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Biochemical investigations into the proteolytic activities in the salivary glands of the tick, *Ornithodoros savignyi*

by

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OUTLINE OF THE DISSERTATION

The dissertation consists of a list of abbreviations and four chapters followed by a summary.

The general introduction, that involves the description of proteases and protease inhibitors, followed by a discussion of the blood clotting in response to an injury is given in chapter 1. This includes the role of the clotting enzymes during clotting, the mechanisms to control clotting and the ticks as sources of antihemostatics.

In chapter two the preliminary investigation into the role of total proteolytic activity present in the salivary glands, the fibrinogenolytic activity in disturbing the normal haemostasis. Azocaseinolytic activity and SDS-PAGE were used to assay the presence of the activities.

In chapter three, the attempted HPLC isolation aimed at purifying the fibrinogenolytic activity is discussed, while in chapter four the role of this partially purified fibrinogenolytic activity in platelet aggregation is investigated.

The concluding discussion in chapter five is followed by a list of references used in this dissertation.

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LIST OF ABBREVIATIONS

A_{405}	Absorbance monitored at 405 nm
ADP	Adenosine diphosphate
ATIII	Antithrombin III
α_2M	α2-macroglobulin
α_1A	α1-antiplasmin
AE-HPLC	Anion exchange chromatography
APC	Activated protein C
APMSF	4-amidinophenylmethanesulphonyl fluoride
Asx	Asparagine/ Aspartic acid
BSA	Bovine serum albumin
CAPS	3-cyclohexyl amino)-propane sulphonic acid
°C	Degree Celsius
CE-HPLC	Cation exchange chromatography
COOH-	Carboxy-terminal
Da	Dalton
ddH_2O	Double distilled water
DEAE	Diethyl aminoethyl
DCI	3,4-Dichloroisocoumarin
DFP	Diisopropyl fluorophosphate
DMSO	Dimethyl Sulphoxide
DTT	Dithiothreitol
E-64	L-trans-Epoxysuccinyl leucylamido (4-guanidino)-butane
EDTA	Ethylenediamine tetraacetate
ES	Excretory/secretory products
Fib	Fibrinogen
FX	Factor X
FXa	Activated factor X

FV	**Factor V**
FVa	**Activated factor V**
F VII	**Factor VII**
FVIIa	**Activated factor VII**
FVIII	**Factor VIII**
FVIIIa	**Activated factor VIII**
FVIIIa	**Activated factor VIII**
FXa	**Activated factor X**
FXIa	**Activated factor XI**
FXIIa	**Activated factor XII**
FXIIIa	**Activated factor XIII**
g	**Relative centrifugal force**
gla	**γ-carboxyglutamate**
Glu	**Glutamine**
Glx	**Glutamine / Glutamic acid**
GPIb	**Glycoprotein Ib**
GPIIb	**Glycoprotein IIb**
GPIIIa	**Glycoprotein IIIa**
gp	**Glycoprotein**
HIC	**Hydrophobic interaction chromatography**
HMW	**High Molecular weight**
HOAc	**Acetic acid**
HPLC	**High performance liquid chromatography**
kDa	**Kilodalton**
K_i	**Inhibition constant**
Km	**Michaelis-Menten constant**
Lys	**Lysine**
LMWH	**Low molecular weight heparins**
ME	**Mercaptoethanol**
M	**Molar**
Mm	**Millimolar**

µl	Microliter
µM	Micro molar
MES	2-(N-Morpholino)ethane sulfonic acid)
Mr	Relative molecular weight
NHS	N-Hydroxy-succinimide
NH$_2$(N-)	Amino terminal
NPGB	p-nitrophenyl-p'-guanidinobenzoate
nm	Nanometers
NMR	Nuclear magnetic resonance
PAGE	Polyacrylamide gel electrophoresis
PBS	Phosphate buffered saline
PCR	Polymerase chain reaction
pI	Isoelectric point
PI's	Proteinase inhibitors
PIP$_2$	Phosphatidylinositol 4,5-bisphosphate
PMSF	Phenyl methane sulphonyl fluoride
PS	Protein S
PPP	Platelet poor plasma
PRP	Platelet rich plasma
PVDF	Polyvinylidene difluoride
RGD	Arg-Gly-Asp
RP-HPLC	Reversed phase High Performance Liquid Chromatography
SD	Standard deviation
SEC	Size exclusion chromatography
SEM	Scanning electron microscopy
SG	Salivary gland
SGE	Salivary gland extract
SDS	Sodium dodecyl sulphate
SDS-PAGE	Sodium dodecyl sulphate-polyacrylamide gel Electrophoresis
SH	Sulfhydryl group

TAP	Tick anticoagulant peptide
TAME	Tosyl-arginine methyl ester
TCA	Trichloroacetic acid
TLCK	N-tosyl-L-lysine chloromethyl ketone
TEMED	N,N,N'N'-Tetramethylene diamine
TRAP	Thrombin receptor activated peptide
Tris	Tris(hydroxymethyl)aminomethane
UV	Ultraviolet
V	Volt
Vmax	maximum velocity
V/V	Volume per volume
Vwf	Von Willebrand factor
W/V	Weight per volume

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CHAPTER 1

LITERATURE OVERVIEW

1.1 INTRODUCTION

Ticks transmit several important diseases to man and animals. These include Lyme disease, relapsing fever and tularemia. Because these diseases are transmitted to humans and to other vertebrates as a result of the ability of arthropods to feed on blood, the study of the biochemical mechanisms and adaptations that arthropods have evolved to facilitate hematophagy may provide insight into how this feeding behavior contributes to the transmission of diseases (Stark and James, 1996).

Ticks manipulate their host's immune system and associated inflammatory and hemostatic reactions to obtain a bloodmeal (Ribeiro, 1985b). Certain tick species secrete noxious substances capable of causing disabling or lethal toxic conditions, termed toxicoses, as well as allergic hypersensitivity reactions in their vertebrate hosts. The best known is tick paralysis, an ascending motor paralysis induced by substances introduced into the host with the saliva when the ticks attach and feed. Cattle have been observed to die within as little as one day from the bites of the tampan, *Ornithodoros savignyi* (Howell *et al.*, 1978).

Failure to control ticks and tick-borne diseases is a major factor limiting livestock production. The cost of worldwide economic loss and the additional burden of protecting livestock against ticks and tick-borne diseases, together with the loss to mankind of significant amounts of animal protein due to cattle death or diminished productivity, are now estimated to be in the billions of dollars annually (Sauer *et al.*, 1995, De Castro and Newson, 1993, Ferreira and Silva, 1998). According to the Food and Agricultural Organization (FAO, 1995) year book, an estimated 80 % of the world's cattle are infested by ticks, giving rise to an annual cost of 7500 million US dollars. Historically, ticks have been controlled by the use of acaricides, but as with other arthropods the greatest problem with the existing tick control procedures

has been the development of resistance to these acaricides (Nolan, 1985; Mulenga *et al.*, 2000) and the presence of the chemical residues in meat and milk show the need for alternative control methods (Kunz and Kemp, 1994). Anti-tick vaccines represent a promising alternative to conventional chemical control and have the advantage of target specificity, environmental friendliness, no side-effects concerning human health and ease of administration (Wikel, 1996 and Mulenga *et al.*, 1999). Antibodies capable of blocking the action of tick antihemostatics could interfere with proper tick feeding. Furthermore, the isolation and biological characterization of these substances will also permit their evaluation with respect to potential therapeutic applications for the treatment or prophylaxis of thrombotic conditions.

Tick antihemostatics enter the host via the salivary secretion. These secretions are the major if not exclusive routes whereby pathogenic organisms and toxins access the host. Salivary glands secrete cement, which anchors the mouthparts to the skin after attachment to the host. They also serve as primary organs of osmoregulation and the regulation of hemolymph volume (Jaworski *et al.*, 1992). Therefore, all medical and veterinary problems related to ticks are mainly associated with the salivary glands (Kaufman, 1989).

Earlier investigations indicated the presence of a fibrinogenolytic activity in the salivary gland extract (SGE) of *O. savignyi* (J. Nienaber, personal communication). These findings prompted the need to investigate the proteolytic activity associated with the salivary glands of this tick species. The present thesis reports on the proteolytic activity and more specifically the fibrinogenolytic activity present in the salivary glands of this tick.

1.2 GENERAL CHARACTERISTICS OF TICKS

Approximately 850 tick species are known, and these are subdivided into two major families. The one comprises the hard ticks or *Ixodidae*, which have a sclerotized dorsal scutal plate while the other, the soft ticks or *Argasidae*, possess a flexible,

leathery cuticle. A third family, the *Nuttalliellidae*, contains only a single species (Sonenshine, 1991).

Ticks infest every class of terrestrial vertebrates, various reptiles and even amphibians (Sonenshine, 1991). Although widely recognized as pests, ticks are best known because of the numerous diseases they transmit. Ticks surpass all other arthropods, except mosquitoes in the variety of pathogenic organisms they transmit, including fungi, viruses, rickettsiae, bacteria, and protozoa (Sonenshine, 1991; Obenchain and Galun, 1982). In addition to the pathogens they transmit, ticks may induce lethal paralysis or severe toxicoses as a result of their bites. In some instances, infestations of ticks may reach such extreme magnitude that animals die of exsanguination or become susceptible to other illness due to their weakened condition (Sonenshine, 1991).

Ticks possess many remarkable features that contribute to their success as vectors of diseases; features distinguishing them from arachnids and insects. These characteristics are summarized briefly in Table 1.1 (adapted from Sonenshine, 1991).

TABLE 1.1: CHARACTERISTICS OF TICKS THAT DISTINGUISH THEM FROM OTHER BLOOD SUCKING ARTHROPODS

Biological characteristics	Ticks	Other blood sucking arthropods
Duration of life cycle	Long, usually years	Relatively short, weeks or months
Blood volume	Large, up to 4 or 5 ml/tick	Relatively, small < 1 ml/individual
Egg production	Large up to 23 000/ female	Smaller, only hundreds/female
Blood meal digestion	Primarily intracellular within the midgut cells	Extracellular, within the midgut lumen
Vector competency	Transmit protozoan, fungal, bacterial, rickettsial, viral, and nematode pathogens	No other arthropod group transmits such a wide variety of pathogens

The salivary glands are the largest, complex and heterogeneous organs in ticks. They consist of a pair of grape-like clusters of acini comprising two major types. Argasid ticks typically have one agranular acini and four types of granular acini.

Within the acini, differentiation occurs during feeding, with important changes in cellular activity (Sonenshine, 1993).

In all stages of ixodid ticks, which feed slowly, the salivary glands are the primary sites for elimination of water and electrolytes, facilitating the concentration of the enormous blood meal. Ticks have developed a highly specialized system of mouthparts and associated structures to gain access to the body fluids of their hosts. Ticks attach to the host skin, using a hypostome as a holdfast, and create a feeding lesion from which they imbibe blood or tissue fluid. Argasid ticks feed rapidly (minutes to hours) from this pool, taking advantage of their flexible, highly expandable body cuticle to take up volumes of fluid 5-10 times their original pre-feeding size.

1.3 BIOACTIVE SUBSTANCES PRESENT IN TICK SALIVARY GLANDS

Tick saliva contains various enzymes and several types of pharmacodynamic modulators that antagonize host hemostatic and inflammatory compounds, facilitating blood meal uptake. Different bioactive substances including anticoagulants, platelet aggregation inhibitors as well as anti-inflammatory agents have been identified in these organisms. In some species, immunosuppressive agents are present that enable these parasites to evade host rejection responses. Ixodid ticks, also secrete cement that binds the tick to the host skin, and excrete excess water and ions accumulated during feeding (Willadsen, 1980, Ribeiro, 1987, Titus and Ribeiro, 1990 and Saito 1991). Table 1.2 (Adapted from Nuttall, 1998) summarizes some of the bioactive peptides or proteins secreted in tick saliva.

TABLE 1.2: SOME BIOACTIVE PEPTIDES AND PROTEINS SECRETED IN TICK SALIVA

Tick species	Component	References
Ornithodoros savignyi	Savignygrin (Inhibits the fibrinogen receptor on platelets)	Mans *et al.*, 2002a
	Savignygen (Inhibits platelet aggregation response to collagen)	Mans, 2002c
	TSGP4 (Causes morbitz type ventricular block)	Mans *et al.*, 2002b
	TSGP2 (Induces ventricular tachycardia in rats)	Mans *et al.*, 2002b
	Inhibitor of the extrinsic blood coagulation	Ehebauer *et al.*, 2002
	Savignin (Thrombin inhibitor)	Nienaber *et al.*, 1999
	Apyrase (Inhibits platelet aggregation)	Mans *et al.*, 1998
	FXa inhibitor	Gaspar *et al.*, 1996
Ornithodoros moubata	Tick anticoagulant peptide (inhibits FXa)	Waxman *et al.*, 1990
	Tick adhesion inhibitor (blocks adhesion to collagen)	Karczewski *et al.*, 1994
	Disaggregin (Platelet aggregation inhibitor)	Karczewski *et al.*, 1994
	Moubatin (Inhibits platelet aggregation to collagen)	Waxman and Connolly, 1993
	Thrombin inhibitor	Friedrich *et al.*, 1993
Rhipicephalus appendiculatus	Histamine binding protein	Peasen *et al.*, 1999
	Anticoagulant (inhibits the prothrombinase complex)	Limo *et al.*, 1991
	Immunoglobulin binding protein	Wang *et al.*, 1998
Amblyomma variegatum	FXa inhibitor	Zhu *et al.*, 1997
Amblyomma americanum	Calreticulin, (Function unknown)	Jawroski *et al.*, 1992
	FXa inhibitor	Zhu *et al.*, 1997
Hyaloma truncutum	FXa inhibitor	Joubert *et al.*, 1995
Ixodes holocyclus	Thrombin inhibitor	Anaspoulos *et al.*, 1991
Ixodes dammini	Inhibitor of the alternative complement pathway	Ribeiro, 1987
Ixodes ricinis	Thrombin inhibitor	Hoffman *et al.*, 1991
Dermacentor andersoni	FV and FVII inhibitor	Gordon and Allen, 1991
Boophilus microplus	FXa and thrombin inhibitor	Anaspoulos *et al.*, 1991
Haemaphysalis longicornis	FXa and thrombin inhibitor	Anaspoulos *et al.*, 1991

1.4 *ORNITHODOROS SAVIGNYI*

The soft tick, *Ornithodoros savignyi*, commonly known as the sand tampan, occurs in the Northwestern part of South Africa and large areas of Namibia and Botswana (Howell *et al.*, 1978). Hosts include humans, domestic animals and a large number of wild animal species.

The sand tampan spends most of its life buried to a depth of 8 cm or more under the shade of large-crowned trees, big bushes or rocks and in kraals. It avoids hot sunlit places (Howell *et al.*, 1978). It is impractical to control sand tampans with pesticides, since it is virtually impossible to kill them when they are on their hosts because they feed so rapidly (first stage nymphs engorge in 10–74 min, later stage nymphs and adults in 15-30 min). Furthermore, it is equally difficult to try and kill them on the ground, not only because it is expensive to spray large areas but also because they quickly sense the presence of any chemicals and do not emerge in the treated areas and so do not come in contact with the pesticides (Howell *et al.*, 1978).

Despite the fact that under natural conditions the sand tampan is not known to act as a vector of pathogenic organisms it is undoubtedly a major pest of domestic stock in the areas in which they occur. The bites are extremely painful and the ticks secrete toxic substances of protein nature that frequently cause death of the hosts, especially young animals such as calves, kids, and lambs. Adult animals may also die from these toxic effects, especially if large numbers of ticks have attacked them. The pathogenic mechanisms and amino acid sequences of the toxins have been determined (Mans *et al.*, 2002b). Humans sometimes become sensitized to these toxins and the bite of even one tick may then be sufficient to provoke a severe allergic reaction, necessitating immediate medical treatment (Howell *et al.*, 1978).

The salivary glands of *O. savignyi* are a rich source of antihemostatics that may be potential anti-tick feeding vaccine candidates and may have therapeutic applications. A factor Xa inhibitor has been identified (Gaspar *et al.*, 1995), isolated and characterized (Gaspar *et al.*, 1996). The gene encoding the factor Xa inhibitor

has been identified and sequenced (Joubert *et al.*, 1998). Savignin, a potent thrombin inhibitor, has been isolated from the salivary glands of the tick (Nienaber *et al.*, 1999) and its gene cloned and sequenced (Mans *et al.*, 2002d). Apyrase activity has been purified and characterized (Mans *et al.*, 1998). This enzyme inhibits platelet aggregation by hydrolyzing ADP, an agonist of platelet aggregation. Savignyigrin, a platelet aggregation inhibitor that possesses the RGD integrin recognition motif, has been purified from this tick. The inhibitor is proposed to inhibit platelets by targeting the platelet integrin $\alpha_{IIb}\beta_3$ (Mans *et al.*, 2002a). An inhibitor of collagen stimulated platelet aggregation has been partially purified (Mans *et al.*, 2002c). Two barium sulphate adsorbing proteins have been isolated from the salivary glands that inhibit the extrinsic pathway of blood coagulation (Ehebauer *et al.*, 2002).

O. savignyi female ticks can be identified by the presence of a large genital opening on the ventral side (Fig 1.1). They are abundant in the field and can be collected in large numbers by sifting of sand. The collected ticks require very little care, their salivary glands are of a reasonable size and can therefore be easily removed by dissection (Fig 1.2). The advantage of using the salivary glands as a source for the isolation of enzymes is that contamination with host blood proteins is avoided. In addition, salivary gland extracts (SGE) are much easier to prepare for high performance liquid chromatography (HPLC) than homogenates of whole ticks would be. Taken together, this makes this tick an ideal model for investigation of anti-hemostatics with the potential use for tick control.
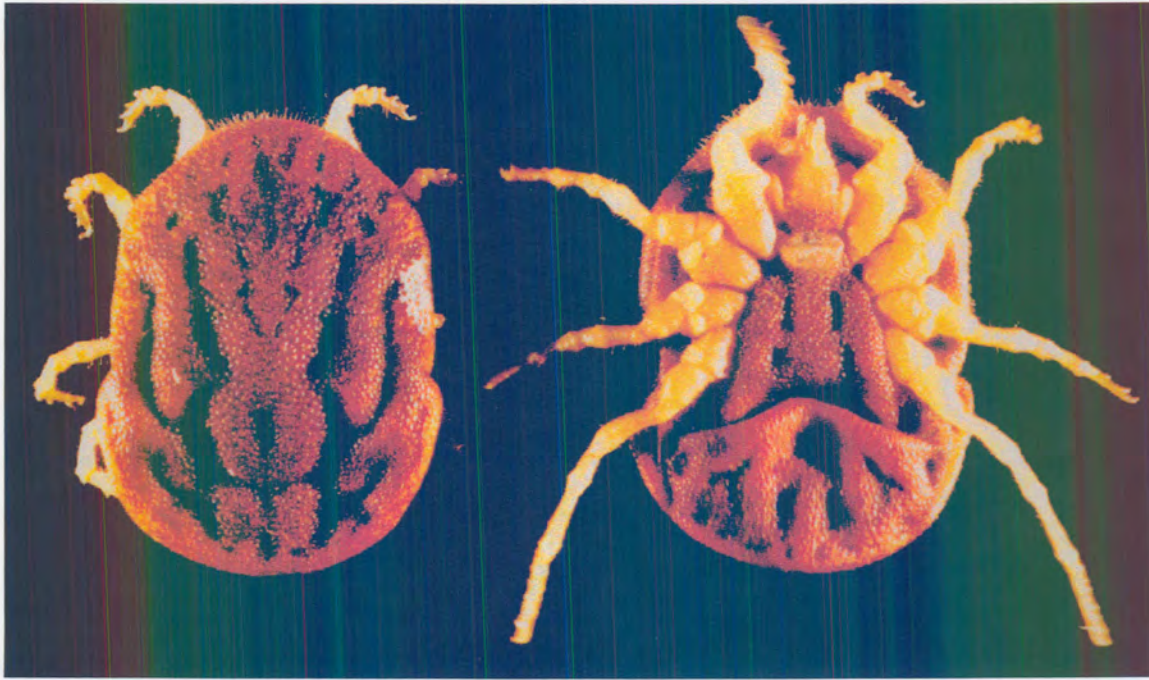
Fig 1.1: The dorsal (left) and ventral (right) view of female *Ornithodoros savignyi*. Photographs: B. J. Mans, University of Pretoria.
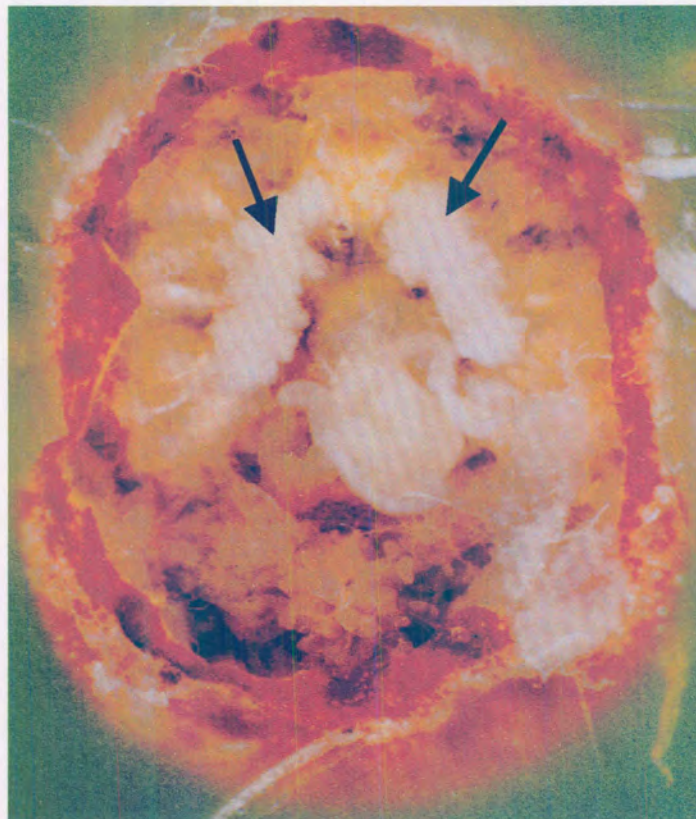
Fig 1.2: Internal organs of *Ornithodoros savignyi*. The paired salivary glands lie in the anterolateral position in the body cavity and resemble two bunches of grapes (indicated by arrows). Photographs: B. J. Mans, University of Pretoria.

Since this thesis describes biochemical investigations into the proteolytic activities and more specifically the fibrinogenolytic activity in the salivary glands, a brief overview on proteases and hemostasis is outlined below.

1.5 PROTEASES

In living organisms, proteolytic enzymes (proteases) are produced to degrade or modify proteins. These enzymes play a key role in blood coagulation, complement activation, fibrinolysis, tumorogenesis, reproduction, cell growth, and other cellular events. Proteases were among the first enzymes to be purified and crystallized, and this helped pave the way for the eventual understanding of protein structure and the catalytic sites on the enzymes. Molecular understanding of biological phenomenon owes much to the use of proteases and protease inhibitors used as tools to dissect cellular processes (Agarwal, 1979). It has been proposed that proteinase activity, inherent in any tissue, is a balance between the protease and its inhibitor (Murphy and Docherty, 1992).

Highly specific limited proteolysis is one of the major regulatory tools in living organisms. The most important systems regulated by limited proteolysis are listed in Table 1.3 (Adapted from Agarwal, 1979).

TABLE 1.3: SYSTEMS REGULATED BY LIMITED PROTEOLYSIS

System	Examples
Hormone production	Insulin, glucagon, Pro-ACTH
Regulatory peptides	Angiotensin, kinins
Defense, protection	Blood coagulation, complement
Invasiveness	Connective tissue penetration, metastasis
Membrane passage	Pre-pro-proteins
Zymogen activation	Pro-enzymes
Transformation, assembly	Pro-collagen, fibrinogen, capside virus polypeptides
Development and differentiation	Fertilization, implantation, surface proteins

Proteolytic enzymes are generally specific for a certain amino acid residue or for a certain sequence in the substrate. Trypsin will cleave in most polypeptides all bonds

next to the carboxyl side of an arginine or lysine residue, regardless of the character of the adjacent amino acid residues, except for proline. Other enzymes of the same bond specificity will on the contrary be highly selective because the substrate and enzyme must bind in several subsites which will not necessarily belong to the sequence around the essential amino acids involved in the catalytic mechanism, but to a polypeptide loop. The bond or sequence specificity is therefore the first prerequisite for the action of a given proteinase but this action will be largely modified by the arrangement of the polypeptidic substrate in space. In a compact tertiary structure of a native protein a proteinase will cleave only accessible bonds on the surface which can fit to the binding site or which are loose enough or mobile to fit (Agarwal, 1979).

All proteolytic enzymes fall within only four groups on the basis of their catalytic mechanism. Three of these classes (serine-, cysteine- and aspartic proteases) are named after a key active site amino acid and the fourth class, the metalloproteinases, are so named because of the requirement of a metal ion for catalysis (Rawlings and Barrett, 1995a). Table 1.4, adapted from Agarwal (1979) summarizes some characteristics of the proteinases.

TABLE 1.4: NOMENCLATURE OF PROTEINASES (ENDOPROTEINASES)

Nomenclature sub-sub-group	Group name	Essential residue in active site	Common inhibitor	Examples
EC 3.4.21	Serine proteinase	Serine, Histidine, Aspartic acid	Fluorophosphates	Trypsin, Chymotrypsin, Blood coagulation cascade, Subtilisin
EC 3.4.22	Cysteine proteinase	Cysteine	Mercurials	Papain, Clostripain
EC 3.4.23	Aspartic proteinase	Aspartic acid	Diazoacetylamino acids	Pepsin, Chymosin
EC 3.4.24	Metalloproteinase	Histidine, Aspartic acid, metal	EDTA	Thermolysin, Collagenases

1.5.1 SERINE PROTEASES

Serine proteases (EC 3.4.21) have a serine, aspartic acid and a histidine in their active center (Rawlings and Barrett, 1994a). These enzymes hydrolyze peptide bonds that are involved in a broad range of biological processes including intra-and

extracellular protein metabolism, digestion, blood coagulation, clot dissolution, immunological response, developmental regulation and fertilization. The serine and histidine residues are known to be required for enzymatic activity based on chemical modification experiments using organophosphates for Ser-195, chloromethyl ketone affinity reagents for His-57 at the active site of the enzyme (Bode and Huber, 2000). The catalytic mechanism of the serine proteases is indicated in Fig 1.3 (adapted from Agarwal, 1979).
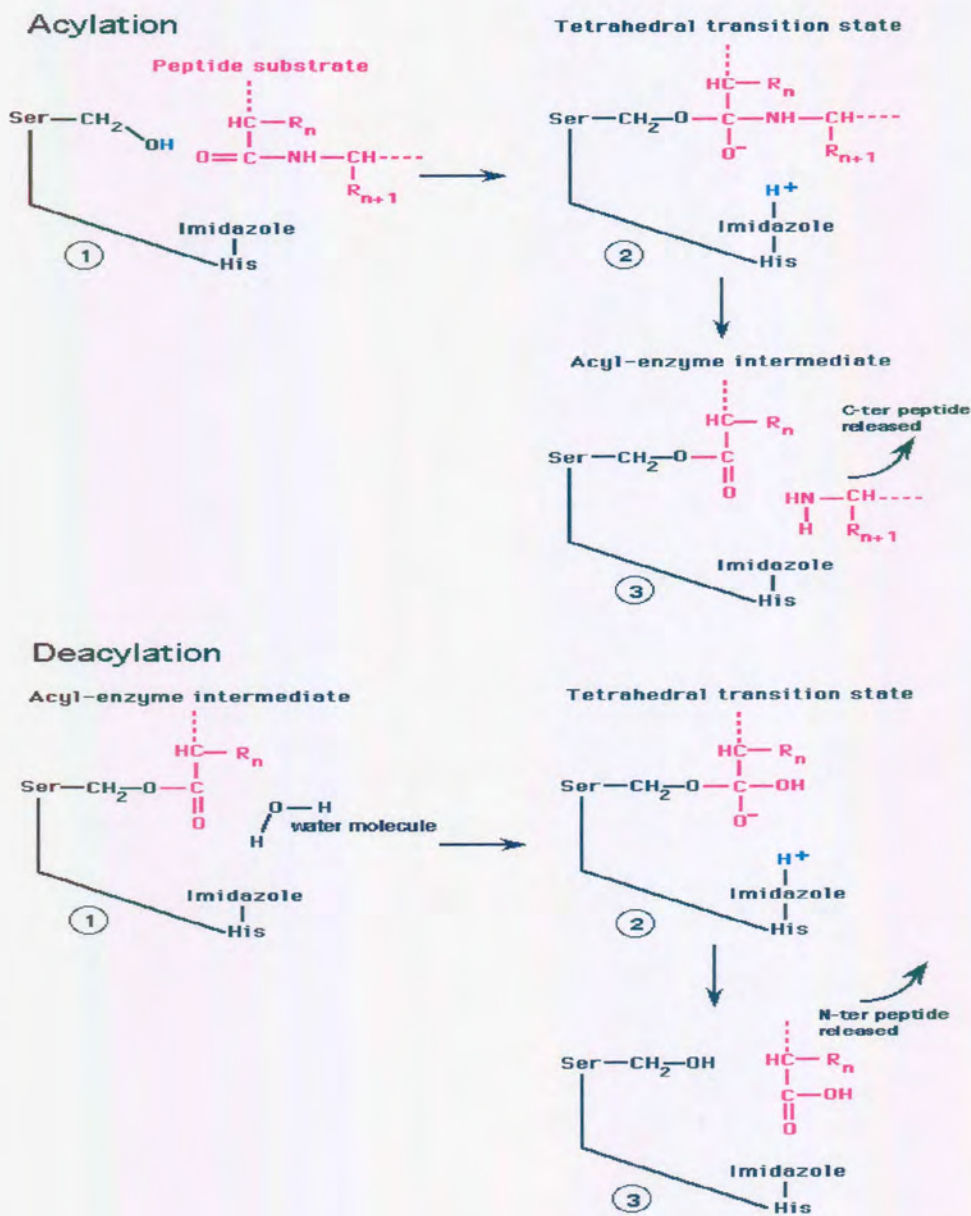
Fig 1.3: Catalytic mechanism of the serine proteases.

1.5.2 CYSTEINE PROTEASES

The cysteine (thiol) proteinases (EC 3.4.22) have a catalytic cysteine and a thiol ester intermediate. Most of these proteases act intracellularly at an acidic pH with rather broad substrate specificity (Rawlings and Barrett 1994b). Their catalytic domain consists of two adjacent sub-domains, which permit a V-shaped active site cleft to accommodate the active site residues Cys 25, His 159 and Asn 175. It has been proposed that the S2 subsite (pocket) of these enzymes is an important specificity determinant to recognize and select peptide substrate (Bode and Huber, 2000). They are important in a wide spectrum of biological processes (Sakanari *et al.,* 1998) and have been shown to play important roles in the pathogenesis of several parasitic protozoa and helminth infections (Mulenga *et al.,* 1999). The catalytic mechanism of the cysteine proteases (adapted from Agarwal, 1979) is indicated in Fig 1.4.
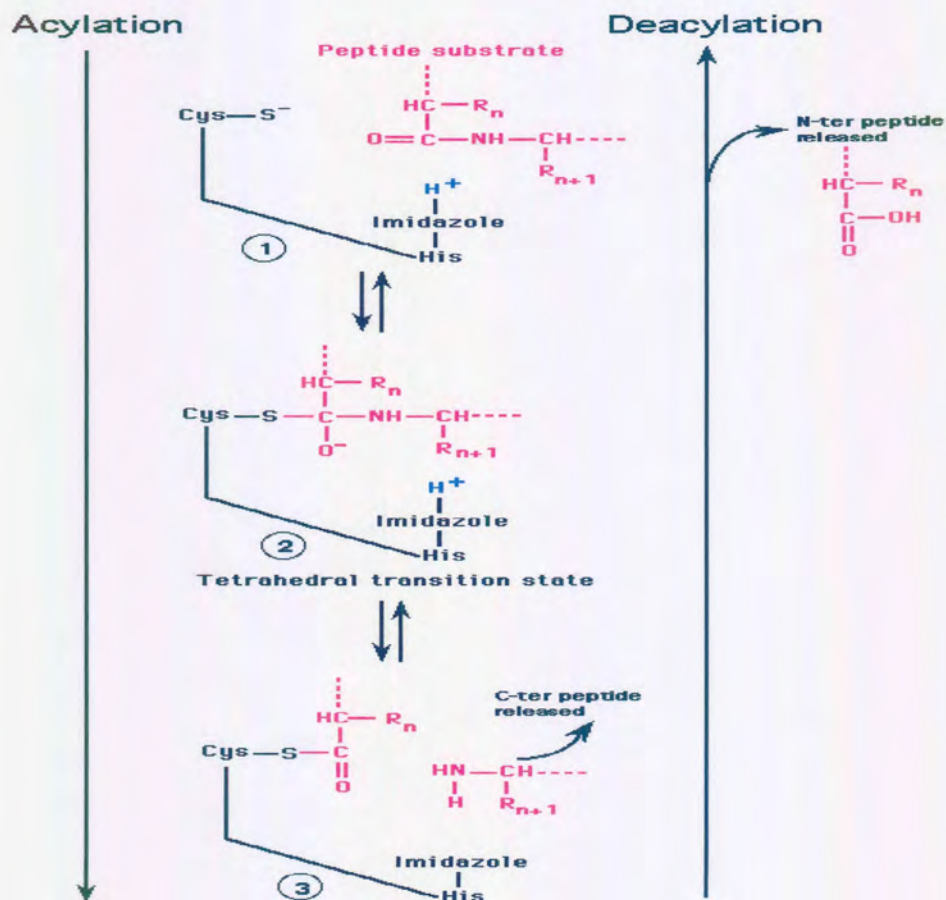
Fig 1.4: Catalytic mechanism of the cysteine proteases.

1.5.3 ASPARTIC PROTEASES

Most aspartic proteinases (EC 3.4.23) have a pH optimum below 5 with an aspartic acid or glutamic acid residue involved in the catalytic process. Eukaryotic aspartic proteases include intracellular acid proteases, digestive enzymes and extracellular regulatory proteases with high substrate specificities (Rawlings and Barrett 1995a). They use two aspartic acid residues during catalysis and are synthesized as pre-pro-enzymes containing a signal sequence for targeting the nascent polypeptide to the endoplasmic reticulum and a propeptide involved in the control of enzyme activity (Marquardt *et al.*, 1987). Proteolytic removal of the pro-peptide by self-hydrolysis results in the activation of the enzyme (Rawlings and Barrett 1995a). The catalytic mechanism of the aspartic proteases (adapted from Agarwal, 1979) is indicated in Fig 1.5.
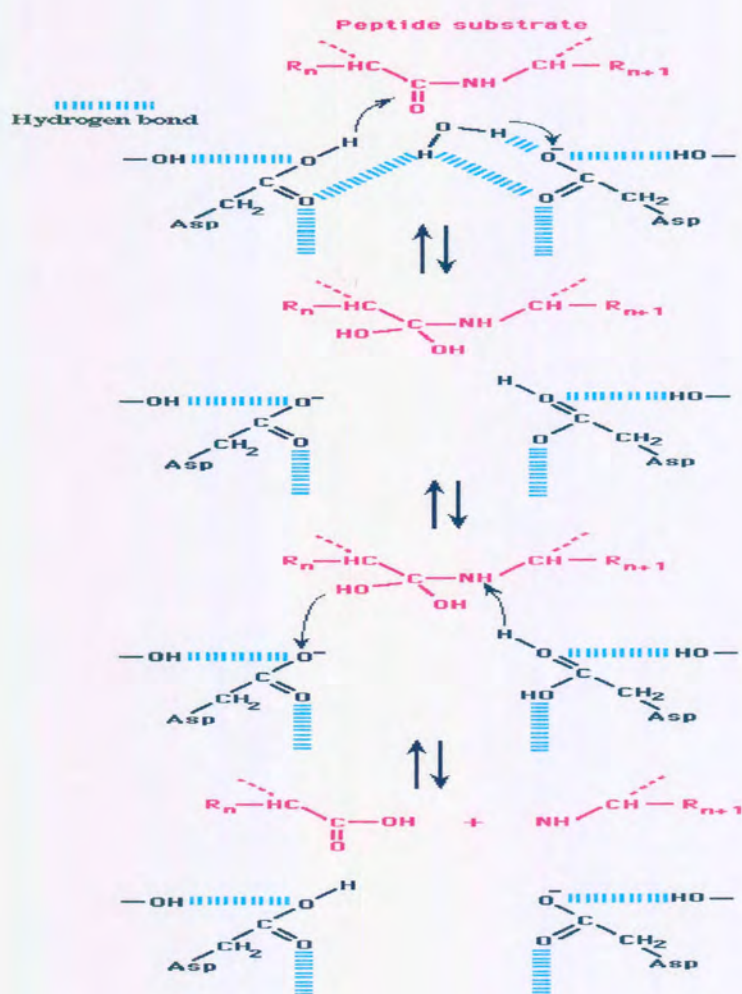
Fig 1.5: Catalytic mechanism of the aspartic proteases.

