nm
Conductivity in mΩ
P1
P2
P3
P4
P5
0
0.04
0.08
3
2
1
0
160
120
80
40
0
20
40
60
80
100
120
140
160
Fraction number (9.5ml)

3.2 IDENTIFICATION OF THE DE-52 PEAKS ASSOCIATED WITH THE HIGHEST FIBRINOLYTIC ACTIVITIES.

The experiments described in detail in section 2.13 were designed to find out which of the peaks resolved by anion exchange chromatography on DE-52 were the most fibrinolytic. Figures 7 and 8 show the results obtained from these experiments.

In the time point assay, the results of which are shown in figure 7, all the tsetse MGE proteases were tested for their ability to solubilise ^{125}I-fibrin clot. From the results obtained in the time point assay, it was evident that the highest amounts of MGE fibrinolytic activity was derived from P3 and P4 of DE-52 fractions. This result was further confirmed by the time course assay which demonstrated very clearly that the highest amounts of fibrinolytic activities in tsetse MGE were associated with P3 and P4, although all tsetse midgut proteases isolated digested fibrin and fibrinogen.

P1 was not tested in the time course experiment (Figure 8) because it was very labile and almost all enzymatic activity had been lost by the time the assay was being performed.

Figure 7. Histogram showing an ^{125}I-fibrin time point assay for tsetse proteases.

Histograms displaying the profile obtained when 80 milliunits of each tsetse protease was used in a time point assay of ^{125}I-fibrin clot digestion. C - Control (No enzyme added); P1 - Tsetse protease P1; P2 - Tsetse protease P2; P3 - Tsetse fibrinolysin P3; P4 - Tsetse fibrinolysin P4; P5 - Tsetse protease P5. The assay (done in quadruplicate for each time point) employed the mixture of 200 ul of unlabelled fibrinogen with 10 ul ^{125}I-fibrinogen. Clotting was initiated by adding 170 milliunits of bovine thrombin. The clot was spun at 12000 rpm for 5 min and washed to remove unclotted protein. Clot digestion was started by adding 80 milliunits of enzyme and left to proceed for 1 hour. At the end of this period, the mixture was spun at 12000 rpm for 10 min. Twenty microlitre aliquots of the supernatants were counted to determine the number of cpm released as ^{125}I-fibrinopeptides during clot digestion.

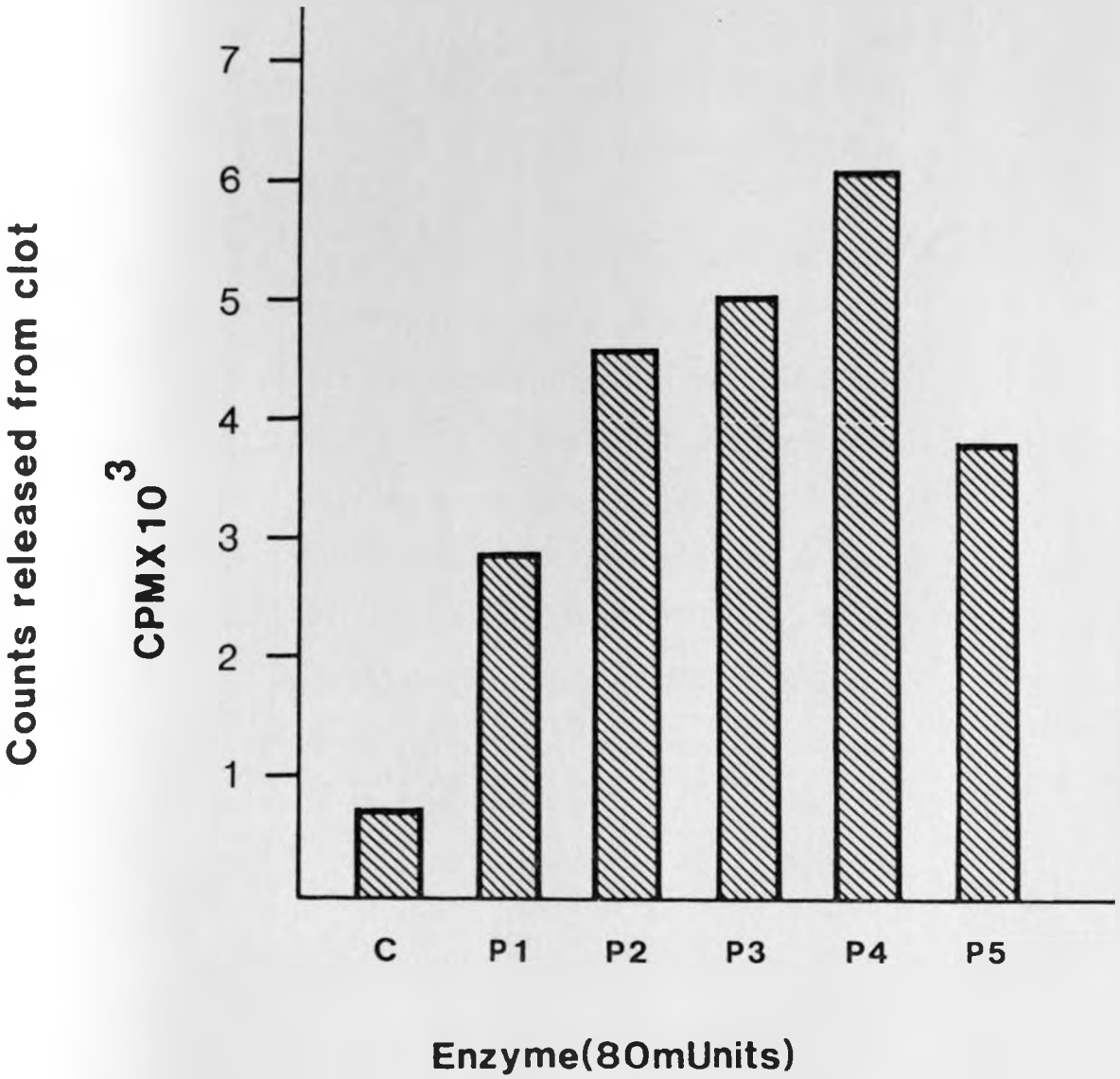
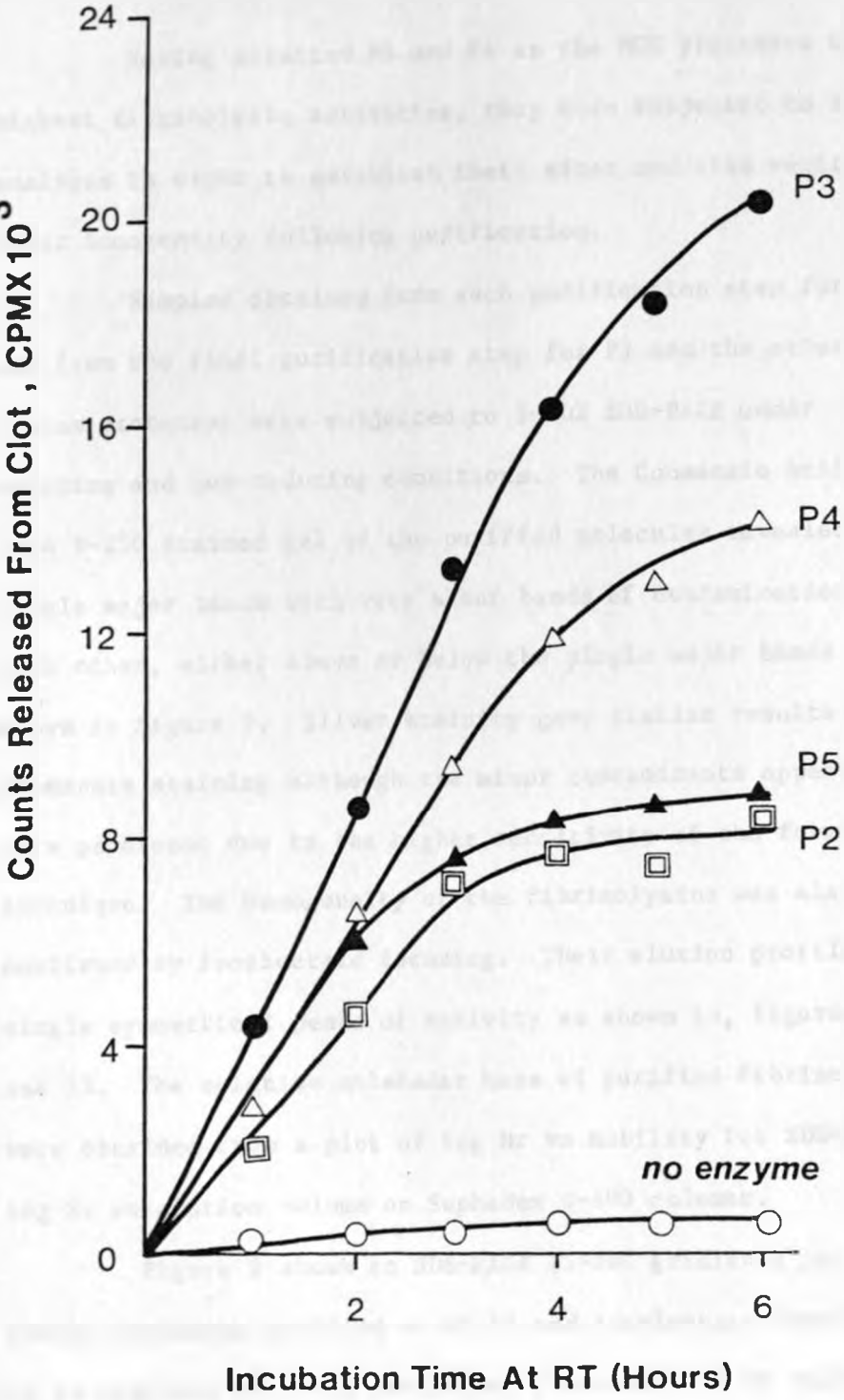

Counts released from clot
CPMX10 3
7
6
5
4
3
2
1
C
P1
P2
P3
P4
P5
Enzyme(80mUnits)

Figure 8. Time course assay of ^{125}I-fibrin clot digestion by tsetse proteases.

Profiles obtained from time course assay of ^{125}I-fibrin clot digestion by tsetse proteases. In each reaction mixture the clot was resuspended in 200 ul of 0.1 M Tris HCl/0.25 M NaCl pH 8.0 containing 80 milli-units of each of the enzymes and incubated at room temperature. Aliquots of 20 ul were withdrawn at the given time points, and the ^{125}I-fibrinopeptide counts in the supernatant determined. Each time point was tested in quadruplicate. The identity of each tsetse protease (P2-P5) is shown on the right.


24
20
16
12
8
4
0
2
4
6
P3
P4
P5
P2
no enzyme
Counts Released From Clot , CPMX10^3
Incubation Time At RT (Hours)

3.3 PHYSICOCHEMICAL CHARACTERISATION OF TWO TSETSE FIBRINOLYSINS.

Having selected P3 and P4 as the MGE proteases with highest fibrinolytic activities, they were subjected to further analyses in order to establish their sizes and also verify their homogeneity following purification.

Samples obtained from each purification step for P4 and from the final purification step for P3 and the other tsetse proteases were subjected to 5-20% SDS-PAGE under reducing and non-reducing conditions. The Coomassie brilliant blue R-250 stained gel of the purified molecules revealed single major bands with very minor bands of contamination from each other, either above or below the single major bands as shown in figure 9. Silver staining gave similar results as Coomassie staining although the minor contaminants appeared more prominent due to the higher sensitivity of the former technique. The homogeneity of the fibrinolysins was also confirmed by isoelectric focusing. Their elution profiles gave single symmetrical peaks of activity as shown in, figures 12 and 13. The relative molecular mass of purified fibrinolysins were obtained from a plot of log Mr vs mobility for SDS-PAGE or log Mr vs elution volume on Sephadex G-100 columns.

Figure 9 shows an SDS-PAGE (5-20% gradient) gel of all tsetse proteases purified on DE-52 and isoelectric focusing. It is apparent that all tsetse MGE proteases had Mr values within the range, 20-30 kDa. From this experiment the apparent

Mr of fibrinolysins P3 and P4 were estimated to be about 26.5 kDa and 24.5 kDa, respectively by plotting log Mr vs mobility of the protein markers and thereafter reading off the corresponding values for P3 and P4.

A Sephadex G-100 column was used to determine the sizes of the two tsetse fibrinolysins P3 and P4. From the elution profiles depicted in figures 10 and 11, the two enzymes eluted at about the same position as chymotrypsinogen-A although P3 comes off the column slightly earlier than P4. The relative molecular mass for these two enzymes was estimated to be 24.8 kDa for P3 and 24.0 kDa for P4, by plotting log Mr vs elution volume.

Figure 9. SDS-PAGE gel analysis of tsetse midgut proteases.

Coomassie brilliant blue R-250 stained SDS-PAGE of purified tsetse midgut proteases.

Plate A: Lane 2, - fibrinolysin P4 recovered after DE-52 chromatography.

Lane 3, - fibrinolysin P4 after DE-52 followed by isoelectric focusing.

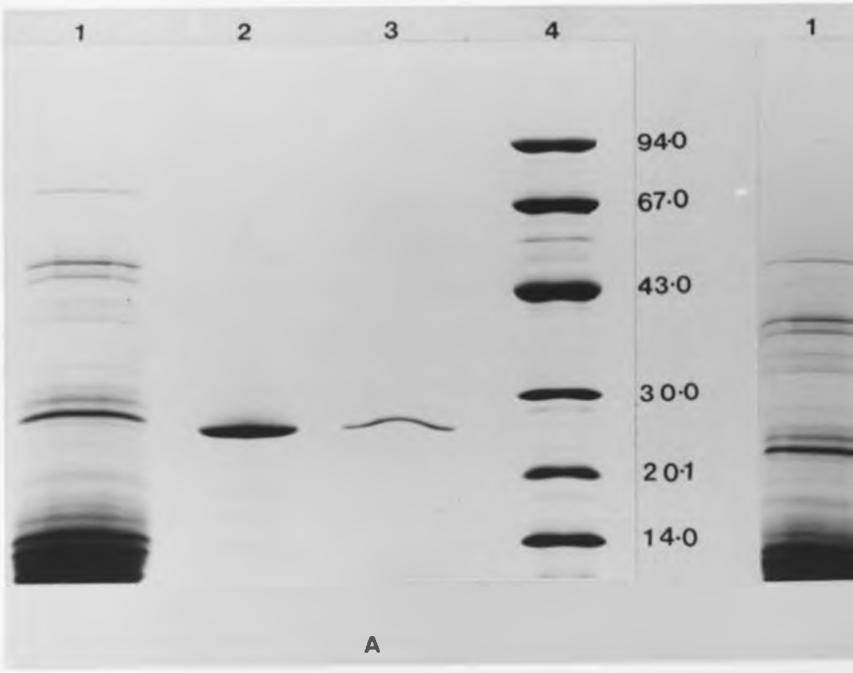
Plate B: Lanes 2 and 3 - represent the two tsetse proteases obtained from focusing tsetse protease P1 obtained from the DE-52.

Lane 4 - tsetse protease P2 after DE-52 and isoelectric focusing.

Lane 5 - tsetse fibrinolysin P3 after DE-52 and isoelectric focusing.

Lane 1 in each of plates A and B contained 15 ug of the crude starting material.

Lanes 4 of plate A and lane 6 of plate B are low molecular weight markers. The markers used in this experiment were; phosphorylase b (Mr, 94.0 kDa), albumin (Mr, 67.0 kDa) ovalbumin (Mr, 43.0 kDa), carboric anhydrase (Mr, 30.0 kDa) trypsin inhibitor (Mr, 20.1) and alpha-lactalbumin (14.0 kDa).