1.5.4 METALLOPROTEASES

Metallo-proteinases (EC 3.4.24) contain a metal, usually Zn^{2+} as an essential part of their active center (Scopes, 1994; Bode and Huber, 2000; Vanaman and Bradshaw, 1999; Rawlings and Barrett 1995b) and show significant substrate specificity (Fortunato *et al.*, 1997). Biologically important eukaryotic metalloproteinases include enzymes that degrade extracellular matrix components (which include collagenases, fibronectin, elastases and laminin) and regulatory proteases (Rosenthal, 1999; Vanaman and Bradshaw, 1999). Fig 1.6 (adapted from Agarwal, 1979) indicates the catalytic mechanism of the metalloproteases.
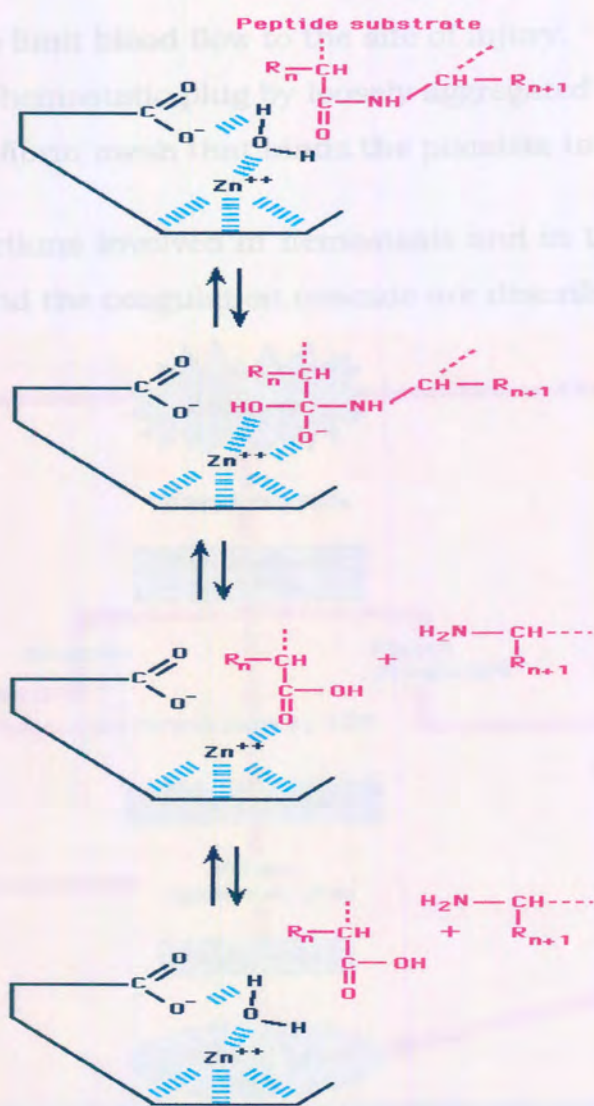
Fig 1.6: Catalytic mechanism of the metalloproteases.

1.6 THE HEMOSTATIC SYSTEM

Hemostasis is the cessation of bleeding at the site of a ruptured vessel (Hoffbrand and Pettit, 1988; Braud *et al.*, 2000), while thrombosis is the formation of a thrombus, i.e. the aggregation of the blood factors, primarily platelets and fibrin with entrapment of cellular elements, frequently causing vascular obstruction at the point of its formation (Braud *et al.*, 2000). The three main components involved in thrombosis and hemostasis are thrombin, platelets, and plasmin. Platelets and thrombin both play pivotal roles in thrombogenesis whereas plasmin dissolves the thrombus. After trauma, three processes halt bleeding: (Murray *et al.*, 1993, Kamiguti *et al.*, 1998).

1) Vasoconstriction to limit blood flow to the site of injury.
2) The formation of a hemostatic plug by loosely aggregated platelets.
3) The formation of a fibrin mesh that binds the platelets to form a more stable clot.

Figure 1.7 shows reactions involved in hemostasis and in the following paragraphs platelet aggregation and the coagulation cascade are described.
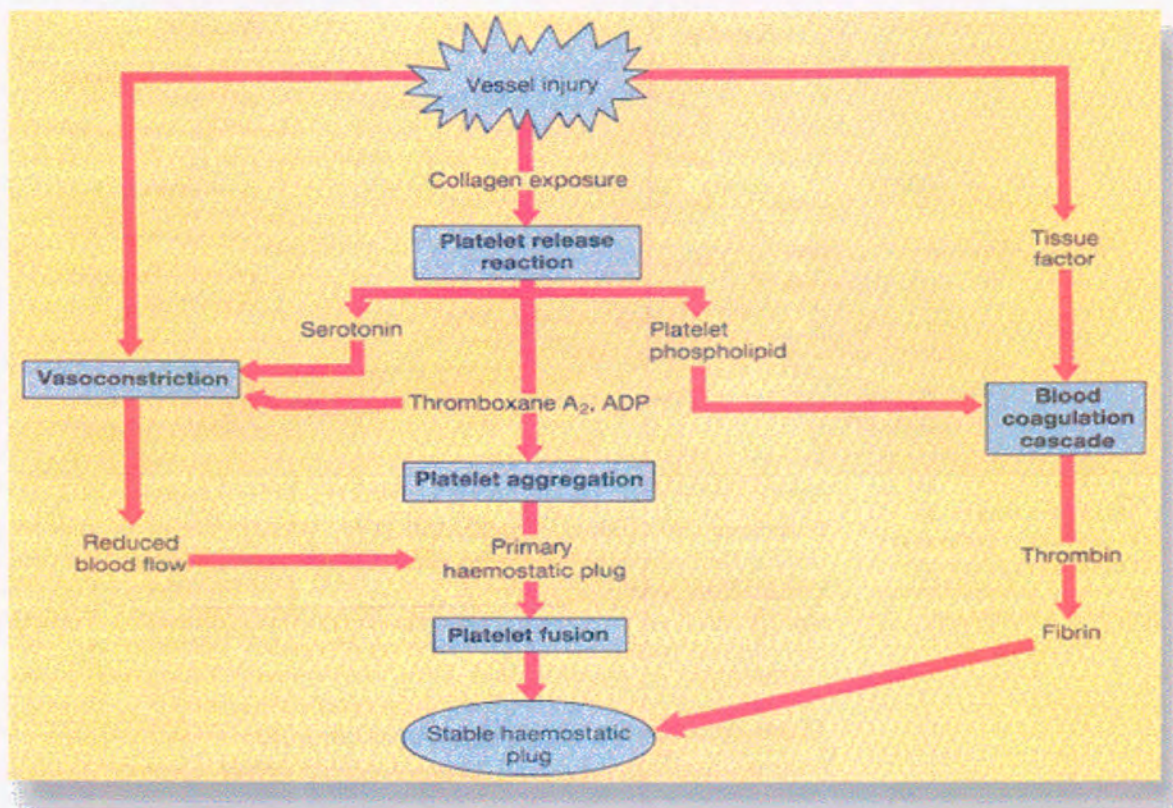
Fig 1.7: Reactions involved in haemostasis. Adapted from Hoffbrand and Pettit, 1988.

1.6.1 PLATELET ACTIVATION AND AGGREGATION

In small blood vessels, platelets alone can arrest bleeding. In their inactive form, platelets are discoid but once activated they become spherical, extend numerous pseudopods and then aggregate (Kamiguti *et al.*, 1998). This occurs when platelets are exposed to ADP, thrombin, adrenaline, collagen, and other agonists. Each agonist stimulates platelets via a specific receptor. The receptor for collagen belongs to the super-family of $\alpha\beta$ dimeric proteins called integrins. The first reaction of platelets to vessel damage is their adhesion to the adhesive proteins, von Willebrand factor (vWF) and collagen on the exposed sub-endothelium (Kamiguti *et al.*, 1998 and Braud *et al.*, 2000). The respective platelet receptors for these proteins are the glycoprotein GPIb/IX complex and $\alpha_2\beta_1$ integrin also known as the GPIa/IIa complex, respectively. Engagement of these receptors stimulates platelets to secrete their granular contents and in particular ADP, which promotes activation of platelet αIIbβ_3 integrin, GPIIb/IIIa. This receptor then binds the RGD-containing ligands (fibrinogen and von Willebrand factor) and thereby promotes platelet aggregation, resulting in the formation of a platelet plug which stops bleeding. Thus, the mechanism by which platelets act, clearly depends on their surface receptors for plasma proteins and on the normal content and release of their granular ADP stores (Kamiguti *et al.*, 1998).

Three sites on the fibrinogen molecule may interact with the fibrinogen receptor on platelets: the NH$_2$-terminal region of the Aα-chain, the COOH-terminal region of the γ-chain, and the COOH-terminal of the Aα-chain. The NH$_2$-terminal peptides of the Aα and Bβ-chains in the central E domain are susceptible to hydrolysis by thrombin, which removes fibrinopeptides A and B, thereby initiating fibrin clot formation (Fig 1.10). After release of these fibrinopeptides, the new NH$_2$-terminal sequence of the Aα-chain is Gly-Pro-Arg-Val. The related tetrapeptide Gly-Pro-Arg-Pro binds tightly to fibrinogen receptor, thereby inhibiting its binding to fibrinogen and consequently prevents aggregation (Seiss, 1989).

The interaction of fibrinogen with platelets is necessary for their aggregation. Fibrinogen binding and aggregability of platelets correlate closely. Fibrinogen binding to activated GPIIb/IIIa is mediated through two types of specific sites. One contains the Arg-Gly-Asp (RGD) sequence including Aα 95-97 and Aα 572-574. The other is the dodecapeptide 400-411 at the carboxy-terminal end of the γ chain. Platelets from patients with severe afibrinogenemia show an abnormal aggregation response in platelet-rich plasma. Platelets from patients that lack the fibrinogen receptor do not aggregate but change shape and secrete normally (Seiss, 1989). The following figure, Fig 1.8 (Adapted from Hoffbrand and Pettit, 1988) illustrates the three steps of platelet aggregation.
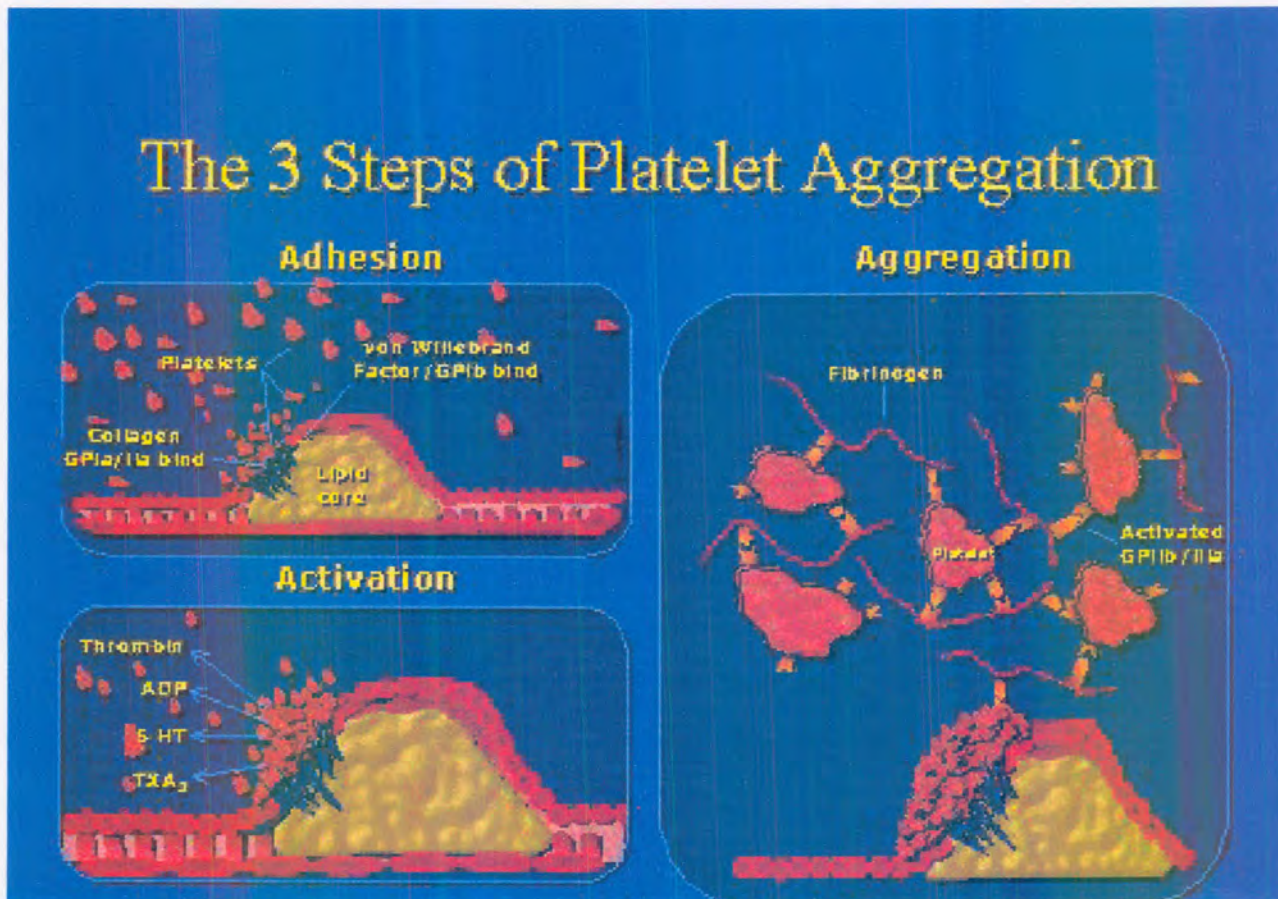
Fig 1.8: Process of platelet aggregation. The binding of glycoprotein GPIb to von Willebrand factor leads to adhesion to the subendothelium and also exposes the GPIIb/IIIa binding sites to fibrinogen and von Willebrand factor leading to platelet aggregation. The GPIa site permits direct adhesion to collagen.

1.6.2 BLOOD COAGULATION

Blood coagulation is vital for the containment of body fluids after tissue injury. Equally vital is the reversal of this process following wound healing. All the proteins necessary for coagulation circulate in the blood awaiting the appropriate signal, i.e., the exposure of tissue factor, an extracellular membrane-anchored protein that forms a "hemostatic envelope" surrounding the vasculature. Exposure of tissue factor, such as occurs upon injury, triggers an activation cascade known as the extrinsic pathway that results in formation of the prothrombinase complex (Factor Xa, Factor Va and prothrombin) and release of active α-thrombin (Stubbs and Bode, 1993, Braud *et al.*, 2000). Fig 1.9 (adapted from Hoffbrand and Pettit, 1988) summarizes the reactions involved in blood coagulation.

One of the central features of the coagulation cascade is that each successive step in the process involves assembly of proteases with regulatory proteins (cofactors) and their substrate(s) on membrane surfaces. These cofactors are essential for rapid activation of the target zymogens (Esmon, 1993). Blood coagulation proceeds through the sequential activation of a number of plasma serine proteases, ultimately resulting in the formation of fibrin, an insoluble protein that is a major component of blood clots.

Coagulation factor Xa plays an important role in this cascade of events, since it converts the zymogen prothrombin into thrombin, the enzyme that catalyses the formation of fibrin from fibrinogen. Fibrin is crosslinked to a stable clot by FXIIIa. Wound healing is completed by the migration of the fibroblasts into the wound area; they start collagen synthesis as soon as they are immobilized by fixation in the fibrin network through FXIII (Lapatto *et al.*, 1997). Table 1.5, adapted from Hoffbrand and Pettit, (1988) summarizes the properties of the blood coagulation factors.
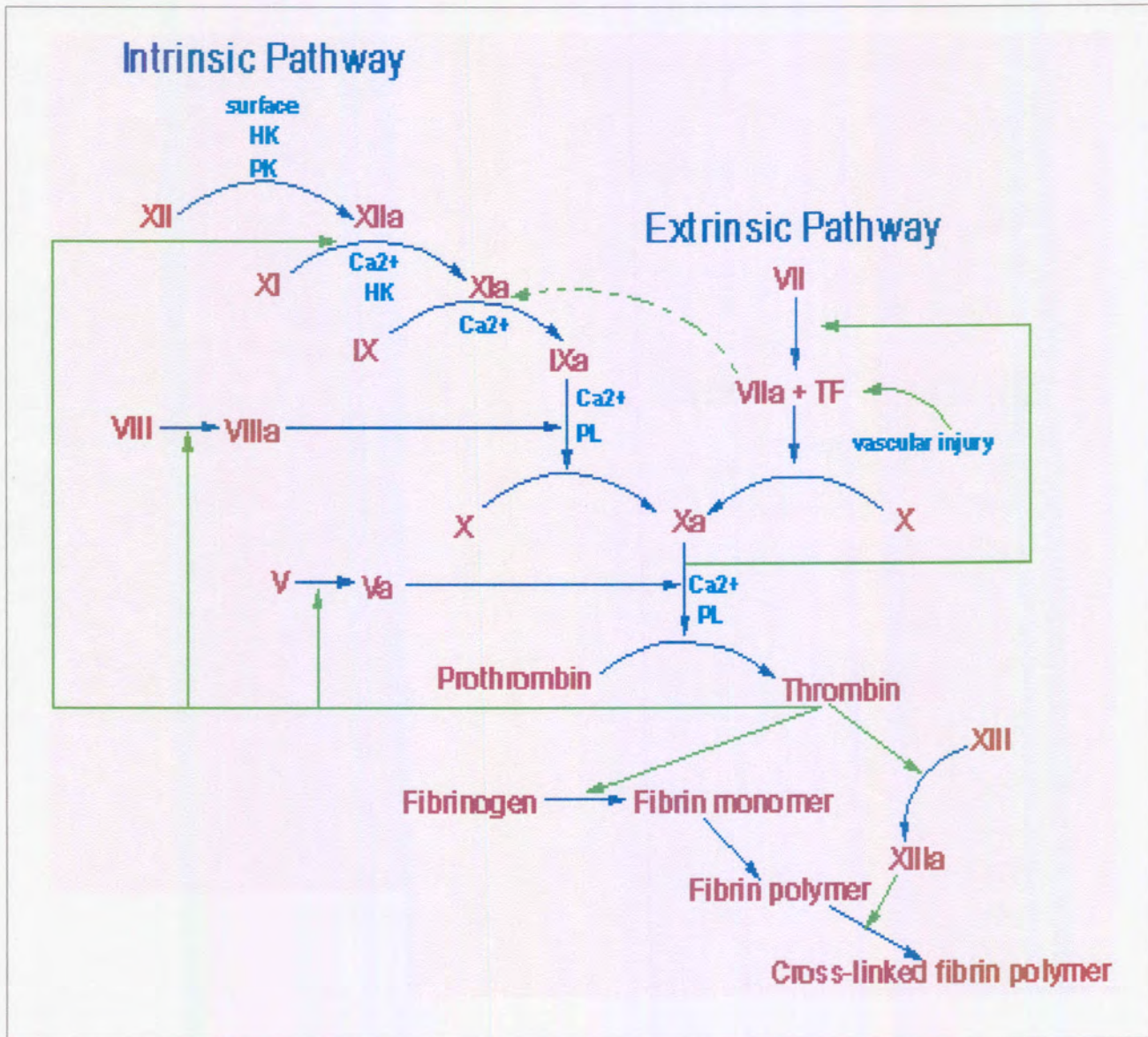
Fig 1.9: The pathways of blood coagulation.

TABLE 1.5: PROPERTIES OF THE BLOOD COAGULATION FACTORS

Roman numeral designation	Common name	Active form
Factor 1	Fibrinogen	Fibrin subunit
Factor 11	Prothrombin	Serine protease
Factor 111	Tissue factor	Receptor/cofactor
Factor 1V	Calcium ions	
Factor V	Proaccelerin	Cofactor
Factor V11	Proconvertin	Serine protease
Factor V111	Antihemophilic factor	Cofactor
Factor 1X	Christmas factor	Serine protease
Factor X	Stuart factor	Serine protease
Factor X1	Plasma thromboplastin antecedent (PTA)	Serine protease
Factor X11	Hageman factor	Serine protease
Factor X111	Fibrin stabilizing factor	Transglutaminase
	Prekallikrein (Fletcher factor)	Serine protease
	HMW kininogen (High molecular weight kininogen, contact activation cofactor, fitzgerald factor, Williams factor, Flaujeac factor)	Cofactor
	Plasminogen	

Fibrinogen is a soluble, hexamer, plasma glycoprotein that consists of three nonidentical pairs of three polypeptide chains (Aα), (64 kDa) (Bβ) (56 kDa) and γ (47 kDa) covalently linked by disulphide bonds at the amino terminal end. Figure 1.10 (adapted from Hoffbrand and Pettit, 1988) depicts a simplified human fibrinogen molecule. The molecule is organized into distinct structural domains. The central domain (E domain) contains the NH$_2$-terminals of all six polypeptide chains. The COOH-terminal regions of the molecule are localized in two distinct D domains (Seiss, 1989). Fibrinogen is the only substrate of the clotting and fibrinolysis pathways. It is attacked by the key enzymes of both systems: thrombin and plasmin (Kelly *et al.*, 1995). Plasmin binds fibrinogen and initially cleaves several sites in the C terminus of the Aα-chain of fibrinogen and the Bβ-chain (Arg42-Ala43) bonds to yield fragment X (240-265 kDa). The cleavage of a γ(Lys62-Arg63) bond in fragment X which has cleaved α(Lys81-Asp82) or α(Arg103-Asp104) and β(Lys122-Asp123) or β(Lys133-Asp134) bonds yields fragment Y (155 kDa) and fragment D (70-100 kDa). Finally the cleavage of the second γ(Lys62-Arg63) bond of fragment Y results in the generation of fragment E (50 kDa) and the second fragment D, both of which

can undergo further degradation (Wu and Diamond, 1995). During fibrin(ogen) degradation, plasmin can cleave over 30 cleavage sites in a fairly specific order while generating additional lysine binding. Thrombin releases fibrinopeptide A with subsequent protofibril extension of staggered monomers with an equivalent of two monomers per unit of protofibril. The slower release of fibrinopeptide B by thrombin is associated with lateral aggregation to form thick fiber bundles containing many protofibrils (Wu and Diamond, 1995).
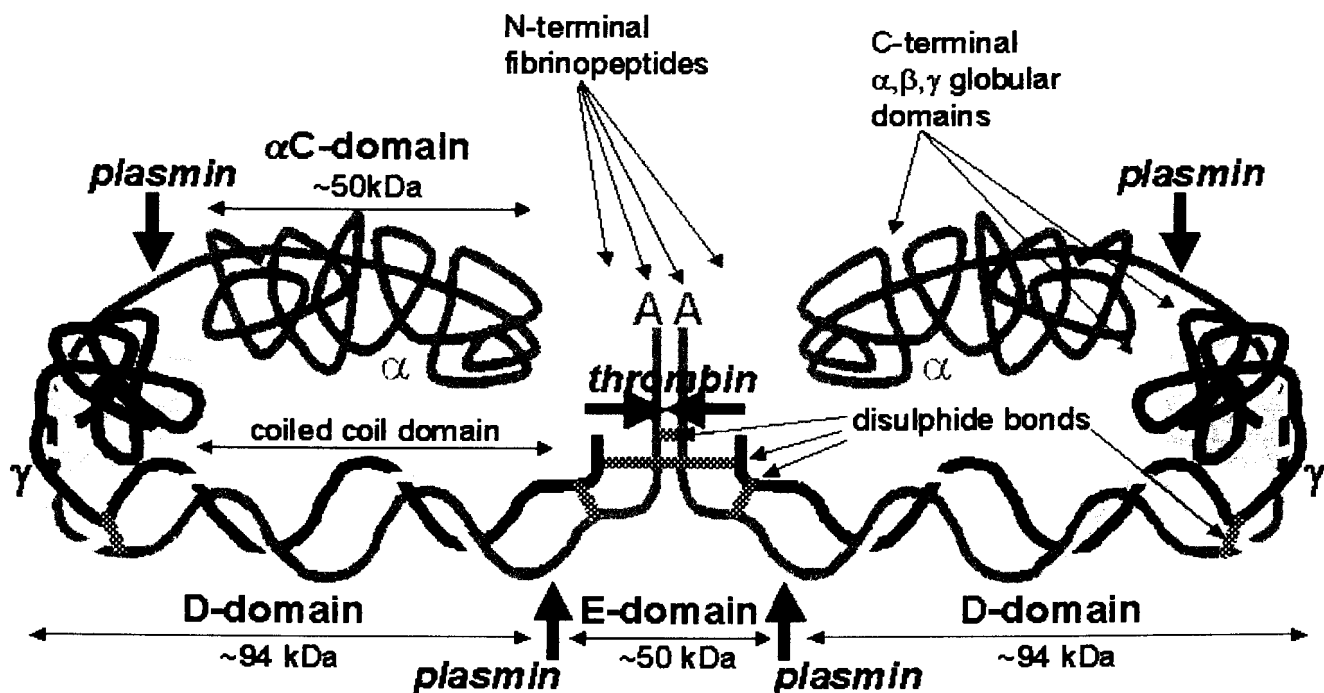
Fig 1.10: A simplified structure of fibrinogen molecule. The thick arrows show sites of cleavage by thrombin and plasmin respectively.

1.6.3 REGULATORY MECHANISMS OF HEMOSTASIS

The clotting process must be precisely regulated. There is a fine line between hemorrhage and thrombosis. Clotting must occur rapidly yet remain confined to the area of injury. There are mechanisms that normally limit clot formation to the site of injury. Firstly, the lability of the clotting factors contributes significantly to the control of clotting. The activated clotting factors have a short life because they are diluted by blood flow, removed by the liver, and are degraded by proteases (Lapatto

et al., 1997). Secondly, the action of proteinases is kept under control by natural proteinase inhibitors (PI).

1.6.3.1 PROTEINASE INHIBITORS

It is the function of proteinase inhibitors to limit the action of clotting proteinases locally and timeously. Human plasma is rich in PI's of different enzyme specificity and these are summarized in Table 1.6 (adapted from Agarwal, 1979). Most of them are able to neutralize more than one proteinase.

TABLE 1.6: PROTEINASE INHIBITORS IN HUMAN SERUM

Inhibitors	Concentration. mean ± SD (mg/100 ml)	MW (Da)
α_1 Antitrypsin	290.0 ± 45.0	54 000
α_1 Antichymotrypsin	48.7 ± 6.5	69 000
Inter-α -trypsin inhibitor	50.0	160 000
Antithrombin III	23.5 ± 2.0	65 000
C1-Inactivator	23.5 ± 3.0	104 000
α_2 Macroglobulin	260.0 ± 70.0	725 000
α_2 Antiplasmin (fast acting)	7.0	70 000
Inhibitors of plasminogen activation	-	75 000

1.6.3.2 PROTEIN C AND PROTEIN S

Human protein C is a vitamin K dependent serine protease zymogen and is homologous with other known vitamin K dependent serine proteases (Berg *et al.,* 1996). Human protein C can be activated by several proteolytic enzymes, such as the factor X activator of Russel's viper venom, trypsin, or thrombin (Marlar *et al.,* 1982). These proteolytic activators can also activate the bovine molecule. Protein C activation is catalyzed on endothelial cells by a complex between thrombin and thrombomodulin. Ca^{2+} stimulates protein C activation in the presence and inhibition in the absence, of thrombomodulin (Rezaie and Esmon, 1992, Berg *et al.,* 1996). Phospholipid membranes accelerate protein C activation, and since thrombin does not interact with membranes directly, and thrombomodulin is an integral

membrane protein, thrombomodulin interacts with thrombin to accelerate protein C activation dramatically, while inhibiting thrombin-clotting activity (Le Bonniec *et al.*, 1991). Activated protein C (APC), acts as a natural anticoagulant and prolongs the partial thromboplastin time by inactivating the activated forms of factor V (factor Va) and factor VIII (factor VIIIa) by limited proteolysis, and in platelet-supported clotting, by inactivating the platelet receptor for factor Xa (Esmon and Owen, 1981, De Stefano *et al.*, 1993). In this process, protein S (PS) serves as a cofactor (Henskens *et al.*, 1995) and phospholipid is needed as a cofactor for the expression of anticoagulant activity (Marlar *et al.*, 1982). Human thrombin may be a major activator *in vivo,* but protein S has been found to accelerate the activation of protein C by four orders of magnitude and suggests that the activation of protein C by thrombin *in vivo* may occur very rapidly (Marlar *et al.*, 1982). Thus, thrombin triggers the deactivation of the clotting cascade in addition to catalyzing the formation of fibrin (Lapatto *et al.*, 1997). The following reaction scheme (Fig 1.11) summarizes processes leading to the activation of protein C.
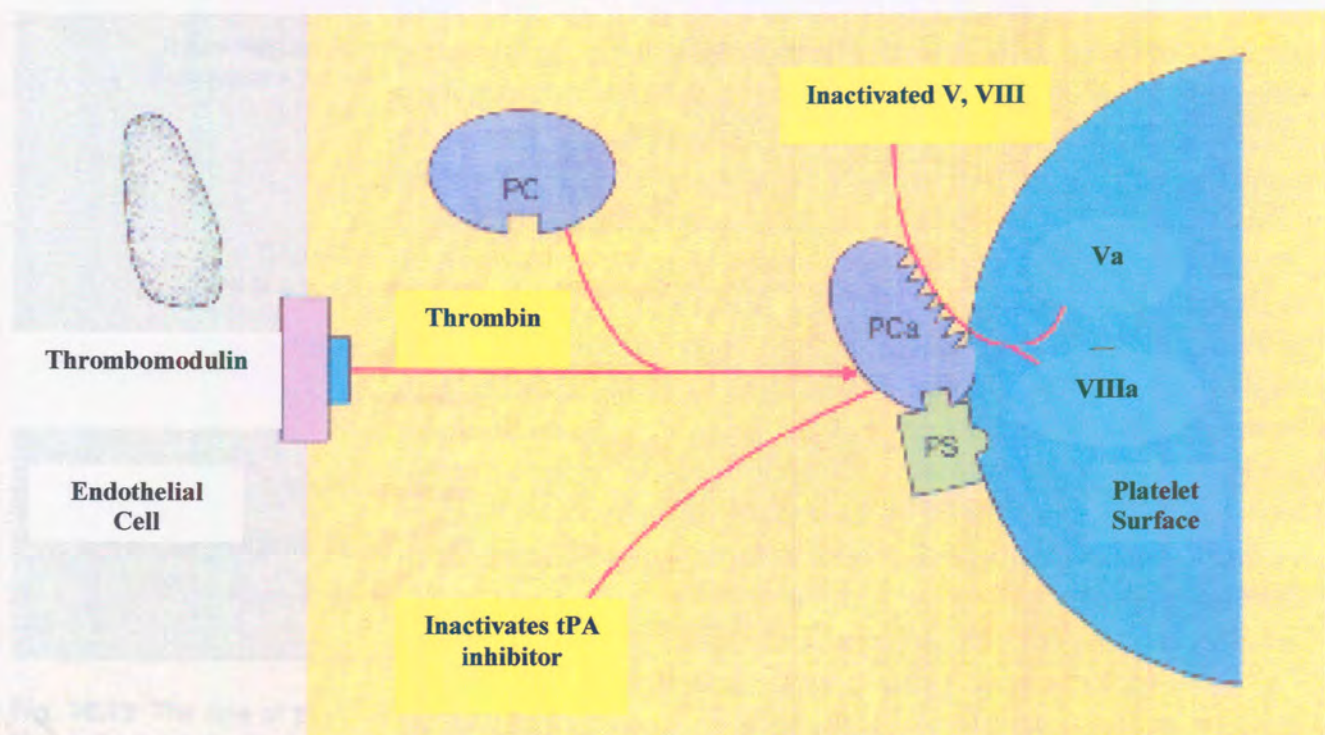
Fig 1.11: Activation and action of protein C (PC) by thrombin which has bound thrombomodulin on the endothelial cell surface. Protein S (PS) is a cofactor, which facilitates binding of activated protein C (PCa) to the platelet surface. The inactivation of factors Va and VIIIa results in the inhibition of blood coagulation. The inactivation of tPA (tissue plasminogen activator) inhibitor (PAI-1) enhances fibrinolysis.

1.6.3.3 FIBRINOLYSIS

Clots are not permanent structures; they are designed to be dissolved when the structural integrity of the damaged area is restored. Fibrin is split by plasmin, a serine protease that hydrolyses peptide bonds in the triple-stranded connector rod regions. Plasmin occurs in plasma in an inert form, plasminogen, which is converted to plasmin by proteolytic activation (Lapatto *et al.*, 1997). The main function of plasmin is the prevention of thromboembolic disorders. Free plasmin in the blood is rapidly inactivated by proteinase inhibitors. α_2-Antiplasmin is the primary inhibitor of plasmin; however, under some conditions plasmin may also react with α-macroglobulin (α_2M) (Anonick *et al.*, 1989). Fig 1.12 (adapted from Hoffbrand and Pettit, 1988) summarizes the reactions involved in the fibrinolytic pathway.
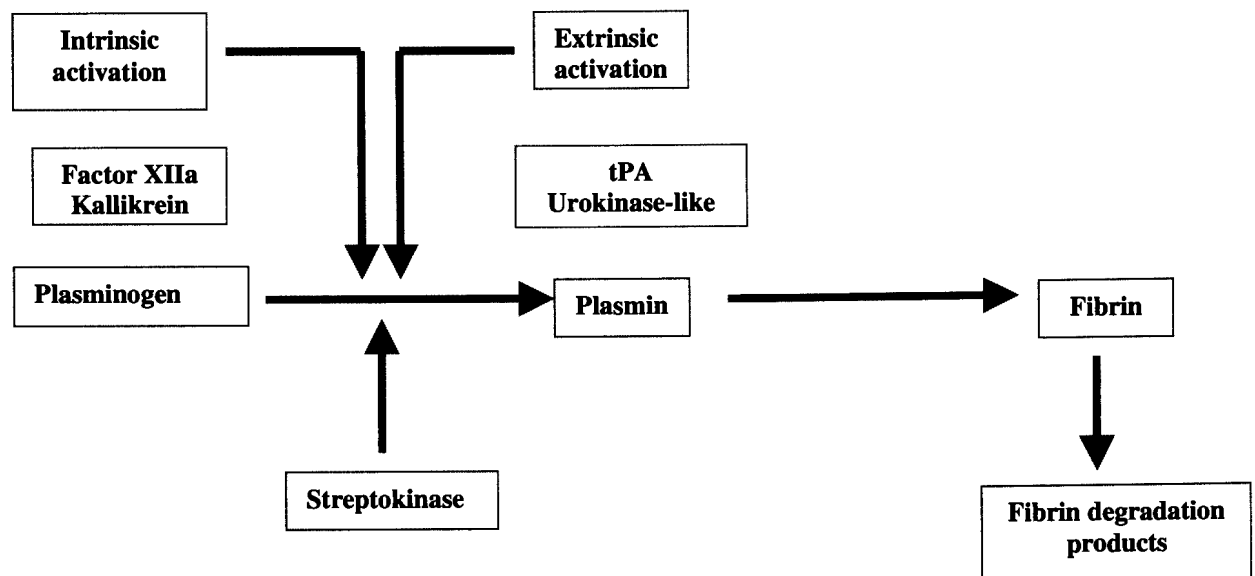
Fig 1.12: The fibrinolytic system.