1
2
3
4
1
94·0
67·0
43·0
30·0
20·1
14·0
A

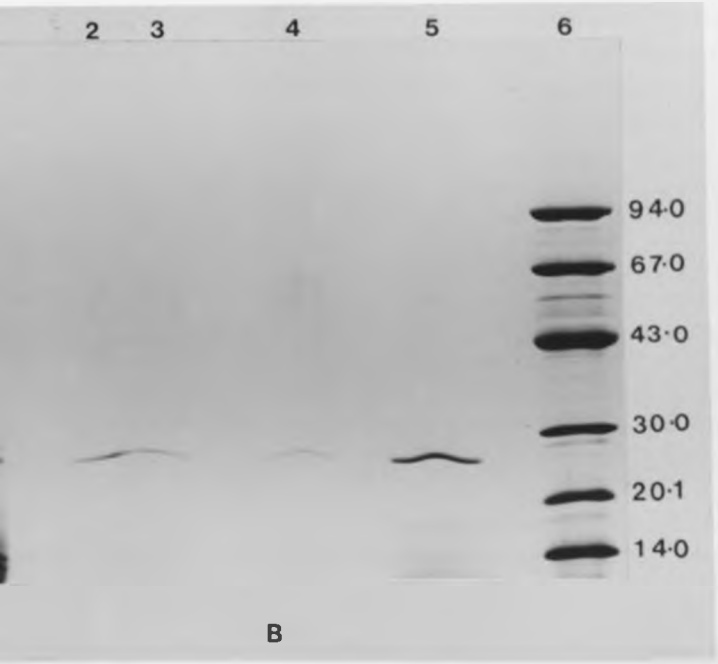
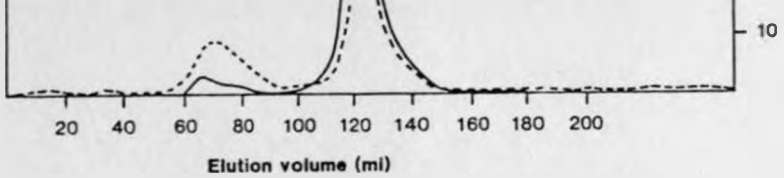

2
3
4
5
6
94·0
67·0
43·0
30·0
20·1
14·0
B

Figure 10. Sephadex G-100 gel filtration chromatography of fibrinolysin P3.

The elution profile obtained for fibrinolysin P3 on Sephadex G-100 gel filtration. The protein standards used were, Bovine serum albumin (Mr 67 kDa); Ovalbumin (Mr 43 kDa); Chymotrypsinogen-A (Mr 25 kDa); and Ribonuclease-A (Mr 13.7 kDa). The numbers 1, 2, 3 and 4 on the figure stand for bovine serum albumin, ovalbumin, chymotrypsinogen-A and ribonuclease-A respectively.


10
20
40
60
80
100
120
140
160
180
200
Elution volume (ml)

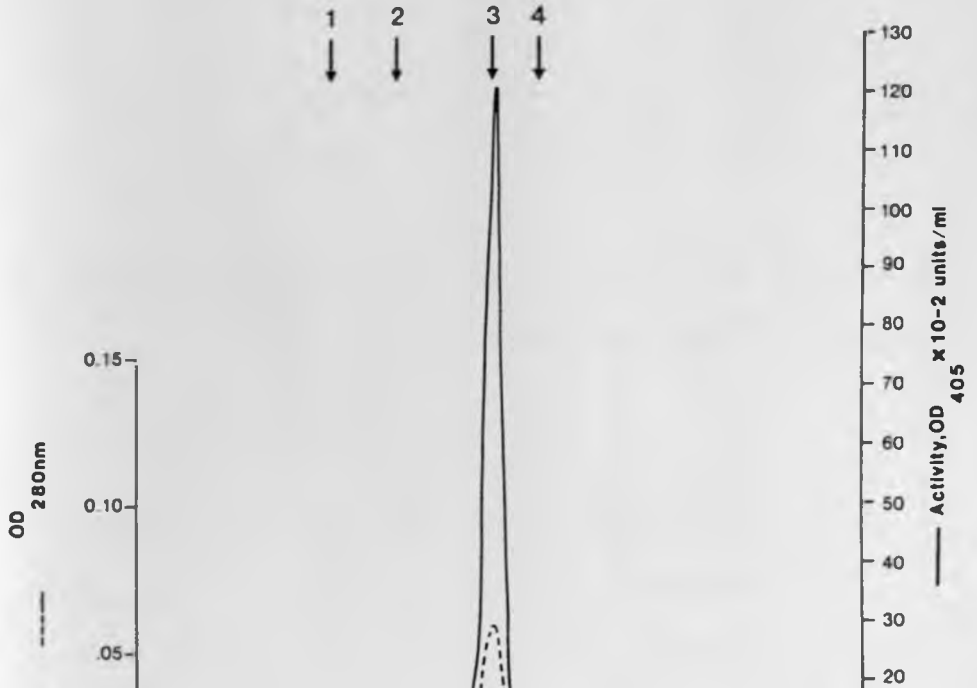
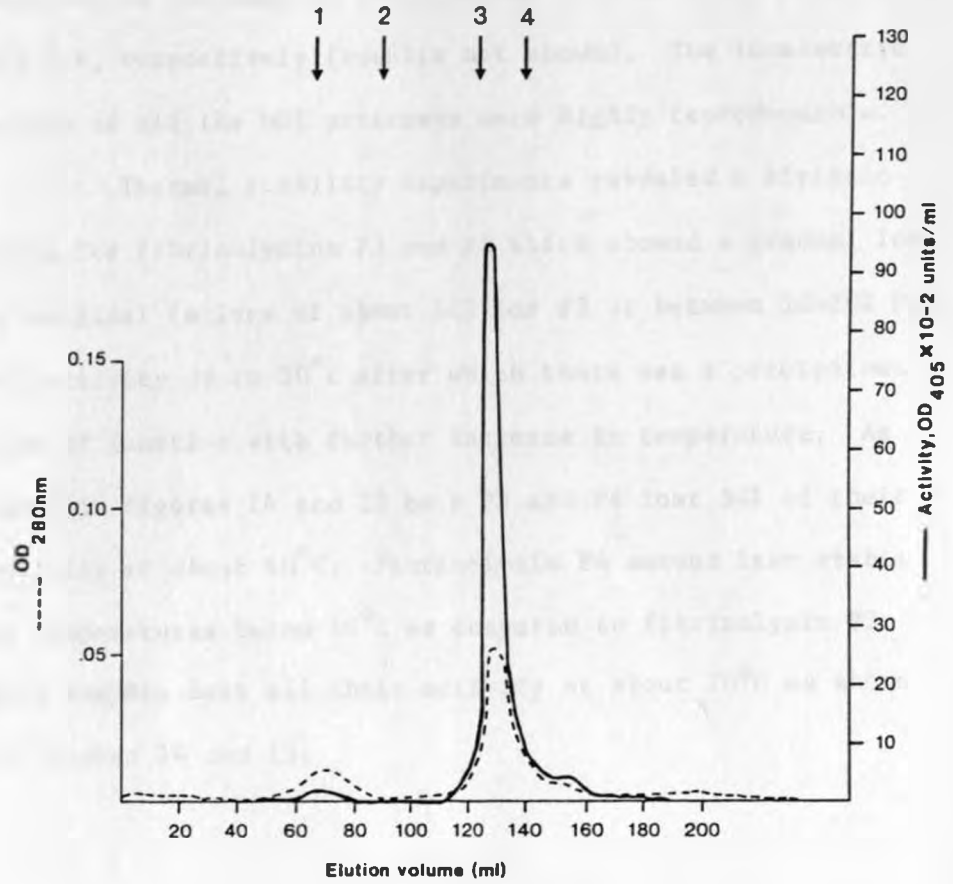

1
2
3
4
0.15
0.10
.05
OD 280nm
130
120
110
100
90
80
70
60
50
40
30
20
Activity, OD 405 ×10-2 units/ml

Figure 11. Sephadex G-100 gel filtration chromatography of fibrinolysin P4.

The elution profile obtained for fibrinolysin P4 on Sephadex G-100 gel filtration. The same sizing markers listed in the legend to figure 10 were used and are indicated by numbered arrows as for figure 10.


1
2
3
4
0.15
0.10
.05
OD 280nm
130
120
110
100
90
80
70
60
50
40
30
20
10
Activity, OD 405 X10-2 units/ml
20
40
60
80
100
120
140
160
180
200
Elution volume (ml)

Isoelectric focusing and thermal stability: Figures 12 and 13 display profiles of P3 and P4, showing that they have apparent isoelectric points of 6.0 and 5.3 respectively. The other tsetse MGE proteases, that is P2, P5 and the two fractions obtained on focusing P1 had apparent pI's of 6.25, 5.15, 8.25 and 7.8, respectively (results not shown). The isoelectric points of all the MGE proteases were highly reproduceable.

Thermal stability experiments revealed a biphasic curve for fibrinolysins P3 and P4 which showed a gradual loss of original (a loss of about 10% for P3 or between 10-20% for P4) activity up to 50^{o}C after which there was a precipitous loss of function with further increase in temperature. As shown in figures 14 and 15 both P3 and P4 lost 50% of their activity at about 60^{o}C. Fibrinolysin P4 seemed less stable at temperatures below 40^{o}C as compared to fibrinolysin P3. Both enzymes lost all their activity at about 70^{o}C as shown in figures 14 and 15.

Figure 12. Isoelectric focusing profile of fibrinolysin P3.

Isoeletric focusing profile of tsetse fibrinolysin P3 using 3.5-10 pH range ampholines on a 110 ml LKB focusing column. The pI of fibrinolysin P3 was estimated as 6.0. Three hundred and fourteen units of enzyme in a volume of 57 ml were loaded onto this column. After focusing only 130 units were recovered.

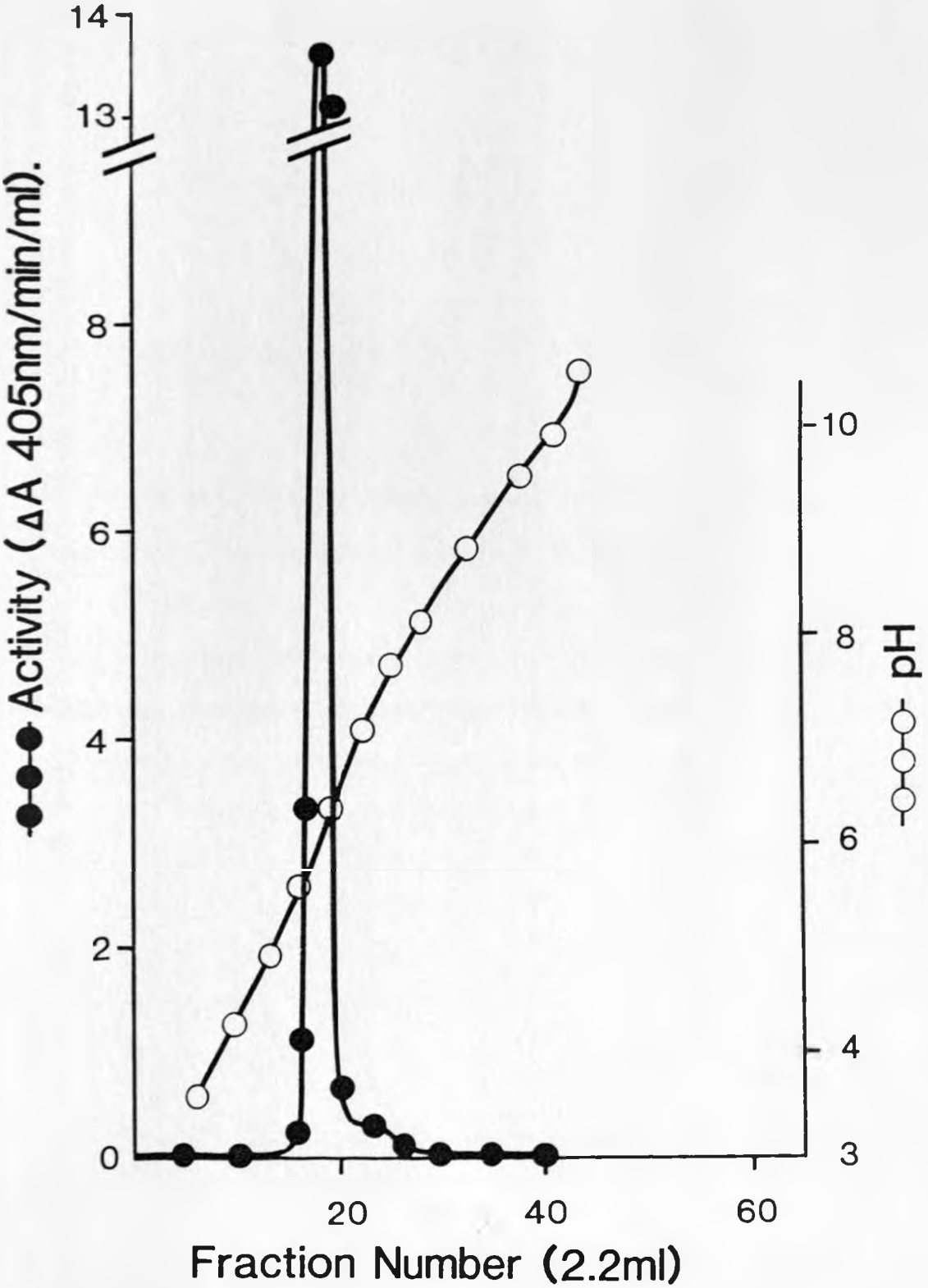

14
13
8
6
4
2
0
Activity (ΔA 405nm/min/ml).
10
8
6
4
3
pH
20
40
60
Fraction Number (2.2ml)

Figure 13. Isoelectric focusing profile of fibrinolysin P4.

Isoelectric focusing profile of tsetse fibrinolysin P4 using 3.5-10 pH range ampholines on a 110 ml LKB focusing column. The pI of fibrinolysin P4 was estimated as 5.3 under these conditions. Five hundred and seventeen units of enzyme in a volume of 110 ml were loaded onto this column, out of which 225.6 units were recovered after focusing.

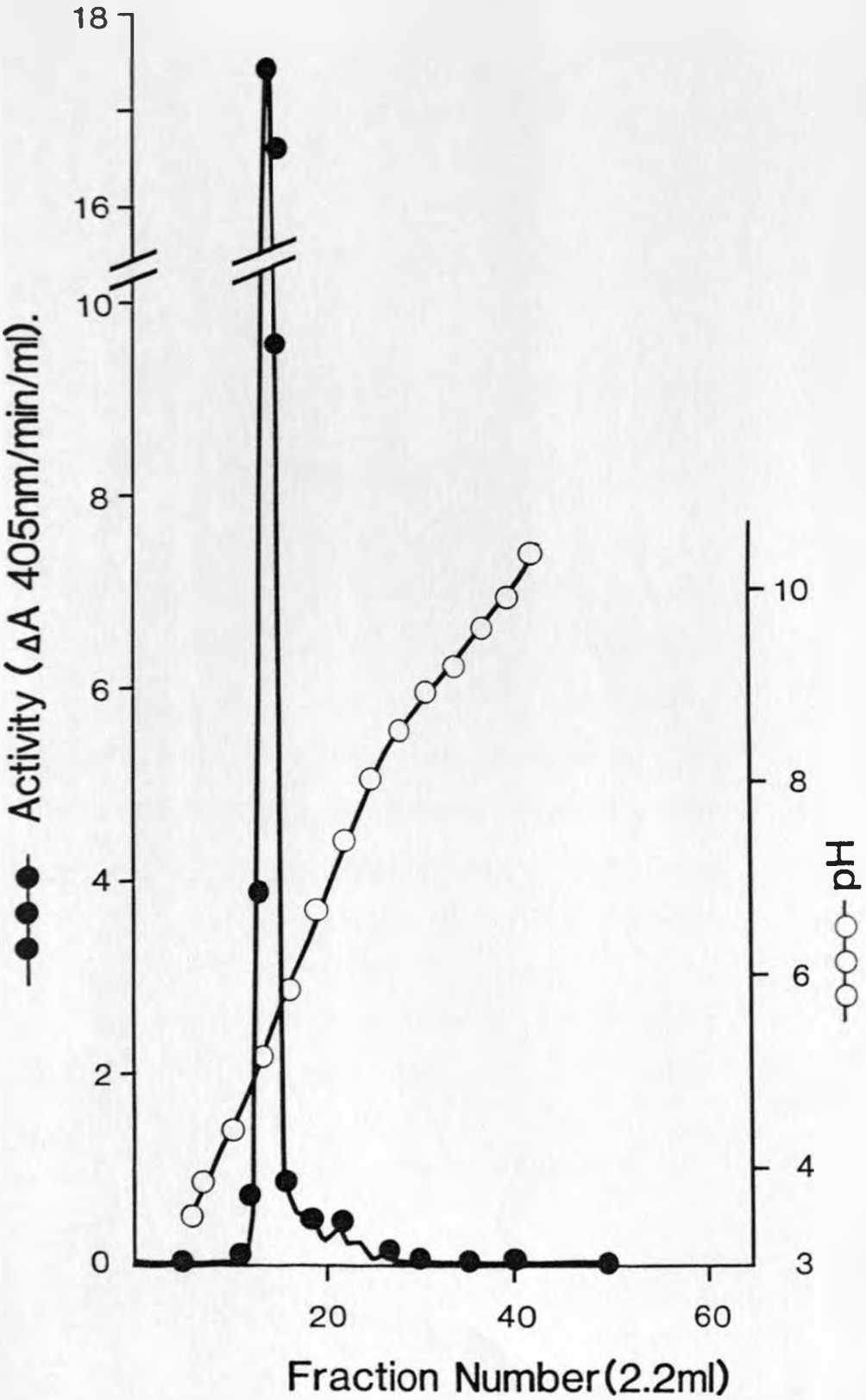

18
16
10
8
6
4
2
0
Activity (ΔA 405nm/min/ml).
10
8
6
4
3
pH
20
40
60
Fraction Number(2.2ml)

Figure 14. Effect of increase in temperature on fibrinolysin P3 activity.

Eighty milliunits of fibrinolysin P3 were incubated for 30 min in a waterbath maintained at different temperatures. Residual enzyme activity was assayed using Chromozym-TH. The results obtained from this experiment were plotted as ΔA_{405nm} vs temperature. Fifty percent of the activity was lost at around $60^{0}C$ for both P3 and P4.

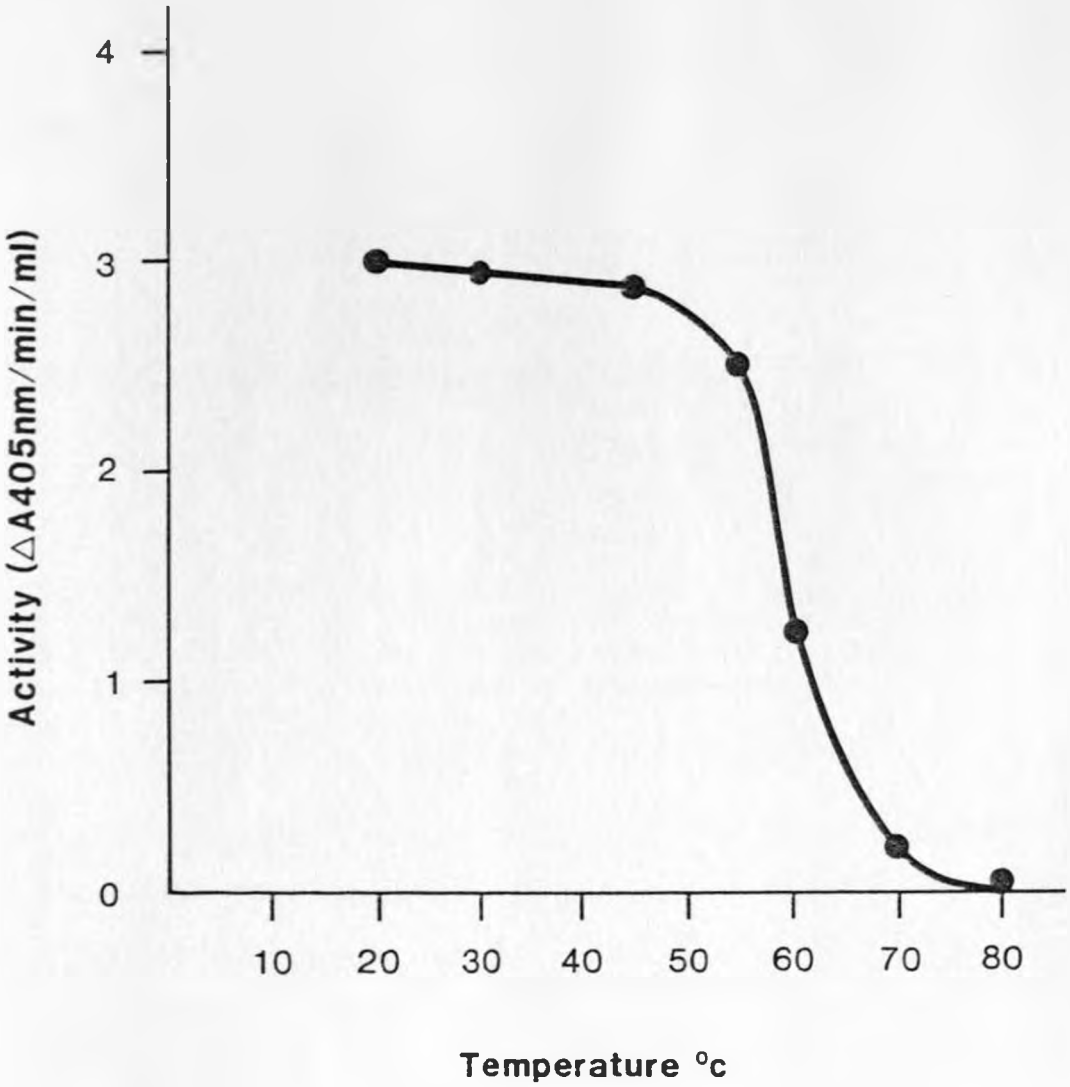
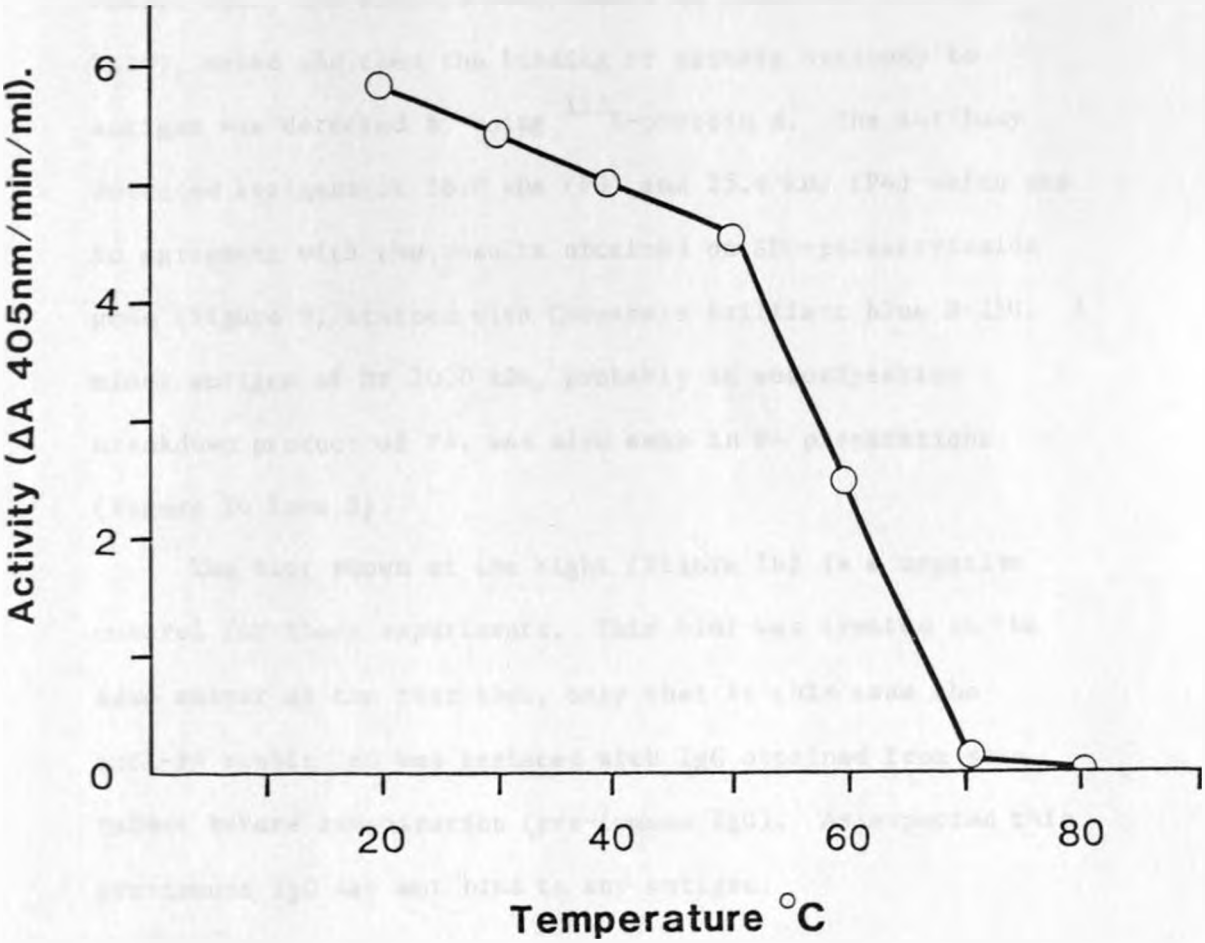

Activity (△A405nm/min/ml)
4
3
2
1
0
10
20
30
40
50
60
70
80
Temperature °c

Figure 15. Effect of increase in temperature on fibrinolysin P4 activity.

The conditions described in the legend to figure 14 were also employed for this experiment. The results obtained are shown in the figure on the facing page.


Activity (ΔA 405nm/min/ml).
6
4
2
0
20
40
60
80
Temperature °C

3.4 IMMUNOBLOT ANALYSIS OF PURIFIED FIBRINOLYSINS P3 AND P4 REACTED WITH ANTI-P4 ANTISERA.

Figure 16 shows the results obtained when P3 and P4 were resolved on SDS-PAGE (5-20% gel), transferred onto nitrocellulose filters and then incubated with purified anti-P4 rabbit IgG. The filters were washed as described (section 2.10), dried and then the binding of primary antibody to antigen was detected by using ^{125}I-protein A. The antibody detected antigens of 26.0 kDa (P3) and 25.4 kDa (P4) which was in agreement with the results obtained on SDS-polyacrylamide gels (Figure 9) stained with Coomassie brilliant blue R-250. A minor antigen of Mr 20.0 kDa, probably an autodigestion breakdown product of P4, was also seen in P4 preparations (Figure 16 lane B).

The blot shown at the right (Figure 16) is a negative control for these experiments. This blot was treated in the same manner as the test blot, only that in this case the anti-P4 rabbit IgG was replaced with IgG obtained from same rabbit before immunization (pre-immune IgG). As expected this pre-immune IgG did not bind to any antigen.

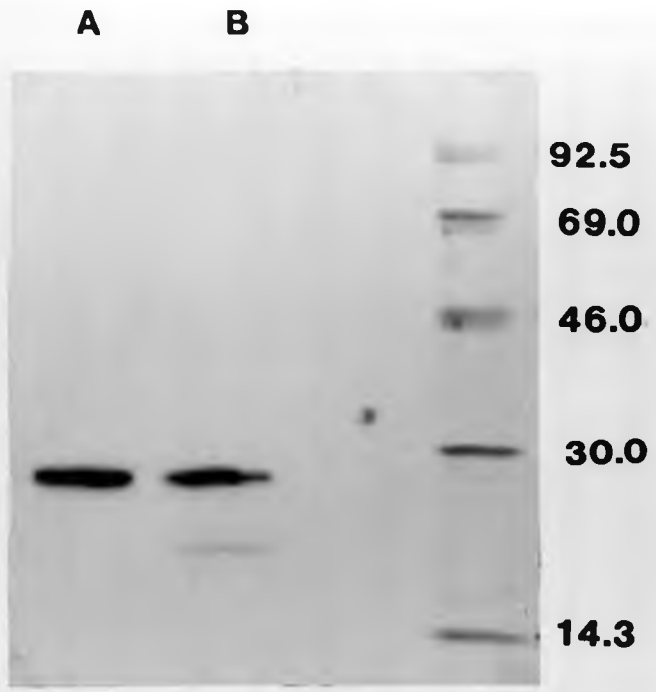
Figure 16. Immunoblot analysis of fibrinolysins P3 and P4.

Immunoblot analysis of tsetse fibrinolysins P3 and P4 using purified immunoglobulins raised against fibrinolysin P4 in a rabbit and pre-immune IgG prepared from the same rabbit from serum obtained before it was immunized against P4.

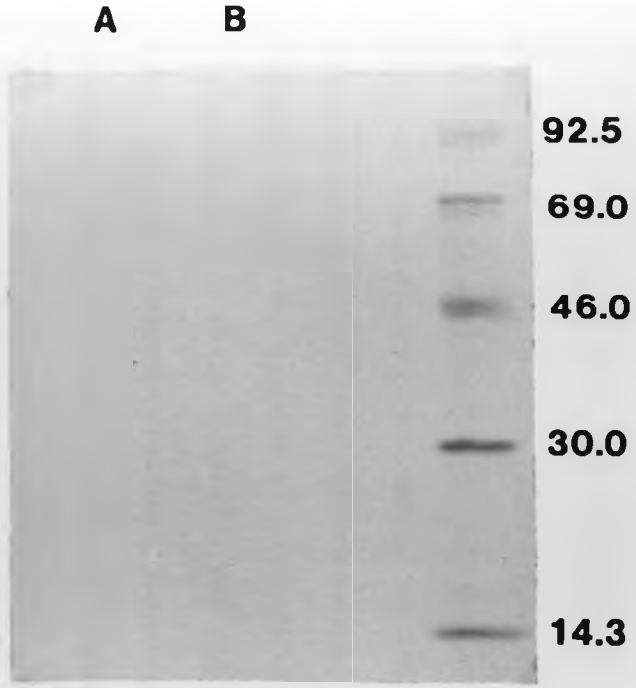
Lane A was loaded with 5 ug of fibrinolysin P4 antigen.

Lane B was loaded with 5 ug fibrinolysin P3 antigen.

Numbers to the right show position of ^{14}C-radiolabelled markers in kDa.



Blot probed with antibody raised against fibrinolysin P4



Negative control probed with preimmune sera

A-Fibrinolysin P3

B-Fibrinolysin P4

3.5 LOCALIZATION OF TSETSE ENZYMES ON SDS-PAGE GELS USING ^{3}H-LABELLED APROTININ.

Attempts were made to affinity purify the midgut proteases of tsetse on p-aminobenzamidine-Sepharose 4B, soybean trypsin inhibitor coupled to Sepharose 4B, and aprotinin coupled to Sepharose 4B. However no activity was ever recovered from these columns. One obvious assumption was that the tsetse enzymes bound too tightly to these immobilized inhibitors thus suggesting that these enzyme(s) had trypsin-like protease specificity. Later, after purifying the enzymes employing ion exchange chromatography, it became necessary to establish beyond doubt that, the protein bands observed on SDS-PAGE corresponded to these protease and that they bound one of these inhibitors. The experiment described in Section 2.11 was, therefore, performed. Aprotinin was selected, since it had been shown to have a high affinity for trypsin-like enzymes (Hjelmeland and Raa, 1982; Steven and Griffin, 1981). ^{3}H-aprotinin-enzyme complexes from individual tsetse MGE proteases, prepared as outlined in Section 2.11, were analysed by SDS-PAGE gradient gels (5-20% gel) prepared as described in section 2.8. Fluorography was performed as outlined under Section 2.11. From this experiment, whose results are shown in figure 17, the following observations were made:

i) Fibrinolysins P3 and P4 (in lanes D and F respectively) labelled strongly with aprotinin, thus suggesting that they are trypsin-like enzymes.

ii) All tsetse MGE proteases obtained by the purification procedure described, displayed sizes of around 25 kDa.

iii) The shift in molecular weight upon binding aprotinin was approximately 6.5 kDa suggesting that all tsetse MGE proteases displayed the typical 1:1 enzyme-inhibitor stoichiometry.

It is noteworthy that free ^{3}H-aprotinin (excess) runs as dimer during electrophoresis and this is the intense band seen slightly below 14.8 kDa on the fluorograph. When ^{3}H-aprotinin's molecular mass of approximately 6.5 kDa is substracted from that of the complexes resolved in this experiment, the molecular mass of fibrinolysins P3 and P4 was estimated at about 26 kDa and 24.5 kDa daltons, respectively, thus implying a single active site for each of the fibrinolysins. In addition to the label seen associated with unbound aprotinin (14.8 kDa) and the complexes with fibrinolysins at an Mr of about 30 kDa, the crude tsetse MGE (especially lane A) shows additional moeities at approximately Mr of 85 and 93 kDa. Tsetse fibrinolysin P5 also has multiple bands of high Mr at about 50 kDa and 85 kDa. The bands may well represent fibrinolysin zymogens. There is precedent for a zymogen, trypsinogen, binding of this inhibitor (Vincent and Lazdunski, 1976).

Figure 17. Localization of tsetse proteases on SDS-PAGE gels using tritium-labelled aprotinin.

Between 5 x 10^5-1 x 10^6 counts were loaded per well.

Lanes A and E, crude tsetse MGE proteases ^{3}H-aprotinin inhibitor complex.

Lane B, ^{3}H-aprotinin - P1 protease inhibitor complex.