1.7 THERAPEUTIC USES OF ANTICOAGULANTS

Thrombosis is the predominant cause of death of humans in industrialized countries. The most common antithrombotic therapeutic agents are coumarins (such as warfarin) and heparin; both are associated with complications. Warfarin is

the most widely used oral anticoagulant in North America because its onset and duration of action are predictable (Breckenridge, 1978 and O'Reilly, 1976). Warfarin is almost always administered orally, although an injectable preparation is available. It is rapidly absorbed from the gastrointestinal tract, reaches maximal blood concentration in healthy volunteers in 90 min (Breckenridge, 1978 and O'Reilly, 1976), and has a half-life of 36-42 hrs. It circulates bound to plasma proteins and rapidly accumulates in the liver (Sutcliffe *et al.*, 1987). The coumarins act by inhibiting vitamin K dependent modifications of Gla domains; thus, their action is unspecific and rather drastic, requiring continuous monitoring of their administration and individual dose adjustment.

Heparin, a heterogenous group of straight-chain anionic mucopolysaccharides, are instantly acting intravenous anticoagulants. They cross the membrane poorly due to their polarity and size. Heparin is administered first because of its prompt onset of action, whereas warfarin takes several days to reach its full effect (Murray *et al.*, 1993). Low molecular weight heparins (LMWH) have antithrombotic effects in animal models as well as in man and have longer half-lives than heparin (Vlasuk *et al.*, 1991). Standard heparin is cleared from the plasma, with the circulating $t_{1/2}$ of about 40 min. LMWH are cleared more slowly with $t_{1/2}$ of 2 hrs (Gallus, 1994). The low molecular weight heparins have been shown to carry a much smaller risk of causing thrombocytopenia. This, coupled with their long $t_{1/2}$, ensure an effective once daily subcutaneous injection for both prevention and treatment of venous thromboembolism. Therefore, LMWH are increasingly preferred in the prophylaxis of post-operative thromboembolism (Sutcliffe *et al.*, 1987). The major drawback of heparin treatment is its inability to inactivate clot-bound thrombin, heightening the risk of reocclusion.

Due to these complications, there is considerable interest in the development of compounds directed against coagulation (Stubbs and Bode, 1994) and in the identification of anticoagulants in hematophagous organisms.

1.8 FIBRIN(OGEN)OLYSIS

Studies on the mechanism of action of anticoagulants isolated from hematophagous organisms show that some interfere with thrombin formation, while others display fibrin(ogen)olytic activities or antiplatelet activity (Markwardt, 1994). Since this thesis concerns fibrinogenolytic enzymes these are discussed below.

Fibrin(ogen)olytic enzymes dissolve fibrinogen/fibrin clots by hydrolyzing fibrinogen/fibrin and act directly, without activating plasminogen. Fibrin(ogen)olytic enzymes have been isolated from various snake venoms, bacteria and some hematophagous organisms. α-Fibrinogenases cleave the Aα-chain of fibrinogen and with increased incubation time and/or concentration of fibrinogenase, the Bβ-chain of fibrinogen is cleaved, while the β-fibrinogenases cleave the Bβ-chain of fibrinogen and the Aα-chain when the concentration and/or incubation time is increased.

The recent interest in the mechanism of action of anticoagulants stems from their use as specific tools for the investigation of blood coagulation and potentially as therapeutic agents in thrombotic disorders.

1.8.1 FIBRINOGENOLYTIC ACTIVITY FROM HOOKWORMS

Hookworms like other hematophagous invertebrates have evolved highly effective anticoagulant strategies to facilitate the acquisition of their required blood meal. These include the inhibition of blood coagulation, platelet aggregation and mediator release and the secretion of fibrinogenolytic enzymes (Furmidge et al., 1995). It is believed that proteolytic enzymes are necessary to adult hookworms for two reasons; they digest host tissue and also impede potentially damaging host-derived coagulation events (Brown et al., 1995).

The hookworm, Necator americanus, degrades human fibrinogen in a time-dependent manner. After incubation for 1 h, degradation of fibrinogen is clearly seen when using SDS-PAGE to analyze degradation products. Prolonged incubation

times cause increasing amounts of degradation, and at 24 h most of the protein sample is degraded. All chains of fibrinogen are degraded, first the Aα, then Bβ-chain and lastly the γ-chain. The fibrinogenolytic activity was shown to be greatest at pH 3.5 (Brown *et al.*, 1995).

In another study, proteinases were found to be released *in vitro* by the parasitic stages of the bovine abomasal nematode, *Ostertagia ostagi*. At alkaline pH, gelatin, casein and fibrinogen were degraded by metallo- and serine proteinases. In contrast, mucin, fibrinogen, albumin and hemoglobin were degraded at acidic pH by aspartyl protease and cathepsin L-like activity (Geldhof *et al.*, 2000).

1.8.2 FIBRINOGENOLYTIC ACTIVITY FROM LEECHES

Hementin, is the fibrinogenolytic enzyme that has been isolated and characterized from the leech, *Haementeria ghilianii*. It has a molecular weight of 120 000 Da and is composed of a single polypeptide chain. It is a neutral metalloproteinase, and not inhibited by plasma protease inhibitors. Hementin cleaves fibrinogen rendering it incoagulable (Malinconico *et al.*, 1984a and Budzynski *et al.*, 1981). It is able to disaggregate platelets by breaking the fibrinogen crosslink between platelets (Sawyer *et al.*, 1991). Hementin differs from the snake venom fibrinogenases in that it cleaves the three chains of fibrinogen at multiple sites at the same time (Malinconico *et al.*, 1984b). Hementerin is another fibrinogenolytic enzyme identified from the salivary glands of the leech *Haementeria lutzi* (Kelen and Rosenfeld, 1975).

1.8.3 FIBRINOGENOLYTIC ACTIVITY FROM BACTERIA AND FUNGI

Fibrinogen, the precursor of fibrin, has been considered to be important in the defense system against microorganisms. The deposition of fibrin in wounds protects the surrounding tissues from bacterial invasion. Some bacteria have been reported to possess fibrinogenolytic activity. The activity might serve to provide a peptide source for bacteria and also to inhibit formation of the matrix of the clot, enhancing dissemination of the bacterial infection. Complement–mediated opsonisation and phagocytosis are also inhibited by fragments of fibrinogen (Whitnack and Beachey,

1985). Thus, the production of fibrinogen-degrading enzymes by microorganisms should be of significance to their virulence (Chen *et al.*, 1995). The presence of the dissemination form of the fungus, containing fibrinogen receptors which could mediate attachment to epithelia via the fibrinogen deposits was demonstrated (Bouchara *et al.*, 1988). In addition, *Aspergillus fumigatus* culture filtrates exhibited a fibrinogenolytic activity, which might play an important role in host tissue invasion (Larcher *et al.*, 1992).

The binding of *Bacteriodes intermedius* to human fibrinogen has been demonstrated. The binding is time dependent, reversible, saturable, and specific. These bacteria also exhibit fibrinogenolytic activity, which they release into the surrounding medium without loss of binding activity. Fibrinogen binding by *B. intermedius* might represent a mechanism of bacterial tissue adherence (Lantz *et al.*, 1985). Strains of *B. gingivalis*, an organism implicated in the etiology of several forms of periodontitis, also binds and degrades fibrinogen. The binding is rapid, reversible, saturable and specific. The fibrinogenolytic activity is activated by dithiothreitol (DTT) and blocked by thiol protease inhibitors (Lantz *et al.*, 1986). Fibrinogenolytic activity has also been isolated from the *Actinomyces*, *Fusobacterium*, *Peptococcus*, *Propionibacterium*, and *Staphylococcus aureus* (Wikstron *et al.*, 1983).

A novel serine-thiol-like protease with a molecular mass of 100 kDa was isolated from the *B. fragilis* strain YCH46. The Aα-chain of fibrinogen was hydrolyzed within 10 hr at 37 °C and a weak hydrolysis of the Bβ-chain occurred after incubation for 24 hr, but cleavage of the γ-chain was not detected under identical conditions and the time required for clotting of protease-treated fibrinogen by thrombin was prolonged. The protease was inhibited by both diisopropyl fluorophosphate (DFP) and phenyl methane sulphonyl fluoride (PMSF), attesting to its serine protease nature (Chen *et al.*, 1995). The purified protein hydrolyzed azocasein, azocoll, casein, elastin, gelatin and fibrinogen (Chen *et al.*, 1995).

Proteolytic enzymes such as brinase from *Aspergillus oryzae* (Fitzgerald *et al.*, 1972) have been used in some thromboembolic disorders. However, their clinical

applications are limited because their fibrinogenolytic effects *in vivo* are inhibited by an excess of protease inhibitors, either antiplasmin (Gaurot, 1967) or α_2-macroglobulin (Nyman and Duckert, 1975).

1.8.4 FIBRINOGENOLYTIC ACTIVITY FROM SNAKE VENOMS

Snake venoms cause hypofibrinogenemia by directly destroying fibrinogen or indirectly through the activation of plasminogen, or through defibrogenation by procoagulant, e.g. thrombin-like enzymes. Hypofibrinogenemia might be used in the treatment of thrombosis. Esnouf and Tunnah (1967) isolated a thrombin-like enzyme from *Agkistrodon rhodostoma* (Malayan pit viper) snake venom, it has been used as a defibrinogenating agent (ancrod, arvin) in thrombosis (Reid and Chan, 1968; Bell *et al.*, 1968).

Fibrinogenases isolated from snakes have been shown to be as effective as conventional anticoagulant treatment with heparin. Fibrinogen degradation by various venom metalloproteases gives rise to similar cleavage products. Cleavage of the Aα chains by venom metalloproteases occurs mainly at the Lys 413-Leu 414 bond, generating 43 kDa and 24 kDa fragments (Takeya *et al.*, 1993) which results in the loss of one RGD sequence, Aα 572-574, thought to be involved in platelet aggregation.

1.8.5 FIBRINOGENOLYTIC ACTIVITY FROM TICKS

To date no fibrinogenolytic activity from ticks has been described in the literature.

1.9 INTRODUCTION TO THE PRESENT STUDY

As part of the ongoing project aimed at identifying potential components in tick salivary glands for use as target tick vaccine antigens, this study was undertaken to obtain some preliminary information on proteolytic enzymes expressed in the tick salivary glands. Since proteinases are involved in key functions in many organisms,

it is logical to assume that these enzymes also play a role in tick physiology, thus making the proteinases attractive as possible target vaccine candidates. In preliminary investigations (J. Nienaber, personal communication) it was found that salivary gland extracts prepared from *O. savignyi* were unable to degrade fibrin clots, but able to hydrolyze fibrinogen. For this reason it was therefore decided to investigate the proteolytic activities present in salivary gland extracts, but more specifically to characterize the fibrinogenolytic activity.

1.10. AIMS OF THE STUDY

The objectives of the present study are:

- Identification and characterization of the general proteolytic activity as well as the fibrinogenolytic activity in tick salivary gland extracts (SGE) of *O. savignyi.*
- Isolation, biochemical and physiological characterization of the fibrinogenolytic activity in SGE.

Chapter 2 describes the identification and characterization of the proteolytic and fibrinogenolytic activities in the crude salivary gland extract as well as the characterization of the partially purified fibrinogenolytic activity with protease inhibitors. Chapter 3 describes the further purification and characterization of the fibrinogenolytic activity in the SGE. In Chapter 4 the effect of the partially purified fibrinogenolytic activity on aggregated platelets is investigated.

Various aspects of this study were reported at scientific meetings:

- (Mahlaku *et al.,*). A poster was presented at the Biochemistry in Africa: 2nd FASBMB and 15th SASBMB Conference in Potchefstroom, South Africa, September 1998.
- (Gaspar *et al.,*). A talk was presented at the 3rd International Conference on Tick and Tick-borne Pathogens: Into the 21st Century, Slovakia, August 1999.

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- (Mahlaku *et al.,*). A poster was presented at the BioY2K Combined Millennium Meeting: 16th SASBMB Conference, of the South African Society of Biochemistry and Molecular Biology (SASBMB), Grahamstown , South Africa, January 2000.

- (Mahlaku *et al.,*). A poster was presented at the 2nd Gauteng Region Annual Biochemistry (GRAB) Symposium, Pretoria, South Africa, September 2000.

- (Mahlaku *et al.,*). A poster was presented at the IUBMB/SASBMS Special Meeting on Biochemical and Molecular Basis of Disease, Cape Town, South Africa, November 2001.

CHAPTER 2

IDENTIFICATION AND CHARACTERIZATION OF THE PROTEOLYTIC AND FIBRINOGENOLYTIC ACTIVITIES IN SALIVARY GLAND EXTRACTS OF *O. SAVIGNYI*

2.1 INTRODUCTION

Proteolytic enzymes are known to be present in a wide range of organisms including vertebrates, bacteria, viruses, fungi (Rao *et al.*, 1998; Mbawa *et al.*, 1992), invertebrates such as ticks (Mulenga *et al.*, 1999; Mendiola *et al.*, 1996; Vundla *et al.*, 1992), protozoans (Rosenthal, 1999) and helminth parasites (Jose *et al.*, 1999). These enzymes have been well studied in mammals and have been shown to execute a variety of key physiological functions, extending from the cellular level to the organ and organism levels, to produce cascades involved in hemostasis and inflammation (Rao *et al.*, 1998). Proteinases from all four major proteolytic enzyme classes are utilized by parasites for diverse functions, such as hydrolysis of host proteins (Chen *et al.*, 1995), tissue penetration (Chen *et al.*, 1995), evasion of host immune responses (Lonsdale-Eccles *et al.*, 1995), inhibition of blood coagulation and thus prolonged availability of nutrients to the parasite (MacLennan *et al.*, 1997) and tissue adherence (Lantz *et al.*, 1985).

The study of parasite proteolytic enzymes has reached a level where well-characterized molecules are now being realistically targeted for selective chemo-and immuno-therapy. Examples of proteolytic enzymes receiving such attention include GP 57/51 cysteine protease of *Trypanosoma cruzi* (cruzipain), the trophozoite cysteine protease of *Plasmoduim falciparum* (Meirelles *et al.*, 1992) and the cathepsin B-like cysteine proteases of *Leishmania mexicana* (Robertson and Coombs, 1983). Each appears to be crucial to the development of the parasite.

2.1.1 TICK PROTEOLYTIC ENZYMES

It is assumed that tick proteolytic enzymes also occupy a central role in tick physiology, just like in other organisms. This makes this group of proteins attractive as possible target vaccine antigens. Data on tick proteolytic enzymes is still very limited. To date only a few publications have appeared in this field of study. There is evidence to show that proteolytic enzymes are involved in the mediation of key physiological functions, such as tick embryonic development, digestion of host red blood cells and proteins and in yolk proteolysis (Mendiola *et al.*, 1998; Fagotto, 1991; Lugullo *et al.*, 1998).

The digestion of a blood meal plays an important role in the biology of the tick and early evidence indicated that the major digestive proteolytic enzyme in the midgut of ticks is similar to the mammalian cathepsin D, an aspartic proteinase mostly localized in acidic lysosomes (Akov, 1982). Studies conducted by Mendiola *et al.* (1996) on midgut extracts from *Boophilus microplus* ticks proved that the main proteolytic activities were associated with aspartic and cysteine proteinases, while Vundla *et al.* (1992) purified two aspartic proteinases from the midguts of *Rhipicephalus appendiculatus*. Renard *et al.* (2000) has since then reported the cloning and functional expression of a *B. microplus* cathepsin L-like cysteine proteinase.

The proteolytic activity present in the midgut of both fed and unfed *Haemaphysalis longicornis* ticks was assessed by using gelatin substrate gel electrophoresis and inhibitor sensitivity analysis. Three predominant (116-, 48-, and 40-kDa) and two weak (55- and 60-kDa) proteinase bands were commonly expressed in both fed and unfed ticks, while a band of 80 kDa was only present in fed ticks. Inhibition studies against a panel of inhibitors showed that the most predominant 40-and 48-kDa bands were cysteine proteinases, while the third 116-kDa band was a member of the serine proteinase gene family (Mulenga *et al.*, 1999). In recent investigations the molecular cloning of two cathepsin L-like cysteine proteinase genes (Mulenga *et al.*, 1999) and two serine proteinase genes (Mulenga *et al.*, 2001) from the tick *H. logicornis* has been described.

2.1.2 ASSAYS OF PROTEOLYTIC ACTIVITY

Proteolytic activity is normally assayed by various methods using either natural substrates, chemically modified proteins, synthetic (chromogenic) substrates, SDS-PAGE zymography (substrate gel electrophoresis) or SDS-PAGE analysis of degradation products.

2.1.2.1 ASSAY OF PROTEOLYTIC ACTIVITY USING CHROMOGENIC SUBSTRATES

Most of these synthetic substrates are composed of a small peptide portion located on the amino terminal of the bond cleaved and a leaving group, which can be measured either directly or indirectly by photometry, flourometry or luminescence, or electrochemically. If the substrate is proteolytically cleaved, the leaving group is released and it can be measured spectrophotometrically by monitoring the increase in absorbance. The selectivity of individual proteases is achieved by designing a suitable peptide portion as the recognition sequence. Leaving groups used more often are for ester substrates: 4-nitrophenol and 4-methylumbelliferone; for amide substrates: 4- nitroaniline and 2-napthylamine (Fritz *et al.*, 1994). The reactions can be performed in 96-well microtiter plates.

2.1.2.2 ASSAY OF PROTEOLYTIC ACTIVITY USING AZOCASEIN

Azocasein is a chemically modified protein (a chromophoric derivative) that was designed as a substrate for the quantitative assay of proteolytic activity. It is synthesized by coupling of the milk protein, casein, with a diazotized aryl amine (Tomarelli *et al.*, 1947). The sulfanilamide groups, the chromophore, are covalently linked to the peptide bonds. As the proteolytic enzyme hydrolyzes peptides bonds, peptides and amino acids are liberated from the polymer. Following incubation, trichloroacetic acid (TCA) is added to stop the enzyme and precipitate macromolecules, including undigested azocasein. These are removed from the reaction mixture by centrifugation. Very short peptide chains and free amino acids

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liberated by the proteolytic enzyme are not precipitated by TCA, and thus remain in solution. The greater the extent of proteolysis, the greater the number of peptide chains and free amino acids in solution, and thus, the more intense orange color. The intensity of the color, measured spectrophotometrically, is used to determine the relative activity of the proteolytic enzyme.

2.1.2.3 SDS-PAGE ZYMOGRAPHY

The method depends upon combining the proteinase-containing sample mixture with SDS, but without boiling the solution. In this form the SDS binds to the proteins, perhaps modifying its conformation slightly and suppressing the activity. The SDS/protein mixture is separated by electrophoresis in a polyacrylamide gel containing a low concentration of gelatin or other protein substrates (less than 1 %). The proteinase/SDS mixture does not bind to the gelatin, as would a free proteinase, and migrates as a narrow band. Subsequent washing of the gel in a non-ionic detergent, such as Triton X-100, removes the SDS from the proteinase and reconstitutes its activity. The reactivated proteinase digests away part of the gelatin and its position can be detected by subsequent staining of the gel. The proteinase-digested gelatin appears as a clear band on the blue stained gel (Dennison, 1999).

2.1.2.4 SDS-PAGE ANALYSIS OF PROTEIN DEGRADATION PRODUCTS

Sodium dodecyl polyacrylamide gel electrophoresis (SDS-PAGE) is a method used to monitor protein purity and to determine molecular mass. It separates proteins on differences in their molecular weights by imposing uniform charge characteristics on the proteins. This is achieved through the use of sodium dodecyl sulphate (SDS), an anionic detergent, that binds strongly to proteins (approximately 1.4 mg SDS/ mg of protein) and gives them similar charge to mass ratios. Separation is achieved by the molecular sieving effect by using poly-acrylamide gels with specific pore sizes. The lower molecular weight proteins migrate faster than the larger ones towards the anode, at rates inversely proportional to their molecular weights

(Garfin, 1990). Protein degradation products can be monitored by comparing the molecular mass of the intact protein to that of the degradation products after exposure of the native protein to proteases.

2.1.3 CHARACTERIZATION OF PROTEOLYTIC ACTIVITY WITH SPECIFIC INHIBITORS

In the laboratory, inhibition of proteinase activity can be used to characterize the proteolytic activity. Inhibitors specific for the four classes of proteolytic enzymes can be used to determine the catalytic type of the peptidase present in crude extracts. For the serine peptides, 3,4 DCI (3,4-dichloroisocoumarin) or APMSF (4-[amidinophenyl]methanesulphonyl fluoride) are normally used, while in the case of metalloproteinases, 1,10-phenanthroline is used. E-64 (L-trans-epoxysuccinyl-leucylamide-[4-guanidino]butane), and pepstatin A are the proteinase inhibitors of the cysteine and aspartic proteinases, respectively. Table 2.1 summarizes the specificities of the different proteinase inhibitors.

TABLE 2.1: CLASSIFICATION OF THE MOST COMMONLY USED PROTEINASE INHIBITORS

Inhibitor	Specificity
1,10-phenanthroline	Metalloproteinase
E-64	Cysteine proteinase
DCI, APMSF	Serine Proteinase
Pepstatin A	Aspartic Proteinase

The class of metalloproteases, which require divalent metals for activity, can often be inhibited by complexing metal ions with EDTA. EDTA is a non specific chelating agent hence its inhibition cannot be taken as a reliable indication that the enzyme is a metalloproteinase since many proteases of other classes are activated by Ca $^{2+}$ ions (Scopes, 1994). 1,10-phenanthroline is the most useful inhibitor with which to recognize metalloproteinases, the very great majority of which are zinc-containing enzymes. The planar molecule of 1,10-phenanthroline binds non-specifically in the active site of some enzymes and it has a vastly higher affinity for zinc than for

calcium, so that it can be used in the presence of up to 10 mM Ca^{2+}, which is required for the stability or activity of a number of metallo- and other peptidases (Barrett, 1994). E-64 reacts rapidly, specifically and irreversibly with the active site of cysteine proteases. It does not react with low molecular mass thiols such as cysteine and dithiothreitol (Barrett, 1994). PMSF (Phenyl methane sulphonyl fluoride) is a serine proteinase inhibitor, which can also inhibit cysteinyl proteases. However, APMSF is a specific inhibitor of trypsin-like serine proteases (Beynon and Bond, 1989). 3,4-DCI reacts rapidly and it is an irreversible, progressive covalent inhibitor of a wide range of serine proteinases, but eventually reach total inhibition (Barrett, 1994). Pepstatin A, is produced by fungi of *Streptomyces* species. It is an extracellular peptide of complex structure which is potent inhibitor of aspartyl proteases such as pepsin, cathepsin, and yeast protease A. It is effective at concentrations as low as 10^{-7} M. It binds tightly to the proteases, but the binding is reversible (Scopes, 1994 and Barrett, 1994).

This chapter describes the identification and characterization of the total proteolytic activity as well as more specifically the fibrinogenolytic activity present in the tick salivary gland extract of *O. savignyi*.

2.2 MATERIALS AND METHODS

2.2.1 MATERIALS USED

All materials used were of analytical grade and double distilled water was used in all experiments. Tris-HCl, NaCl, EDTA (ethylenediamine tetraacetate), sodium azide, DTT, methanol, acetic acid, glycine, ammonium persulphate, bromophenol blue and TEMED were obtained from Merck, Darmstadt, Germany. $CaCl_2$, and $MgCl_2$ were obtained from BDH Chemicals Ltd. Poole, England. Low molecular weight marker proteins were obtained from Pharmacia, U.S.A. Acrylamide, bisacrylamide, 2 β-mercaptoethanol and SDS were purchased from BDH Laboratory suppliers LTD., England. Coomassie Blue was obtained from Bio-Rad Laboratories, U.S.A. PVDF (Polyvinylidene-difluoride) membranes were supplied by Merck, Germany. CAPS (3-(cyclohexylamino)-1-propane sulphonic acid) was supplied by Sigma, U.S.A and

Triton X-100 from Boehringer Mannheim, Germany. Azocasein, fibrinogen, 1,10 phenantholine, pepstatin A, E-64, DCI, APMSF, DMSO (dimethyl sulphoxide) and MES (2-[N-Morpholino]ethane sulfonic acid) were purchased from Sigma-Aldrich Co U.S.A. Molecular mass marker proteins were obtained from Pharmacia U.S.A. Slide-A-Lyzer dialysis cassettes were purchased from Pierce, U.S.A.

2.2.2 COLLECTION OF TICKS AND PREPARATION OF THE CRUDE SALIVARY GLAND EXTRACT

O. savignyi ticks were collected from the Upington district in the Northern Cape Province of South Africa by sifting of sand. The salivary glands of female ticks were dissected under a binocular stereomicroscope (10x magnification) using the method of Brown *et al.*, (1984). They were embedded in molten wax with their dorsal parts visible. The integument was removed by lateral dissection of the cuticle with a #11 scalpel under a 0.9 % NaCl solution. After dissection, the salivary glands were placed in an Eppendorf tube frozen immediately in liquid nitrogen and stored at –70 °C. The glands were suspended in the specific buffer used for an experiment and sonicated for 3 x 6 pulses (30 % duty cycle and an output control of 3) with a Branson Sonifier (Branson Sonic Power Co.) with 1 min intervals on ice. After centrifugation for 5 minutes in an Eppendorf microcentrifuge at 10 000 g, the supernatants were filtered through a 0.22 μm membrane (Millipore), diluted as required and used immediately.

2.2.3 DETERMINATION OF THE PROTEOLYTIC ACTIVITY

Proteinase activity was determined by using azocasein as the substrate according to the method of Walker *et al.* (1998) with slight modifications. Salivary gland extract (SGE) representing an equivalent of 1.25 salivary glands in 50 μl of 50 mM of Tris-HCl pH 7.6 and 10 μl of azocasein solution (1 % w/v) in 0.05 % SDS were mixed. The reaction mixture was then incubated at 37 °C for 4 hours. Thirty μl of chilled 10 % TCA solution was added to stop the reaction and the tubes were placed on ice for 30 min, before centrifuging at 14 000 g for 5 min to remove precipitated

proteins. The supernatant (60 µl) was then withdrawn and added to the well of a microtiter plate containing 40 µl of 1 M NaOH. All incubations were done in triplicate and the released peptides were measured spectrophotometrically at 405 nm in a microtiter plate reader along with those for appropriate blanks and controls. The experiment was repeated with 0.03, 0.06, 2.5 and 5 salivary glands, respectively.

2.2.4 pH DEPENDENCE OF THE PROTEOLYTIC ACTIVITY

The buffers used to analyze the pH profile of the proteolytic activity were: 50 mM sodium acetate (pH 3 and 5), 50 mM Tris-HCl (pH 7 and 9) and 50 mM sodium phosphate pH 11. SGE was prepared as described in section 2.2.2 in the experimental buffer (1.25 SG/50 µl) and then incubated with azocasein at 37 °C overnight. Except for the buffers the rest of the procedure was followed as described in section 2.2.3.

2.2.5 CHARACTERIZATION OF THE PROTEOLYTIC ACTIVITY WITH SPECIFIC INHIBITORS

The effects of proteinase inhibitors on the proteolytic activity were determined using azocasein as a substrate. Chemical inhibitors were made up as aqueous solutions (100 mM E-64, 100 µM pepstatin A, 10 mM 1,10-phenanthroline). A 10 mM DCI solution was prepared fresh in DMSO. An equivalent amount of DMSO was added to the control. The SGE (representing an equivalent of 1.25 salivary glands) was prepared in different buffers: 50 mM sodium acetate pH 3 and 5, 50 mM Tris-HCl pH 7 and 9 and 50 mM sodium phosphate pH 9 and 11. Inhibitor solutions (15 µl) were preincubated with the enzyme (15 µl SGE) at 37 °C for 2 hours prior to the addition of the substrate. Azocasein (10 µl of 10 % w/v in 0.05 % SDS solution) was added to start the reaction and the incubation was done overnight at 37 °C. The rest of the procedure was followed as described in section 2.2.3.

2.2.6 DETERMINATION OF THE FIBRINOGENOLYTIC ACTIVITY BY SDS-PAGE ANALYSIS

The method of Chen *et al.* (1995) was used to assess the fibrinogenolytic activity in crude salivary gland extracts. SGE (1 SG/50 μl of 50 mM Tris-HCl, pH 7.6) was incubated with human fibrinogen (1.2 mg/200 μl buffer) for various times at 37 °C and the proteolytic activity stopped by addition of 100 μl of 6 M urea containing 3 % SDS. Controls in which only fibrinogen or SGE was present were also included. Samples (50 μl), were diluted in a 1:2 ratio with Laemmli sample buffer (0.06 M Tris-HCl, pH 6.8; 2 % SDS [w/v]; 0.1 % glycerol [v/v]; 0.05 % β-mercaptoethanol [v/v] and 0.025 % bromophenol blue [w/v]) and boiled at 94 °C for 5 minutes. Low molecular mass protein standards, were prepared as described for the sample and applied to the gel (5 μl/well). The standard mix contained phosphorylase b (94 kDa), BSA (76 kDa), ovalbumin (43 kDa), carbonic anyhdrase (30 kDa), trypsin inhibitor (20 kDa) and α-lactalbumin (14 kDa).

Electrophoresis was performed according to the method described by Laemmli (1970). A 12 % separating gel (0.5 M Tris HCl, pH 8.8, 0.1 % SDS) and a 4 % stacking gel (1.5 M Tris HCl, pH 6.8, 0.1 % SDS) were prepared from a 30 % (w/v) acrylamide, 0.8 % (w/v) N' N'-methylene bisacrylamide stock solution. The solutions were degassed for 10 min prior to polymerization by 50 μl of 10 % ammonium persulphate and 10 μl TEMED. The gel was cast in a 1 mm vertical gel system (Biometra, Göttingen, Germany) and allowed to polymerize overnight at room temperature. The electrophoresis buffer consisted of 0.02 M Tris-HCl, 0.1 M glycine and 0.06 % SDS, pH 8.3. A constant voltage of 60 V was maintained until samples concentrated on the stacking gel. The voltage was then adjusted to 100 V and kept constant for the remaining separation period. Proteins were visualized by staining in 0.1 % Coomassie Brilliant blue (40 % methanol, 10 % acetic acid) and were destained in an excess of destaining solution (40 % methanol, 10 % acetic acid).

2.2.7 EFFECT OF METAL IONS ON THE FIBRINOGENOLYTIC ACTIVITY

SGE (1 SG/50 µl of 50 mM Tris-HCl, pH 7.6) was incubated with human fibrinogen (1.2 mg/200 µl) in the presence of EDTA (2 mM); EDTA (2 mM) and $MgCl_2$ (6 mM); or EDTA (2 mM) and $CaCl_2$ (6 mM) for 16 hours. Fibrinogen and SGE controls were included. Fibrinogenolytic activity was determined by SDS-PAGE as described in section 2.2.6.

2.2.8 pH DEPENDENCE OF THE FIBRINOGENOLYTIC ACTIVITY

SGE, (equivalent of 1 salivary gland in 50 µl of each respective buffer) was incubated with human fibrinogen (1.2 mg/200 µl of respective buffer) for 4 h at 37 °C. The buffers used were: 50 mM sodium acetate pH 3 and 5, 50 mM Tris-HCl pH 7 and 9 and 50 mM sodium phosphate, pH 11. The reaction was stopped by adding 100 µl of 6 M with 3 % SDS. Samples (50 µl), were diluted in a 1:2 ratio with Laemmli sample buffer and then heated at 95 °C for 5 min. Samples (30 µl) were loaded onto the gel and electrophoresis was performed as described in section 2.2.6.

2.2.9 CHARACTERIZATION OF THE FIBRINOGENOLYTIC ACTIVITY WITH SPECIFIC INHIBITORS

SGE (1 gland/50 µl) was prepared in different buffers: 50 mM sodium acetate pH 3 and 5: 50 mM Tris-HCl pH 7 and 9 and 50 mM sodium phosphate pH 11. SGE (50 µl) and protease inhibitor solutions (50 µl) were pre-incubated at 37 °C for 2 hours. Fibrinogen (50 µl of a 200 µg/µl solution) was added and incubation continued at 37 °C for 4 hours. The fibrinogenolytic activity was determined as described in section 2.2.6. The inhibitors were made up as aqueous solutions (100 mM E-64, 100 µM pepstatin A, 10 mM 1,10-phenanthroline) or ethanol (50 µM APMSF) and were prepared fresh for each experiment. APMSF was always used within the first 5 min of its preparation due to its short half-life of 6 min.

2.2.10 ZYMOGRAPHIC ANALYSIS OF THE FIBRINOGENOLYTIC ACTIVITY

The method used for the detection of proteinase activity in polyacrylamide gels was a modification of the methods described by Feitosha *et al.* (1998). A 10 % SDS-PAGE gel was prepared as described in section 2.2.6 and polymerized with fibrinogen at a final concentration of 2 mg/ml. Samples of crude SGE (equivalent of 1 salivary gland/ lane) or 1.2 mg plasmin/lane (positive control) were dissolved in a 1:2 ratio of Laemmli sample buffer in the absence of reducing agents and without heating. Electrophoresis was performed at 60 V at room temperature. After electrophoresis, gels were washed in 3 changes of 2.5 % Triton X-100 (v/v) over a 45 min period to remove the SDS. The gels were cut into strips and incubated at 37 °C in 50 mM Tris-HCl pH 7.6, 200 mM NaCl, 5 mM $CaCl_2$ and 1 mM DTT. Zones of proteolysis were visualized as clear bands against a blue background by Coomassie blue staining (0.1 % Coomassie blue R-250 in 30 % methanol and 10 % acetic acid) followed by destaining in 20 % methanol with 10 % acetic acid. The experiment was repeated with various changes which included, running gels at 4 °C, excluding 1 mM DTT in the incubation buffers, using different buffers at different pH values (50 mM sodium acetate pH 3 and 5; 50 mM Tris-HCl pH 7 and 9 and 50 mM sodium phosphate pH 11), various amounts of salivary glands/lane and total exclusion of SDS from the gels.

2.2.11 CATION EXCHANGE HPLC OF SALIVARY GLAND EXTRACTS

High performance liquid chromatography was performed with a Beckman 110B solvent delivery system, 406 analog interface and 166-detector module and Beckman 340 organizer with injector. Data were collected using the System Gold™ Software (Beckman, Polo Alto, CA, U.S.A). Forty salivary glands were sonicated in 400 µl ml of 20 mM MES, pH 6.1 and centrifuged at 10 000 g for 5 min. The supernatant was diluted to 1 ml and filtered through a 0.22 µm membrane before loading onto a cation exchange column (TSK-GEL SP-5PW, 7.5 mm ID x 7.5 cm), which had been pre-equilibrated with the same buffer. A linear gradient elution of 0

to 1 M NaCl was performed with buffer (20 mM MES, 1 M NaCl, pH 6.1) at a flow rate of 1 ml/min. Individual fractions (1 ml) were collected and assayed for the total proteolytic activity against azocasein as described in section 2.2.3. Fractions were also tested for fibrinogenolytic activity as described in section 2.2.6.

2.2.12 CHARACTERIZATION OF THE PROTEOLYTIC ACTIVITY PRESENT IN CATION–EXCHANGE HPLC FRACTIONS WITH SPECIFIC INHIBITORS

Cation exchange fractions were freeze dried and individual fractions from five different runs were reconstituted in different pH buffers as described in section 2.2.4. Azocasein (10 μl of 1 % w/v in 0.05 % SDS) was added and proteolytic activity was then assayed as in section 2.2.3. above. The effect of proteinase inhibitors on proteolytic activity was studied by incubating 15 μl of the proteinase inhibitor with 15 μl of the various reconstituted HPLC fractions for 2 hours at 37 °C. Inhibitors (100 μM E-64, 100 μM pepstatin A, 10 mM 1,10-phenanthroline, 10 mM DCI) were made up as described in section 2.2.5.

2.2.13 CHARACTERIZATION OF THE FIBRINOGENOLYTIC ACTIVITY PRESENT IN CATION EXCHANGE HPLC REGIONS A, B, C WITH SPECIFIC INHIBITORS

Fractions in region A (from 5 separate CE-HPLC runs, Fig 2.14) were pooled, freeze-dried and reconstituted in different pH buffers (50 mM sodium acetate pH 3 and 5; 50 mM Tris-HCl pH 7 and 9 and 50 mM sodium phosphate pH 11). Regions B and C were prepared in the same manner. Proteinase activity in each individual CE-HPLC region was characterized by pre-incubating 50 μl reconstituted region A, B or C with 50 μl proteinase inhibitor [APMSF (50 μM); 1,10 Phenanthroline (10 mM), pepstatin A (100 μM), or E-64 (100 μM)] for 2 h at 37 °C. Thereafter, fibrinogen (50 μl) was added and the samples were further incubated for 4 h at 37 °C. Fibrinogenolytic activity was assayed as described in 2.2.6.

2.3 RESULTS

2.3.1 DETERMINATION OF PROTEOLYTIC ACTIVITY IN SALIVARY GLAND EXTRACTS

2.3.1.1 CONCENTRATION-DEPENDENCE OF PROTEOLYTIC ACTIVITY

Preliminary results showed that the crude SGE exhibits proteolytic activity against the general protease substrate, azocasein. Fig 2.1 indicates that the hydrolysis of azocasein by the SGE is concentration-dependent.
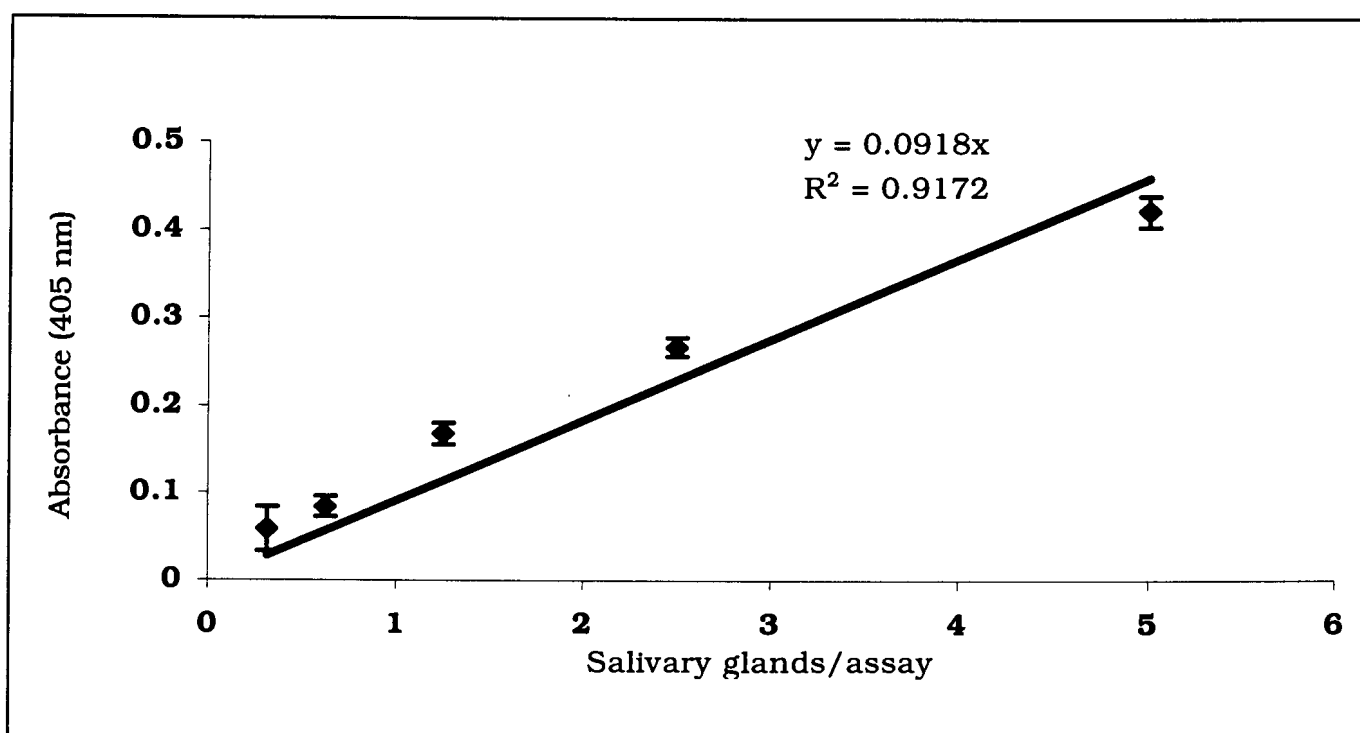
Fig 2.1: Concentration-dependent hydrolysis of azocasein by SGE at pH 7.6. Azocasein was incubated with various concentrations of SGE at 37 °C and the released peptides were measured spectrophotometrically as described in section 2.2.3. Absorbance values were corrected by subtraction of reagent blank containing no SGE. Each analysis was performed in triplicate and symbols represent the mean ± SD.

Extracts prepared from 1.25 salivary glands were used in the azocasein assay for subsequent experiments.

2.3.1.2 pH-DEPENDENCE OF THE PROTEOLYTIC ACTIVITY

The effect of pH on SGE proteinase activity with azocasein as substrate was determined using buffers with different pH values in the range of 3 to 11. The highest proteolytic activity was measured at pH 9 and this was followed by that at pH 7 (46 % relative to activity at pH 9). Minimal proteolytic activity was observed in the more acidic and basic pH range.
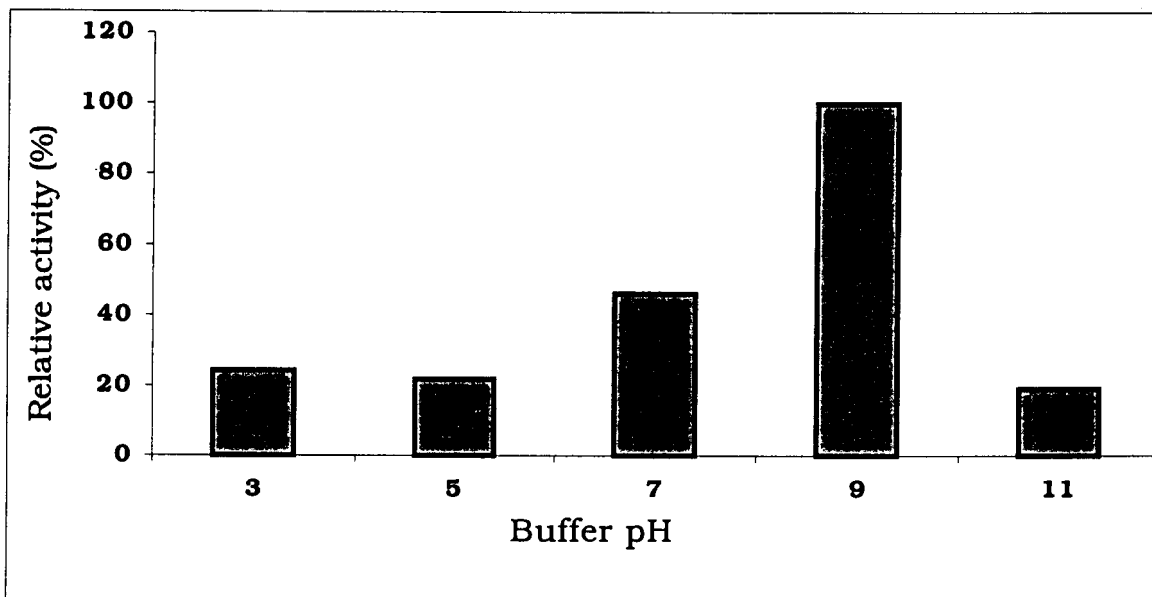
Fig 2.2: The effect of pH on azocasein degradation by crude SGE (1.25 SG/50 µl buffer). Values are the average of three individual determinations. The activity is expressed relative to that of pH 9, the highest activity. Controls for non-proteolytic hydrolysis of azocasein at different pH conditions were subtracted from the sample values. Bars represent the mean ± SD of triplicate measurements (SD values are smaller than bar sizes).

2.3.1.3 CHARACTERIZATION OF THE PROTEOLYTIC ACTIVITY WITH SPECIFIC INHIBITORS

In order to determine to which class of proteolytic enzyme the activity may belong, the effect of inhibitors over the pH range of 3-11 was studied (Table 2.2). At pH 7 and 9 the proteolytic activities were found to be mainly due to the presence of serine and metalloproteases, as DCI and 1,10-phenanthroline had the greatest inhibitory effect on the activities. The metalloproteinase inhibitor, 1,10-

phenanthroline, showed the most statistically significant inhibition (57 % and 83 %) at pH 7 and 9, respectively, and this was followed by the serine proteinase inhibitor (50 % and 56 % inhibition at pH 7 and 9). The metalloproteinase was found to be less active at pH 11. In the presence of 1,10 phenanthroline an inhibition of 17 % ($p < 0.01$) was observed. The serine proteinase activity observed at pH 7 and 9, was no longer present at pH 11 as no inhibition was observed in the presence of the serine proteinase inhibitor, DCI. The proteolytic activity was enhanced in the presence of this inhibitor (relative activity of 122 %, $p < 0.05$). This activation of activity was also observed at pH 5 (relative activity of 103 %, $p < 0.01$).

At pH 3 the observed activity may be associated with both cysteine and aspartic proteases as E-64 and pepstatin A had the greatest effect, but these values were found not to be statistically significant. At pH 5, inhibition by pepstatin A (27 %, $p < 0,05$) was observed. In addition to the inhibition observed by pepstatin A at this pH, the aspartic proteinase inhibitor also showed to have an effect although minimal on the proteolytic activity at pH 9 (12 % inhibition, $p < 0,05$) and at pH 11 (17 % inhibition, $p < 0.01$).

TABLE 2.2: THE EFFECT OF PROTEASE INHIBITORS ON SALIVARY GLAND PROTEOLYTIC ACTIVITY

pH	Inhibitor (Concentration)	A_{405} * mean	Standard deviation	Relative activity (%)
3	CONTROL	**0.079**	**0.001**	**100**
	1,10-phenanthroline (10 mM)	0.072	0.012	91
	E-64 (100 mM)	0.052	0.007	75
	DCI (10 mM)	0.082	0.002	104
	Pepstatin A (100 μM)	0.062	0.007	79
5	CONTROL	**0.071**	**0.003**	**100**
	1,10-phenanthroline (10 mM)	0.067	0.004	94
	E-64 (100 mM)	0.061	0.016	86
	DCI (10 mM)	0.073**	0.003	103
	Pepstatin A (100 μM)	0.052*	0.006	73
7	CONTROL	**0.150**	**0.011**	**100**
	1,10-phenanthroline (10 mM)	0.064***	0.008	43
	E-64 (100 mM)	0.162	0.011	108
	DCI (10 mM)	0.072**	0.005	50
	Pepstatin A (100 μM)	0.155	0.008	103
9	CONTROL	**0.325**	**0.013**	**100**
	1,10-phenanthroline (10 mM)	0.055***	0.001	17
	E-64 (100 mM)	0.321	0.018	99
	DCI (10 mM)	0.144**	0.027	44
	Pepstatin A (100 μM)	0.286*	0.018	88
11	CONTROL	**0.063**	**0.002**	**100**
	1,10-phenanthroline (10 mM)	0.052**	0.001	83
	E-64 (100 mM)	0.060	0.001	95
	DCI (10 mM)	0.077*	0.004	122
	Pepstatin A (100 μM)	0.052**	0.001	83

*A_{405} are means of triplicate values and activity is expressed relative to the control (no inhibitor). A_{405} values that are significantly different from the controls are shown as *$p < 0.05$, **$p < 0.01$, ***$p < 0.001$. Other differences are insignificant. See sections 2.2.4 and 2.2.5 for details.

2.3.2 DETERMINATION OF THE FIBRINOGENOLYTIC ACTIVITY IN SALIVARY GLAND EXTRACTS

2.3.2.1 FIBRINOGENOLYTIC ACTIVITY TIME STUDY

Fibrinogenolytic activity was measured by following the extent of hydrolysis using fibrinogen as a substrate. Fig 2.3 shows the results of SDS-PAGE analysis of

incubation mixtures of human fibrinogen with SGE. As shown in the control lane, reduced human fibrinogen was separated into A(α)-, B(β)-, and γ-chains. When incubated with SGE, the Aα-chain started to disappear within 2 hours. The B(β)- and γ-chains were resistant to hydrolysis by SGE even after 24 hours of incubation period since the intensity of the B(β) and γ-bands remained the same with time.
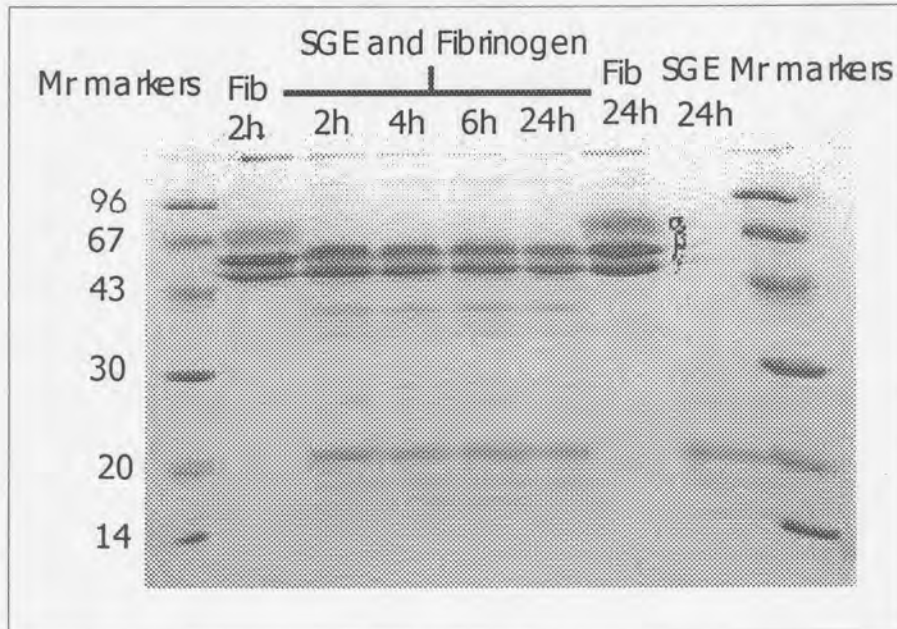
Fig 2.3: Time course study of the fibrinogenolytic activity of the crude SGE. Fibrinogen (1.2 mg in 200 µl of 50 mM Tris-HCl, pH 7.6) was incubated with the SGE (equivalent of 1 salivary gland in 50 µl of buffer) for different time intervals. Degradation products were monitored on the Coomassie blue stained gels. The low molecular mass markers were phosphorylase b (94 kDa), BSA (67 kDa), ovalbumin (43 kDa), carbonic anyhydrase (30 kDa), trypsin inhibitor (20 kDa) and α-lactalbumin (14 kDa). Fibrinogen (Fib) controls were incubated for 2 and 24 hours, respectively. A control containing only SGE incubated for 24 h in buffer alone was also included.

2.3.2.2 EFFECT OF METAL IONS ON THE FIBRINOGENOLYTIC ACTIVITY

Dependence of bivalent metal ions was investigated by measuring the enzymatic activity in the presence of EDTA. After EDTA pre-treatment of the SGE, the fibrinogenolytic activity was completely inhibited. Addition of metal ions (Ca^{2+} and Mg^{2+}) restored proteolytic activity only partially with apparent preference for Ca^{2+} (Fig 2.4).
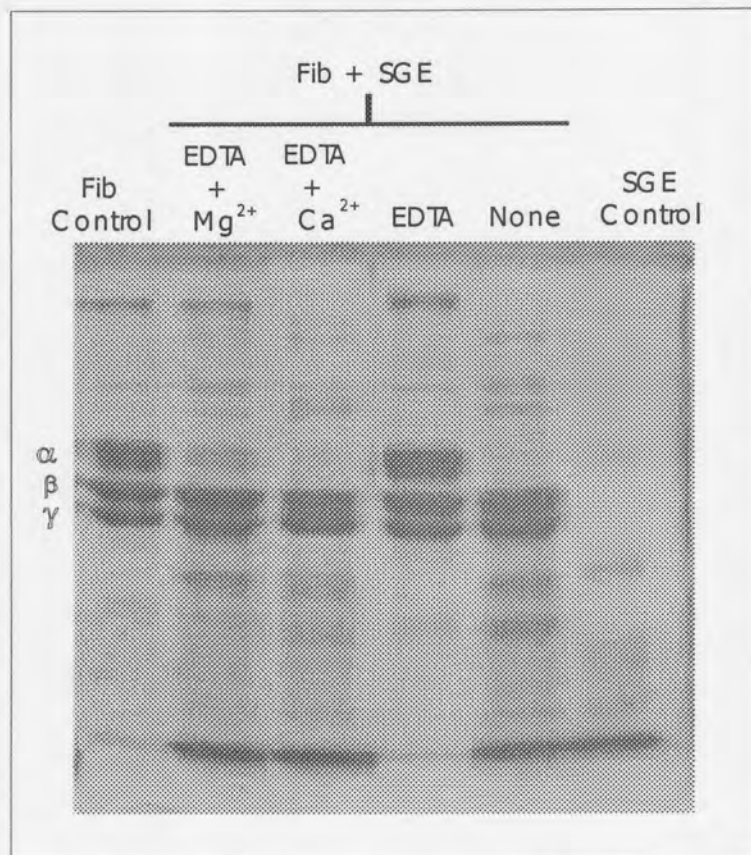
Fig 2.4: The effect of metal ions on the fibrinogenolytic activity. Fibrinogen (Fib) was incubated with SGE in the presence of the indicated compounds. See text for details (section 2.3.2.2).

2.3.2.3 pH-DEPENDENCE OF THE FIBRINOGENOLYTIC ACTIVITY

The pH dependence of the fibrinogenolytic activity in SGE was determined by incubating fibrinogen with extracts prepared in different pH buffers. In Fig 2.5 A is indicated the fibrinogen control at different pH values which indicated that fibrinogen is stable over a wide pH range of 3-11, while Fig 2.5 B indicates the SGE control under different pH values used for the experiment.
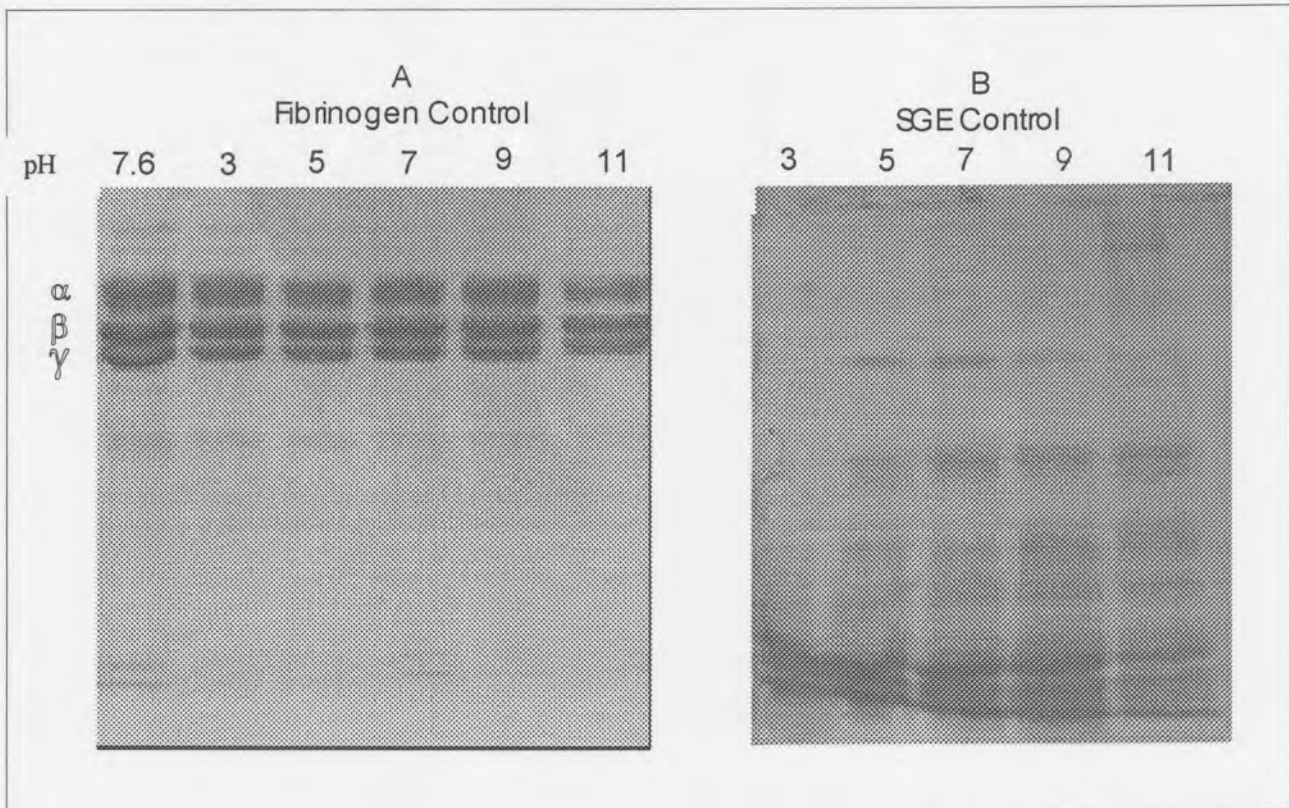
Fig 2.5: Fibrinogen (A) and SGE (B) controls at different pH conditions.

Fibrinogenolytic activity was detected in the SGE and the results showed a potent cleaving activity on the Aα-chain of fibrinogen in the pH range of 3 to 9, but to a lesser extent at pH 5 (Fig 2.6). No fibrinogenolytic activity was observed at pH 11.
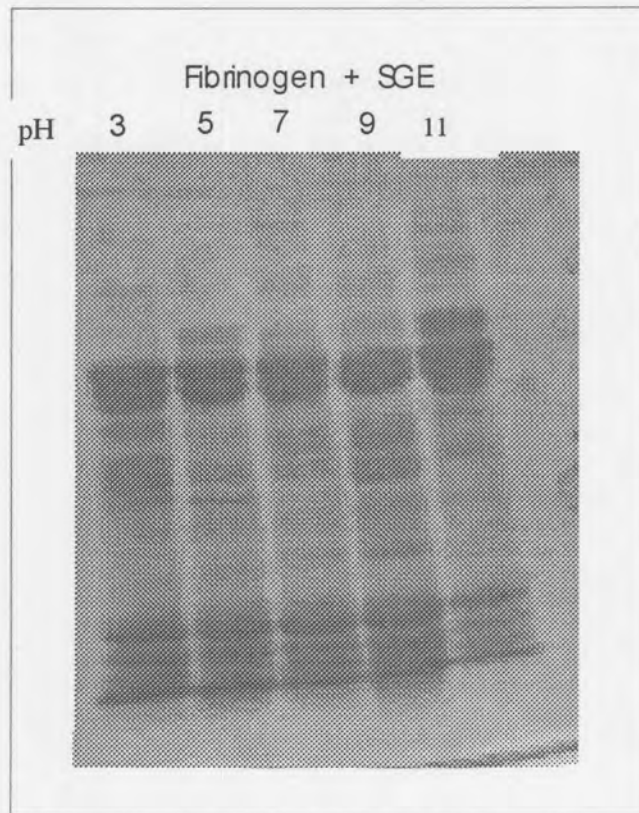
Fig 2.6: pH dependence of the fibrinogenolytic activity. The SGE was prepared in the respective buffers (see section 2.2.8). The reaction was started by adding fibrinogen prepared in the same buffer with a further 4 hours incubation at 37 °C. Activity was assayed by analyzing the degradation of fibrinogen on Coomassie blue stained gels.

2.3.2.4 CHARACTERIZATION OF THE FIBRINOGENOLYTIC ACTIVITY WITH SPECIFIC PROTEASE INHIBITORS

In an attempt to identify the type of proteolytic enzyme/s associated with the fibrinogenolytic activity, the pH dependent assays were repeated in the presence of the four individual protease inhibitors.

The fibrinogenolytic activity observed over the pH 3-9 range in the SGE was mainly inhibited at pH 3 and 5 by the aspartic proteinase inhibitor, pepstatin A, with very slight inhibition at pH 9. The results are indicated by the undegraded Aα-chain of fibrinogen at pH 3 and 5 in Fig 2.7.
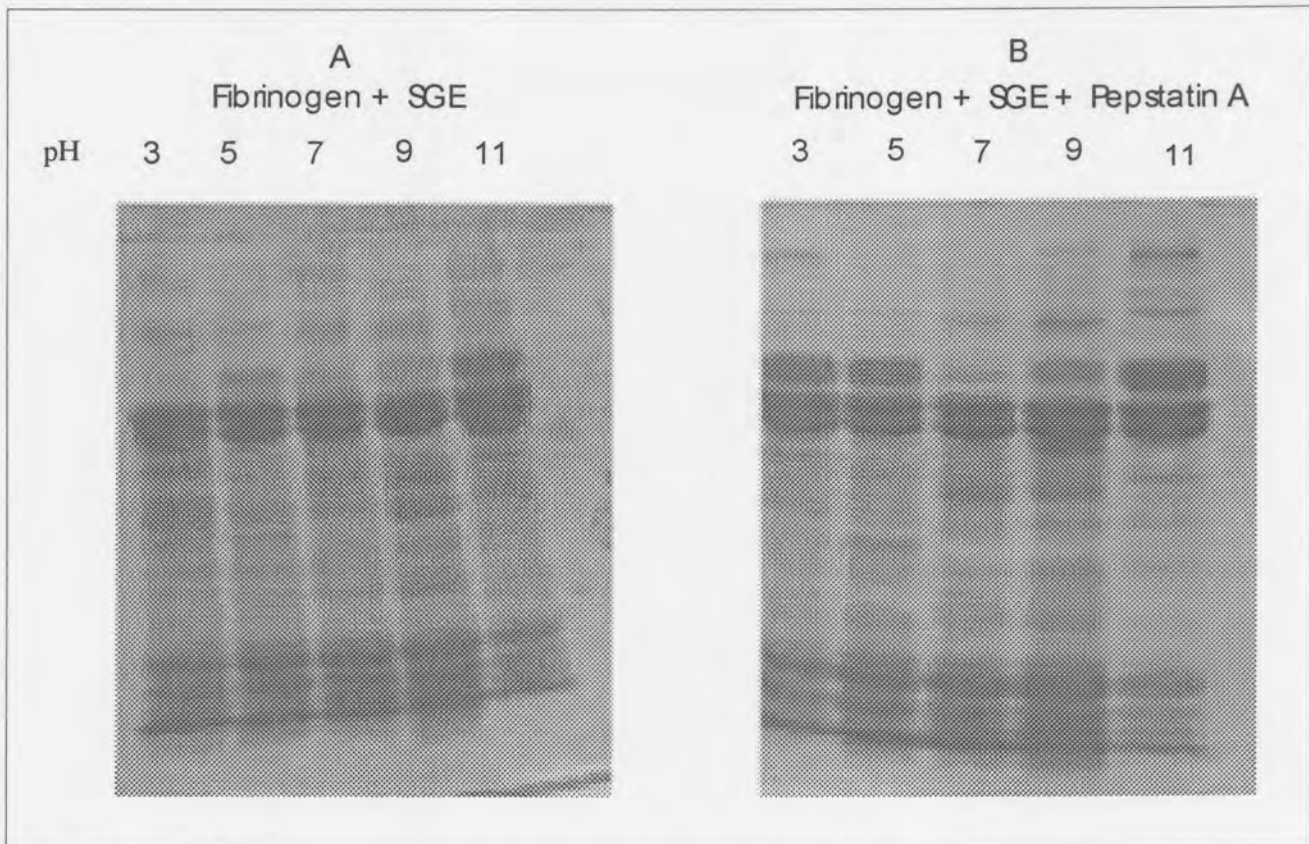
Fig 2.7: Effect of pepstatin A on the fibrinogenolytic activity at different pH values. (A) Fibrinogen was incubated with SGE (prepared in different pH buffers) for 4h at 37 °C. (see 2.2.8 for rest of details). (B) SGE (prepared in different pH buffers) was pre-incubated with pepstatin A (100 μM) for 2h at 37° C. Thereafter, fibrinogen was added and incubated further for 4h at 37 °C. (see 2.2.9 for rest of details).

The fibrinogenolytic activity observed over the pH 3-9 range in the SGE was not affected by the serine proteinase inhibitor, APMSF, suggesting that there was no contribution by this class of proteases. The results are indicated in Fig 2.8.
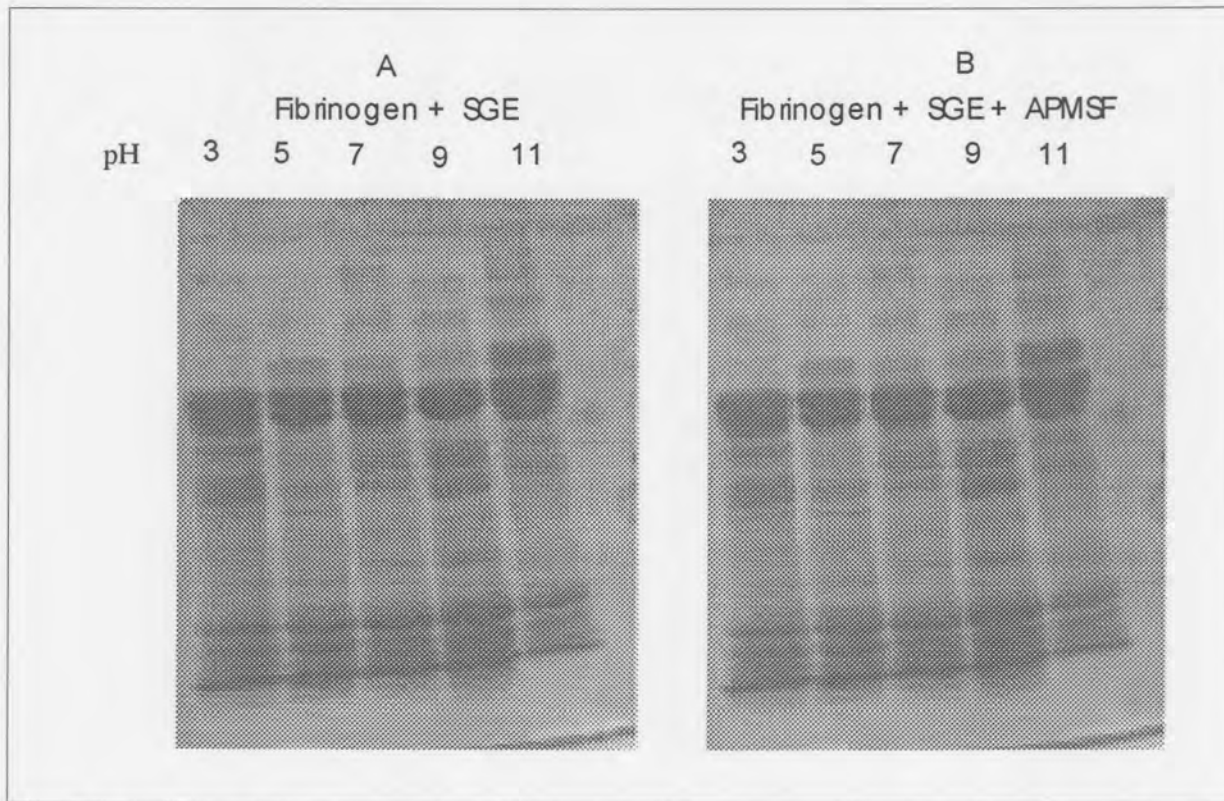
Fig 2.8: Effect of APMSF on the fibrinogenolytic activity at different pH values. (A) Fibrinogen was incubated with SGE (prepared in different pH buffers) for 4h at 37 °C. (see 2.2.8 for rest of details). (B) SGE (prepared in different pH buffers) was pre-incubated with APMSF (50 μM) for 2h at 37° C. Thereafter, fibrinogen was added and incubated further for 4h at 37 °C. (see 2.2.9 for rest of details).

The fibrinogenolytic activity observed over the pH 3-9 range in the SGE was completely inhibited by the metalloproteinase proteinase inhibitor, 1,10-phenanthroline. This indicates that the activity observed at pH 3-9 is due to the action of a metalloproteinase. The results are indicated in Fig 2.9.
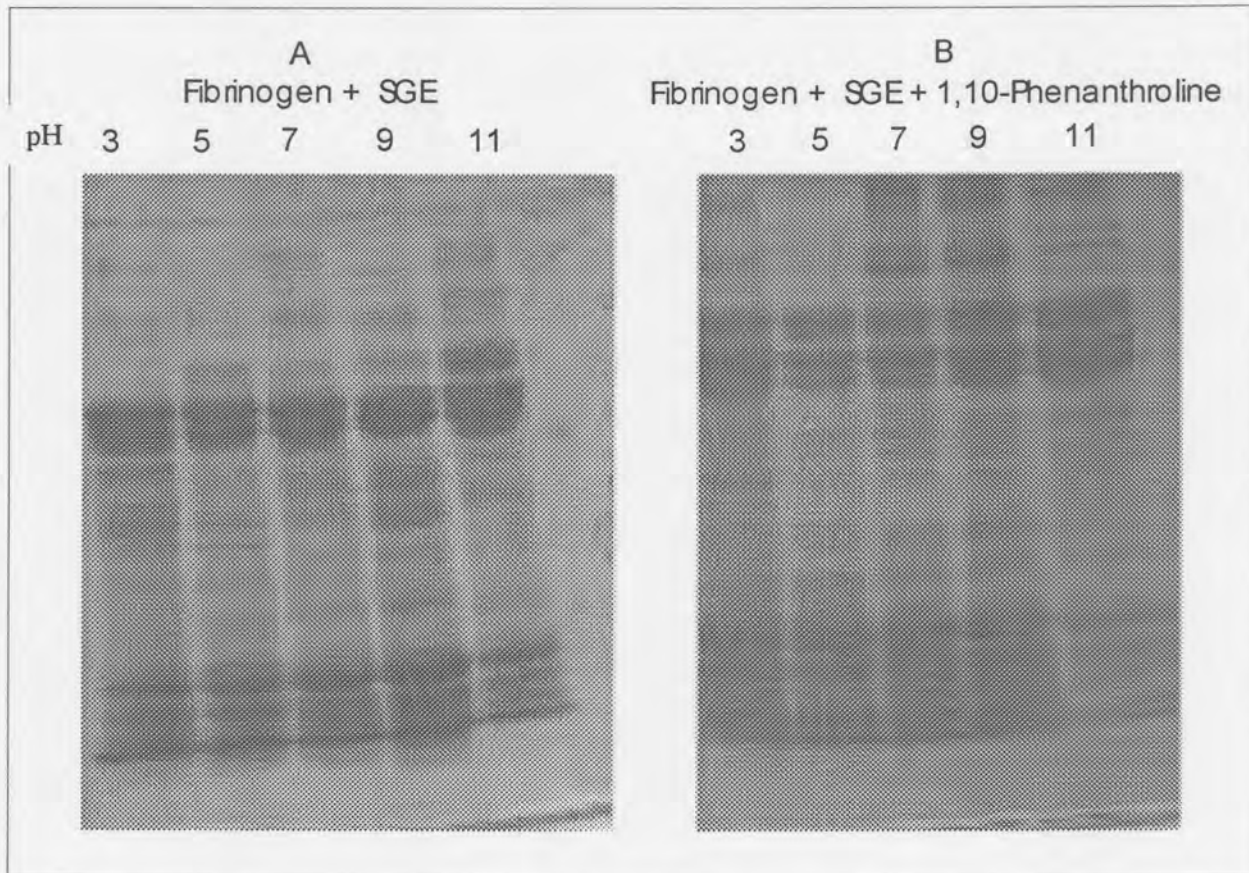
Fig 2.9: Effect of 1,10-phenanthroline on the fibrinogenolytic activity at different pH values. (A) Fibrinogen was incubated with SGE (prepared in different pH buffers) for 4h at 37 °C. (see 2.2.8 for rest of details). (B) SGE (prepared in different pH buffers) was pre-incubated with 1,10-phenanthroline (10 mM) for 2h at 37° C. Thereafter, fibrinogen was added and incubated further for 4h at 37 °C. (see 2.2.9 for rest of details).