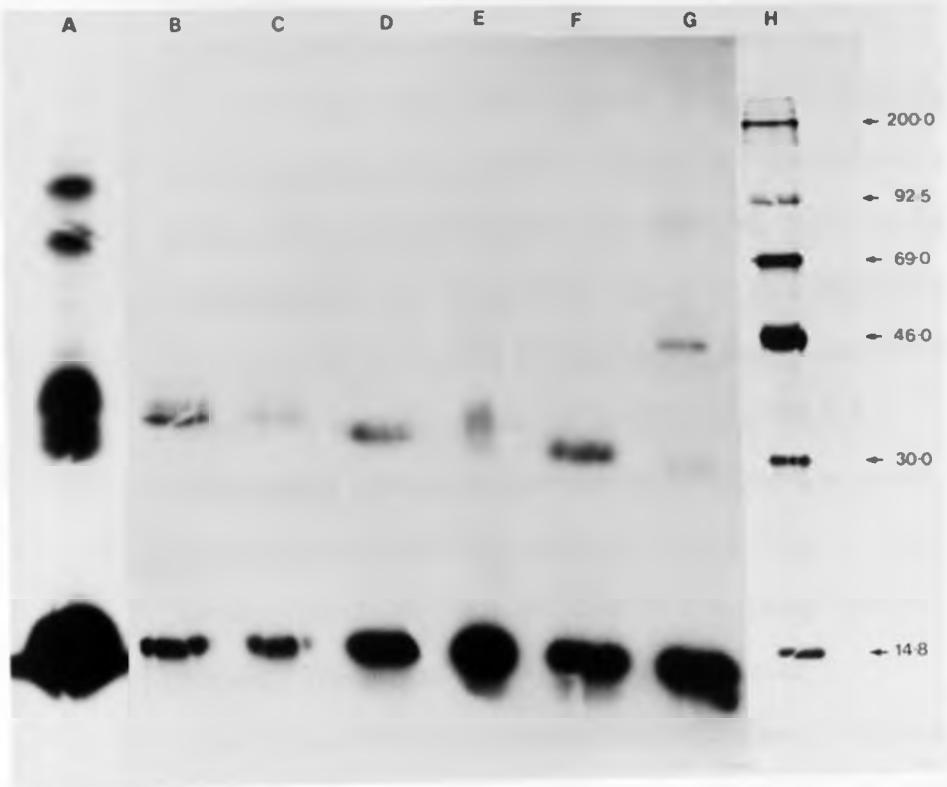
Lane C, ^{3}H-aprotinin - P2 protease inhibitor complex.

Lane D, ^{3}H-aprotinin-fibrinolysin P3 inhibitor complex.

Lane F, ^{3}H-aprotinin-fibrinolysin P4 inhibitor complex.

Lane G, ^{3}H-aprotinin - P5 protease complex.

Lane H, ^{14}C protein markers.


A
B
C
D
E
F
G
H
200·0
92·5
69·0
46·0
30·0
14·8

3.6 ENZYME DOSE RESPONSE STUDIES OF ^{125}I-FIBRIN CLOT SOLUBILIZATION.

This experiment was designed to serve two purposes:-

a) To establish the optimum amount of enzyme (fibrinolysin) needed for other ^{125}I-fibrin(ogen) assays.

b) To demonstrate the disappearance of ^{125}I-counts from the clot, into the supernatant following incubation with increasing amounts fibrinolysins.

It is clearly seen from figures 18 and 19 that the clot digestion reaction is linear between 0-160 milliunits of fibrinolysin P4 and 0-80 milliunits for fibrinolysin P3. From this assay it was decided that 80 milliunits of each fibrinolysin was a reasonable amount of enzyme to use in the fibrin clot or fibrinogen lysis assay since most cleavage sites recognized by these enzymes in fibrin(ogen) would not be completely hydrolysed within the assay period chosen, therefore facilitating an easier interpretation of intermediate breakdown fibrinogen products which are generated during the course of the reaction.

Figure 18. Enzyme dose response curves of ^{125}I-fibrin digestion by fibrinolysin P3.

Enzyme dose response curves generated when fibrinolysin P3 was used in digesting ^{125}I-fibrin. The assay mixture contained 300 ul of unlabelled fibrinogen and 10 ul ^{125}I-fibrinogen. Clotting was initiated by adding 0.17 units of thrombin. After spinning (12000 rpm) and drying of the fibrin clot, the following doses were added to each test to start clot digestion; 40, 80, 160 or 320 milliunits. After one hour, 20 ul each of supernatant and pellet were counted and the values obtained plotted against the amount of enzyme. Each enzyme dose was tested in triplicate, at room temperature.

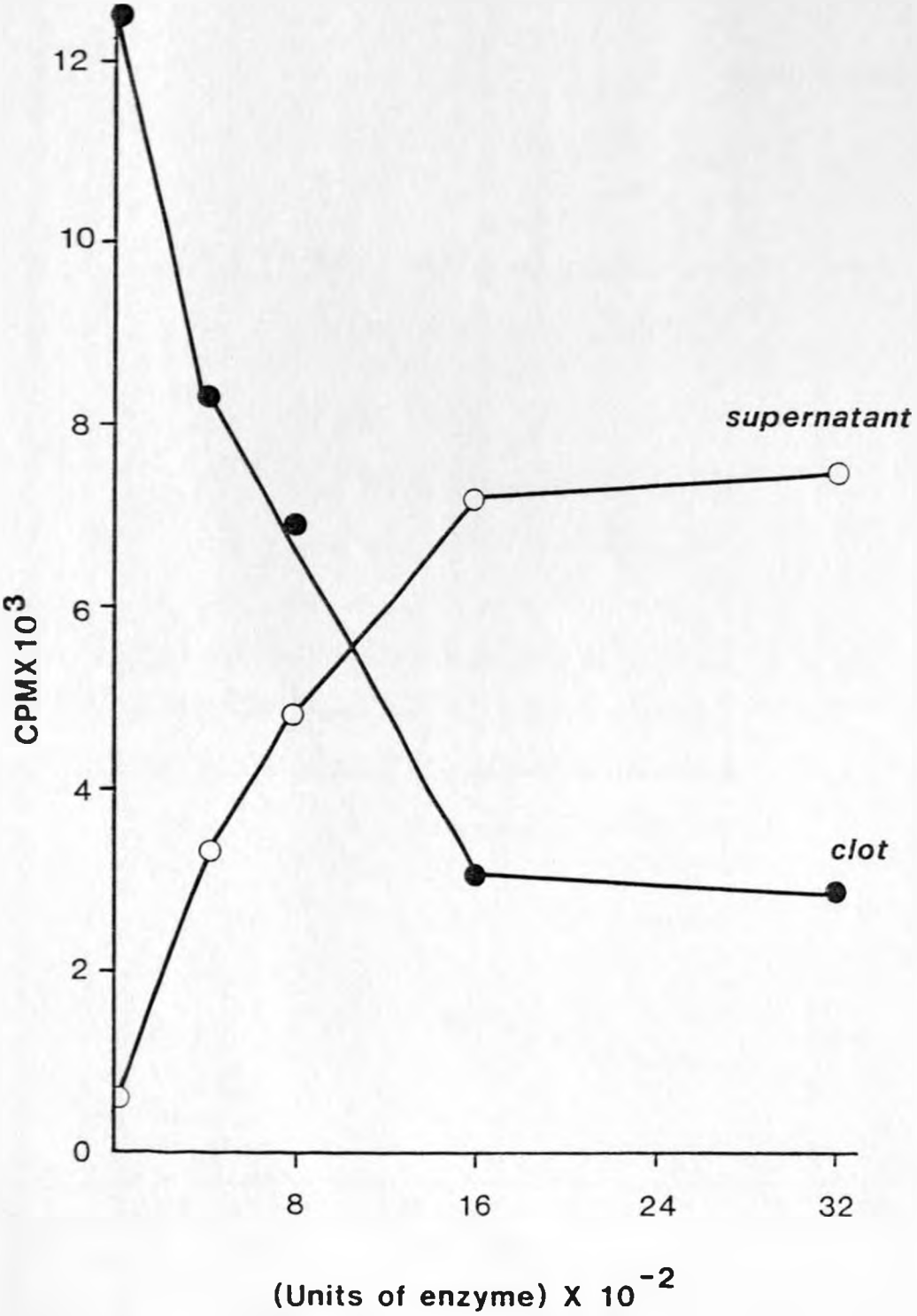
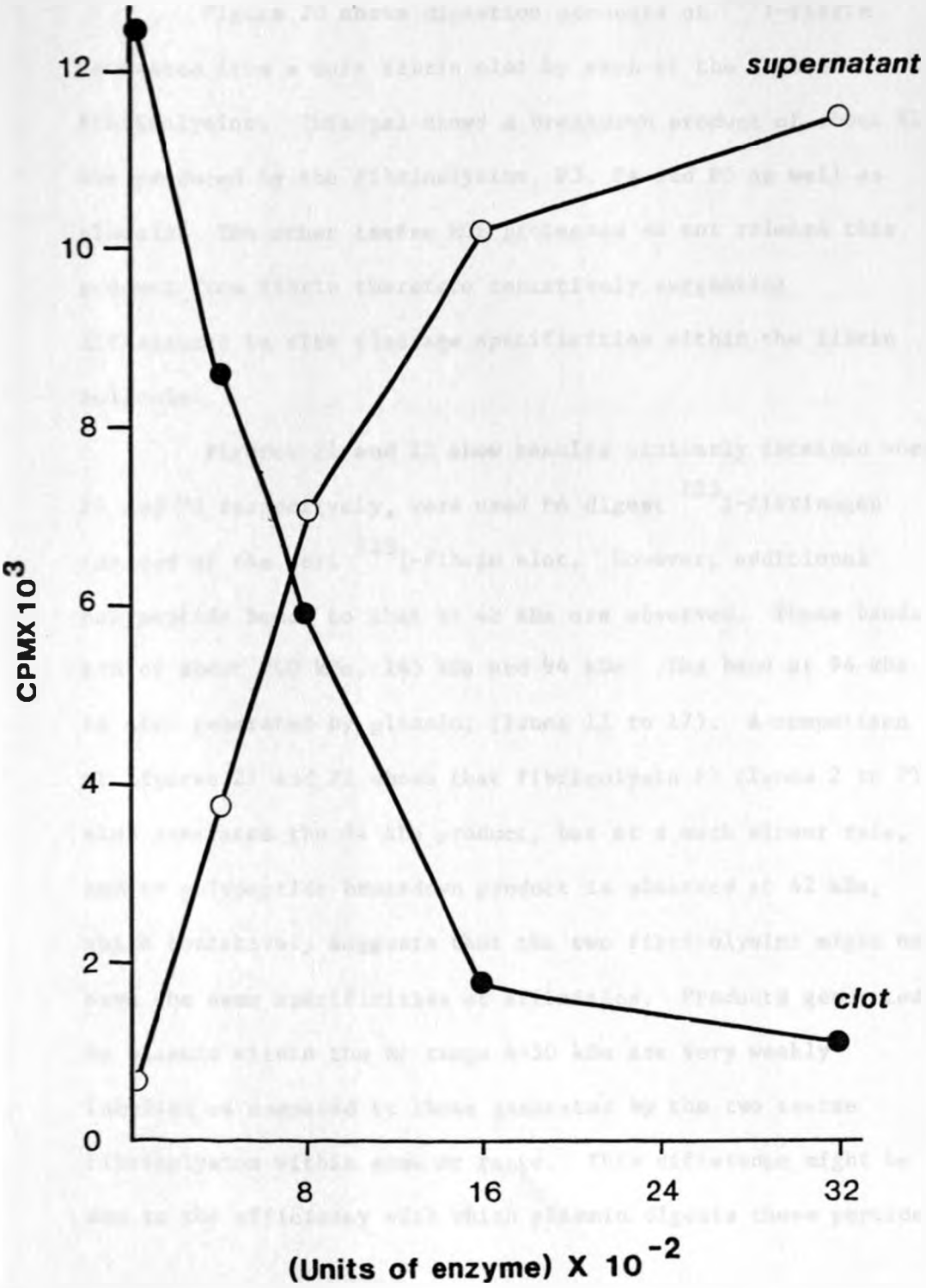

12
10
8
6
4
2
0
CPMX10^3
supernatant
clot
8
16
24
32
(Units of enzyme) X 10^{-2}

Figure 19. Enzyme dose response curves of ^{125}I-fibrin digestion by fibrinolysin P4.

Enzyme dose response curves generated when fibrinolysin P4 was used in digesting ^{125}I-fibrin. This result was obtained by counting 20 ul aliquots of resuspended clot or supernatant for each test point, the rest of the experimental set up was similar to that described in the legend to figure 18.


supernatant
clot
CPMX10^3
12
10
8
6
4
2
0
8
16
24
32
(Units of enzyme) X 10^{-2}

3.7 ANALYSIS OF CLEAVAGE PRODUCTS GENERATED FROM ^{125}I-FIBRIN(OGEN) BY THE TWO TSETSE FIBRINOLYSINS AND PLASMIN ON SDS-PAGE GELS.

Figure 20 shows digestion products of ^{125}I-fibrin generated from a soft fibrin clot by each of the tsetse fibrinolysins. This gel shows a breakdown product of about 42 kDa produced by the fibrinolysins, P3, P4 and P5 as well as plasmin. The other tsetse MGE proteases do not release this product from fibrin therefore tentatively suggesting differences in site cleavage specificities within the fibrin molecule.

Figures 21 and 22 show results similarly obtained when P4 and P3 respectively, were used to digest ^{125}I-fibrinogen instead of the soft ^{125}I-fibrin clot. However, additional polypeptide bands to that at 42 kDa are observed. These bands are of about 200 kDa, 145 kDa and 94 kDa. The band at 94 kDa is also generated by plasmin, (lanes 11 to 17). A comparison of figures 21 and 22 shows that fibrinolysin P3 (lanes 2 to 7) also generates the 94 kDa product, but at a much slower rate, and no polypeptide breakdown product is observed at 42 kDa, which tentatively suggests that the two fibrinolysins might not have the same specificities or affinities. Products generated by plasmin within the Mr range 6-30 kDa are very weakly labelled as compared to those generated by the two tsetse fibrinolysins within same Mr range. This difference might be due to the efficiency with which plasmin digests these peptides

to very small peptides which run into the buffer during electrophoresis or it might also be due to differences in cleavage sites resulting in plasmin releasing weakly labelled peptides or no peptides at all within this molecular weight range.

Fibrinogen was chosen as substrate in this experiment, because unlike fibrin, it is simpler to use. It is more soluble and it involves fewer processing steps to attain the final material needed for SDS-PAGE analyses. However, the assumption used also in selecting fibrinogen is that it is the precursor of fibrin and should therefore provide the same enzyme binding sites based within the same domain in both molecules.

Figure 20. SDS-PAGE gel analysis of fibrin breakdown products generated by various tsetse proteases and human plasmin.

SDS-PAGE (5 - 20% gel) gradient on which non-reduced fibrin breakdown products generated by various tsetse proteases and human plasmin were resolved. Breakdown products from four time points (1, 2, 3 and 4 hours) were analysed for each protease. One hundred and sixty milliunits were used in generating the fibrin peptides.

Plate A Lanes 1 and 2 are controls onto which undigested fibrin (zero time point) was loaded.

Lanes 3-6 were loaded with digests generated by protease P1.

Lanes 7-10 were loaded with digests generated by protease P2.

Lanes 11-14 were loaded with digests generated by fibrinolysin P3.

Lane 15 - low molecular weight markers.

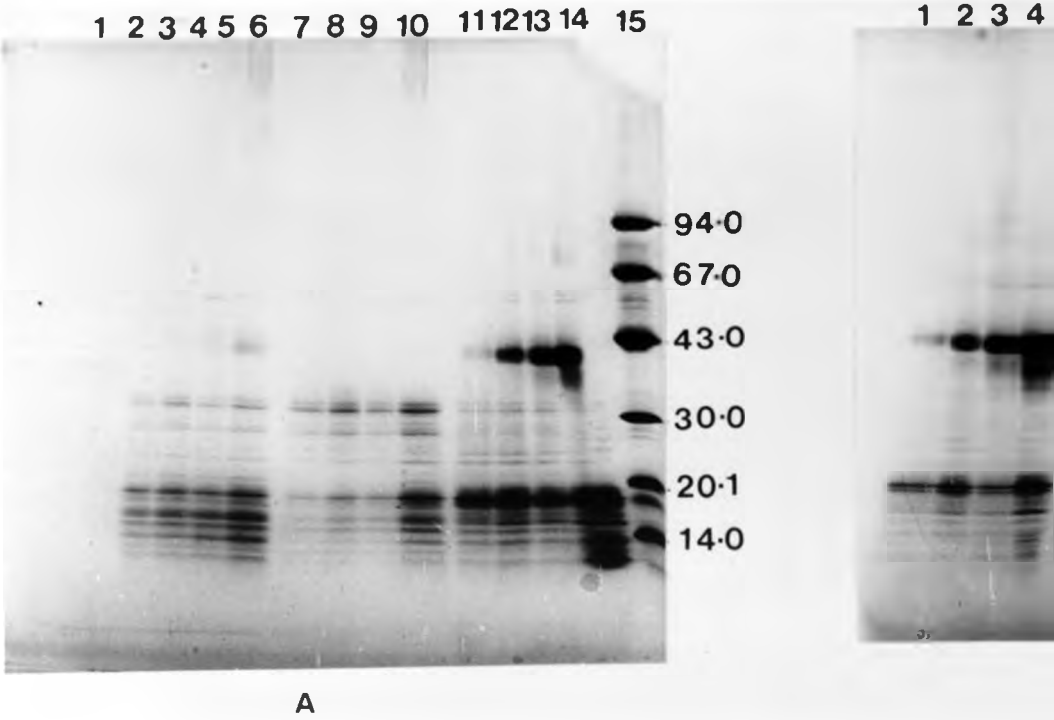
Plate B Lanes 1-4 were loaded with digest genereated by fibrinolysin P4.

Lanes 5-8 were loaded with digest generated by protease P5.

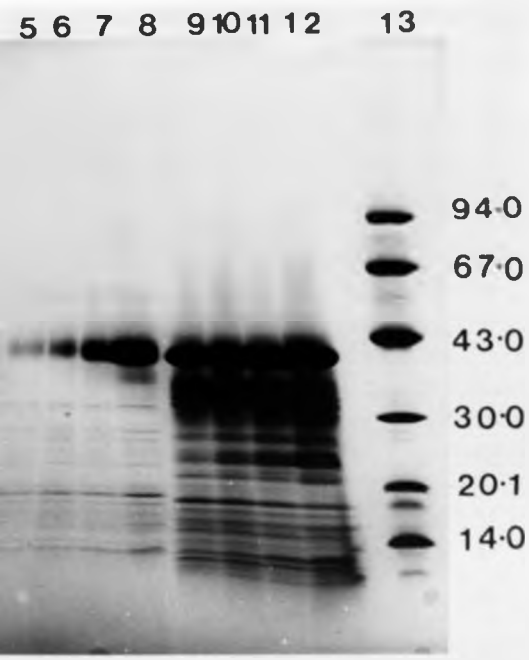
Lanes 9-12 were loaded with digests generated by human plasmin.

Lane 13 - low molecular weight markers.

The amount of digest loaded onto each lane was equivalent to about 10 ug of protein in the original reaction mixture before spinning to retrieve the supernatant.



A



B

Figure 21. SDS-PAGE gel analysis of ^{125}I-fibrinogen breakdown products generated by fibrinolysin P4 and plasmin.

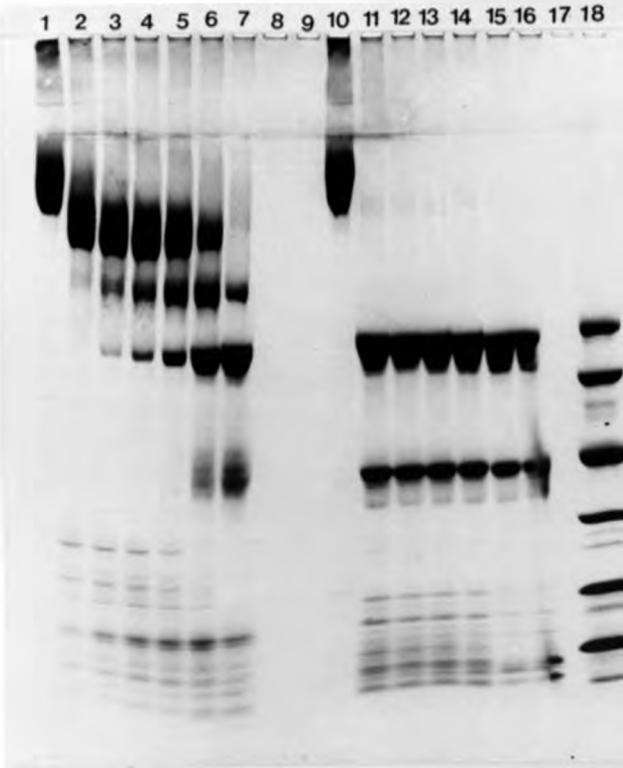
Coomassie brilliant blue R-250 stained SDS-PAGE (5-20% gel). (A), stained gradient gel and it's corresponding autoradiograph (B), displaying the degradation products obtained when ^{125}I-fibrinogen was incubated with 160 milliunits of fibrinolysin P4 or plasmin. Four hundred microlitres of cold fibrinogen to which was added 32 ul of ^{125}I-fibrinogen was used in the experiment. At the end of each reaction, 15 ug equivalent of fibrinogen was analysed on a 5-20% SDS-PAGE gel. Time points tested varied from 0 to 180 min. The lanes were loaded as follows:-

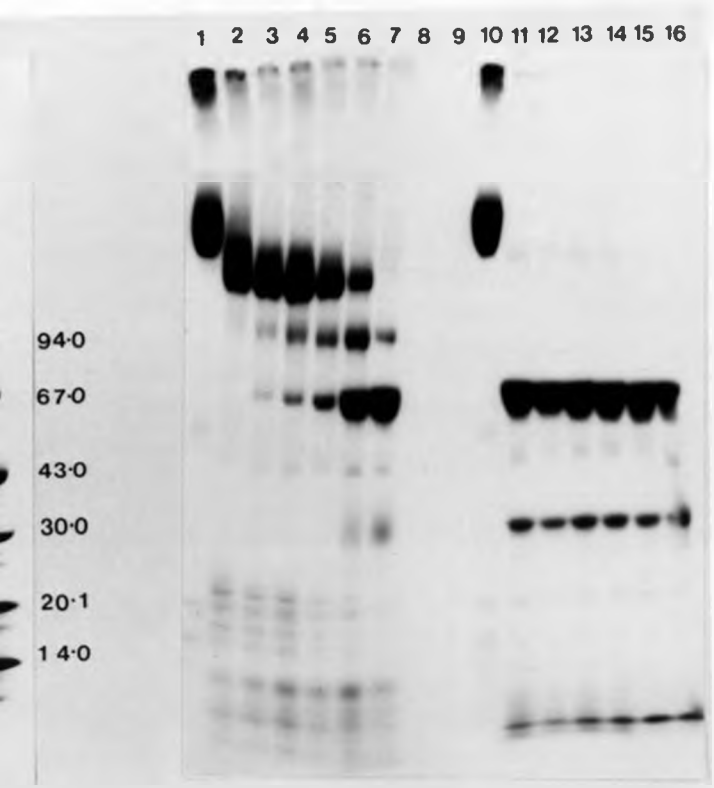
1 and 10 with undigested ^{125}I-fibrinogen (controls no enzymes was added).

Lanes 2-7 were loaded with various ^{125}I-fibrinogen digests generated by fibrinolysin P4, in an increasing order of time: 15 min; 30 min; 45 min; 60 min; 120 min and 180 min.

Lanes 11 - 17 were loaded with ^{125}I-fibrinogen digests generated by human plasmin. Time points were analysed in an increasing order as already described for fibrinolysin P4.

Lane 18 contained low molecular weight protein markers.


1 2 3 4 5 6 7 8 9 10 11 12 13 14 15 16 17 18
A
Coomassie stained



B

Autoradiograph

Figure 22. SDS-PAGE gel analysis of ^{125}I-fibrinogen breakdown products generated by fibrinolysin P3 and plasmin.

Coomassie brilliant blue R-250 SDS-PAGE (5-20% gel). (A), stained gradient gel and its corresponding autoradiograph (B), displaying the degradation products obtained when ^{125}I-fibrinogen is incubated with 160 milliunits of fibrinolysin P3 or plasmin. Four hundred microlitres of cold fibrinogen to which was added 32 ul of ^{125}I-fibrinogen was used in the experiment shown. At the end of each reaction, 15 ug equivalent of fibrinogen was analysed on a 5-20% SDS-PAGE gel. Time points tested varied from 0 to 180 min. The lanes were loaded as follows:-

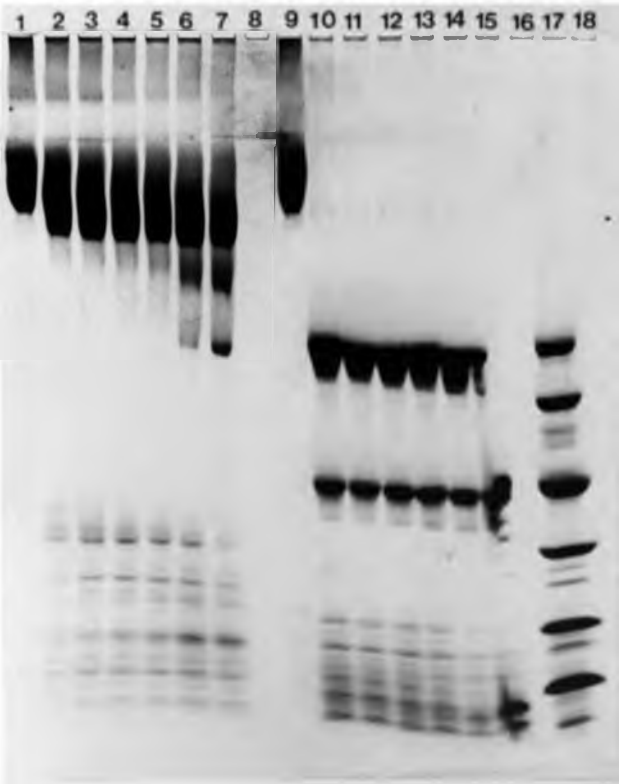
1 and 9 with undigested ^{125}I-fibrinogen (controls, no enzyme was added).

Lanes 2-7 were loaded with various ^{125}I-fibrinogen digests generated by fibrinolysin P3, in an increasing order of time:-

15 min; 30 min; 45 min; 60 min; 120 min and 180 min.

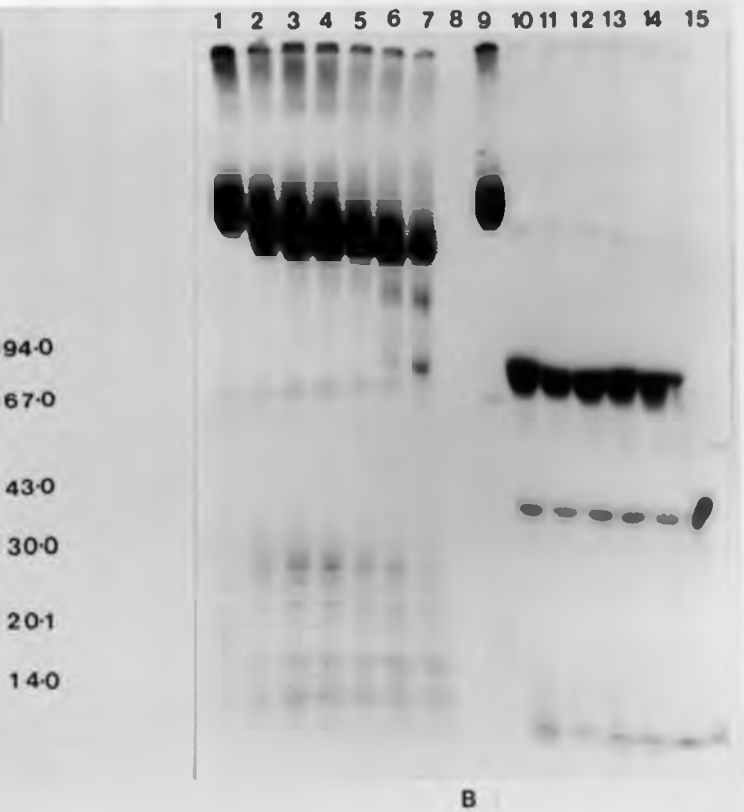
Lanes 11 - 17 were loaded with ^{125}I-fibrinogen digests generated by human plasmin. Time points were analysed in an increasing order as already described for fibrinolysin P3.

Lane 18 - low molecular weight protein markers.



A

Coomassie stained



Autoradiograph

3.8 AMIDASE HYDROLYTIC ACTIVITIES OF TSETSE FIBRINOLYSINS AND PLASMIN TOWARDS VARIOUS p-NITROANILIDE SUBSTRATES.

All the substrates listed in table 4 (shown below) were used in this experiment but, only eight substrates were hydrolysed by P3, P4 or plasmin (see Figure 23). Because only substrates that have Arg or Lys in the P1 position were hydrolysed, it can be deduced that the two tsetse fibrinolysins have higher affinities for chromogenic substrates with the basic amino acids arginine and lysine in the P1 position. From figure 23 it appears that the P2 position of the chromogenic substrate should preferably be occupied by bulky hydrophobic amino acids like phenylalanine or proline. None of the three enzymes hydrolysed single amino acid chromogenic substrates that were blocked at the N-terminal end, regardless of whether the amino acid residue was an arginine or lysine.

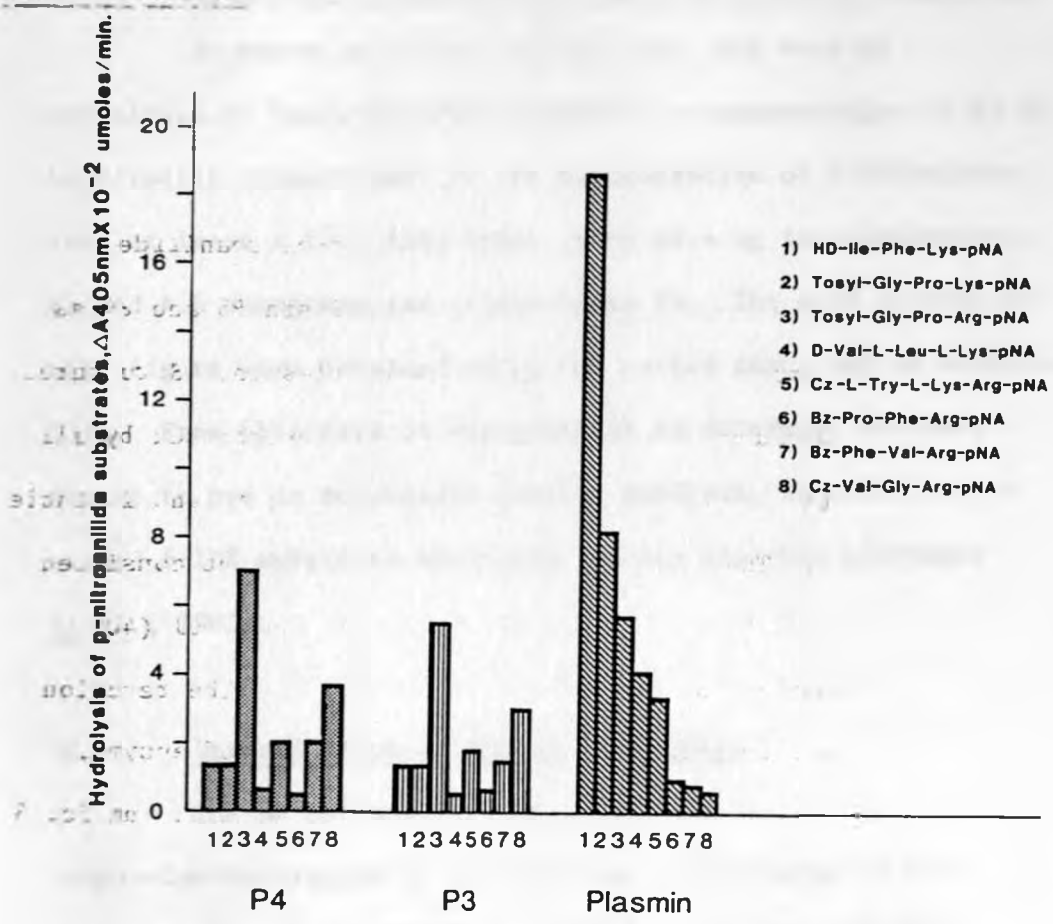
Table 4: p-Nitroanilide substrates and their enzyme specificities.