The fibrinogenolytic activity was inhibited by E-64, a cysteine protease inhibitor, at pH 5. This indicates that the activity observed at pH 5 is also due to the presence of a cysteine protease. The results are indicated in Fig 2.10.
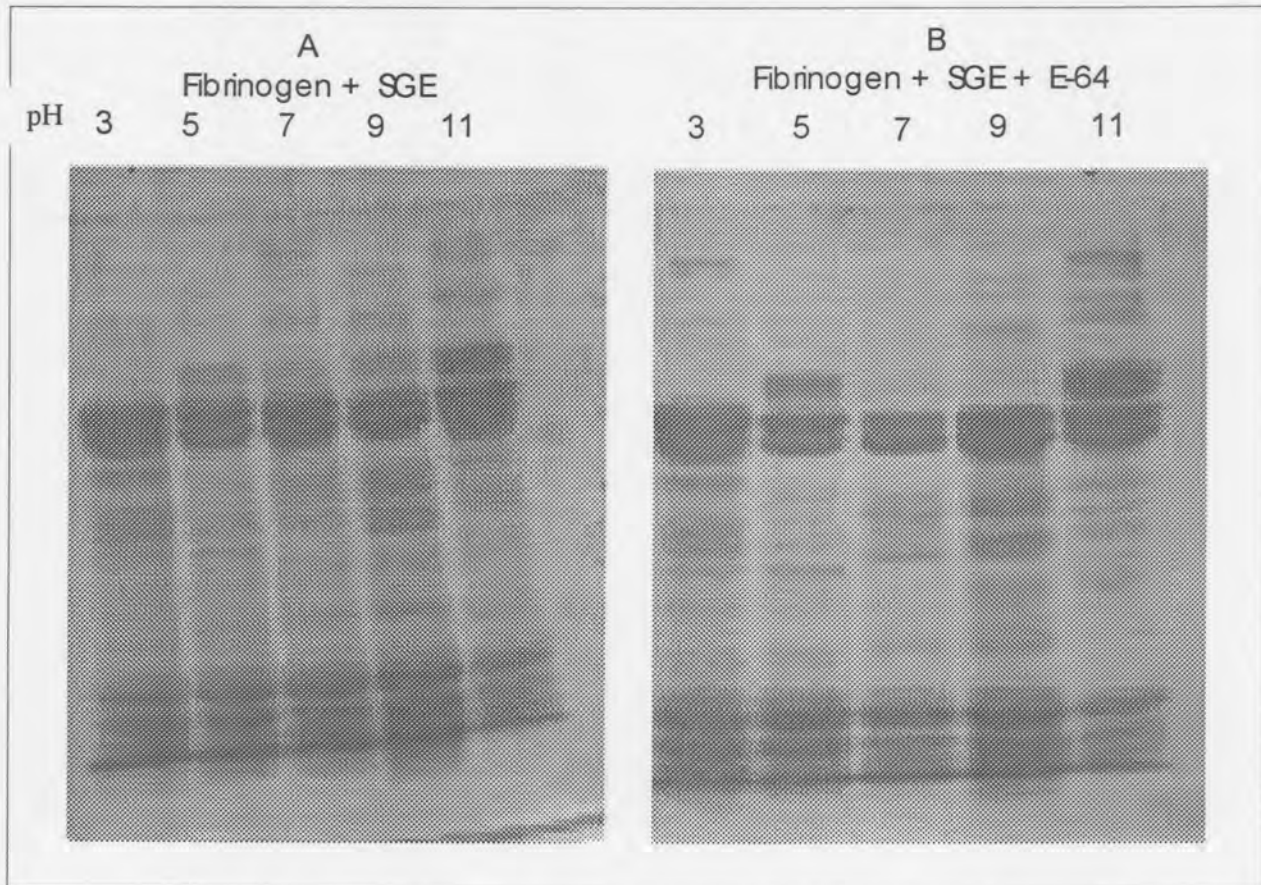
Fig 2.10: Effect of E-64 on the fibrinogenolytic activity at different pH values. (A) Fibrinogen was incubated with SGE (prepared in different pH buffers) for 4h at 37 °C (see 2.2.8 for rest of details). (B) SGE (prepared in different pH buffers) was pre-incubated with E-64 (100 mM) for 2h at 37° C. Thereafter, fibrinogen was added and incubated further for 4h at 37 °C. (see 2.2.9 for rest of details).

2.3.2.5 ZYMOGRAPHIC ANALYSIS OF THE FIBRINOGENOLYTIC ACTIVITY

To get an indication of the molecular mass of the proteins associated with the fibrinogenolytic activity the zymographic method was employed. The positive control (plasmin) showed activity when incubated at different pH conditions (3-11). The results obtained for pH 7 are shown in Fig 2.11 A. No fibrinogenolytic activity was observed in the presence of the SGE (Fig 2.11 E). No activity was observed even if electrophoresis was done at 4 °C.

Exclusion of SDS in the sample and electrophoresis buffer, with addition of 1 mM DTT in the incubation buffers presented little fibrinogenolytic activity at pH 3 (Fig 2.11 C) and a smear of activity at pH 9 (Fig 2.11 D). No activity was observed under these conditions at pH 5, 7 and 11. As expected, the plasminolytic activity was found at the top of the gel because of the poor resolution of proteins in the absence of SDS during electrophoresis (Fig 2.11 B).
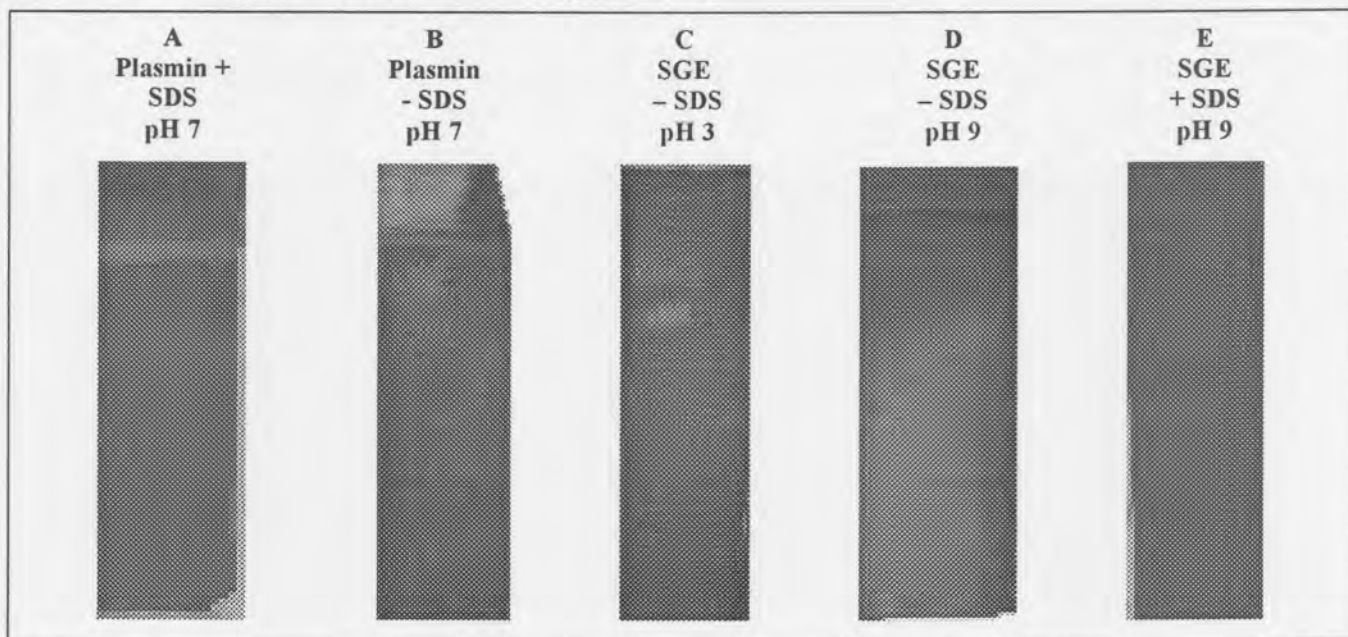
Fig 2.11: **Zymographic analysis of plasmin and SGE on the fibrinogen substrate gel**. Human fibrinogen was co-polymerized with the 12 % polyacrylamide gel and plasmin or SGE was loaded onto the gel. Different conditions were used as described in section 2.2.10. A) and B) gel strips indicates the plasmin in the presence and in the absence of SDS, respectively. C indicates the detected fibrinogenolytic activity at pH 3, while D) indicates the smear of activity at pH 9. The gel strip E) indicates the lost fibrinogenolytic activity in the presence of SDS.

2.3.3 CATION-EXCHANGE HPLC AND TESTING OF FRACTIONS FOR PROTEOLYTIC AND FIBRINOGENOLYTIC ACTIVITY

2.3.3.1 DETERMINATION OF PROTEOLYTIC ACTIVITY

SGE was subjected to cation-exchange HPLC and proteolytic activity in fractions assayed using azocasein as described in section 2.2.3. Three regions of general proteolytic activity were found when azocaseinolytic activity was determined at pH 6.1 of the eluting buffer. Region A was from 2-7 min, region B was from 10–18 min while region C was at 19–28 min (line with stars).
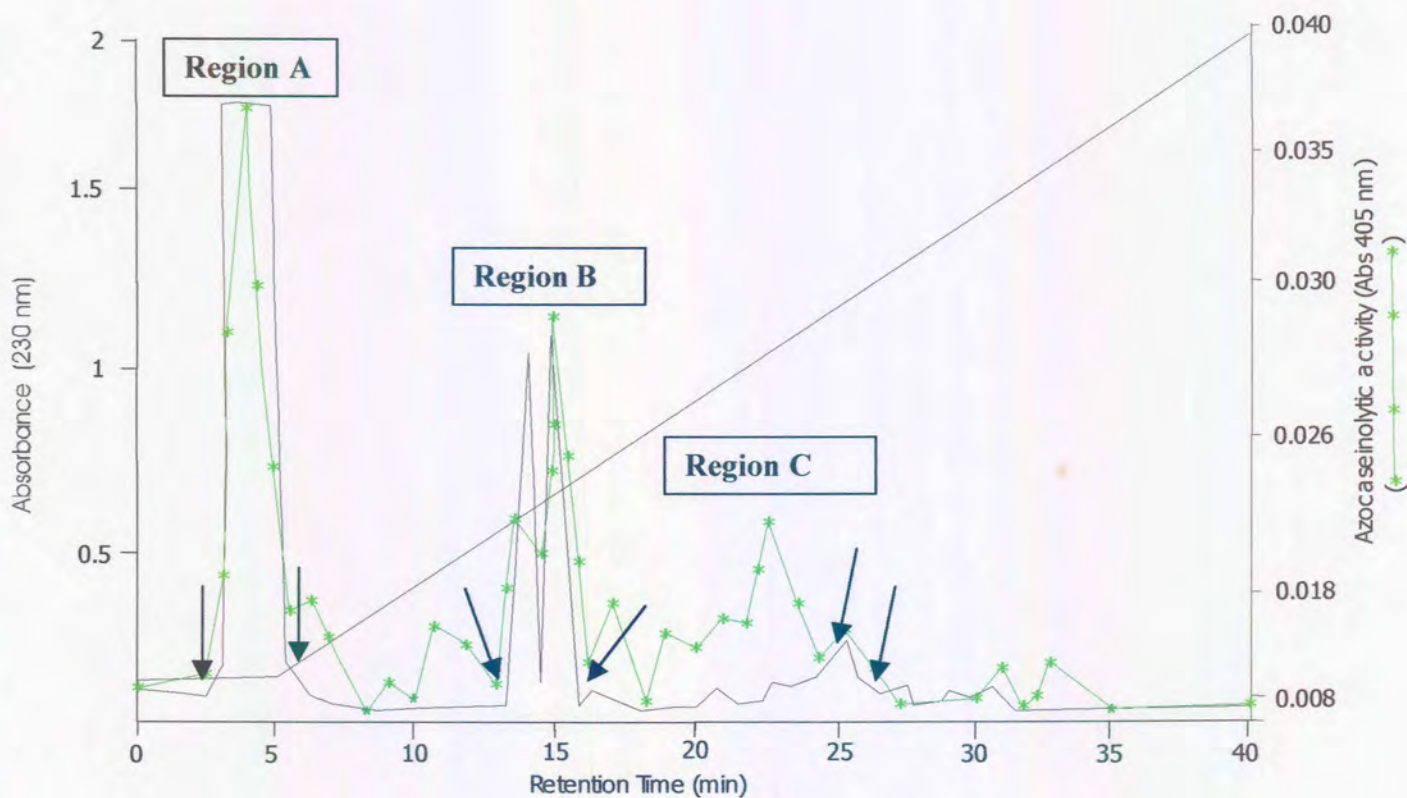
Fig 2.12: Cation exchange HPLC of the SGE. SGE (40 salivary glands prepared in 20 mM MES, pH 6.1) was applied to a cation exchange column, which had been equilibrated with the same buffer. A gradient elution was performed with buffer B, (20 mM MES, 1 M NaCl, pH 6.1) from 5-40 min. One-ml fractions were collected and 30 µl aliquots were assayed for azocaseinolytic activity and assays were done in triplicate. Arrows indicate regions with fibrinogenolytic activity (see 2.3.3.2) and line (with stars) indicates proteolytic activity (see 2.3.3.1).

2.3.3.2 DETERMINATION OF FIBRINOGENOLYTIC ACTIVITY

In an attempt to purify the fibrinogenolytic activity, SGE was subjected to cation exchange HPLC, and three regions of fibrinogenolytic activity were observed, indicated in Fig 2.12 as regions A, B and C (indicated with arrows). Region A represents fractions with retention times 2-6 min, region B represents fractions with retention times 13-15 min, while region C represents fractions with retention times 25-26 min.

2.3.4 CHARACTERIZATION OF PROTEOLYTIC AND FIBRINOGENOLYTIC ACTIVITY ASSOCIATED WITH CE-HPLC REGIONS A-C

2.3.4.1 CHARACTERIZATION OF THE PROTEOLYTIC ACTIVITY WITH SPECIFIC INHIBITORS

Fractions from the cation exchange HPLC containing proteolytic activity were treated with inhibitors at different pH's to determine the type of protease present in each region. Only the data for the fractions in a specific region exhibiting the highest proteolytic activity at various pH values are given. The results are shown in Table 2.3.

Assaying of the cation exchange fractions for proteolytic activity at pH 3 showed that the predominant proteolytic activity is associated with region A (fraction with retention time 1-2 min). Characterization of the activity in this fraction with the four different inhibitors showed that pepstatin A, the inhibitor of aspartic proteases, had the greatest inhibitory effect (56 %, $p < 0.001$), followed by E-64, the protease inhibitor of the cysteine proteases, which reduced the activity by 22 % ($p < 0.001$). This is indicative of the presence of aspartic and cysteine proteases in this region. Although the pepstatin A inhibitory effect was the highest at pH 3, the results also show that pepstatin A inhibition although minimal was always observed for fractions with retention time (1-2 min in region A at pH 5, 7, 9 and 11. Besides the

main aspartic proteinase activity associated with Region A, it can be seen from Table 2.3 that the other three classes of proteinases also contributed over the whole pH range tested to the total proteolytic activity, but to a lesser degree. However, at pH 11 the contribution by all four proteinase classes was equally significant.

At pH 5 and 7, proteolytic activity was also observed in region B. Serine protease activity was found to be the major activity present in region B (retention time 9-10 min at pH 5 and 10-11 min at pH 7) as the serine protease inhibitor DCI, led to an inhibition of 34 % ($p < 0.001$) at pH 5 and 36 % ($p < 0.001$) at pH 7. However, DCI showed the greatest effect at pH 9 (50 % inhibition, $p < 0.0001$). The 27 % inhibitory activity for DCI in region B at pH 11 was found to be statistically insignificant. In addition to the serine protease activity observed in region B, it was found that this region is also associated with metalloproteinase activity, as at both pH 7 and 9, the inhibitor, 1,10-phenanthroline, reduced proteolytic activity. The highest inhibition was observed at pH 9 (50 %, $p < 0.0001$). Characterization of proteolytic activity of region B at pH 11 with inhibitors showed that 1,10-phenanthroline had the highest inhibitory effect (32 % inhibition, $p < 0.001$) and thus the activity at this pH can be largely ascribed to the presence of the metalloprotease. Although aspartic and cysteine proteases were found to be mainly associated with region A, the results in Table 2.3 also indicate that pepstatin A inhibited proteolytic activity in region B at pH 5 (31 % inhibition, $p < 0.001$), but caused activation at pH 7 (45 % increase in activity, $p < 0.001$). Activation of activity was also observed in the presence of E-64 for region B at pH 11. In contrast, at pH 9 the cysteine proteinase caused 41 % inhibition (p, 0.001) of the proteolytic activity.

Activity in region C could be detected only when HPLC fractions were analyzed at pH 9. Characterization of this activity indicated that a metalloproteinase was mainly associated with this region (44 % inhibition by 1,10-phenantholine, $p < 0.001$).

TABLE 2.3: CHARACTERIZATION OF THE PROTEOLYTIC ACTIVITY PRESENT IN CATION-EXCHANGE HPLC FRACTIONS AT pH 3-11

pH of assay	Region (retention time)	Inhibitor	A_{405} * mean	Standard deviation	Relative activity (%)
pH 3	Region A (1-2 min)	Control	**0.097**	**0.001**	**100**
		1,10-phenanthroline	0.093	0.002	96
		E-64	0.076***	0.002	78
		DCI	0.082***	0.001	85
		Pepstatin A	0.043***	0.001	44
pH 5	Region A (1-2 min)	CONTROL	**0.098**	**0.001**	**100**
		1,10-phenanthroline	0.084***	0.001	86
		E-64	0.081	0.005	83
		DCI	0.081***	0.001	83
		Pepstatin A	0.065***	0.001	66
	Region B (9-10 min)	CONTROL	**0.067**	**0.001**	**100**
		1,10-phenanthroline	0.066	0.001	99
		E-64	0.054	0.004	81
		DCI	0.044***	0.001	66
		Pepstatin A	0.046***	0.002	69
pH 7	Region A (1-2 min)	CONTROL	**0.095**	**0.005**	**100**
		1,10-phenanthroline	0.098	0.003	103
		E-64	0.074*	0.001	78
		DCI	0.081*	0.001	85
		Pepstatin A	0.062**	0.001	65
	Region B (10-11 min)	CONTROL	**0.067**	**0.001**	**100**
		1,10-phenanthroline	0.053***	0.001	79
		E-64	0.063*	0.001	94
		DCI	0.043***	0.001	64
		Pepstatin A	0.097***	0.001	145
pH 9	Region A (1-2 min)	CONTROL	**0.090**	**0.001**	**100**
		1,10-phenanthroline	0.087*	0.001	97
		E-64	0.074***	0.001	82
		DCI	0.072***	0.001	80
		Pepstatin A	0.065***	0.001	72
	Region B (10-11min)	CONTROL	**0.096**	**0.001**	**100**
		1,10-phenanthroline	0.048***	0.001	50
		E-64	0.057***	0.002	59
		DCI	0.048***	0.001	50
		Pepstatin A	0.096	0.002	100
	Region C (18-19 min)	CONTROL	**0.084**	**0.001**	**100**
		1,10-phenanthroline	0.047***	0.002	56
		E-64	0.095	0.004	113
		DCI	0.077**	0.001	92
		Pepstatin A	0.082	0.002	98
pH 11	Region A (1-2 min)	CONTROL	**0.088**	**0.001**	**100**
		1,10-phenanthroline	0.059***	0.001	67
		E-64	0.067***	0.001	76
		DCI	0.058***	0.002	66
		Pepstatin A	0.063***	0	72
	Region B (10-11 min)	CONTROL	**0.066**	**0.001**	**100**
		1,10-phenanthroline	0.045***	0.001	68
		E-64	0.073**	0.001	111
		DCI	0.048	0.005	73
		Pepstatin A	0.060**	0.001	91

*A_{405} are means of triplicate values and activity is expressed relative to the control (no inhibitor). A_{405} values that are significantly different from the controls are shown as *$p < 0.05$, **$p < 0.01$, ***$p < 0.001$. Other differences are insignificant. See section 2.2.12 for details.

2.3.4.2 CHARACTERIZATION OF THE FIBRINOGENOLYTIC ACTIVITY WITH SPECIFIC INHIBITORS

As shown previously (Fig 2.6) no fibrinogen degradation was observed in the presence of SGE at pH 11. In Fig 2.13 the Aα-, Bβ- γ-chains of reduced fibrinogen at pH 11 serves as a control for this experiment. In the absence of inhibitors the fibrinogenolytic activity in region A was found mainly at pH 7 with slight activity at pH 9. In the presence all four inhibitors, the activity was completely inhibited (Fig 2.13) indicating the presence of all four proteinase classes in region A.
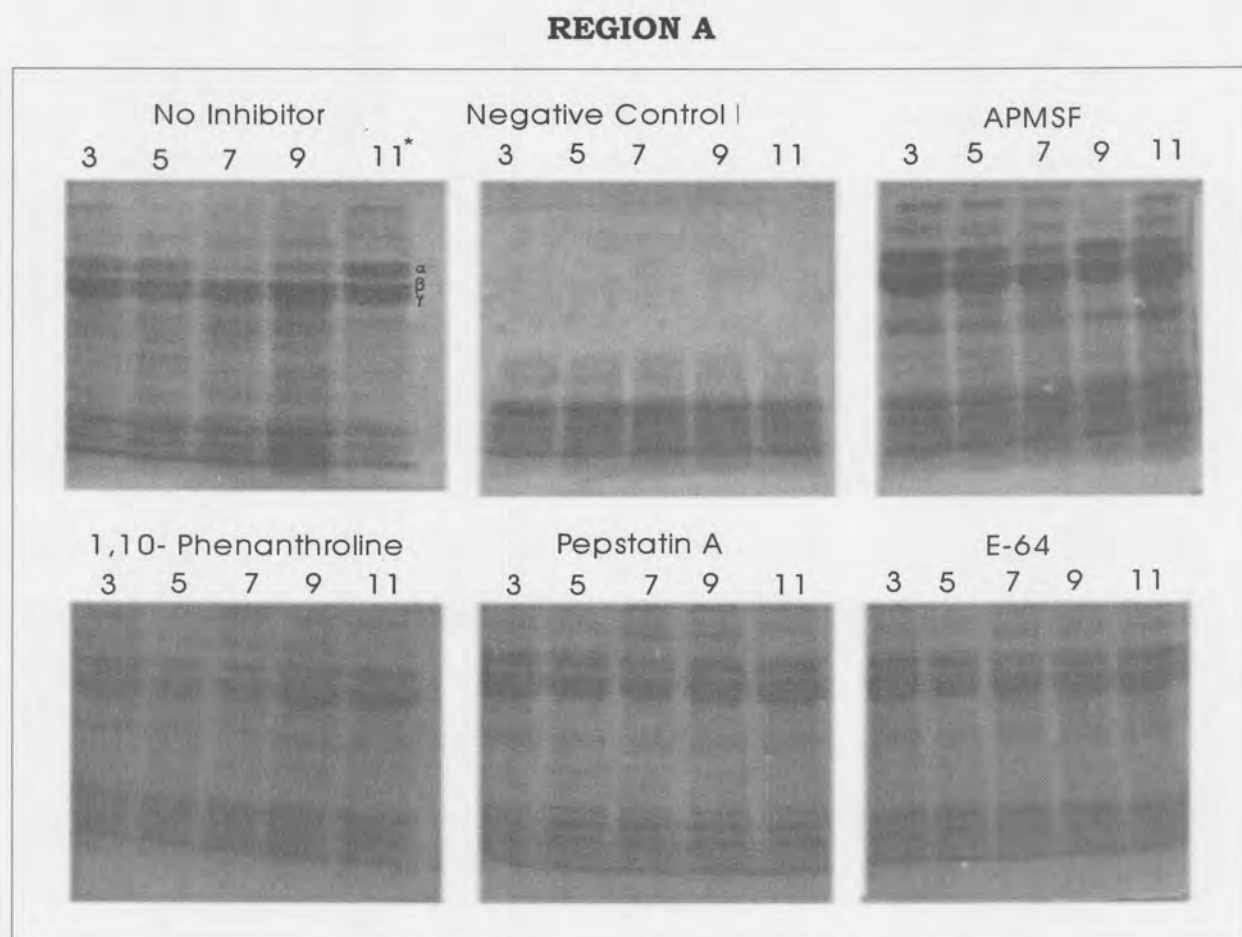
REGION A

Fig 2.13: Effect of pH and proteinase inhibitors on the fibrinogenolytic activity of region A after cation exchange HPLC. Fibrinogenolytic activity was studied at different pH values. The front peak (region A) of the cation exchange column was freeze-dried and reconstituted in different pH buffers and pre-incubated with inhibitors for 2 hours at 37 °C. The reaction was started by adding fibrinogen with a further 4 hours incubation at 37 °C. Activity was assayed by analyzing the degradation of fibrinogen on the Coomassie blue stained gels. Lanes 1-5 correspond to pH 3-11. * Lane 5 (pH 11) is the control for undegraded fibrinogen. Negative controls: In the absence of fibrinogen.

In region B (Fig 2.14), the activity was found at pH 7 and this activity was unaffected by the aspartic proteinase inhibitor, pepstatin A. The activity was completely inhibited by the serine, metallo- as well as the cysteine proteinase inhibitors, APMSF, 1,10-phenanthroline and E-64, respectively, attesting to the contribution of these proteases to the observed activity.

REGION B
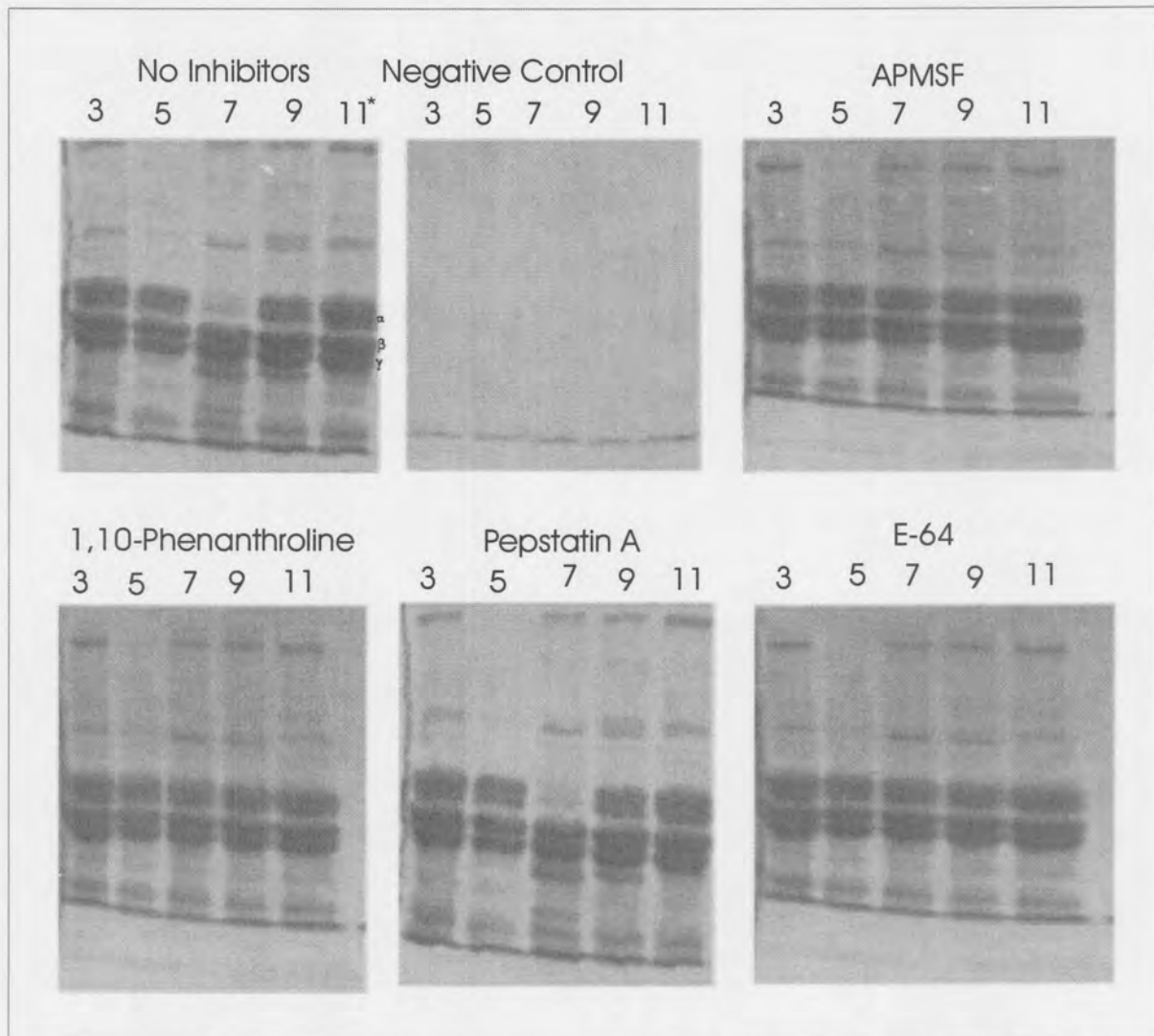
Fig 2.14: Effect of pH and proteinase inhibitors on the fibrinogenolytic activity of region B after cation exchange HPLC. Fibrinogenolytic activity was studied at different pH values. Region B of the cation exchange column was freeze-dried and reconstituted in different pH buffers and pre-incubated with inhibitors for 2 hours at 37 °C. The reaction was started by adding fibrinogen with a further 4 hours incubation at 37 °C. Activity was assayed by analyzing the degradation of fibrinogen on the Coomassie blue stained gels. Lanes 1-5 correspond to pH 3-11. * Lane 5 (pH 11) is the control for undegraded fibrinogen. Negative controls: In the absence of fibrinogen.

The activity of region C was found at pH 7 and 9 (Fig 2.15). This activity was unaffected by pepstatin A and E-64 at pH 9, but some inhibition was observed for E-64 at pH 7. Almost complete inhibition was observed by the metalloproteinase inhibitor, 1,10-phenanthroline (Fig 2.15). The serine proteinase inhibitor, APMSF, inhibited the activity at pH 7 with slight inhibition at pH 9. The metalloproteinase inhibitor, 1,10-phenanthroline, inhibited the activity at pH 7 and 9. The results are indicated in (Fig 2.15).

REGION C
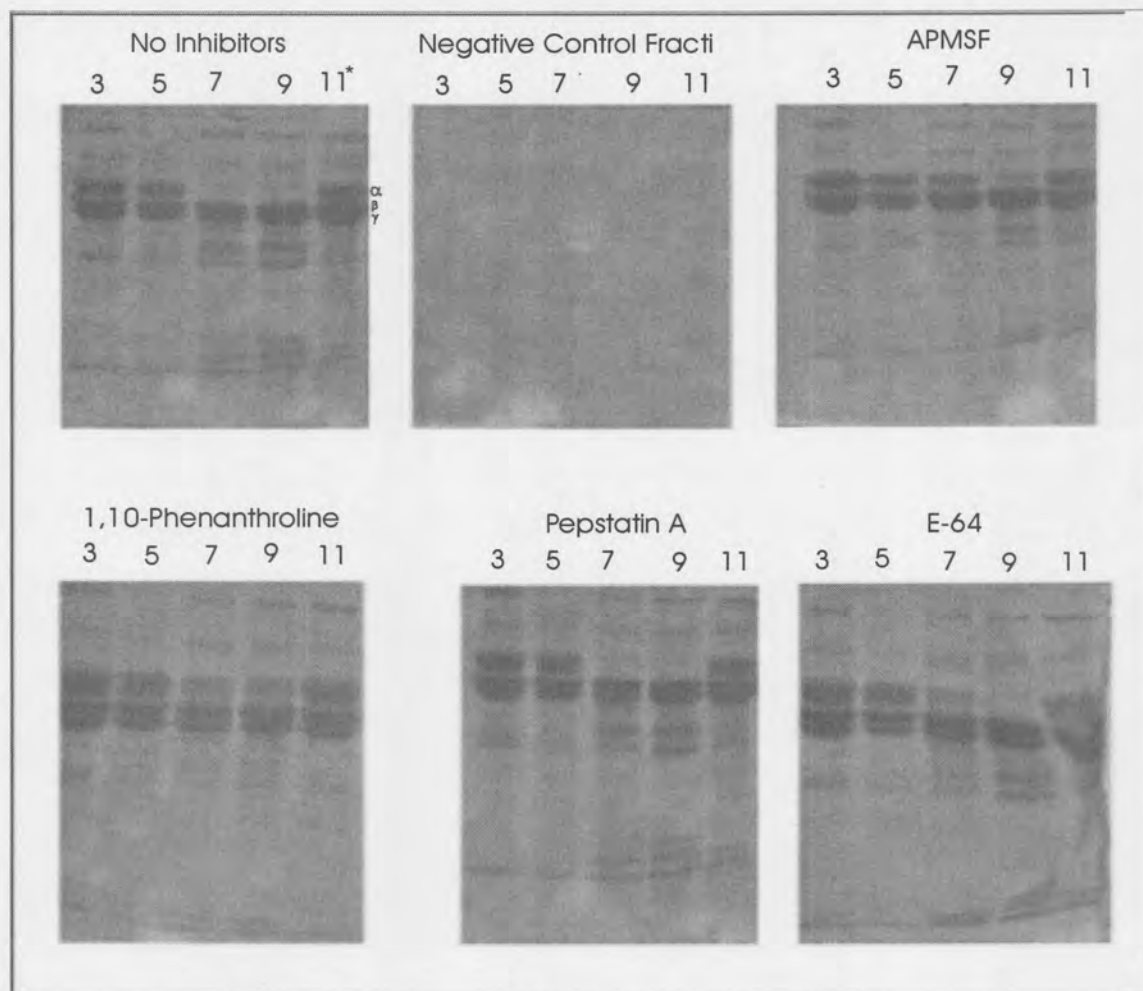
Fig 2.15: Effect of pH and proteinase inhibitors on the fibrinogenolytic activity of region C after cation exchange HPLC. Fibrinogenolytic activity was studied at different pH values. Region C of the cation exchange column was freeze-dried and reconstituted in different pH buffers and pre-incubated with inhibitors for 2 hours at 37 °C. The reaction was started by adding fibrinogen with a further 4 hours incubation at 37 °C. Activity was assayed by analyzing the degradation of fibrinogen on the Coomassie blue stained gels. Lanes 1-5 correspond to pH 3-11. * Lane 5 (pH 11) is the control for undegraded fibrinogen. Negative controls: In the absence of fibrinogen.