Substrate	Trade Name	Enzyme Specificity	Reference
Tosyl-Gly-Pro-Arg-pNA	Chromozym-TH	Plasmin and Thrombin	Quoted in Boehringer Manheim Biochemicals Catalogues 1985/86 pp55
Tosyl-Gly-Pro-Lys-pNA	Chromozym-PL	Plasmin	Quoted in Boehringer Manheim Biochmeicals Catalogues 1985/86 pp55
BZ-Pro-Phe-Arg-pNA	Chromozym-PK	Plasma Kallikrein	Quoted in Boehringer Manheim Biochemicals Catalogues 1985/86 pp54
CBZ-Val-Gly-Arg-pNA	Chromozym-TRY	Trypsin	Quoted in Boehringer Manheim Biochemicals Catalogues 1985/86 pp54
N CBZ-Arg-pNA		Trypsin	Somorin et al., 1978
H-D-Val-Leu-Lys-Arg-pNA		Plasmin and Plasminogen activators	Quoted in Bachem Catalogue 1983 pp12
N-Acetyl-Phe-Gly-pNA		Papain	Bendall et al., 1977
CBZ-Arg-Arg-pNA		Cathepsin B	Quoted in Bachem Catalogue 1983, pp6
L-Arg-pNA		Cathepsin B	Quoted in Bachem Catalogue 1982, pp8
Acetyl-Arg-pNA		Trypsin and papain	Nishi and Nogushi 1972
Bz-Phe-Val-Arg-pNA		Thrombin	Lottenberg et. al. 1981
Bz-Arg-pNA		Trypsin and papain	Fritz et. al. 1966
H-D-Ile-Phe-Lys-pNA		highly specific for human plasmin	Quoted in Bachem Catalogue; pp12, 1983
Acetyl-Lys-pNA		Typsin and Plasmin	Quoted in Bachem Catalogue; pp 12, 1983

Figure 23. Amidase hydrolytic activity of p-nitroanilides using fibrinolysins P3, P4 and plasmin.

Amidase-hydrolytic activity of p-nitroanilide substrates displayed by fibrinolysin P3 and P4 as compared to that obtained with plasmin. Structures of substrates that were at least hydrolysed by all the enzymes are indicated on the figure and in table 4. The assay mixture in this experiment consisted of 2.5 ml 0.1 M Tris-HCl, pH 8.0 and 25 ul (40 mmoles) of the substrate under test. The reaction was initiated by adding 26 milliunits of enzyme. p-Nitroanilide release was monitored at 405 nm for 3 min.

ΔA_{405nm} values attained after 1 minute were plotted for each substrate, and these are presented in figure 23. The incubations were performed at 37°C. The assay was carried out in quadriplicate for each substrate.


Hydrolysis of p-nitroanilide substrates, ΔA405nmX10⁻² umoles/min.
20
16
12
8
4
0
1) HD-Ile-Phe-Lys-pNA
2) Tosyl-Gly-Pro-Lys-pNA
3) Tosyl-Gly-Pro-Arg-pNA
4) D-Val-L-Leu-L-Lys-pNA
5) Cz-L-Try-L-Lys-Arg-pNA
6) Bz-Pro-Phe-Arg-pNA
7) Bz-Phe-Val-Arg-pNA
8) Cz-Val-Gly-Arg-pNA
1 2 3 4 5 6 7 8
1 2 3 4 5 6 7 8
1 2 3 4 5 6 7 8
P4
P3
Plasmin

3.9 KINETICS OF CHROMOZYM-TH HYDROLYSIS BY TSETSE FIBRINOLYSINS.

a) Effect of enzyme concentration on rates of hydrolysis.

As shown in figures 24A and 24B, the rate of hydrolysis of Tosyl-Gly-Pro-Arg-pNA at a concentration of 80 uM is directly proportional to the concentration of fibrinolysin over at least a four fold range, upto 10.4 ng for fibrinolysin P3 and 6.6 nanograms for fibrinolysin P4. The data plotted in this figure were obtained using the method described in section 2.15. From this data it was possible to determine how much enzyme to use in subsequent kinetic analyses, in order not to exceed 5-10% substrate hydrolysis during analyses (Erlanger et al., 1961).

b) Determination of kinetic constants.

The pH optimum for the hydrolysis of Tosyl-Gly-Pro-Arg-pNA by the two tsetse fibrinolysins was determined according to the procedure of Simpson and Hard (1984), which is described in section 2.17. Both fibrinolysins were found to have pH optimum of 8.0 (Figure 25).

The classical Michaelis kinetic constant, Km, for the hydrolysis of Tosyl-Gly-Pro-Arg-pNA was determined from a Lineweaver-Burk plot which relates reaction rates to substrate concentration. Figure 26 shows such a result obtained for

fibrinolysin P4. The kinetic data was generated using the method described in section 2.15. Vmax, the maximal velocity at saturating substrate concentration, was determined from an Eddie-Scatchard plot shown in figure 28.

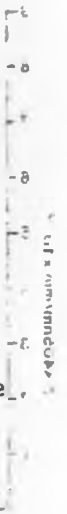
The inhibition of the hydrolysis of Tosyl-Gly-Pro-Arg-pNA by benzamidine was found to be competitive for both fibrinolysins. The inhibiton constant, Ki, was determined for fibrinolysin P4 (Figure 27). The Dixon plot shown in this figure also confirms the competitive inhibition by benzamidine of the P4 hydrolysis of Tosyl-Gly-Pro-Arg-pNA. These data were obtained from the experimental method described under sections 2.16. It should be pointed that the kinetic data for fibrinolysin P3 is not presented here because, it was highly variable although, in general, slightly higher Km, lower Vmax and lower Ki values were observed. Table five shows the Kinetic Parameters obtained for the fibrinolysins P3 and P4.

Table 5. Kinetic parameters generated for fibrinolysins P3 and P4 using Tosyl-Gly-Pro-Arg-pNA and Benzamidine.

FIBRINOLYSIN	Km(uM)	Ki(uM)	Vmax (nmoles/min)
P3	15-20	20-25	4.0 - 6.0
P4	6.0	32.5	8.0

Figures 24a and 24b.

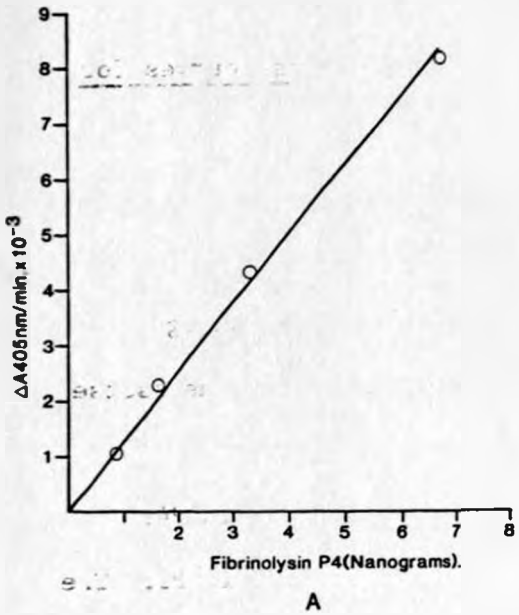
Chromozym-TH hydrolysis dose-response curves for fibrinolysins P3 and P4.



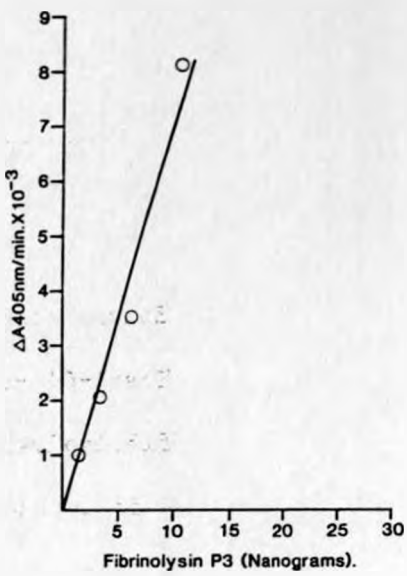
Enzyme dose-response curves generated using Tosyl-Gly-Pro-Arg-pNA as substrate for the tsetse fibrinolysins P3 and P4.

A is a result obtained using fibrinolysin P4.

B is a result obtained using fibrinolysin P3. The substrate (40 mmoles) in 2.5 ml 0.1 M Tris-HCl pH 8.0, was used in each assay. The reactions were carried out for 4 min at 37°C, after which ΔA_{405nm} values attained after 1 minute were plotted against the corresponding enzyme dose. Each point was tested in triplicate.


ΔA405nm/min.x10⁻³
Fibrinolysin P4(Nanograms).

A


ΔA405nm/min.X10⁻³
Fibrinolysin P3 (Nanograms).

B

Figure 25 A and B.

pH optima profiles of fibrinolysins P3 and P4.

A) Profile of pH optimum for fibrinolysin P3 using Tosyl-Gly-Pro-Arg-pNA as substrate and 80 milliunits of enzyme.

B) Profile of pH optimum for fibrinolysin P4 using Tosyl-Gly-Pro-Arg-pNA as substrate and 80 milliunits of enzyme.

Composition of buffer solutions:

a) pH 2-6: 0.2 M citrate-HCl (pH 2.0), citrate-NaOH (pH 4.0), and citrate-NaOH (pH 6.0).

b) pH 6-8: 0.2 M NaH_2PO_4/Na_2HPO_4 (pH 7.0), (pH 7.5) and (pH 8.0).

c) pH 7-9: 0.2 M Tris-HCl (pH 7.0), (pH 7.5), (pH 8.0), (pH 8.5) and (pH 9.0)

d) pH 9-12: 0.2 M Borate-HCl (pH 9.0), (pH 9.5), (pH 10.0), (pH 11.0) and (pH 12.0)

Activity at each selected pH value was assayed as follows: 2.5 ml of the buffer with that particular pH was incubated with 80 milliunits of enzyme for 15 min at 37°C in a waterbath. This mixture was withdrawn and put in a 3 ml cuvette. To this mixture was added 40 mmoles of chromozym-TH and the reaction monitored for 4 min. ΔA_{405nm} attained after 1 minute were plotted against pH, see figure 25 A and B, on the next page. The assays were carried out in triplicate for each tested pH.

4 8 12 14
pH at 37 °C
A
4 8 12 14
pH at 37 °C
B

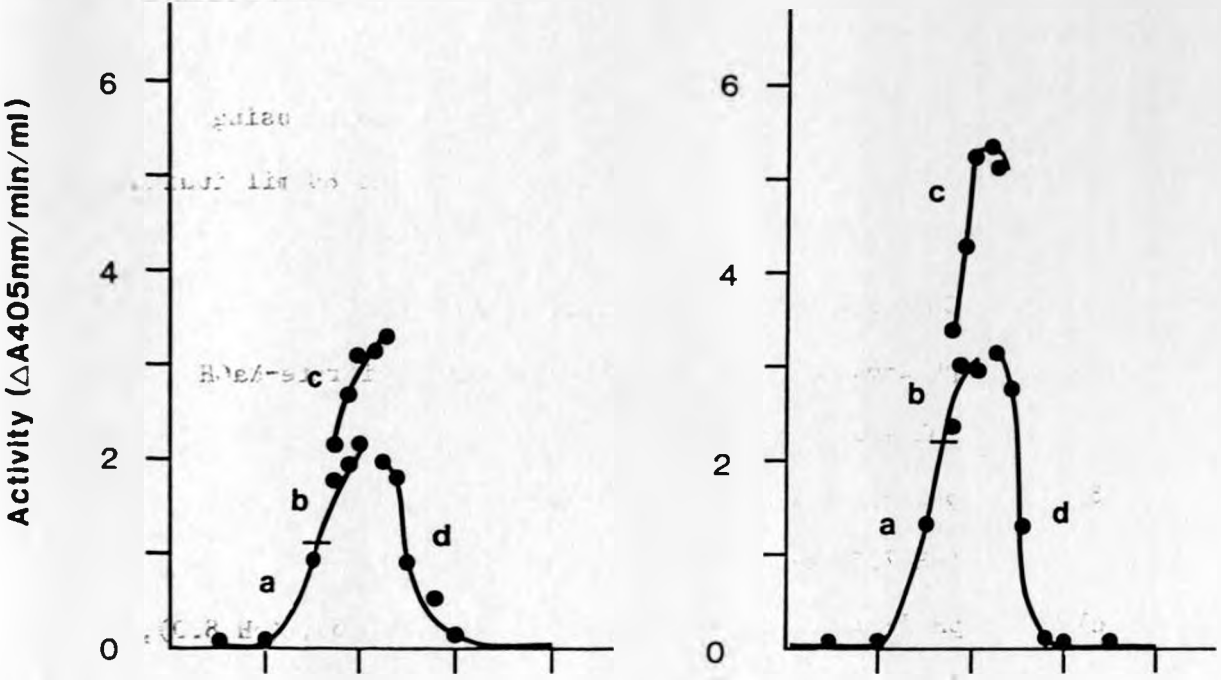

Activity (ΔA405nm/min/ml)
6
4
2
0
a
b
c
d
6
4
2
0
a
b
c
d

Figure 26. Lineweaver-Burk double reciprocal plot for Km and Vmax estimation.

A Lineweaver-Burk double reciprocal plot of velocity against substrate concentration. This figure depicts competitive inhibition of hydrolysis of Tosyl-Gly-Pro-Arg-pNA by fibrinolysin P4 when benzamidine was used as inhibitor. Each point was tested in triplicate and on the same occassion.

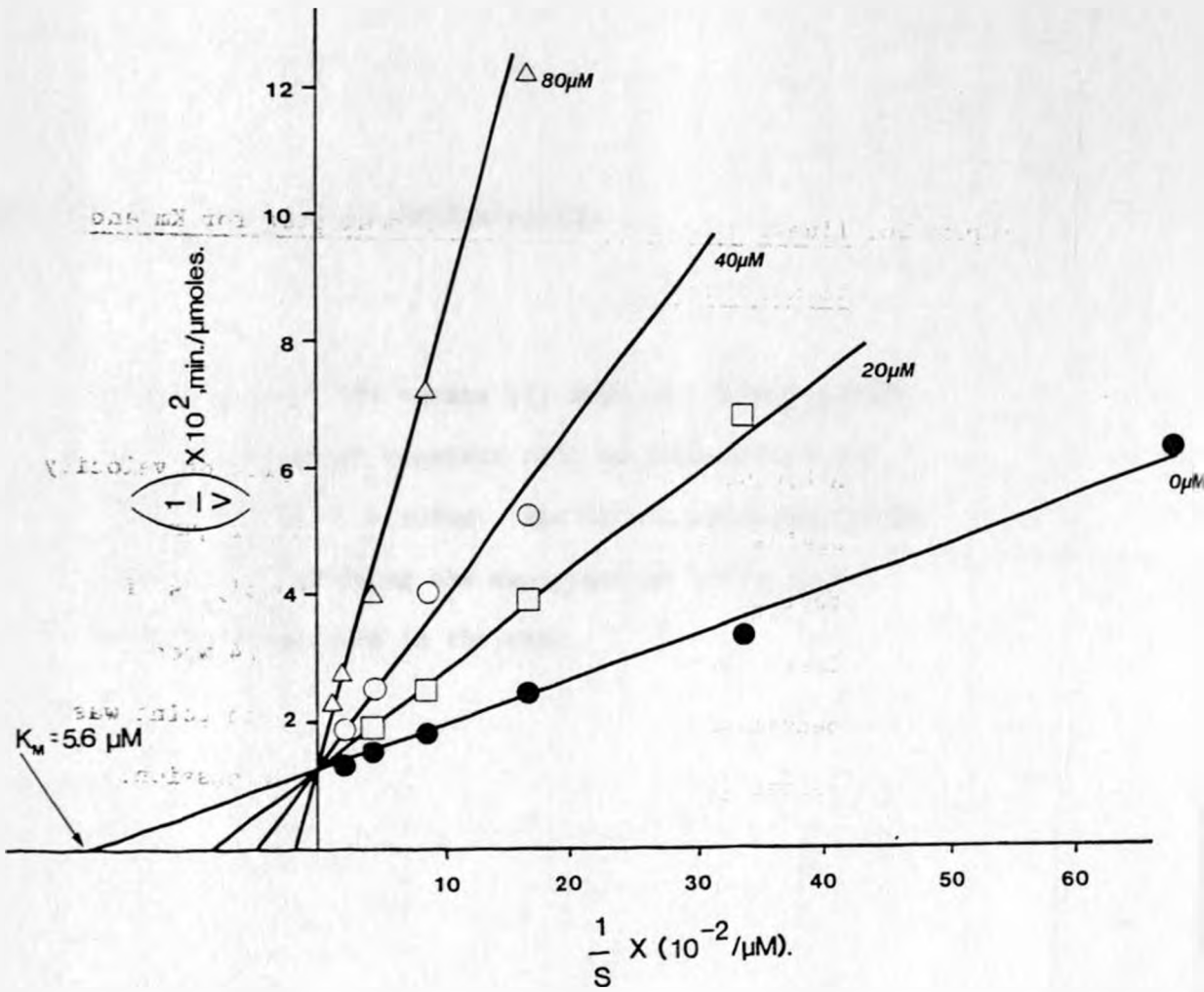
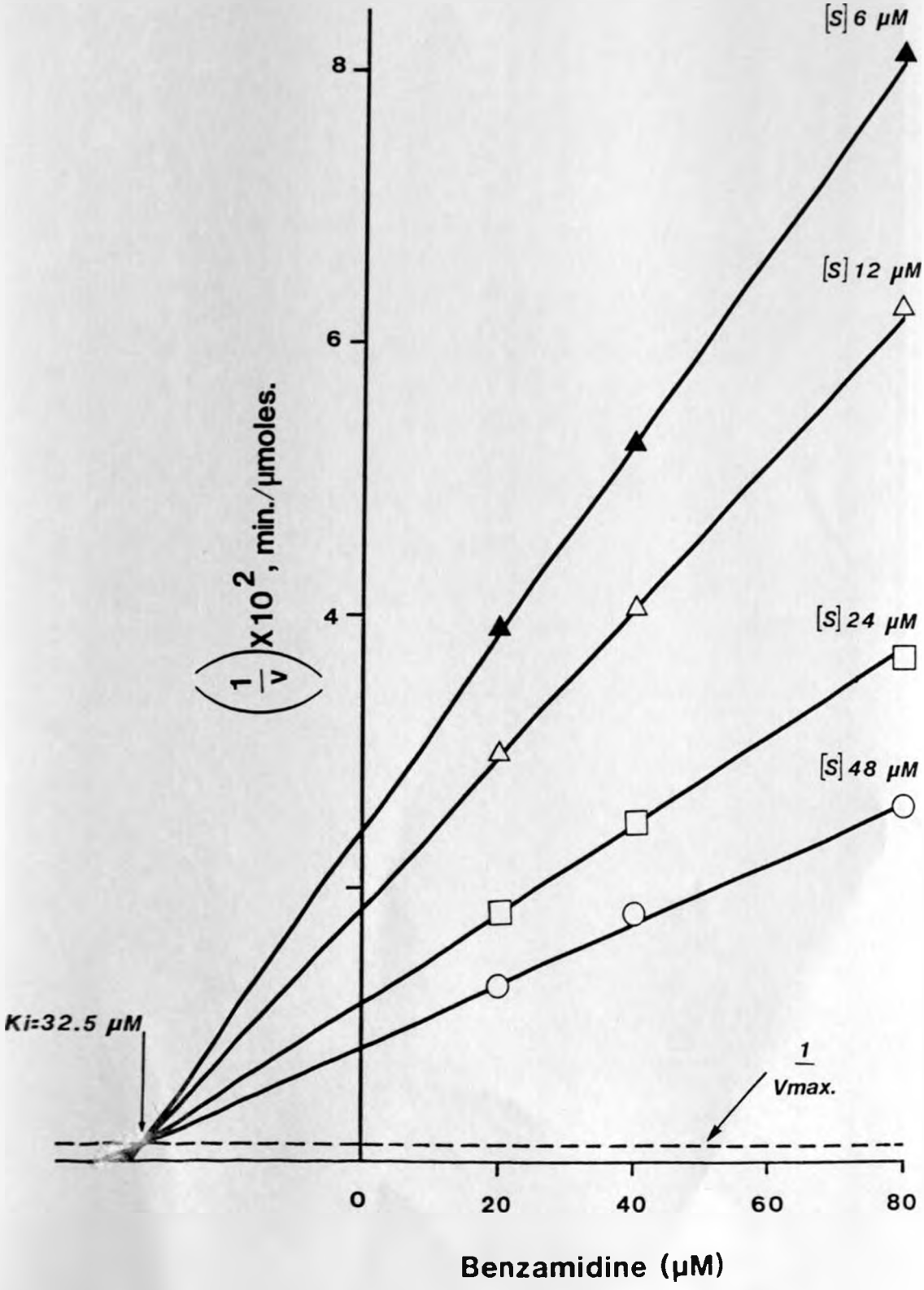

80μM
40μM
20μM
0μM
12
10
8
6
4
2
$\frac{1}{V}$ X 10^2,min./μmoles.
K_M = 5.6 μM
10
20
30
40
50
60
$\frac{1}{S}$ X (10^{-2}/μM).

Figure 27. Dixon plot for Ki determination.

Dixon plot of 1/v versus [I] used for determination of the inhibitor constant (Ki) of benzamidine and fibrinolysin P4 binding. The Ki was estimated to be about 32.5 uM using the experimental conditions already described in the text.


[S] 6 μM
[S] 12 μM
[S] 24 μM
[S] 48 μM
8
6
4
0
20
40
60
80
$\left(\frac{1}{v}\right) X 10^2$, min./μmoles.
Ki=32.5 μM
1/Vmax.
Benzamidine (μM)

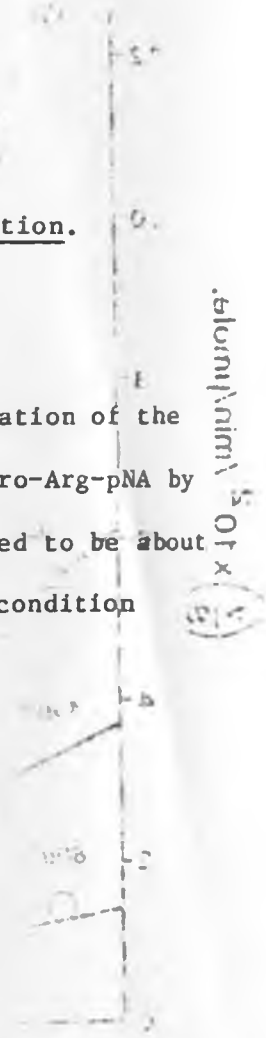
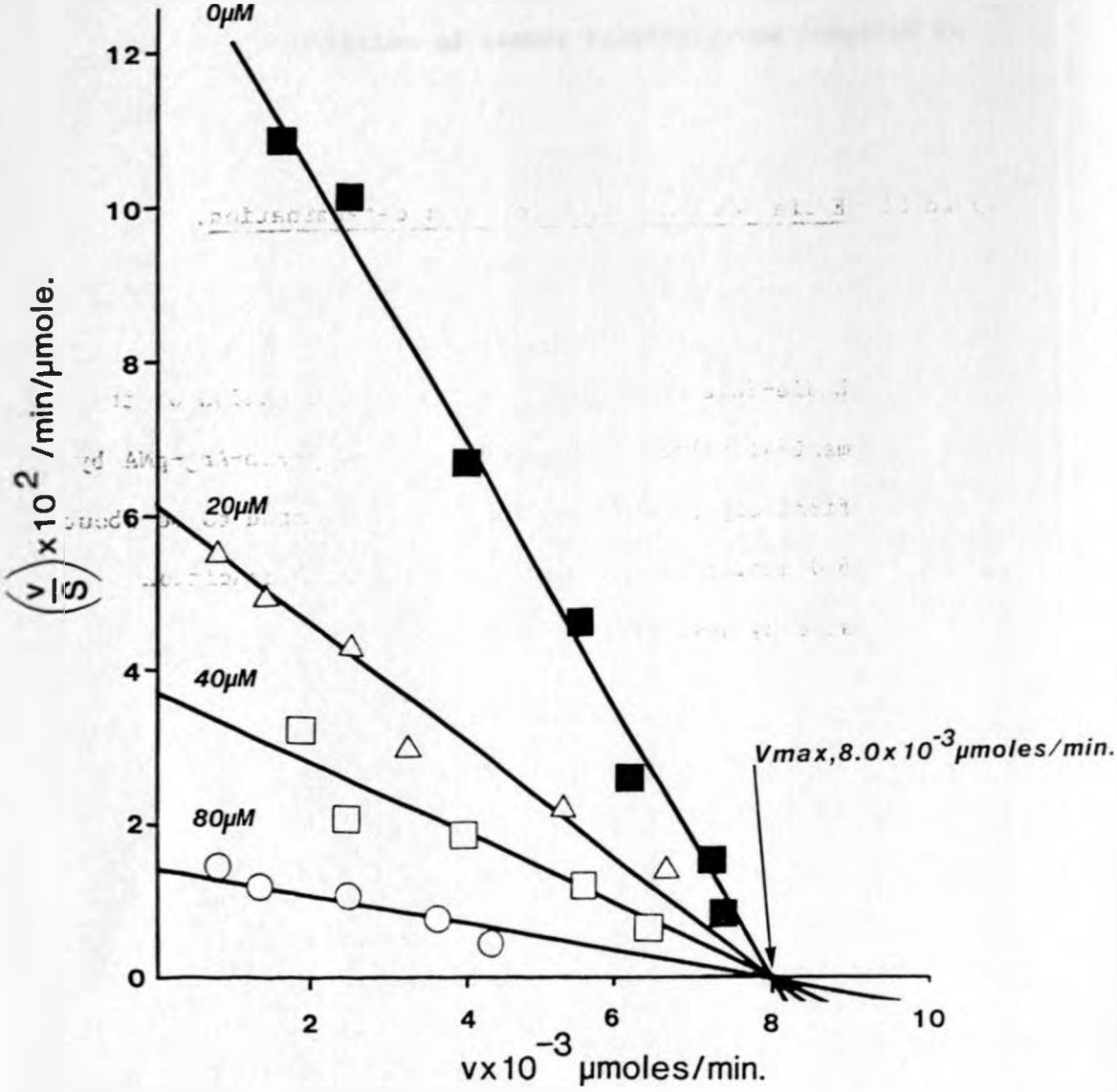


Figure 28. Eddie-Scathard plot for Vmax determination.

Eddie-Scatchard plot used for determination of the maximal velocity (Vmax) of Tosyl-Gly-Pro-Arg-pNA by fibrinolysin-P4. The Vmax was estimated to be about 8.0 nmoles/min using the experimental condition already described in the text.


0μM
20μM
40μM
80μM
Vmax,8.0x10-3μmoles/min.
v/s x10^2 /min/μmole.
vx10-3 μmoles/min.
0
2
4
6
8
10
12

3.10 INFLUENCE OF VARIOUS INHIBITORS ON TSETSE FIBRINOLYSINS.

The fibrinolysins were inhibited by classical serine protease inhibitors and also, to a limited extent, by the thiol protease inhibitors PCMB and iodoacetamide. Table 6 shows the results of inhibition of tsetse fibrinolysins compared to plasmin.

Table 6. Effects of a few selected inhibitors against tsetse fibrinolysins and plasmin.

INHIBITOR	% INHIBITION OF FIBRINOLYSIN (P4)	% INHIBITION OF FIBRINOLYSIN (P3)	% INHIBITION OF PLASMIN
700 uM DFP	100	100	45
700 uM TLCK	74	54.2	15.5
700 uM TPCK	34.6	13	15
1.4mM IODOACETAMIDE	14	29	26
700 uM PMSF	29	11	22.1
700 uM BENZAMIDINE	95.8	93.9	46
40ug SBTI	100	100	97
40ug APROTININ	100	100	97
40ug $alpha_1$-PI	100	100	72
700 uM pCMB	35	21	30

CHAPTER 4

D I S C U S S I O N.

4.1 IDENTIFICATION AND PURIFICATION OF TSETSE MIDGUT EXTRACT FIBRINOLYTIC PROTEASES.

The presence of fibrinolytic activities within the midgut of tsetse flies as well of other haematophagous insects is well documented. However little work has been performed to elucidate the nature and characteristics of these fibrinolytic molecules. In this study, the fibrinolytic activities of the tsetse fly G. morsitans centralis were shown to originate mainly from two trypsin-like proteases out of a total of the five midgut proteases isolated.

Based on fibrinogen digestion patterns on SDS-polyacrylamide gels, three of these proteases behave as if they may be exopeptidases, since they generate a diagonal shift in molecular weight of fibrinogen as digestion proceeds. Thus these enzymes may be the same as those described by Gooding and Rolseth (1976) who, using exopeptidases specific substrates, identified an amino peptidase and two carboxypeptidases in tsetse midgut extracts. Indeed they isolated five midgut proteases, which they identified as an aminopeptidase (Mr, 100 kDa), carboxypeptidase A (Mr, 30 kDa), carboxypeptidase B (Mr, 22 kDa), trypsin (Mr, 20 kDa), a trypsin-like enzyme (Mr, 19 kDa) and a chymotrypsin-like enzyme (Mr, 35.5 kDa).

In this study the two enzymes that had the highest fibrinolytic activities were identified and characterised further.

The DE-52 cellulose chromatography profile (Figure 6) reveals the presence of at least five major protease peaks which hydrolyse the chromogenic substrate, Tosyl-Gly-Pro-Arg-pNA at different rates. These are probably distinct proteases, which have different affinities towards Tosyl-Gly-Pro-Arg-pNA. This substrate was selected as the substrate of choice for screening these enzyme activities at various purification stages because it was the best p-nitroanilide substrate among those tested (Figure 23). Having resolved five distinct peaks of proteolytic activity on DE-52 cellulose, it was necessary to decide which of the five hydrolases contributed most towards the clot lysis or fibrinogen digestion activities of tsetse which have been previously shown to be present within the crude MGE by way of fibrin plate lysis assay, Hawkins (1966) and Parker and Mant (1979). Thus a fibrin single time point and time course assays were designed and performed. It was subsequently established by the results obtained from these two assays that all tsetse proteases, (P1 to P5) obtained using DE-52 cellulose chromatography fractionation, had at least some ^{125}I-fibrinolytic activity. However they did not hydrolyse fibrin to a similar extent, when equal units of each enzyme were used in the assay (Figure 7). It should be noted that peak one (P1) obtained from DE-52 column was very labile on freeze-thawing and it was therefore only used in the

^{125}I-fibrin time point assay. Furthermore, this fraction did not hydrolyse fibrin as fast as the other four proteases, (Figure 9). From these two fibrin digestion assays, it was established that fibrinolysins P3 and P4 had the highest fibrinolytic activity.

Having established which of the five proteases had the highest fibrinolytic activity, two proteases were selected and subjected to further purification by employing isoelectric focusing. The isoelectric points of the two tsetse fibrinolysins, P3 and P4, were determined to be about 6.0 and 5.3 respectively. The activity of the enzymes was reduced drastically during purification by isoelectric focusing.

4.2 CHARACTERISTICS OF TSETSE FIBRINOLYSINS P3 AND P4.

The results presented here demonstrate that the two tsetse fibrinolysins generally have properties in common with most serine proteases of typsin-like specificity isolated from other species.

Physical properties: Fibrinolysins P3 and P4 from G.m. centralis, were most stable at pH 8.0 and displayed pH optima within the broad pH range 7.5-9. Bovine trypsin has a pH optimum of 8.2 but displays the highest stability at pH 3.0 (Erlanger et al., 1961). Human plasmin, which produces similar digestion products from fibrin and fibrinogen as does P4, is most stable at pH's below 4 (similar to bovine trypsin) and displays highest activity around pH 7.4-8.0 (Walsh, 1970). The

findings presented here are similar to observations made by others working on serine proteases from other species (Hjelmeland and Raa, 1982; Gates and Travis, 1969; Jany, 1976; and Chen et al., 1978). These observations suggest that trypsin-like molecules from invertebrates are generally unstable at acid pH but stable at alkaline pH, whereas the opposite is observed for enzymes from higher vertebrates, like bovine trypsin and plasmin or ovine trypsin (Vithayathil et al., 1961). Regardless of their source, all the trypsin-like molecules are optimally active in the alkaline pH range.

The thermal lability properties displayed by both fibrinolysins are quite similar to those observed for other serine proteases, in that they reveal a gradual loss of activity up to 50°C after which there is a precipitious loss of function with further increase in temperature. This agrees well with the anionic eel trypsin whose activity remains steady upto 50°C, then decreases sharply thereafter (Yoshinaka et al., 1985).

The molecular mass estimates for fibrinolysins P3 and P4, namely, 26 kDa and 24.5 kDa on both SDS-PAGE and gel filtration techniques are similar to values reported in the literature for trypsins from other sources such as: starfish 24.4 kDa (Winter and Neurath, 1970); shrimp, 25 kDa (Gates and Travis, 1969); and eel, 25 kDa (Yoshinaka et al., 1985). Hence the tsetse fibrinolysins fall within the general range reported for molecular masses of trypsins - from 20 kDa to 25 kDa (Kiel, 1971). The Mr values reported in this study for P3 and P4 from G. morsitans centralis (26 and 24.5 kDa respectively) differ

slightly from those reported by Gooding and Rolseth (1976) for G. morsitans morsitans Westwood (20 and 19 kDa, respectively).