2.5 DISCUSSION

During the present study the general proteolytic and fibrinogenolytic activities were successfully identified in the crude extracts by means of assaying for azocasein degradation, SDS-PAGE analysis of the fibrinogen degradation products and fibrinogen substrate gels. This study indicated that the SGE contains a heterogeneous mixture of proteolytic activities. Using azocasein as substrate, proteolytic activity was found to be dependent on the number of salivary glands used in the assay. The proteolytic activity was found over a broad range pH of 3-11 with the highest activity at pH 9 followed by that at pH 7. Some tissue proteases have shown activity above pH 9. The activity of Lebetase, a fibrinogenolytic activity from the venom of *Vipera lepetina* snake venom was maintained at pH values of 6.0-10.8 (Siigur *et al.*, 1998), while, Piscivorase I and II, the fibrinolytic enzymes isolated from the venom of *Agkistrodon piscivorus piscivorus* were stable in the pH range of 7-10, (Hahn *et al.*, 1995). Hotez *et al.* (1990) observed the pH optimum of the two metalloproteases of infective *Ancyclostoma* hookworm to be between 9 and 10, with 87.5 % residual activity remaining at pH 11 relative to the optimum pH.

Characterization of the proteolytic activity in the salivary glands with specific inhibitors indicated that at pH 3, the low level of proteolytic activity may be due to the presence of an aspartic and a cysteine protease. However, the results were not found to be statistically different from the control. The low levels of proteolytic activity at the extreme pH conditions (relative activity of ~ 20 %) makes characterization studies difficult. At pH 5, the activity was mainly due to the aspartic protease as pepstatin A, the inhibitor diagnostic for aspartic protease activity, had the largest effect. At pH 7 and 9, the proteolytic activity was found to be due to the presence of a metallo- and serine protease as the inhibitors specific for these two enzyme classes had the most significant effect. Inhibition by DCI and 1,10-phenanthroline was however, more pronounced at pH 9. The low level of activity at pH 11 is most likely due to the presence of the metalloprotease activity as well as the aspartic proteinase as both 1,10 phenanthroline and pepstatin A resulted in some inhibition. The low level of inhibitory activity observed for pepstatin A at pH 9 and pH 11 was unexpected as aspartic proteases are normally

active at a low pH and the significance of such a broad pH range of activity is uncertain. Brown *et al.* (1995) investigated the proteolytic activities secreted by the adult hookworm, *Necator americanus*, and also found that pepstatin A inhibited proteolysis of both hemoglobin and fibrinogen in the pH 3.7- 7.0 range. In a more recent publication, Wang and Ng (2002) purified a proteinase designated pleureryn from the fresh fruiting bodies of the edible mushroom, *Pleurotus eryngii*. The activity of the protease was adversely affected by pepstatin A, indicating that it is an aspartic proteinase. It exhibited a pH optimum of 5 with substantial activity remaining at pH 4 and 12.

Geldhof *et al.* (2000) identified aspartic proteases released *in vitro* by the parasitic stages of the bovine nematode *Ostertagia ostertagi* that were active in an acidic pH range and had great activity against fibrinogen, albumin, hemoglobin and IgG. It is known that the blood meal digestion in tick midgut occurs at an acidic pH, involves lysosomes and the major activities have been shown to be due to aspartic and cysteine proteinases (Mendiola *et al.*, 1996). This combination of proteases is commonly found in the well-studied lysosomes of mammals, which is in accordance with the acidic nature of both catalytic mechanisms. Cysteine proteases have been found to play an important role in the pathogenesis of several parasitic protozoa and helminth infections (Coombs and Mottram, 1997; Mckerrow *et al.*, 1993; Rosenthal, 1995). For instance, cysteine proteinases are involved in the degradation of hemoglobin by the malaria parasite, *Plasmodium falciparum* (North *et al.*, 1990) and the growth and development of *Trypanosoma cruzi* (Franke De Cazzulo *et al.*, 1994; Harth *et al.*, 1993; Meirelles *et al.* 1992). While it cannot be taken for granted that observations on cysteine and aspartic proteinases in other parasites will be consistent in ticks, it is logical to assume that tick proteinases are also involved in many biological functions. Potential functions of tick proteolytic enzymes may include, involvement in molting processes (Richer *et al.*, 1993) and hydrolysis of both host immunoglobulins (Carmona *et al.*, 1993) and C3 component of complement (Reed *et al.*, 1989), as an escape mechanism from the host immune response.

Fibrinogen degradation by SGE was investigated by analyzing fibrinogen degradation products by SDS-PAGE. Under reducing conditions, fibrinogen

separated into Aα, Bβ, and λ chains. When fibrinogen was incubated with crude SGE, fibrinogenolytic activity in the extract digested the Aα-chain of fibrinogen within 2 hours of incubation. No degradation of the Bβ- nor the γ-chain was observed, even after 24 hours of incubation at 37 °C. The intensity of the Bβ-and γ-bands remained the same with time. If the Bβ- and γ-chains were hydrolyzed and the bands seen at these positions were due to degradation products corresponding to the same molecular masses as the undegraded Bβ and γ-chains, the intensity of the original bands would change. Degradation products can be seen in the lower region of the electrophoresis gels. Although fibrinogen was hydrolyzed, no fibrin clot was ever observed in the incubation tubes, indicating that the fibrinogenase is different from the typical blood clotting enzyme, thrombin. However this should be verified in future investigations using an appropriate assay. The Aα-chain which is more exposed was found to be the most susceptible to degradation by the fibrinogenolytic activity when compared to the Bβ- and γ- chains. Higher concentrations and longer incubation times are most probably required to degrade the Bβ and the γ-chains as well and these will only be degraded after the Aα-chain has been degraded.

The fibrinogenolytic activity in the SGE was inhibited by the chelating agent, EDTA. Reconstitution experiments with Mg^{2+} and Ca^{2+} showed that an increase in the original concentration of magnesium and calcium ions did restore activity to some extent, indicating that Ca^{2+} and Mg^{2+} ions play an important role in the fibrinogenolytic activity of the SGE. It will also be interesting to do reconstitution experiments with Zn^{2+} ions since the latter are required by metalloproteases for their activity (Markland, 1991 and 1998). The requirement for Ca^{2+} and Mg^{2+} ions could be indicative of the presence of metallo-proteases. However, as metal ions are often required for the activity of other classes of protease (Bond and Butler, 1987), the presence of metalloproteases should be confirmed by using an inhibitor that is specific for this class e.g. 1,10-phenanthroline. It is impossible from these results to distinguish between cysteine/serine-dependent proteolytic enzymes whose activities are stabilized or activated by metal ions and purely metal–dependent proteases present in the SGE.

Aα-specific-fibrinogenolytic activity in the SGE was observed over a wide pH range of 3-9. Slight fibrinogenolytic activity was observed at pH 5, whilst no activity occurred at pH 11. A fibrinogen control in which the protein was exposed to various pH conditions in the absence of SGE, showed that fibrinogen was stable under these conditions. This confirmed thus that the proteolytic activity observed was due to the SGE and not as a result of the assay conditions. Characterization of the fibrinogenolytic activity in the SGE showed that the metalloproteinase activity was active over the pH range of 3-9. In contrast, when azocasein was used as a substrate, the metalloproteinase activity in the SGE was found to be active at pH 7-11. These results seem to suggest that fibrinogen is the preferred substrate for the metalloproteinase. An aspartic proteinase activity degraded the fibrinogen at pH 3 and 5, while a cysteine proteinase-dependent fibrinogenolytic activity was present at pH 5 and no serine proteinase-dependent activity was found under all pH conditions.

Although activity could be detected using SDS-PAGE gels to analyze degradation products, no reproducible results with the zymographic method could be obtained. This method, in which proteins are first separated by SDS-PAGE, should give an indication of the molecular weights of the proteins responsible for the fibrinogenolytic activity. However, enzyme activity was absent in all zymograms. The difficulty appeared to reside in the inability to remove all of the SDS from the gels when washing with water-Triton X-100. However, this was found not to be the case as a clear band of the fibrinogenolytic activity was observed for the plasmin control. It appears that the fibrinogenolytic activity is sensitive to the Triton X-100 treatment, however many proteins retain their activity in 1-3 % Triton X-100 (Bollag et al., 1996). Distilled water-ethanol is also known to be very good in removing SDS from gels (Bollag et al., 1996). It is however a very effective protein denaturant and could therefore not be useful in solving this problem. In another attempt, SDS was omitted from both the sample and electrophoresis buffers and using these conditions, a smear of activity was observed for the plasmin control. Under these conditions, a smear of activity was also observed for the SGE at pH 3 and 9. It is not clear at this stage why no activity was observed at pH 5 and 7. It was also decided to include 1 mM DTT to the incubation buffer, as DTT is known to enhance

Barrett, 1994). The DTT also ensures that no disulphide bonds form during the experiment. For proteins containing both free –SH groups and disulphide bonds, disulphide interchange can occur and this may lead to irreversibility (Pace *et al.*, 1989). Addition of DTT was found to have no effect.

From the behavior of the activity during CE-HPLC, it was found that there are three regions of proteolytic activity, one associated with the acidic pI range and two are associated with the basic pI range (indicated in Fig 2.4 as regions A, B and C respectively). The predominant proteolytic activity is associated with region A and is ascribed to the aspartic proteases, since pepstatin A was the best inhibitor in this region. The aspartic protease activity was present in this region at all the pH values tested. The aspartic proteinase activity was most prominent at pH 3, but exhibited some activity at pH 5-11. As mentioned before similar results were obtained for the SGE. It was suprising to find that at pH 11, region A was also associated with other proteolytic activities. This can be ascribed to the presence of metallo-, serine- and cysteine proteases that did not completely adsorb to the cation-exchange column and eluted together with other acidic proteins in the front peak. Region B was found to be associated with serine, cysteine and metalloprotease activity. The serine proteinase activity in this region was active in the pH 5-9 range, while the metalloproteinase activity was active in the 7-11 range. The cysteine proteinase activity was found to be active only at pH 9. The presence of cysteine proteinase activity in regions A and B was unexpected, since no cysteine proteinase activity was detected in the crude extract. The presence of endogenous cysteine proteinase inhibitors in the crude extract could possibly explain this result. The proteolytic activity in region C was detected at pH 9 and it was found to be associated mainly with the metalloprotease activity.

Assaying of the CE-HPLC fractions for fibrinogenolytic activity also resulted in three regions of activity and in all cases the activity was found to be more active at pH 7-9. Attempts to characterize the fibrinogenolytic activity associated with the three regions using the protease inhibitors indicated that the activity under given conditions was inhibited by more than one protease class. Region A was found to be associated with fibrinogenolytic activity at pH 7 and to a lesser extent at pH 9. All four inhibitors had an effect on the fibrinogenolytic activity of region A. The wide

range of proteolytic activity associated with region A could be due to unadsorbed proteins (basic pI) considering that the binding capacity of the column could have been exceeded. In region B, the fibrinogenolytic activity was found at pH 7. There was no effect with pepstatin A, the protease inhibitor of the aspartic proteases and the fibrinogenolytic activity observed was found to be due to the combined action of the serine- cysteine- and metalloproteases. In the crude extract no serine proteinase degradation of fibrinogen was detected and the serine proteinase activity observed in region B is most likely as a result of the separation of an endogenous serine proteinase inhibitor from the proteinase by the chromatography step. In region C, the fibrinogenolytic activity was observed at pH 7 and 9 and was not affected by pepstatin A. The fibrinogenolytic activity in region C was found to be due to metalloproteinase activity that was active at pH 7 and 9 as well as cysteine and serine proteinases that were mostly active at pH 7. Contrary to the results obtained for the crude extract in which fibrinogenolytic activity was observed over the whole pH 3-9 range, all three regions exhibited fibrinogenolytic activity only at pH 7 and 9. The activity at pH 3 and 5 in the crude extracts could possibly be as a result of non-specific proteolysis by proteinases that are active in an acidic environment. These proteinases may be degraded by other proteolytic enzymes during the purification procedure and lead to loss of fibrinogenolytic activity in the pH 3-5 range. In some instances it was found that DCI, pepstatin A and E-64 led to enhancement of proteolytic activity. These inhibitors may be inactivating enzymes that degrade other proteolytic enzymes and thus lead to enhancement of the activity.

Findings reported in this chapter have shown that all four proteinase classes are present in the SGE. All four classes are capable of cleaving fibrinogen, but the results suggest that the metalloproteinase activity may be the main fibrinogenolytic enzyme. Addition of the metalloproteinase inhibitor, 1,10-phenanthroline, had the largest effect on both the fibrinogenolytic activity in the SGE as well as on CE-HPLC fractions. The metalloproteinase activity was found to be active over a broad pH range of 3-9 and most active at pH 7 and 9. Furthermore, these findings laid the foundation for the purification and characterization of the fibrinogenolytic and other individual proteases present in the salivary glands. The isolation of pure proteases

should ultimately allow us to characterize these molecules further and to determine the biological functions of these molecules.

The next chapter describes the HPLC purification of the fibrinogenolytic activity.

PARTIAL ISOLATION AND CHARACTERIZATION OF THE FIBRINOGENOLYTIC ACTIVITY FROM THE SALIVARY GLANDS OF THE TICK *O. SAVIGNYI*

3.1 INTRODUCTION

The explosion of work and interest in molecular biology in recent years has made protein purification something of a lost art, especially among younger biochemists and molecular biologists. At the same time, many of the more interesting biological problems have reached a stage that requires work with purified proteins and enzymes. Purifying an enzyme is rewarding all the way, from first starting to free it from the mob of proteins in a broken cell to having it finally in splendid isolation. It matters that, upon removing the enzyme from its snug cellular niche, one cares about many inclemencies: high dilution in unfriendly solvents, contact with glass surfaces and harsh temperatures, and exposure to metals, oxygen, etc (Srere and Mathews, 1990).

Why isolate proteins? To gain insight, the knowledge of which could then be used practically as in the design of medicines, diagnostics, pesticides, or industrial processes (Dennison 1999). The ultimate role in purifying proteins is to fully characterize them and determine how they fulfill their unique functional roles *in vivo*. The goal of conventional enzyme purification is therefore to obtain a single protein responsible for catalyzing a single chemical reaction (Srere and Mathews, 1990).

In biochemistry there is no means for directly quantitating the purity of a protein sample. Demonstration of purity of a protein preparation always involves an assessment of the level of particular types of impurities. It is this concept that is meant whenever the term purity is used. In order to assess the purity of a sample,

one must first identify the type of impurity that is to be measured, then identify a characteristic property, which can distinguish the protein of interest from the putative contaminants. Purity is then the demonstration that the sample is free of detectable quantities of contaminant. Methods that can be used to determine the level of impurities include; chromatography, electrophoresis, sedimentation, etc (Rhodes and Laue, 1990).

The most widely used method of evaluating the size of a protein molecule is SDS-PAGE. The method is simple, inexpensive, rapid, and reasonably accurate for a very wide range of proteins. For these reasons it is the method of choice for most protein systems, and almost always included in characterization studies. SDS-PAGE is the most widely used method for determining apparent molecular weights of denatured proteins, but electrophoretic methods for obtaining size, shape, and molecular weight information are not limited to just this approach. Despite its popularity, it is not necessary to include SDS in the gel formulation; native gel electrophoresis of protein samples may be carried out under almost any buffer condition required for determination of molecular mass. In addition, the sensitivity of the current staining procedures allows these approaches to be applied to very small amounts of proteins (Rhodes and Laue, 1990).

3.1.1 PROTEIN PURIFICATION BY CHROMATOGRAPHY

Chromatographic resolution of biological macromolecules in all cases, except size exclusion chromatography, is a surface mediated process, i.e. there is differential adsorption of solutes at the surface of the chromatographic packing material. Because biological macromolecules differ physically in their size and shape, charge, hydrophobicity, and arrangement of functional groups within their three dimensional structure, it is not suprising that the major chromatographic modes by which bio-polymers can be fractionated are by ion exchange chromatography (charge distribution), size exclusion (molecular mass), hydrophobic interaction chromatography (surface hydrophobicity), reversed phase chromatography (general hydrophobicity), immobilized metal affinity chromatography (surface-available

histidines), and bio-affinity chromatography (distribution of specific amino acids at the surface of proteins (Chicz and Regnier, 1990).

Proteins contain both charged and neutral external amino acids which are solvent accessible and can interact with chromatographic supports. Ion exchange separations are performed on either anion exchange chromatography or cation exchange chromatography sorbents depending on the pI and surface charge distribution of the protein. Protein separations based on surface hydrophobicity are accomplished on either hydrophobic interaction or reversed phase sorbents depending on the method of elution. When proteins are eluted with organic solvents, the technique is termed reversed-phase chromatography while separations achieved with ascending salt gradients have been designated hydrophobic interaction chromatography (Chicz and Regnier, 1990).

The popularity of the reversed phase chromatography is attributed to a number of factors which includes: the excellent resolution achieved for closely related as well as structurally disparate compounds; the ease with which chromatographic selectivity can be manipulated through the addition of mobile modifiers; the generally high recoveries, even at ultramicroanalytical levels; and the excellent reproducibility that can be achieved (Aquilar and Hearn, 1996). For these reasons reversed phase chromatography has become a common method for the analysis and purification of peptides and proteins. The loss of enzymatic activity after reversed phase chromatography has been attributed to the high density of n-alkyl chains on the column packing material surface and the harsh mobile-phase conditions, the use of the organic modifiers for elution and the low pH (Aquilar and Hearn, 1996).

This loss of enzymatic activity after reversed phase chromatography was solved by using hydrophobic interaction chromatography. Like reversed phase chromatography, this method relies on hydrophobic interactions with the stationary phase to effect separation (Mant and Hoges, 1991) but the activity of the protein is maintained due to the much lower hydrophobic ligands bound to the stationary phase and because the mobile phase is an aqueous buffer of high salt concentrations and the pH is neutral (Wu and Karger, 1996). These are native, non-

denaturing conditions for most proteins and there is therefore no loss of biological activity (Mant and Hodges, 1991).

3.1.2 PURIFICATION OF FIBRINOGENASES

The fibrinogenolytic enzymes isolated from various sources fall into two groups, based on their molecular weights. They have molecular weights of approximately 25 000 and 60 000 respectively. The most characteristic aspect of the amino acid composition of these proteases is the very high level of Asx and Glx residues (Hahn *et al.*, 1995). Their potential use as thrombolytic agents is being investigated in the medical fields (Hahn *et al.*, 1995).

These enzymes have been isolated by combinations of ion exchange, size exclusion, reversed phase and fibrinogen-affinity columns. The activity in these studies have been determined by analyzing fibrinogen degradation products with SDS-PAGE, general protease assays using substrates like casein, azocasein, dimethylcasein, tosyl-arginine methyl ester (TAME), etc. Some of the enzymes have been cloned and expressed recombinantly. Ancrod (EC 3.4.21.28) from *Agkistrodon Rhodostoma* venom (Esnouf and Tunnah, 1967) and batroxobin (EC 3.4.21.29) from *Bothrops atrox* venom (Stocker and Barlow, 1976) are serine proteinases which have fibrinogenolytic action. Both have been used in the treatment of deep vein thrombosis and peripheral vascular occlusive diseases (Stocker and Barlow, 1976 and Sharp *et al.*, 1968). They are also administered prophylactically, to reduce the incidence of postoperative deep vein thrombosis and the recurrence of thrombosis after vascular surgery (Braud *et al.*, 2000). The widespread clinical utility of ancrod has been limited by immunological reactions in patients as well as the availability and thus cost. While immunoreactivity could be due to trace contaminants in the commercial preparations, the heavy glycosylation (29 % by weight; 96 moles of non-nitrogenous sugars, 60 moles of hexosamine, 18 moles of sialic acid) could contribute to the reactivity (Stocker and Barlow, 1976).

Characterization of other fibrinogenases from other organisms might identify novel antithrombotic agents. To date many fibrinogenases have been purified from various snake venoms and a diverse range of hematophagous organisms.

This chapter describes the partial isolation and characterization of the fibrinogenolytic activity from the salivary glands of *O. savignyi.*

3.2 MATERIALS AND METHODS

3.2.1 MATERIALS

All materials were of analytical grade and deionized water was used in all experiments. Tris, NaCl, HCl, NaOH, sodium azide, DTT, methanol, acetic acid, ammonium sulphate, ammonium persulphate, TEMED, bromophenol blue, 3-(cyclohexyl amino)-propane sulphonic acid (CAPS) were supplied by Sigma, U.S.A and Polyvinylidene difluoride (PVDF) membranes were purchased from MERCK, Darmstadt, Germany. Acrylamide, N, N'-methylene bisacrylamide, glycerol, 2β-mercaptoethanol and calcium chloride were obtained from BDH chemicals Ltd., Poole, England. The acetonitrile-R Chromasolv and tricine were purchased from Fluka-Riedel-deHaen, Buchs, Germany. Low molecular peptide standards were obtained from Amersham Pharmacia Biotech, Little Chalfont, England. Trifluroacetic acid (TFA) was purchased from Sigma-Aldrich, U.S.A. SDS and Triton X-100 were from Boehringer Mannheim GmbH, Germany. HiTrap affinity columns (1 ml) were purchased from Amersham Pharmacia Biotech. Purified human fibrinogen was purchased from Sigma. Coomassie Brilliant Blue R-250 was from Bio-Rad, Richmond, U.S.A. Molecular mass marker proteins were obtained from Pharmacia. The Slide-A-Lyzer dialysis cassettes were purchased from Pierce, U.S.A. *O. savignyi* ticks were from Upington, in the Northwestern parts of South Africa. All the HPLC work was performed with a Beckman 110B solvent delivery system, 406 analog interface and 166 detector module and data were collected using the System Gold™ Software (Beckman, Palo Alto, CA, U.S.A).

3.2.2 PREPARATION OF THE CRUDE SALIVARY GLAND EXTRACT

The SGE was prepared as described in section 2.2.2.

3.2.3 DETERMINATION OF ENZYME ACTIVITY

Fibrinogenolytic activity was assayed by SDS-PAGE as described in section 2.2.6.

3.2.4 CATION EXCHANGE HPLC (CE-HPLC)

All samples were filtered through a 0.22 μm membrane (Millex GV4, Millipore Corporation, U.S.A) prior to injection. For initial fractionation of the salivary gland extracts 40 salivary glands were prepared in Buffer A, (20 mM MES, pH 6.1) as described in section 2.2.2. The extracts were applied to the cation exchange column (TSK-GEL SP-5PW, 7.5 mm ID x 7.5 cm Tosohaas), pre-equilibrated with Buffer A. Proteins were eluted with a linear gradient (0-1 M NaCl) with Buffer B (20 mM MES, 1 M NaCl pH 6.1) over 35 minutes. The flow rate was 1 ml/min and the absorbance was monitored at 230 nm. The proteins were eluted as set out in Table 3.1. One-milliliter fractions were collected and assayed for fibrinogenolytic activity. Excess NaCl was removed by dialyzing the fractions for one hour against 1.5 mM $CaCl_2$ and 1.5 mM $MgCl_2$ using Pierce dialysis cassettes prior to their application to the anion exchange column.

TABLE 3.1: CONDITIONS FOR CE-HPLC

Time(min)	Flow rate (ml/min)	% A	% B	Duration (min)
0	1	100	0	5
5	1	0	100	35
40	1	100	0	0.5
40.5	1	100	0	9.5
50	END			

Buffer A: 20 mM MES pH 6.1

Buffer B: 20 mm MES, 1 M NaCl, pH 6.1

3.2.5 ANION EXCHANGE HPLC (AE-HPLC)

The fractions containing the fibrinogenolytic activity from the front un-adsorbed peak from the cation exchange column were pooled and applied to an anion exchange column (TSK gel DEAE 5PW, 7.5 mm x 7.5 cm, Tosohaas), pre-equilibrated with Buffer A (20 mM Tris-HCl, 2 mM $MgCl_2$, 2 mM $CaCl_2$, pH 7.6). The proteins were eluted with a linear gradient (0-1 M NaCl) with Buffer B (20 mM Tris-HCl, 2 mM $MgCl_2$, 2 mM $CaCl_2$, 1 M NaCl, pH 7.6) over 35 min. The flow rate was 1 ml/min and the absorbance was monitored at 230 nm. One-ml fractions were collected and assayed for fibrinogenolytic activity. Before each sample injection a blank run was performed to elute any adsorbed proteins. The fractions containing the highest fibrinogenolytic activity were dialyzed and lyophilized. The dried fractions were dissolved in 1 ml ammonium sulphate buffer [1.7 M $(NH_4)_2SO_4$;20 mM Tris-HCl, pH 7.6] for hydrophobic interaction chromatography.

TABLE 3.2: CONDITIONS FOR AE-HPLC

Time (min)	Flow rate (ml/min)	% A	% B	Duration (min)
0	1	100	0	5
5	1	0	100	35
40	1	100	0	0.5
40.5	1	100	0	9.5
50	END			

Buffer A: 20 mM Tris-HCl, 2 mM $CaCl_2$, 2 mM $MgCl_2$, pH 7.6

Buffer B: 20 mm Tris-HCL, 2 mM $CaCl_2$, 2 mM $MgCl_2$, 1 M NaCl, pH 7.6

3.2.6 HYDROPHOBIC INTERACTION CHROMATOGRAPHY (HIC)

Fractions exhibiting fibrinogenolytic activity were adjusted to 1.7 M ammonium chloride and applied to the HIC column. Hydrophobic interaction chromatography was performed using a TSK-Phenyl-5-PW (75 mm x 7.5 mm) column from Bio-Rad laboratories, Richmond, CA, U.S.A. Buffer A was a 1.7 M $(NH_4)_2SO_4$; 20 mM Tris-HCl (pH 7.6) solution and Buffer B a 20 mM Tris-HCl (pH 7.6) solution. The column

was pre-equilibrated with Buffer A and the proteins eluted as set out in Table 3.3. The flow rate was 1 ml/min and the absorbance was measured at 230 nm.

TABLE 3.3: CONDITIONS FOR HIC

Time (min)	Flow rate (ml/min)	% Buffer A	% Buffer B	Duration (min)
0	1	100	0	5
5	1	0	100	35
40	1	0	100	20
60	END			

Buffer A: 1.7M $(NH_4)_2SO_4$;20 mM Tris-HCl (pH 7.6)

Buffer B: 20 mM Tris-HCl (pH 7.6)

3.2.7 SIZE EXCLUSION HPLC (SE-HPLC)

Active fractions collected from the HIC column were desalted by loading them onto the size exclusion column. A G3000SW$_{XL}$ size exclusion column (TSK gel G3000 SWxl, Tosohaas) which had been equilibrated with Buffer A (20 mM Tris, 15 mM NaCl, 2 mM $CaCl_2$, 2 mM $MgCl_2$, pH 7.6). Samples were applied to the column in 1 ml quantities and eluted isocratically at a flow rate of 1 ml/min. The eluate was monitored at 230 nm. Fractions (respective peaks) were collected and assayed for fibrinogenolytic activity.

TABLE 3.4: CONDITIONS FOR SE-HPLC

Time (min)	Flow rate (ml/min)	% A	% B	Duration (min)
0	1	100	0	20
20	END			

Buffer A: 20 mM Tris-HCl, 15 mM NaCl, 2 mM $CaCl_2$, 2 mM $MgCl_2$, pH 7.6

3.2.8 FIBRINOGEN AFFINITY CHROMATOGRAPHY

A fibrinogen affinity column was prepared by coupling purified human fibrinogen to a HiTrap NHS-activated Sepharose™ high performance column according to the manufactures instructions and equilibrated with Buffer A (20 mM Tris, 15 mM NaCl,

2 mM MgCl₂, 2 mM CaCl₂ pH 7.6). The fractions with fibrinogenolytic activity from the SE-HPLC were pooled and loaded onto the fibrinogen affinity column. The column was washed with Buffer A (2 x 5 ml) followed by elution of the adsorbed peak by 6 ml of Buffer B (0.1 M Na acetate pH 4, containing 1 M NaCl). Samples were collected at 30-sec intervals. Fibrinogenolytic activity was assayed as described in section 2.2.6.

3.2.9 REVERSED PHASE HPLC (RP-HPLC)

Reversed phase was performed using a Jupiter 5 µ C5 300A (250 mm x 4.60 mm) column from Phenomenex. Eluent A was a 0.1 % TFA; 0.1 % CH₃CN solution and Eluent B a 0.1 % TFA; 60 % CH₃CN solution. The column was pre-equilibrated with Eluent A. The fibrinogenolytic activity obtained from either the size exclusion or the fibrinogen affinity column was applied to a C5-reversed phase column. Proteins were eluted with a linear gradient of acetonitrile, which was applied over 60 min as set out in Table 3.5. A flow rate of 1 ml/min was maintained and the absorbance was measured at 230 nm. The collected fractions were dried in a vacuum concentrator (Bachoffer). The dried fractions were either resuspended in buffer (20 mM Tris-HCl, 15 mM NaCl, 2 mM MgCl₂ 2 mM CaCl₂, pH 7.6) for fibrinogenolytic activity assays or dissolved in the SDS-PAGE sample buffer for electrophoretic analysis.

TABLE 3.5: CONDITIONS FOR RP- HPLC

Time (min)	Flow rate (ml/min)	% Buffer A	% Buffer B	Duration (min)
0	1	100	0	5
5	1	0	100	60
65	1	0	100	5
70	END			

Buffer A: 0.1 % TFA; 0.1% CH₃CN

Buffer B: 0.1 % TFA; 60 % CH₃CN

3.2.10 GLYCINE SDS-PAGE ANALYSIS

Electrophoresis was performed according to the method described by Laemmli (1970). A stock solution containing 30 % (w/v) acrylamide and 0.8 % (w/v) N, N'-methylene bisacrylamide were used to prepare a 12 % separating gel (pH 8.8) and a 4 % stacking gel (pH 6.8). The gel mixtures were degassed for 30 min at room temperature and polymerized with 1 % ammonium persulphate and 0,1 % TEMED. The electrophoresis buffer consisted of 0.02 M Tris-HCl, 0.1 M glycine and 0.06 % SDS. A constant voltage of 60 V was maintained until samples concentrated on the stacking gel. The voltage was then adjusted to 100 V and kept constant for the remaining separation period. The gels were either stained with Coomassie Blue [0.1 % (w/v) Coomassie Brilliant Blue R-250 in 40 % methanol (v/v); 10 % acetic acid (v/v)] followed by destaining with a large excess of 40 % methanol (v/v) 10 % acetic acid (v/v) or electro-blotted onto PVDF membranes (see section 3.2.12).

TABLE 3.6: STACKING AND SEPARATING GEL COMPOSITION FOR GLYCINE SDS-PAGE

Reagent	Stacking gel (4 %)	Separating gel (12 %)
Acrylamide/bisacrylamide (ml)	1.3	4
Buffer (ml)	2.5 ml Tris-HCl, pH 6.8	2.5 ml Tris-HCl, pH 8.8
10 % SDS (μl)	100	100
Ammonium persulphate (μl)	50	50
TEMED (μl)	5	5
H$_2$O (ml)	6.1	3.4

3.2.11 TRICINE SDS-PAGE

In order to identify the molecular mass of proteins in the range of 1 to 100 kDa, Tricine SDS-PAGE was performed according to the method of Schagger and von Jagow (1987). An acrylamide, N, N'-methylene bisacrylamide stock solution (49,5 % T, 3 % C) as well as a gel buffer stock solution (3 M Tris, 0.3 % SDS, pH 8.45) were used to prepare a 4 % stacking gel and a 16 % separating gel (pH 8.45). The gel mixtures were degassed and the solution was polymerized with the addition of 0.05 % ammonium persulphate and 0.1 % TEMED. For the anode electrophoresis buffer

0.2 M Tris (pH 8.9) was used and for the cathode electrophoresis buffer 0.1 M Tris, 0.1 M tricine, 0.1 % SDS (pH 8.25) was used. A constant voltage of 30 V was maintained until the samples concentrated in the stacking gel. Thereafter, the voltage was adjusted to 80 V and kept constant for the remaining separation period. Gels were stained and destained as described in section 3.2.10.

TABLE 3.7: STACKING AND SEPARATING GEL COMPOSITION FOR THE TRICINE SDS-PAGE

Reagent	Stacking gel (4 %)	Separating gel (16 %)
Acrylamide/bisacrylamide solution (ml)	0.5	3.34
Buffer, 3M Tris-HCl pH 8.45 (ml)	1.55	3.34
10 % SDS (μl)	10	10
Ammonium persulphate (μl)	50	50
TEMED (μl)	5	5
H_2O (ml)	4.2	3.32

3.2.12 ELECTROBLOTTING

After separation of proteins, the gel was equilibrated in 1 mM CAPS buffer (pH 9), for 15 min. A PVDF membrane was equilibrated in methanol and then washed with CAPS buffer. The separated proteins from the gel were transblotted to the membrane with a Bio-Rad Transblot-BP semi-dry transfer cell (power supply ESPS 2000/300 from Pharmacia Biotechnology) at a constant voltage (20 V) for 60 min. Blotted membranes were stained with a solution of 0.25 % Coomassie blue, 40 % methanol, 10 % acetic acid and 50 % double distilled water for 10 min. Destaining was achieved using a solution of 40 % methanol containing 10 % acetic acid and 50 % double distilled water.

3.3 RESULTS

3.3.1 PURIFICATION USING SE-, AE-, AND RP-HPLC.

For initial fractionation of the SGE, extracts from 40 salivary glands were prepared and applied to the size exclusion column. The chromatogram in Fig 3.1 reveals that the majority of proteins in the crude extract eluted between 7 and 12 min. Testing of the fractions for fibrinogenolytic activity showed that the activity was present in fractions with retention times 10-12 min.
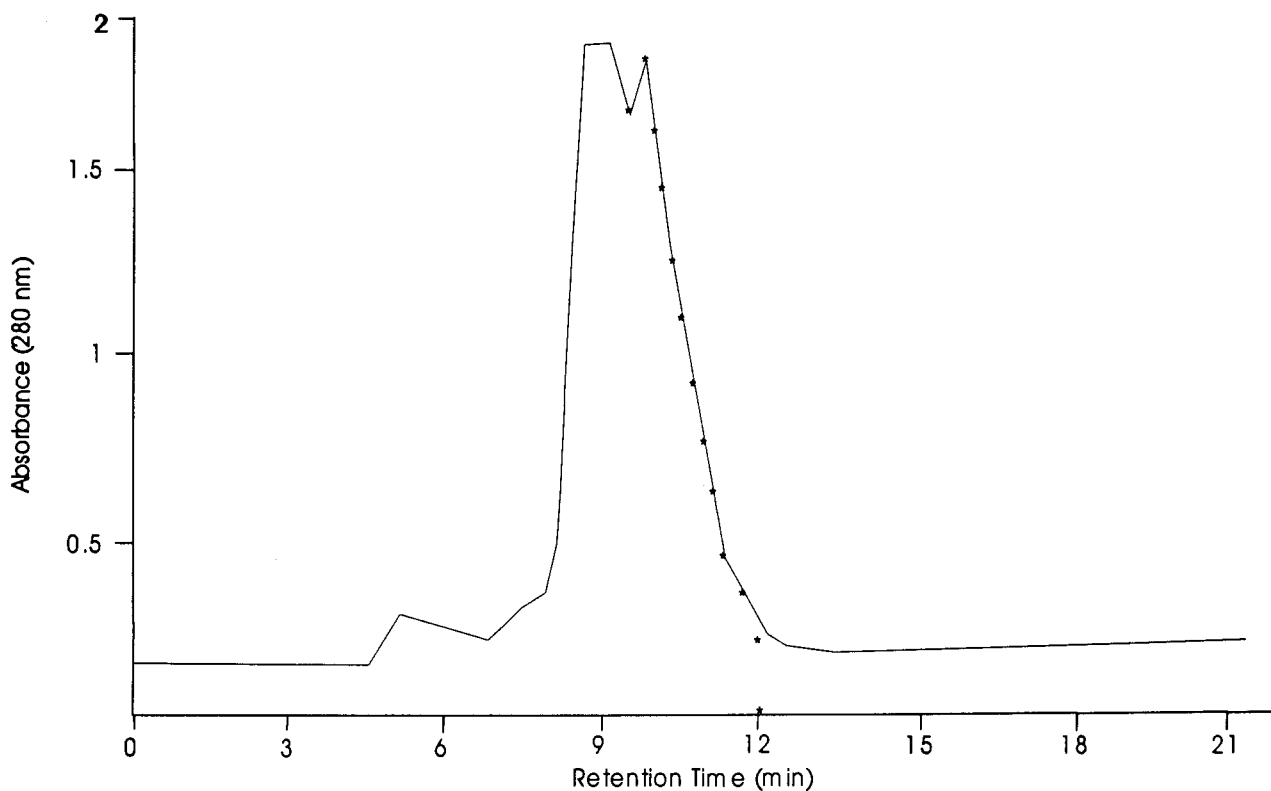
Fig 3.1: Size exclusion HPLC of SGE. The extract was prepared in elution buffer (20 mM Tris, 15 mM NaCl, 2 mM CaCl$_2$ and 2 mM MgCl$_2$ pH 7.6) and applied to the column. Elution was performed with the same buffer at a flow rate of 1 ml/min. Elution was monitored at 280 nm and 1 ml fractions were collected and assayed for fibrinogenolytic activity as described in section 2.2.6. Stars indicate the region of fibrinogenolytic activity.

The SE-HPLC fractions containing fibrinogenolytic activity were pooled and applied to the AE column (see Fig 3.2). Fractions were collected and assayed for enzyme

activity (see Fig 3.3). Activity was observed in fractions with retention times 2 to 4 min (proteins with a basic pI) and 13-17 min (proteins with an acidic pI).
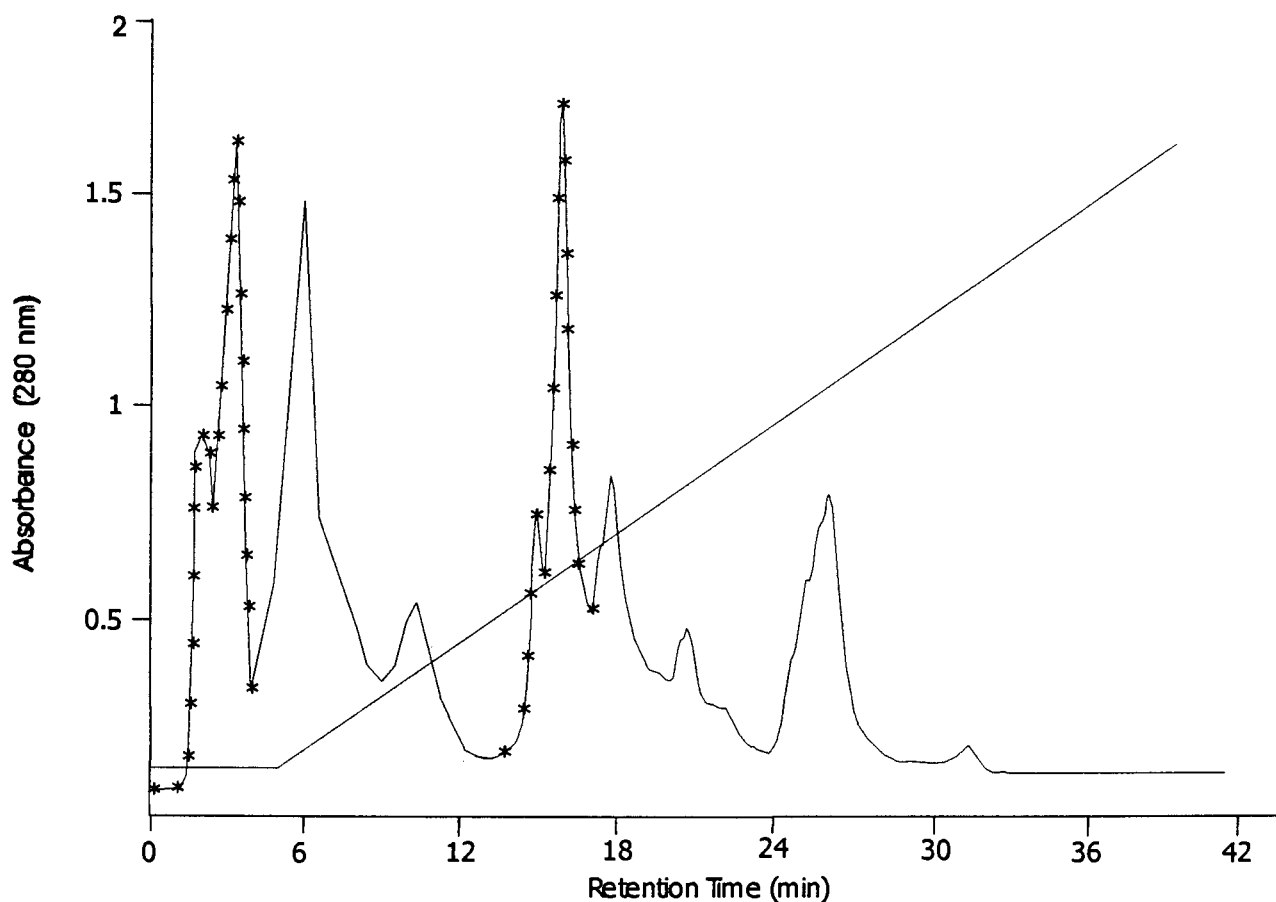
Fig 3.2: DEAE anion exchange HPLC of the active fibrinogenolytic fractions after SE-HPLC. After fractionation of SGE on the size exclusion column (Fig 3.1), the fractions were dialyzed against 2 mM $CaCl_2$ and 2 mM $MgCl_2$ in 2L ddH_2O and applied to the DEAE anion exchange column. Proteins were eluted with a linear gradient of 0-1 M NaCl over 35 min. Protein elution was monitored at 230 nm at a flow rate of 1 ml/min and 1-ml fractions were collected. Fibrinogenolytic activity was determined as in section 2.2.6. Stars indicate the two regions of fibrinogenolytic activity.

Fig 3.3: SDS-PAGE analysis of the degradation of human fibrinogen by the anion exchange HPLC fractions. Activity was assayed by incubating the anion exchange fractions with human fibrinogen at 37 °C and analyzing for fibrinogen degradation on the Coomassie blue stained gels. Lane 2 corresponds to the active fibrinogenolytic fractions with retention time 2- 4 min while lanes 7-10 correspond to fractions with retention times 13-17 min.

The activity in the fractions with retention times 2-4 min was directed at the Aα-chain, whilst fractions with retention time 13-17 min degraded the Aα- and Bβ-chains. The latter fractions were subsequently loaded onto the C5 reversed phase column so as to determine the complexity of the mixture. The resulting chromatogram showed that several proteins are present in the pooled sample (Fig 3.4). The fractions were dried *in vacuo* and reconstituted in assay buffer for determination of fibrinogenolytic activity. As expected no activity was present after the RP-HPLC step.
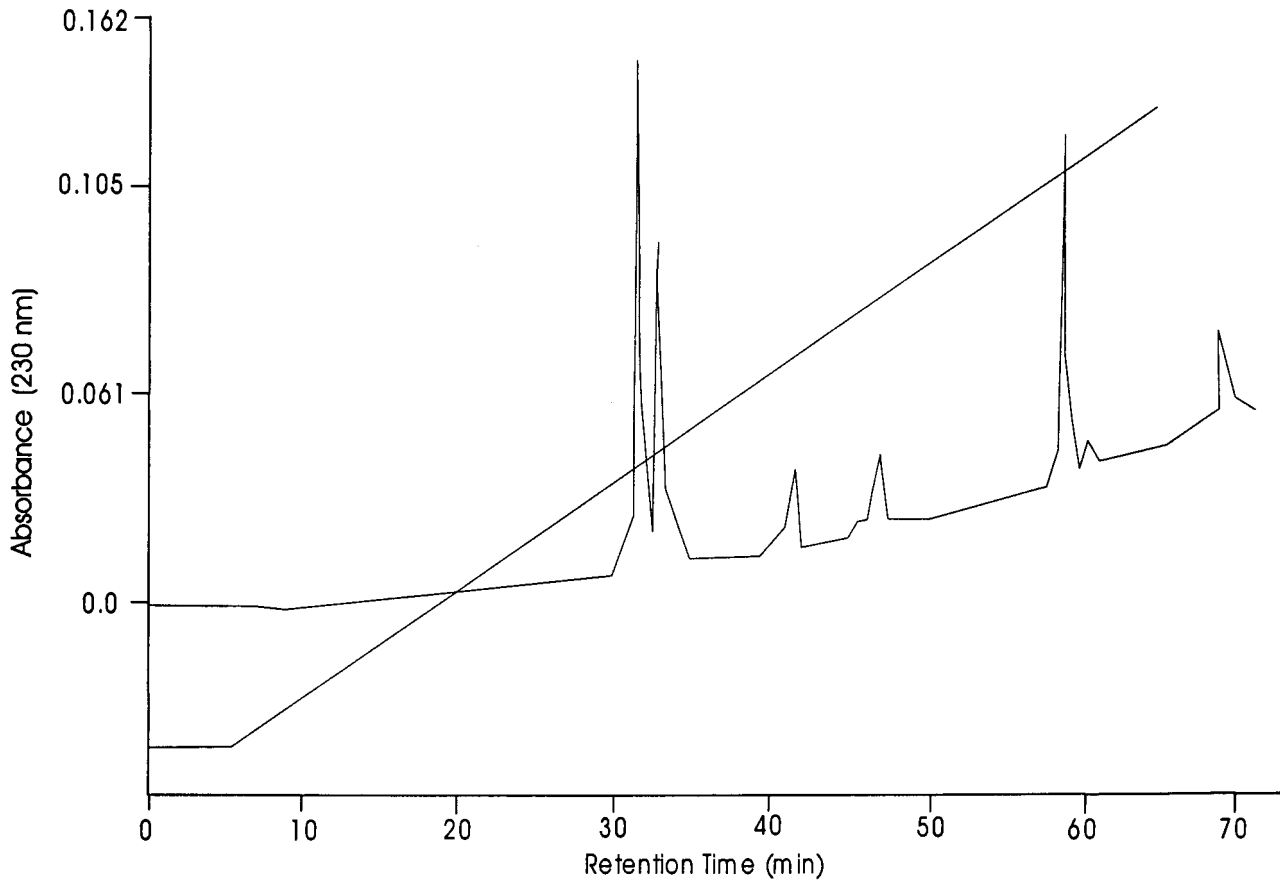
Fig 3.4: RP-HPLC profile of the anion exchange HPLC fractions (retention time 13-17 min). A linear gradient of 0.1-60 % acetonitrile was applied over 60 min. Individual peaks were collected, dried *in vacuo* and assayed for fibrinogenolytic activity.

3.3.2 PURIFICATION USING CE- AND SE-HPLC

SGE (prepared from 40 salivary glands) was applied to the cation-exchange column and the collected fractions were assayed for fibrinogenolytic activity. The activity was present in three regions (Fig 3.5). Region A corresponds to fractions with a retention time of 2-6 min, region B corresponds to fractions with retention times 13-15 min and region C corresponds to fractions with retention times 25-26 min.
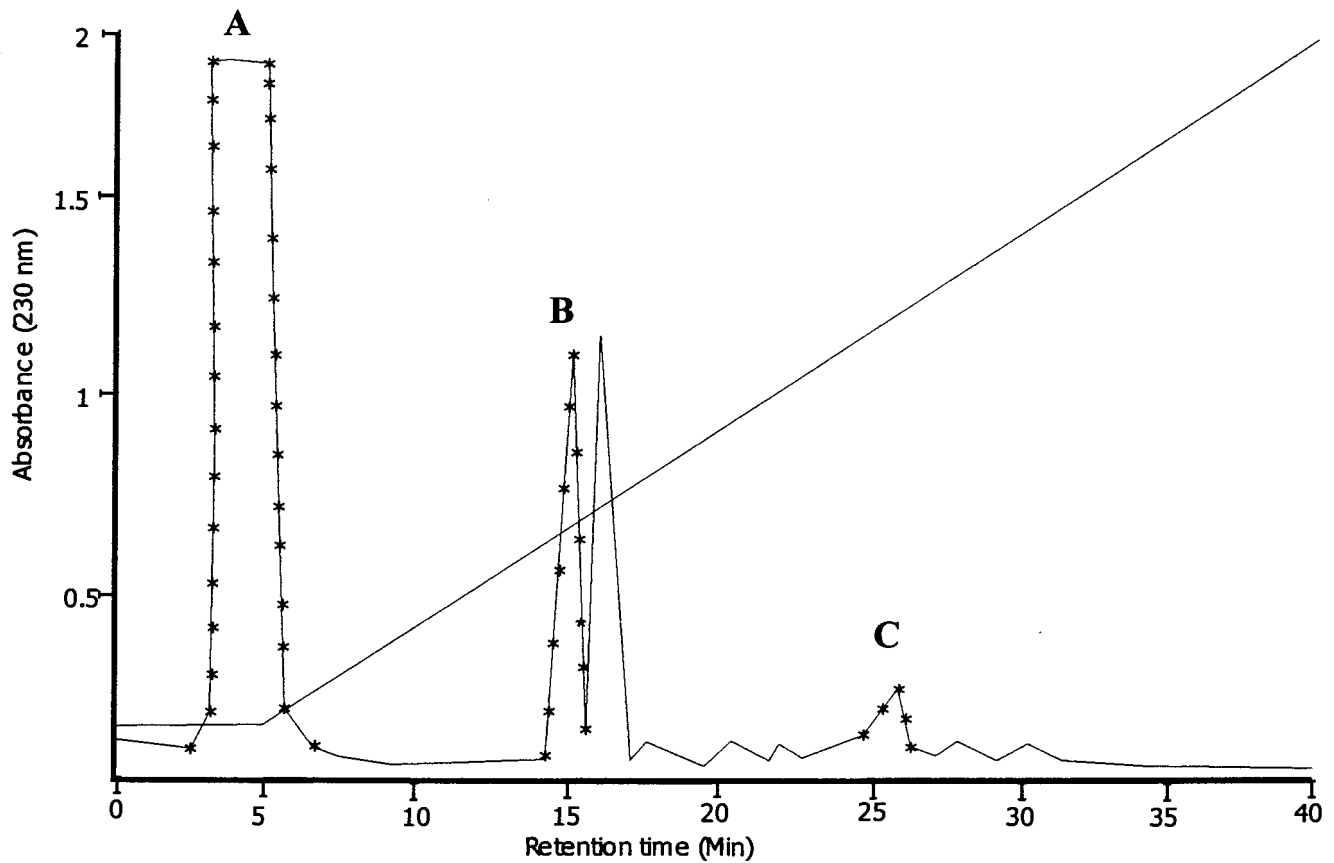
Fig 3.5: Cation exchange HPLC of the crude SGE. A linear gradient of 0-1 M NaCl was applied over 35 min. Protein elution was monitored at 230 nm and 1 ml fractions were collected and assayed for the fibrinogenolytic activity as described in section 2.2.6. Stars indicate the three regions exhibiting fibrinogenolytic activity.

The activity was Aα-chain specific in all the 3 regions. The fibrinogenolytic activity is indicated by the Aα-chain degradation of human fibrinogen in Fig 3.6.

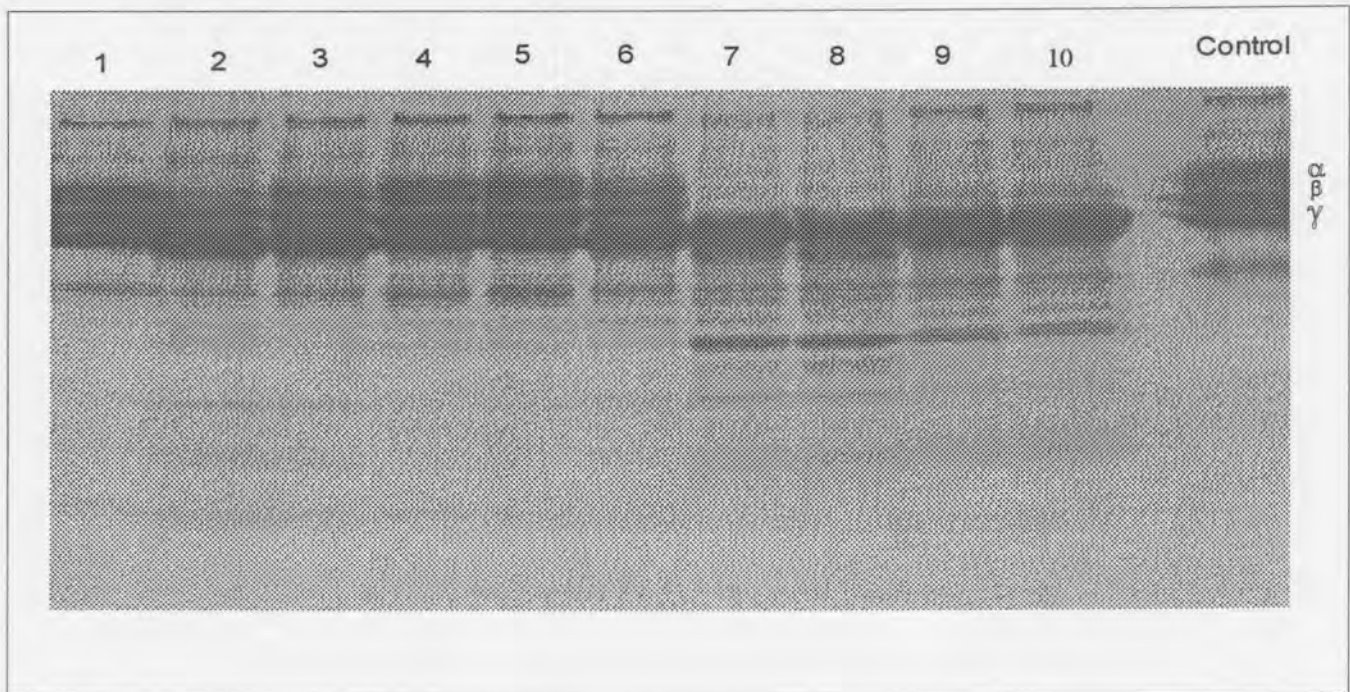**Fig 3.6: SDS-PAGE analysis of fibrinogen degradation by the cation exchange HPLC fractions.** Activity was assayed by incubating the cation exchange fractions with human fibrinogen at 37 °C and analyzing for fibrinogen degradation on the Coomassie blue stained gels. Lanes 1-30 correspond to fractions with retention times 2-33 min in Fig 3.5. Activity was observed on region A (lane 1-4), region B (lanes 12 and 13) and region C (lanes 23).

Region C from several cation-exchange HPLC runs were pooled and dialyzed prior to re-chromatography on the cation exchange column. Fig 3.7 indicates the resultant chromatogram, and the stars on the chromatogram indicate the activity (retention time 23-28 min). SDS-PAGE analysis of the activity in the CE-HPLC rechromatography fractions is indicated in Fig 3.8.
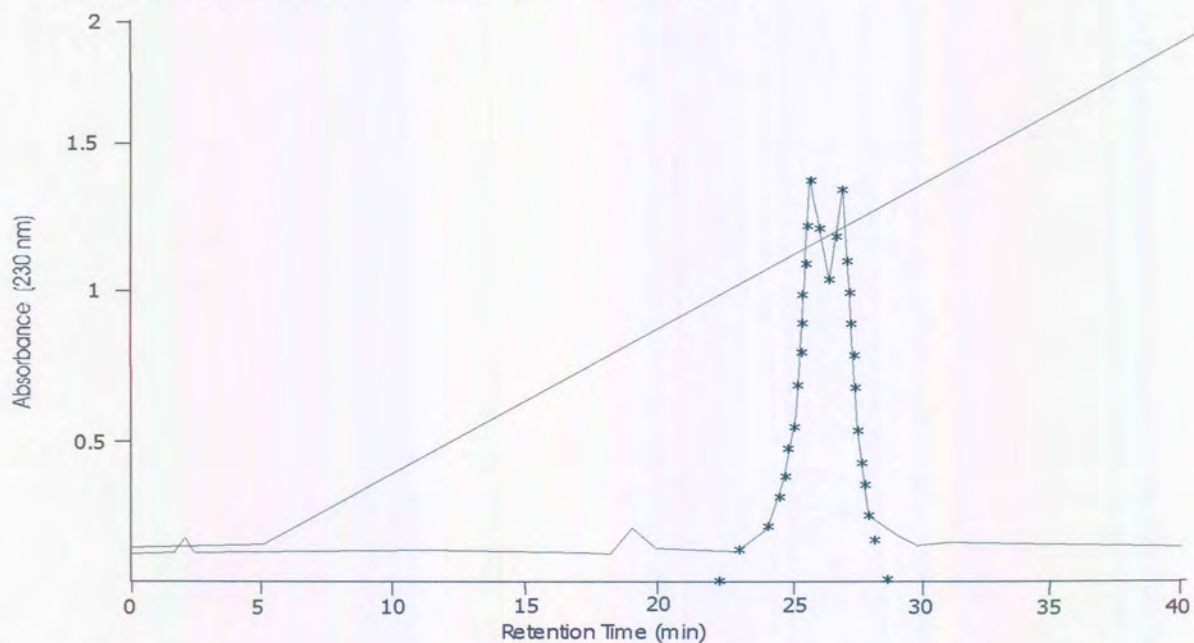
Fig 3.7: Rechromatography of fibrinogenolytic activity of region C on the cation exchange column. A linear gradient of 0-1 M NaCl was applied over 35 min and protein elution was monitored at 230 nm. Fractions of 1 ml were collected and assayed for the fibrinogenolytic activity as described in section 2.2.6. The stars indicate the region of activity.
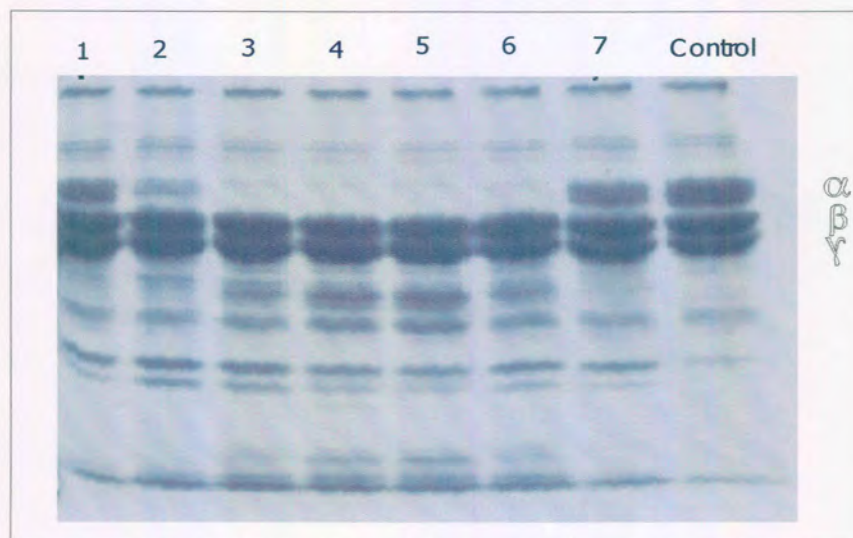
Fig 3.8: SDS-PAGE analysis of fibrinogen degradation by the fractions after cation exchange rechromatography of region C. Activity was assayed by incubating the cation exchange fractions with human fibrinogen at 37 °C and analyzing for fibrinogen degradation on the Coomassie blue stained gels. Lanes 1-7 corresponds to fractions with retention times 22-29 min in Fig 3.7.

The active fibrinogenolytic fractions (retention time 23-28 min) after cation exchange re-chromatography were subjected to SE-HPLC. The resultant chromatogram is shown in Fig 3.9 and the fibrinogenolytic activity in Fig 3.10.
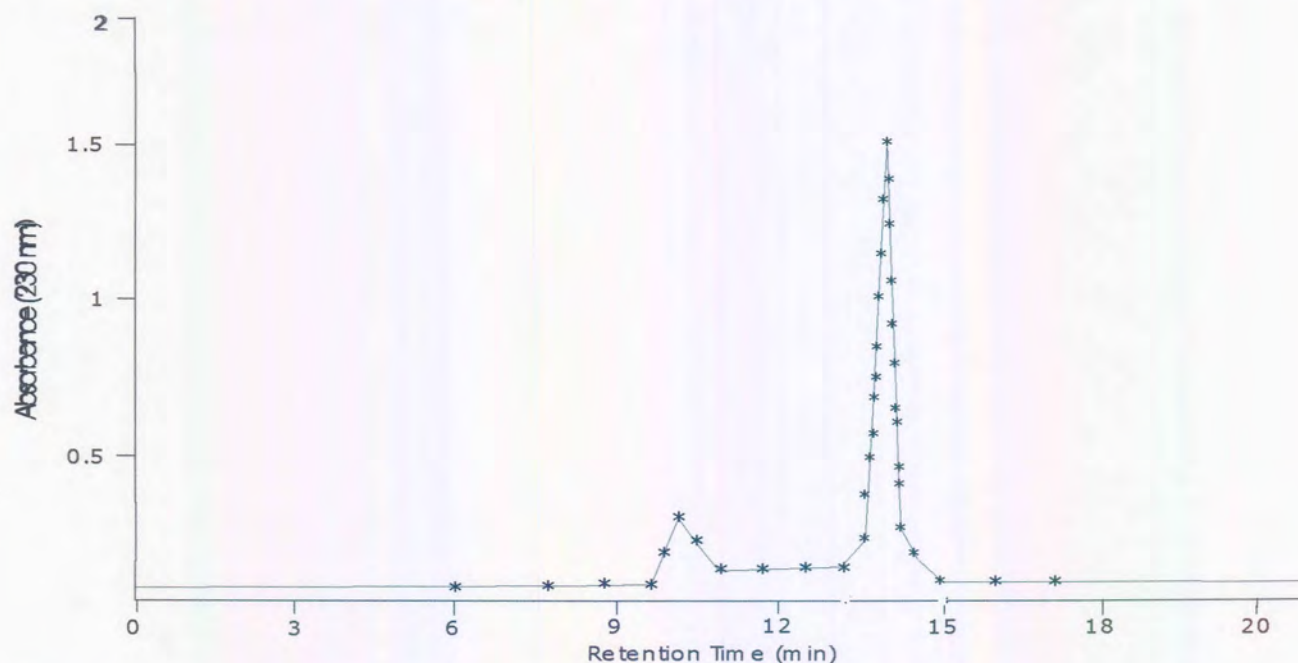
Fig 3.9: Size exclusion HPLC of the fibrinogenolytic activity after cation exchange rechromatography. Protein elution was monitored at 230 nm at a flow rate of 1 ml/min and 1-ml fractions were collected and activity was assayed as in section 2.2.6. Stars indicate the region of activity.
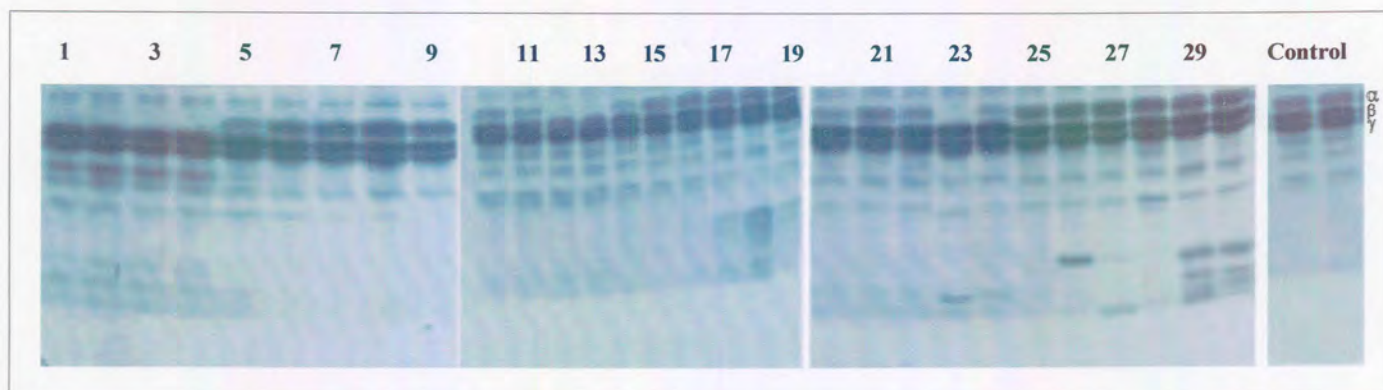
89
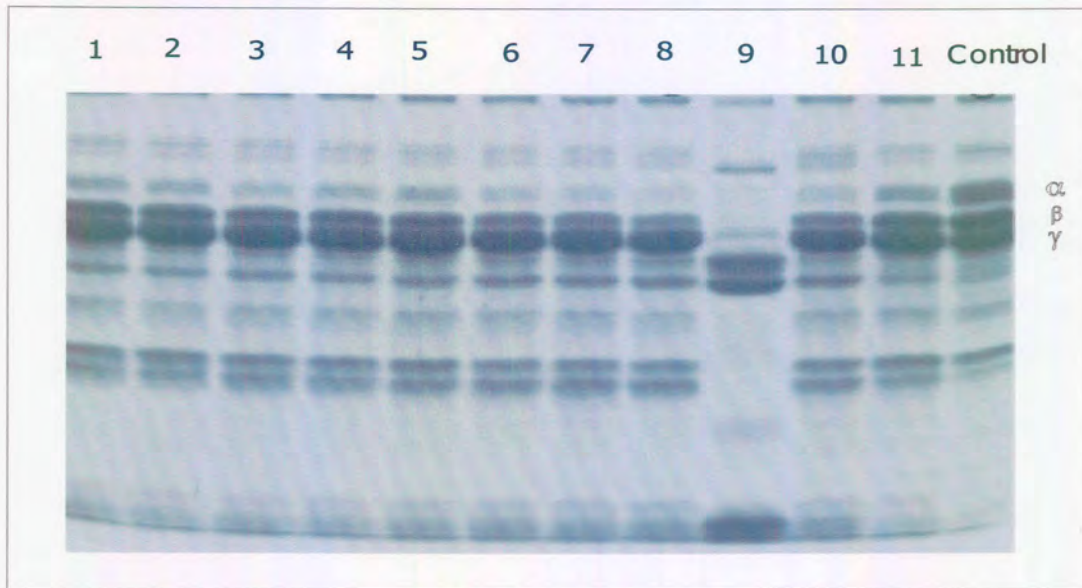
Fig 3.10: SDS-PAGE analysis of fibrinogen degradation by the SE-HPLC fractions. Activity was assayed by incubating the size exclusion fractions with human fibrinogen at 37 °C and analyzing for fibrinogen degradation on the Coomassie blue stained gels. Lanes 1-11 corresponds to fractions with retention times 6-17 min in Fig 3.9.

An α-specific activity was found in fractions with retention time 6-17 min (Fig 3.8) and the highest fibrinogenolytic activity was found in the fraction loaded in lane 9 which corresponds to retention time 14-15 min, indicated by stars in Fig 3.9. This activity was different from the expected Aα-chain degradation and all the fibrinogen chains were degraded (Aα-, Bβ-, and the γ-chain). In order to test whether proteolysis is concentration-dependent, this fraction was serially diluted and the activity assayed by SDS-PAGE analysis. The results (Fig 3.11) indicated that as the fraction dilution increased the Bβ- and γ-fibrinogenase activity in the fraction decreased.
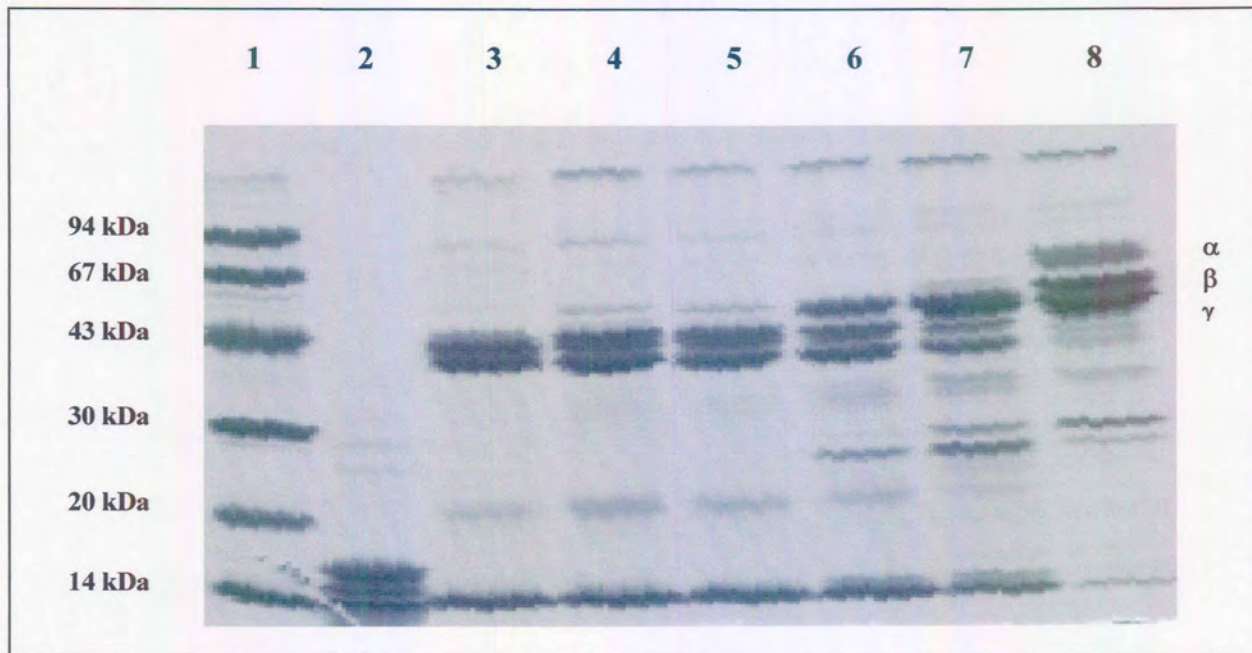
Fig 3.11: Concentration-dependence of the fibrinogenolytic activity. A serial dilution of the active fibrinogenolytic fraction with retention time 14-15 min in Fig 3.10 was done. Lane 1 is the molecular mass marker proteins, lane 2 is the peptide markers, lane 3 = 50 μl, lane 4 = 40 μl, lane 5 = 30 μl, lane 6 = 20 μl, lane 7 = 10 μl of the fraction. Lane 8 is the fibrinogen control. The proteins were Coomassie blue stained on the 12 % separating gel.