Amidase activity: The trypsin-like specificity of these fibrinolysins was shown by using trypsin specific chromogenic p-nitroanilide substrates which these enzymes hydrolysed fairly rapidly. The amidase hydrolysis of Tosyl-Gly-Pro-Arg-pNA by the fibrinolysins obeyed Michaelis-Menten kinetics over the range of substrate concentrations examined. The low Km values for fibrinolysin P4 with Tosyl-Gly-Pro-Arg-pNA as substrate indicate that the association constant for the formation of enzyme-substrate complex is low (Table 5). This implies that the affinities of fibrinolysin P3 and P4 for Tosyl-Gly-Pro-Arg-pNA are high, although slightly lower in the case of P3. This observation was further supported by comparing the amidase hydrolytic rates of various chromogenic p-nitroanilide substrates. In this study both tsetse fibrinolysins hydrolysed Tosyl-Gly-Pro-Arg-pNA faster than any of the other p-nitroanilide substrates.

The amidase activities of the two tsetse fibrinolysins and bovine plasmin were almost equally inhibited by the same concentrations of the inhibitors, SBTI, aprotinin and alpha$_1$-proteinase inhibitor. Blow et al. (1974) have shown that SBTI's are proteins which bind very tightly to trypsin, blocking it's active site in the process. Similar studies showing the inhibition of trypsins by SBTI have been done by Stambaugh and Buckley (1972), Gates and Travis (1969), Hjelmeland and Raa (1982) and by Bundy and Gustafson, (1973).

Aprotinin inhibition of trypsin and trypsin-like enzymes has been demonstrated by investigators like Kassell (1970), Hjelmeland and Raa (1982) and Steven and Griffin (1981). One of the major proteinase inhibitors in plasma is alpha$_1$-proteinase inhibitor, which acts as a general scavenger for tissue serine proteinases in blood including plasmin. Alpha$_1$-PI also inhibited P3 and P4. The demonstration in this study that equal amounts of these protein inhibitors, inhibit purified tsetse fibrinolysins and bovine plasmin to the same extent indicates that the two fibrinolysins, and human plasmin, probably have substrate binding sites similar to trypsin. Nevertheless their substrate binding affinities are clearly distinct as was shown by their different rates of hydrolysis of p-nitroanilides and fibrinogen. Complete inhibition was observed when DFP was used to inhibit P3 and P4. This strongly suggests that these fibrinolysins have a serine amino acid residue at their active site. Since TPCK is considered to be a better inhibitor of enzymes with chymotrypsin-like specificity whereas TLCK is considered to inhibit best those enzymes which exhibit trypsin-like specificity, higher inhibition of both fibrinolysins by TLCK as opposed to TPCK is a strong indication of trypsin-like specificity. It should be noted that the low inhibition exhibited by phenylmethanesulfonylfluoride (PMSF) towards both fibrinolysins is not surprising inview of the poor inhibition observed with TPCK.

Light (1971) had previously observed inhibition of bovine trypsin by thiol reagents although there is no thiol group near the active site of this enzyme.

Proteolytic activty: The chemistry of human fibrinogen has been extensively studied (Blomback et al., 1981) and the molecule (Mr 340 kDa) has been shown to consist of three pairs of chains (two each of A-alpha, B-beta, and gamma) which are connected by disulphide bridges. The molecular weights of the chains are approximately 70, 60 and 50 kDa, respectively. The general scheme for fibrinogen digestion by plasmin was originally proposed by Marder et al. (1969) and was later supported by the results of other investigators (Furlan and Beck, 1972; Gaffney, 1972; Mills, 1972; Pizzo et al. (1972) From such experiments it is apparent that fibrinogen is degraded through several intermediates, X (Mr 240 kDa), Y (Mr 160 kDa), and to the final large molecular weight products, D (Mr 80-100 kDa) and E (Mr 60 kDa). All these products, are heterogeneous populations of proteins rather than discrete protein intermediates, which is a reflection of the lack of clear-cut specificity for plasmin.

Studies done using P3 and P4 on the native substrates of plasmin, non-reduced fibrinogen and fibrin, showed that the two enzymes generate fibrin(ogen) breakdown products at different rates. When ^{125}I-fibrinogen was used as the substrate for tsetse fibrinolysin P4, two conspicuous fibrinogen breakdown products of about 42 and 94 kDa were generated via intermediate breakdown products (Mr 200 and 130 kDa) similar to those generated by plasmin, as already

described (Harfenist and Canfield, 1975). These result suggest that tsetse fibrinolysin P4 might be the protease which is used by tsetse flies to prevent (or dissolve) any clots from forming within the gut and proboscis. This observation is further supported by the fact that fibrinolysin P4 generated the two (42 and 94 kDa) fibrinogen breakdown products, similar to those generated by plasmin, faster than any of the other tsetse proteases that were examined. After a period of about 45 to 60 minutes these two fibrinogen breakdown products could be observed in the fibrinogen digests of fibrinolysin P4 on gels, whereas hardly any products within these molecular weight range were generated by the other tsetse enzymes within the same period of time. Generation of the 42 kDa intermediate by fibrinolysin P4 implies that this tsetse enzyme digests the B chain of fibrinogen since this has been shown (Ferguson et al., 1975) to be a fibrinogen B chain breakdown product. There is also indirect evidence for the breakdown of the fibrinogen alpha-chain by both fibrinolysin P3 and P4. The breakdown product observed at about 25 kDa on gels is identical to similar products generated by plasmin from the fibrinogen A alpha-chain (Budzynski et al., 1974; Harfenist and Canfield, 1975). Thus one would infer from these results that the tsetse fibrinolysin P4, an endopeptidase, seems to cleave fibrinogen and fibrin around the same domains as does bovine plasmin, the classical fibrinolysin. Fibrinolysin P3 initially generates very low molecular weight products (Mr=14-20 kDa) slowly, but with time it also starts generating a breakdown product of Mr around 94 kDa. The slow action of fibrinolysin P3 would imply

that the binding affinity of this enzyme to fibrin(ogen) is lower than that of P4 or plasmin and the possibility of limited sites of cleavages can also not be ruled out. The pattern of fibrinogen cleavage by P3, suggests that this enzyme may posses exo- as well as endo-peptidase activity.

4.3 INDUCTION OF ANTIBODIES AGAINST INSECT TISSUES AND PURIFIED MOLECULES.

Immunological resistance in rabbits against the tsetse fly G.morsitans morsitans (Westwood) has already been demonstrated (Parker and Gooding, 1979). This acquired host resistance was observed in rabbits used for maintaining and breeding tsetse colonies. It affected female fly mortality, pupal weights and to a lesser extent productivity, but not male mortality or emergence from pupae. Thus a specific mechanism, which was possibly immunological in nature, was implicated. Parker and Mant (1979) showed that sera obtained from rabbits used in maintaining tsetse contained precipitating antibodies against saliva. These antibodies did not neutralize the *in vitro* activity of tsetse salivary gland anticoagulant. Otieno *et al*. (1984), maintained G. morsitans morsitans on rabbits immunized with crude tsetse midgut proteases and made several observations: newly emerged flies fed on these rabbits showed a depressed trypsin/proteinase VI activity; six and a half percent of these flies failed to empty their crops as readily

as controls and approximately 8% of the cases stayed as long as eleven days with an undigested bloodmeal.

Other investigators have also been able to demonstrate increased mortality rates in other insects such as in Anopheles stephensi (Alger and Cabrera, 1972) and Stomoxys (Schlein and Lewis, 1976), when gut tissue and whole mosquitoes were used to induce antibodies or when rabbits were immunized with homogenate from various tissues of Stomoxys calcitrans. It should be noted that acquired resistance to tick infestation by bovids suggests that immunological principle(s) are involved (see review by Wickel, 1982), but the manner in which the antibodies interfere with these functions has not yet been elucidated.

The fibrinolysins isolated in this study have been shown to be immunogenic. However the purified IgG and Fab fragments obtained from the immune sera did not inhibit the hydrolytic activity of these enzymes towards Tosyl-Gly-Pro-Arg-pNA. This suggests that these antibodies might not be active-site directed. Such antibodies may thus bind to non-active site epitopes on the enzymes and did not affect the capacity of the fibrinolysins to bind and hydrolyse the peptide substrate. By western blotting it was shown that antibodies made against fibrinolysin P4 reacted with fibrinolysin P3, thus suggesting that the two share common antigenic determinants.

CONCLUSION.

Further research is required in the area of host immune responses to various tsetse antigens, with particular emphasis directed towards developing a viable immunization scheme using potential vector immunogens. Another area of interest that should be pursued is that of why the trypanosomes are able to co-exist comfortably within the tsetse midgut with very highly proteolytic activities without being susceptible to lysis by these enzymes. An attempt should also be made to develop specific proteinase inhibitors of tsetse digestive enzymes, with a view of incorporating these inhibitors into insecticides.

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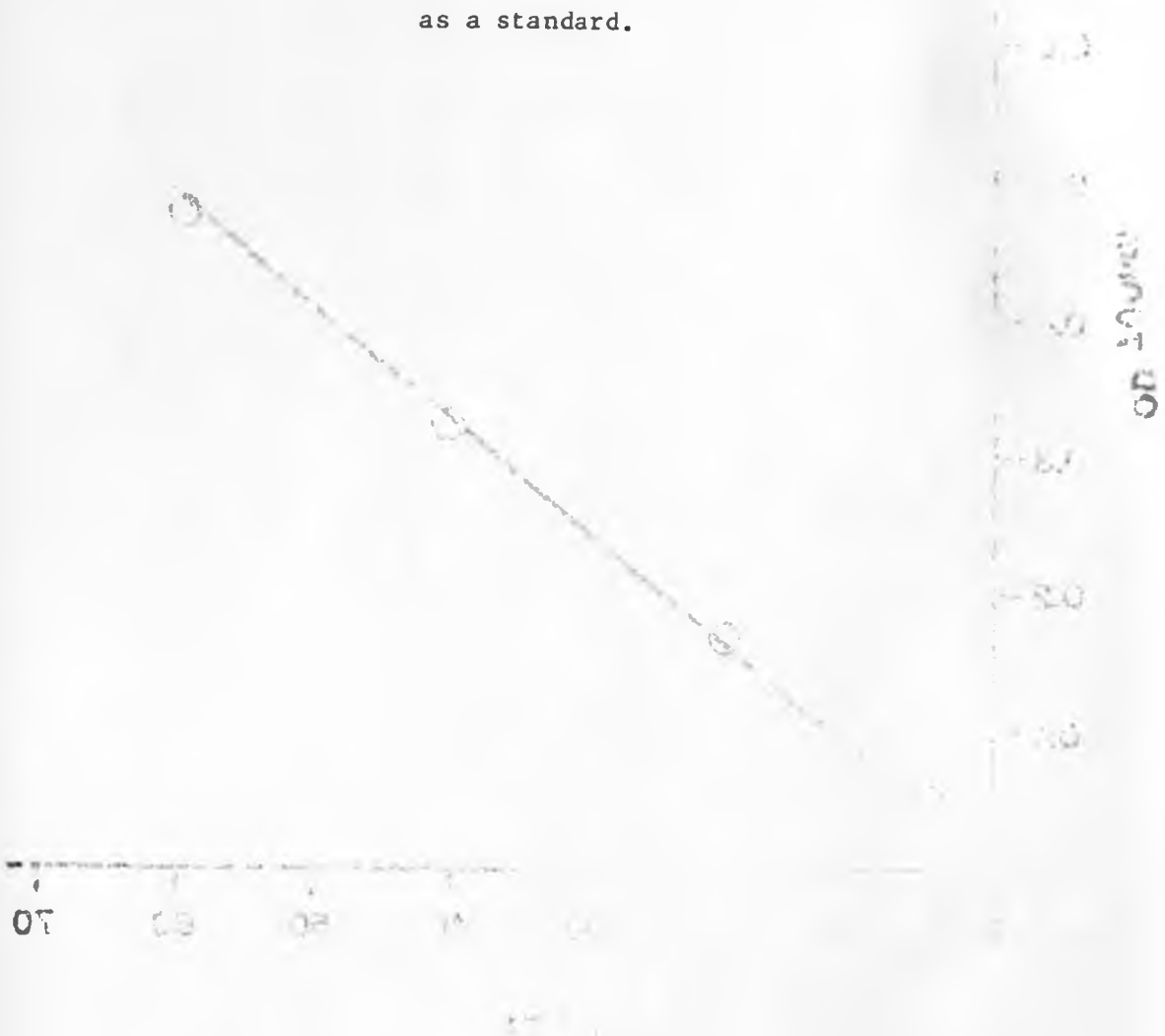
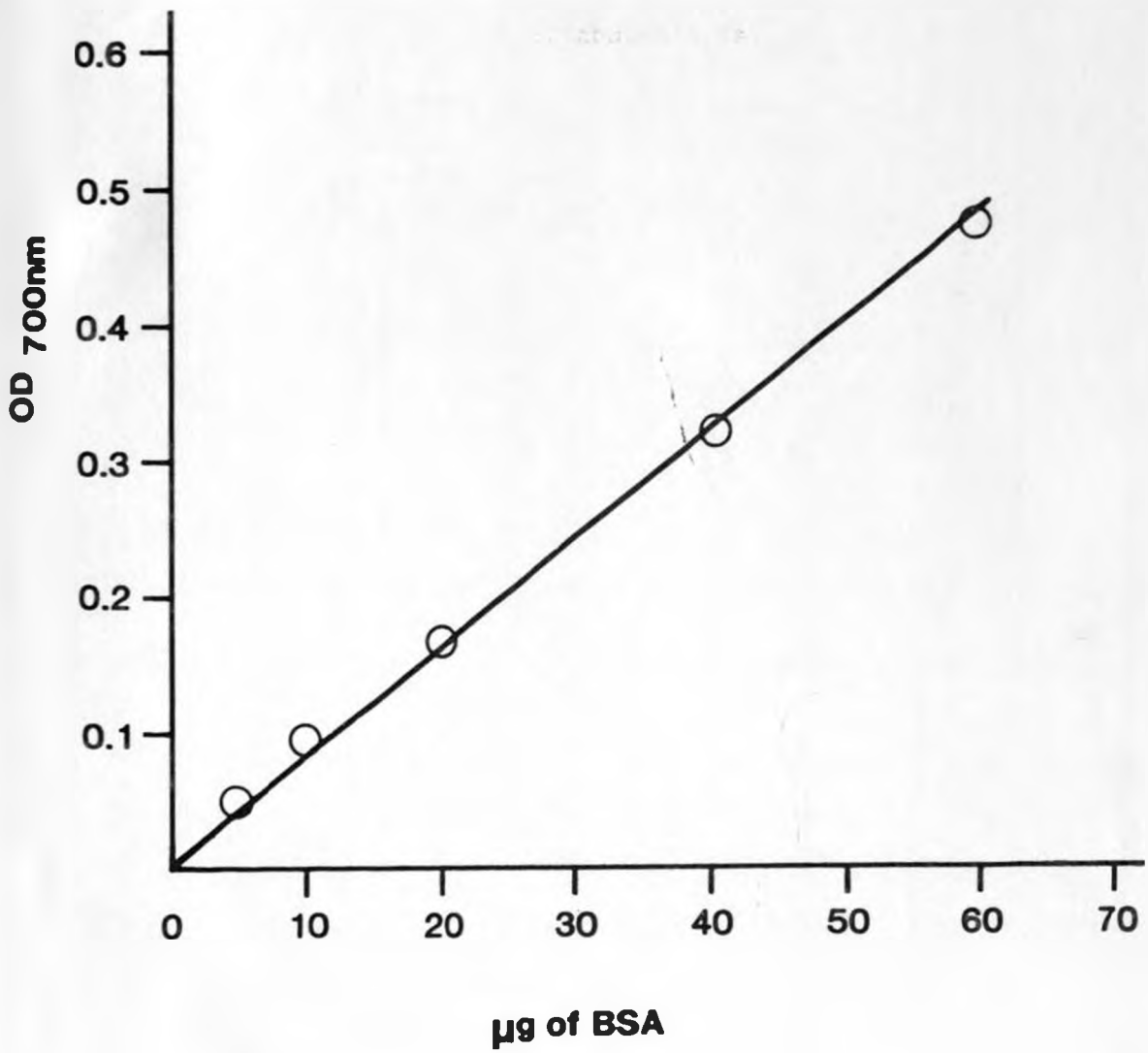
Appendix.

Figure 5 Lowry's standard protein curve used for quantitative estimation of proteins employing bovine serum albumin as a standard.




0.6
0.5
0.4
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10
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µg of BSA