The purity of fraction with retention time 14-15 min in Fig 3.9 containing the fibrinogenolytic activity was assessed by tricine SDS-PAGE (Fig 3.12). These results showed that the fraction with retention time 14-15 min contains at least 5 different proteins in the molecular mass range of > 25 kDa.
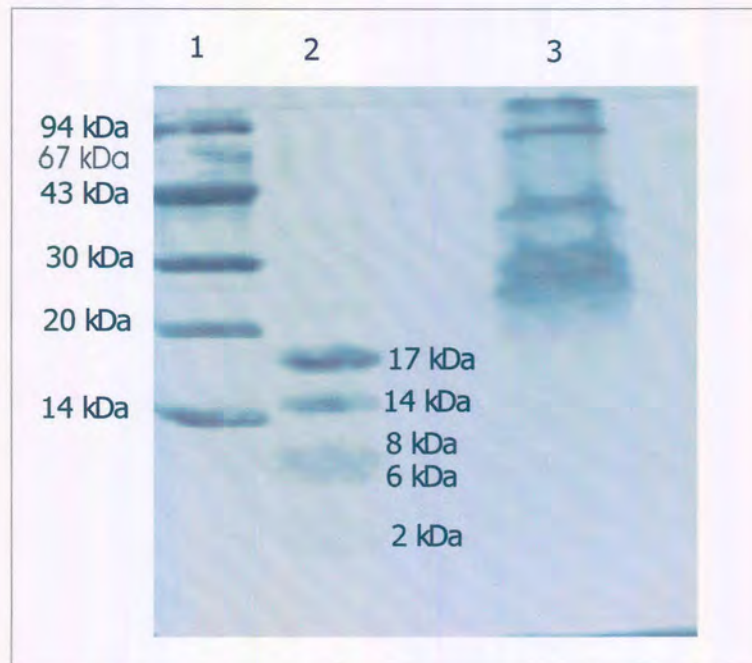
Fig 3.12: Tricine SDS-PAGE analysis of SE-HPLC fraction. The fibrinogenolytic activity containing fraction (retention time 14-15 min) in Fig 3.10 was dialyzed for 2 hours against water and freeze dried prior to dissolution in the Laemmli sample buffer. Lane 1 indicates, the low molecular mass marker proteins, lane 2 indicates the peptide molecular mass marker proteins, lane 3, indicates fraction (retention time 14-15 min). The low molecular mass markers were phosphorylase b (94 kDa), BSA (67 kDa), ovalbumin (43 kDa), carbonic anyhydrase (30 kDa), trypsin inhibitor (20 kDa) and α-lactalbumin (14. kDa).

3.3.3 PURIFICATION OF THE FIBRINOGENOLYTIC ACTIVITY USING CE-, SE- AND FIBRINOGEN-AFFINITY CHROMATOGAPHY

In another attempt to purify the fibrinogenolytic activity, salivary glands were once again subjected to CE-HPLC and fractions in region C were pooled from four individual runs and applied to the SE-column. The SE-HPLC fraction with retention time 14-15 min was then subjected to the affinity column and the chromatogram is shown in Fig 3.13. Testing of fractions for fibrinogenolytic activity using SDS-PAGE analysis of fibrinogen degradation products indicated that the fibrinogenolytic activity was present in fractions corresponding to retention times 12-14.5 min as shown by the activity gel in Fig 3.14.
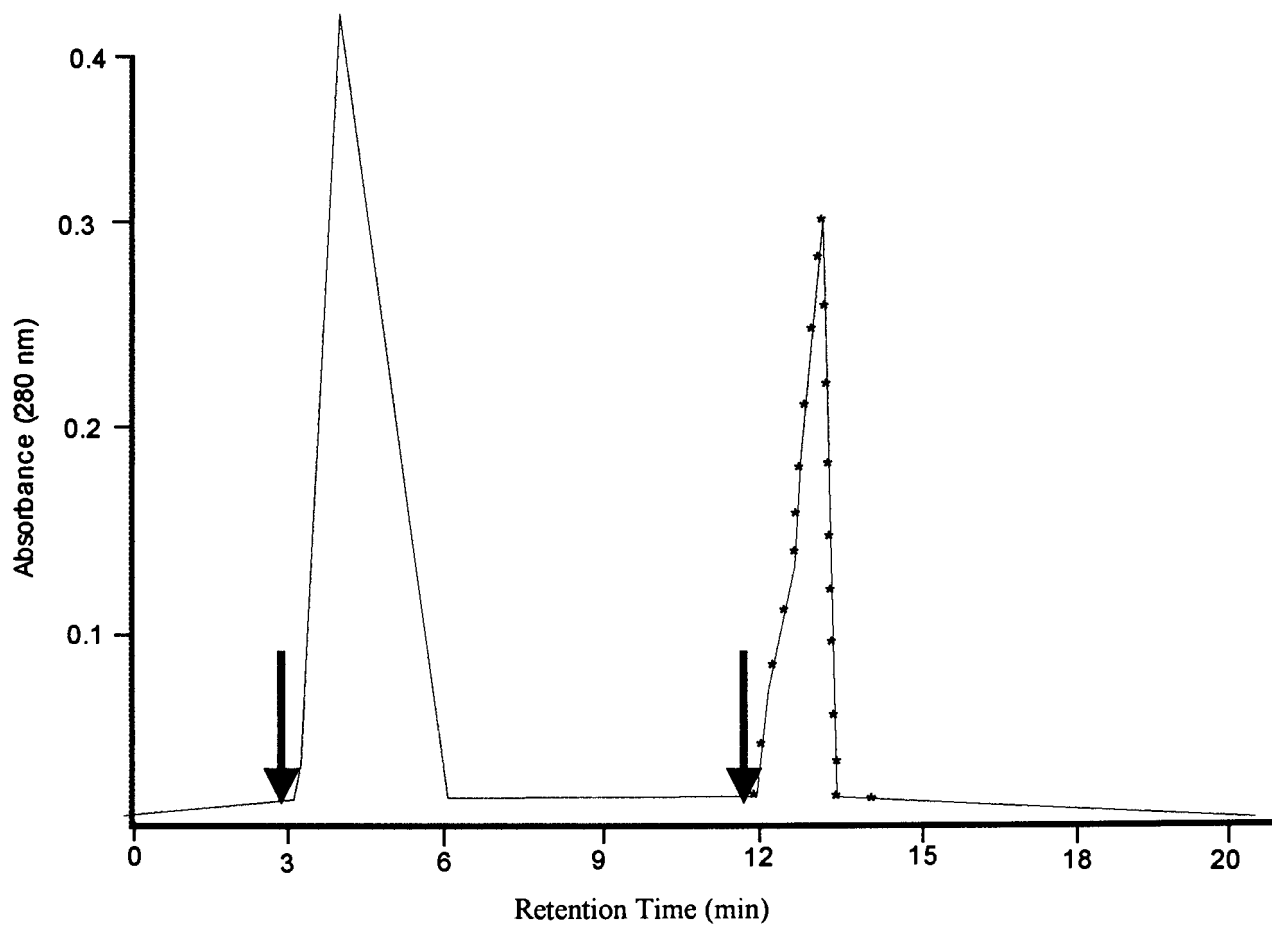
Fig 3.13: Affinity chromatography of the active fibrinogenolytic activity after SE-HPLC. First arrow indicates the beginning of the wash step (see section 3.2.8) and second arrow indicates the beginning of the elution of the adsorbed proteins (see section 3.2.8). Fractions 1-12 were collected at 1-min intervals and fractions 13-22 were collected at 30-sec interval and the flow rate was 1 ml/ min. The eluted fractions were assayed for the fibrinogenolytic activity as described in section 2.2.6 and stars indicate activity (retention time 12-14.5 min).
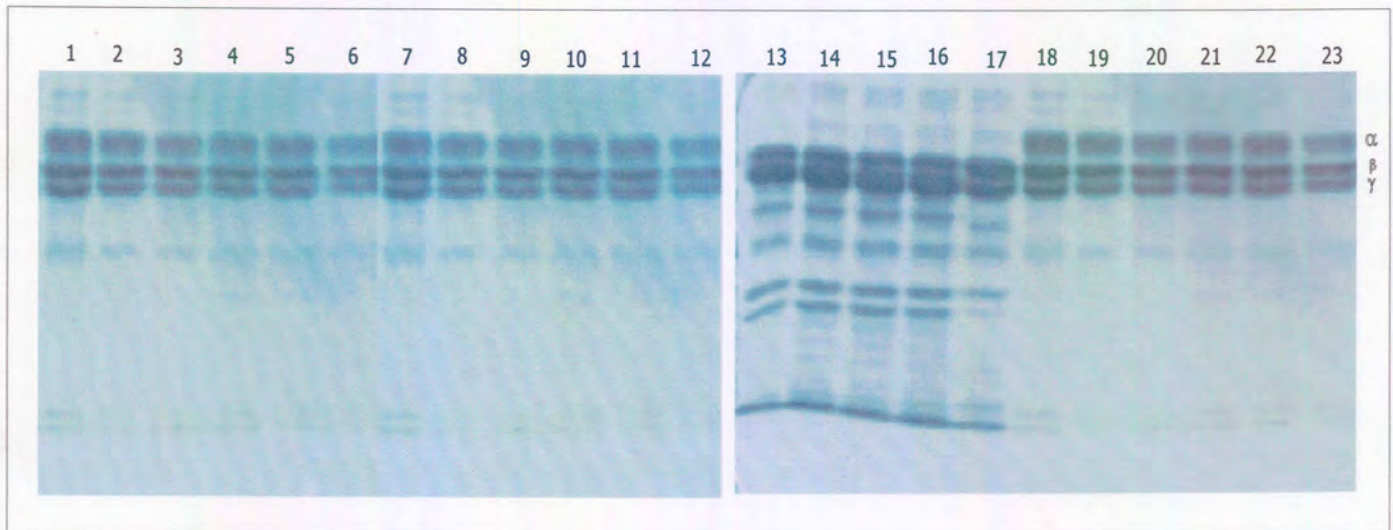
Fig 3.14: SDS-PAGE analysis of fibrinogen degradation by affinity chromatography fractions. Activity was assayed by incubating the fractions and analyzing for fibrinogen degradation on the Coomassie blue stained gels. Lanes 1-12 corresponds to fractions collected in Fig 3.13 with retention times corresponding to 0-12 min, while lanes 13-22 corresponds to fractions with retention time 12-17 min. Fibrinogen control is in lane 23.

To assess the purity of the fibrinogenolytic activity after affinity chromatography, fractions corresponding to retention time 12-14.5 min were pooled, dialyzed for 2 hours against ddH_2O, lyophilized and subjected to tricine SDS-PAGE analysis. The gel was blotted onto a PVDF membrane after electrophoresis so as to facilitate sequencing in case enough of the pure protein would be found after this purification step. The results are indicated in Fig 3.15 and from these it was found that two proteins had adsorbed to the fibrinogen affinity column. The proteins had molecular masses of 31 and 39 kDa, respectively, as shown in lane 1 of Fig 3.15.
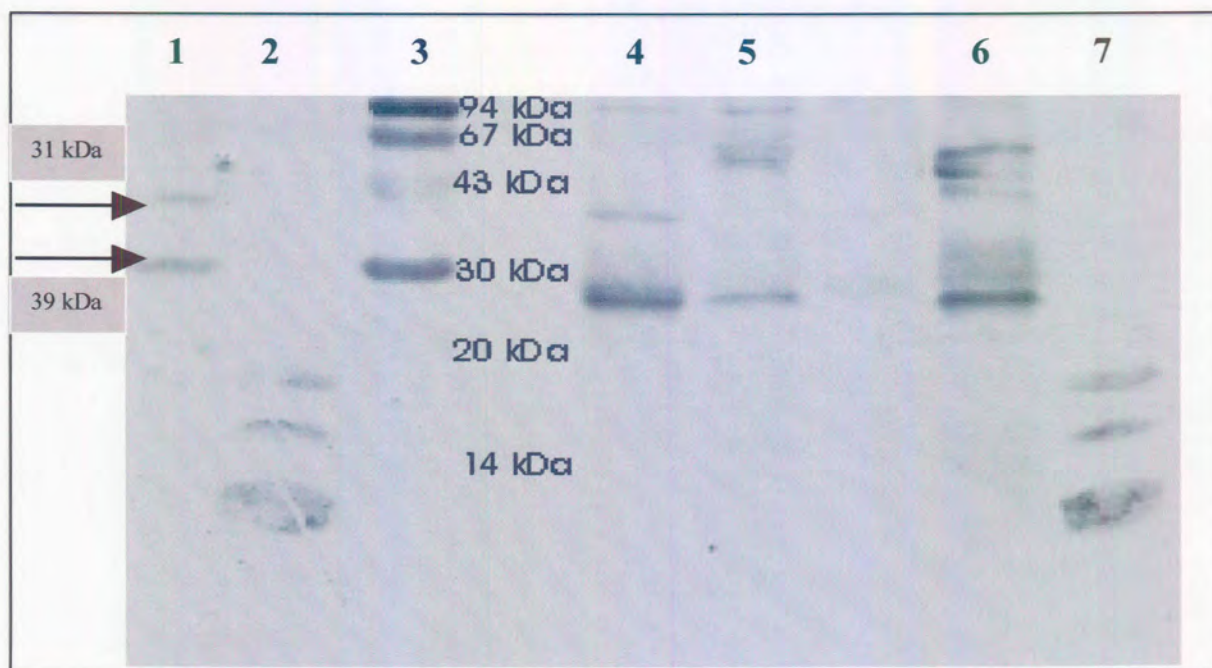
Fig 3.15: Tricine SDS-PAGE analysis of the fibrinogenolytic enzyme purity after affinity chromatography. Lane 1, indicates the partially purified fibrinogenolytic enzyme after affinity chromatography; lanes 2 and 7 are the peptide molecular mass marker proteins; lane 3 is the low molecular mass marker proteins; lanes 4 and 5 are the flow through (proteins not adsorbed to the column) and lane 6 is fraction corresponding to retention time 12-14.5 obtained from SE-HPLC. The gel was stained with Coomassie blue.

The molecular masses of the proteins in the active fraction were estimated from the polyacrylamide gel in Fig 3.15, using the low molecular weight markers. A calibration curve of the log molecular weight against the Rf value (Rf is distance migrated by protein / distance of migrating front) was drawn and an equation describing the curve gave a linear relationship (Fig 3.16). Table 3.8 indicates the molecular masses of the standard proteins together with the calculated masses of the unknown proteins and the Rf values as determined from Fig 3.15.

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$$y = -1.0673x + 5.1003$$
$$R^2 = 0.9952$$

Rf value (y-axis: 0, 0.2, 0.4, 0.6, 0.8)

Log molecular mass (x-axis: 4.1, 4.2, 4.3, 4.4, 4.5, 4.6, 4.7)
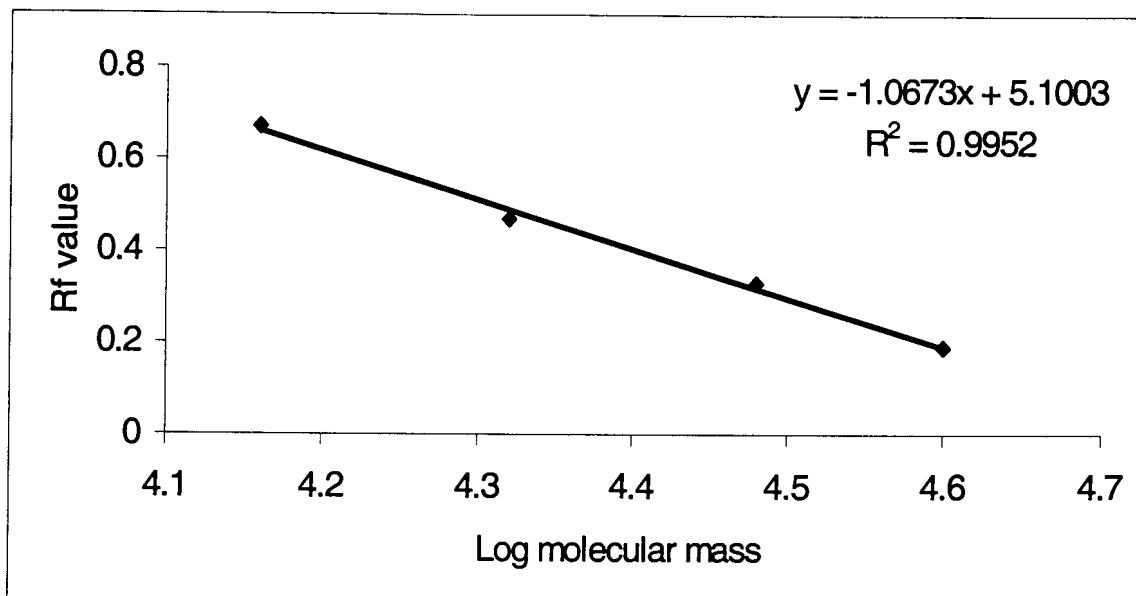
Fig 3.16: Calibration curve for low molecular mass markers. The graph shows the relative mobility of molecular mass markers under reducing conditions. The equation describing the regression line is given.

TABLE 3.8: MOLECULAR MASS OF PARTIALLY PURIFIED FIBRINOGENASE FROM CE-HPLC REGION C AS DETERMINED BY TRICINE SDS-PAGE ANALYSIS

Molecular mass markers	Rf values	Log molecular mass	Molecular mass (kDa)
Phosphorylase B	0.07	4.97	94
Bovine serum albumin	0.11	8.82	67
Ovalbumin	0.19	4.63	43
Lactosidase	0.33	4.48	30
Chymotrypsin	0.47	4.30	20.1
Ribonuclease	0.67	4.16	14.1
Protein 1	0.2		31
Protein 2	0.3		39

Due to the low concentrations of the fibrinogenolytic enzyme after affinity HPLC, the two bands (31 and 39 kDa respectively, in lane 1 Fig 3.15) could not be sequenced so as to get the N-terminal amino acid sequence. In order to test that the isolated proteins were not in fact fibrinogen degradation products, the SGE was incubated with fibrinogen at 4 °C, for 2 hours and fibrinogen degradation products were analyzed on SDS-PAGE (results not shown). It was observed that under identical conditions there were insignificant levels of proteolysis and this excluded the

possibility that the 31 and 39 kDa proteins were in fact fibrinogen degradation products.

3.3.4 FURTHER PURIFICATION OF CATION EXCHANGE REGION A USING AE-, HIC, SE- AND RP-HPLC.

In an attempt to isolate the protein responsible for the activity in region A (Fig 3.5) active cation-exchange fractions were dialyzed against 1.5 mM $MgCl_2$ and 1.5 mM $CaCl_2$ in $2l$ of ddH_2O. Salts were included because the activity was lost on dialysis in their absence. The dialyzed fractions were concentrated on the anion exchange HPLC column and a gradient elution was performed with 0-1 M NaCl. Fig 3.17 indicates the anion exchange HPLC elution profile. The fibrinogenolytic activity was associated with fractions with retention time of 12-15 min as indicated in Fig 3.17. The SDS-PAGE analysis of the activity in the anion-exchange fractions is shown in Fig 3.18.
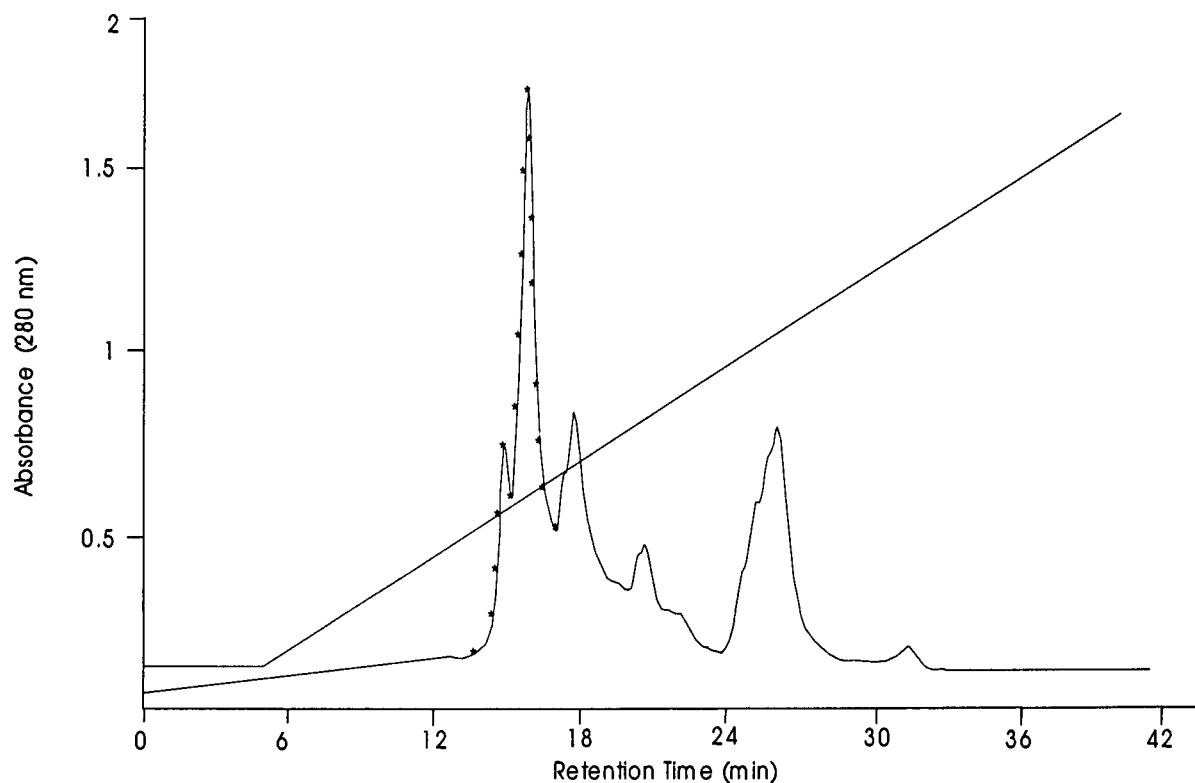
Fig 3.17: DEAE anion exchange HPLC of cation exchange chromatography region A (Fig 3.5). A linear gradient of 0-1 M NaCl was applied over 35 minutes. Protein elution was monitored at 230 nm. One-ml fractions were collected and assayed for fibrinogenolytic activity (2.2.6). The stars indicate the region exhibiting fibrinogenolytic activity (retention time 12-15 min).
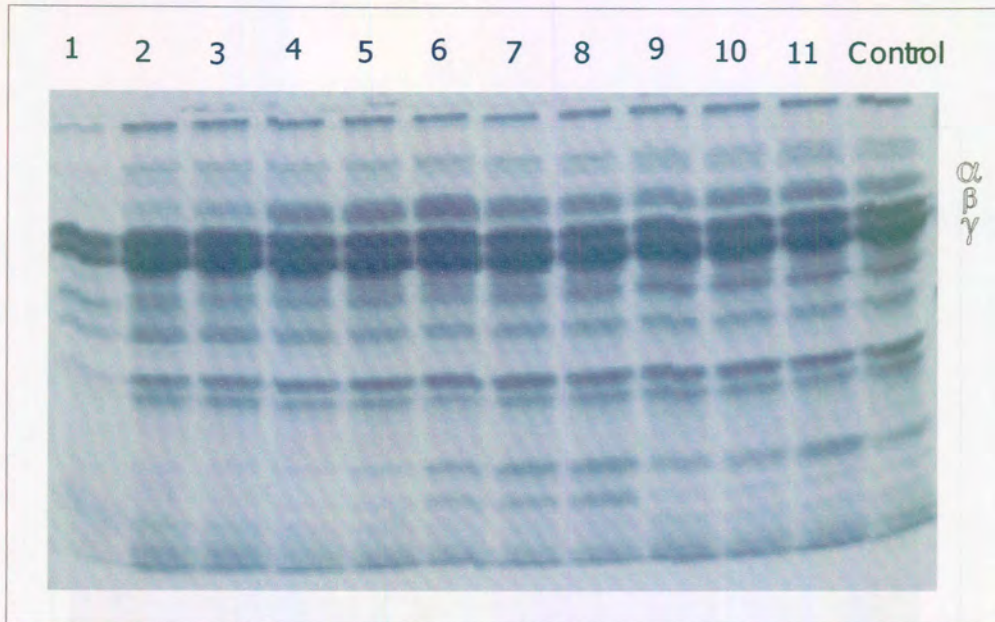
Fig 3.18: SDS-PAGE analysis of fibrinogen degradation by anion exchange HPLC fractions. Activity was assayed by incubating the collected fractions with human fibrinogen at 37 °C and analyzing for fibrinogen degradation on the Coomassie blue stained gels. Lanes 1-11 correspond to fractions with retention times 12-23 min collected at 1min intervals.

Active fractions from AE-HPLC were pooled and subsequently applied to the HIC column (Fig 3.19). The fibrinogenolytic activity was lost after the HIC column. However, when the fractions were desalted by size exclusion chromatography, the activity could be restored. The activity of the individual peaks after desalting on the SE-HPLC column is shown in Fig 3.20.

Fig 3.19: Hydrophobic interaction chromatography of the active fibrinogenolytic activity after anion exchange chromatography. Anion exchange fractions were diluted 1:10 with HIC buffer A prior to injection. Absorbance was measured at 230 nm and a gradient elution was performed over 35 minutes at a flow rate of 1 ml/min. Individual peaks (1-8) were collected and desalted by SE-HPLC before assaying for fibrinogenolytic activity (2.2.6).

The fibrinogenolytic activity was distributed all over the HIC column, since all the peaks possessed the α-fibrinogenolytic activity as indicated in Fig 3.20.
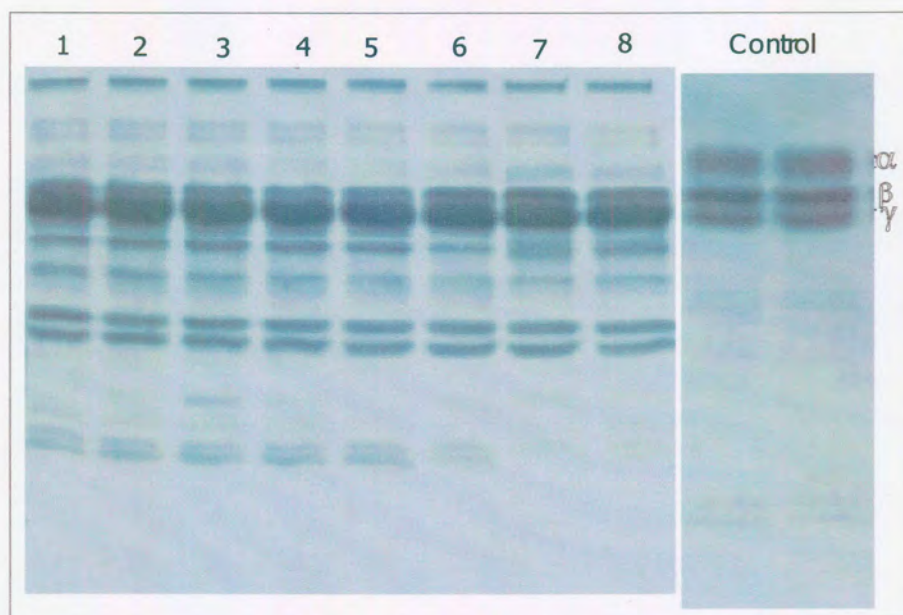
Fig 3.20: Assay of the fibrinogenolytic activity of the HIC fractions after SE-HPLC. HIC fractions (individual peaks) were pooled and desalted on the SE-HPLC column prior to assaying for fibrinogenolytic activity. Activity was assayed by incubating the collected fractions with human fibrinogen at 37 °C and analyzing for fibrinogen degradation on the Coomassie blue stained gels. Lanes 1-8 corresponds to peaks 1-8 collected in Fig 3.19.

Reversed phase HPLC of the active fibrinogenolytic fractions (peaks 1-8 pooled) was performed to further purify the fibrinogenolytic activity. The method was however, futile in the sense that more than one peak was obtained (Fig 3.21) and as the enzyme lost activity after the RP-HPLC treatment, it was impossible to determine which protein component is associated with the fibrinogenolytic activity. However it gave an indication of the number of components associated with the fraction exhibiting activity. Fig 3.22 shows the tricine SDS-PAGE of the eluted proteins after RP-HPLC.
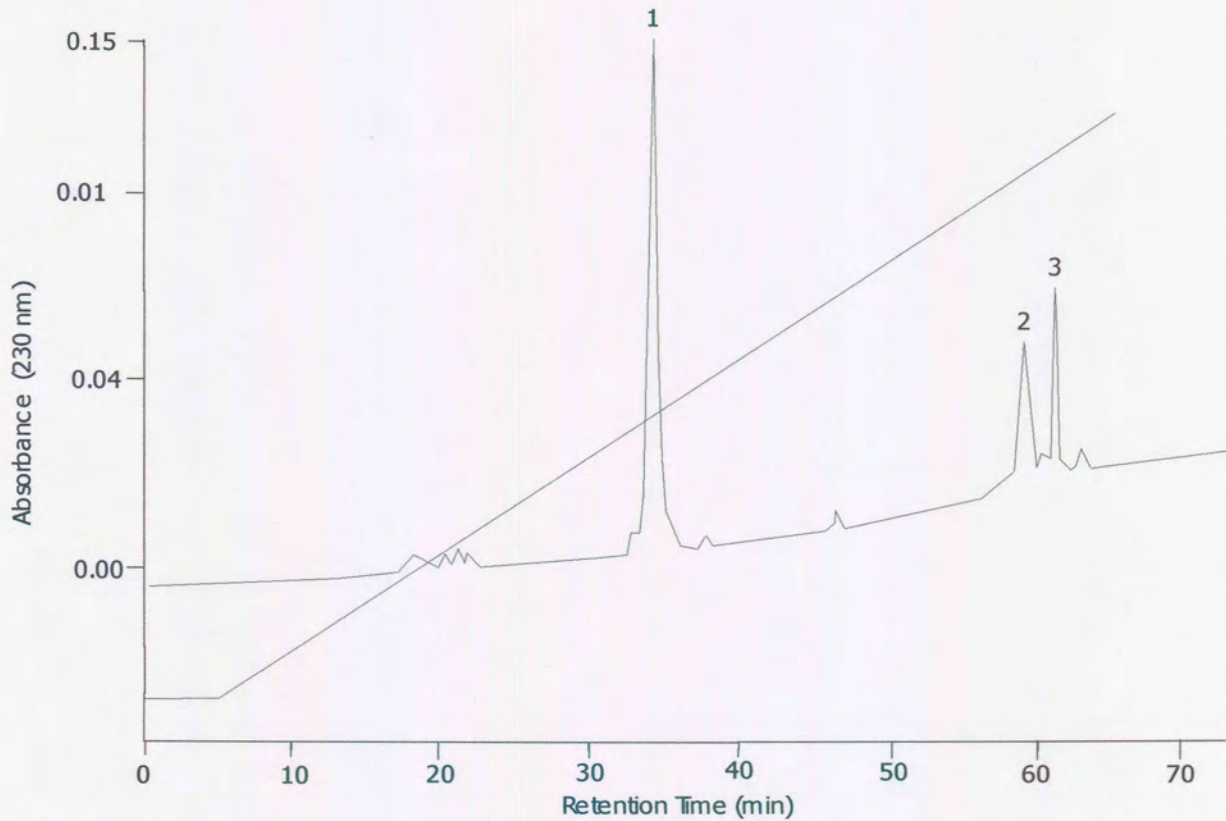
Fig 3.21: RP-HPLC profile of the fibrinogenolytic activity after AE-HPLC. Absorbance was measured at 230 nm and a linear gradient of 0.1-60 % acetonitrile was applied over 60 min. Individual peaks (1-3) were collected, dried *in vacuo* and analyzed for purity.
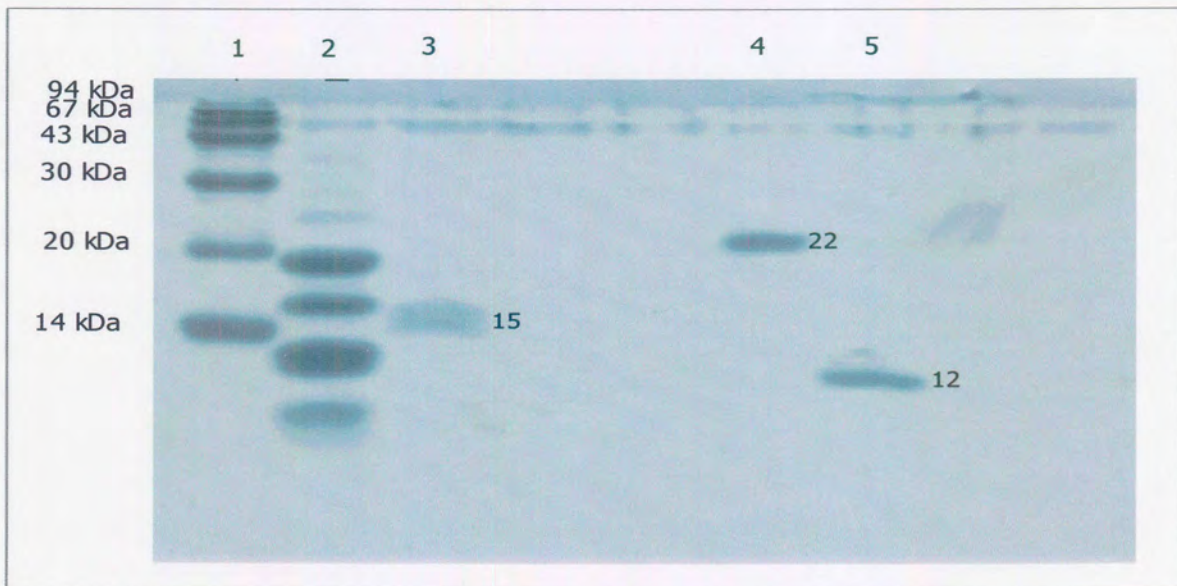
Fig 3.22: Tricine SDS-PAGE analysis of the three major RP-HPLC peaks. Lane 1 contains the low molecular mass marker proteins and lane 2 contains the peptide molecular mass marker proteins. In lanes 3, 4, and 5 are the major protein peaks from Fig 3.21 (indicated as 1, 2, and 3). The gel was stained with Coomassie blue.

A calibration curve of the log molecular weight against the Rf value gave a linear relationship (Fig 3.23). The three RP-HPLC peaks were found to be associated with molecular masses of 15, 22 and 12 kDa, respectively.
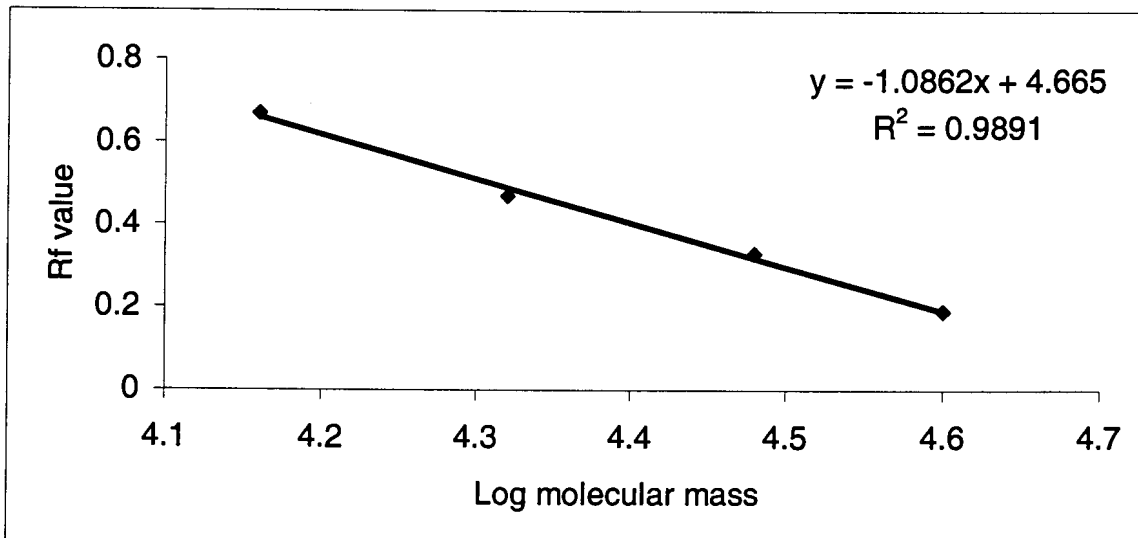
Fig 3.23: Calibration curve for low molecular mass markers. Relative mobility of molecular mass markers under reducing conditions.

TABLE 3.9: MOLECULAR MASS OF PARTIALLY PURIFIED FIBRINOGENASE FROM CE-HPLC REGION A AS DETERMINED BY TRICINE SDS-PAGE ANALYSIS

Molecular mass markers	Rf values	Log molecular mass	Molecular mass (kDa)
Phosphorylase b	0.07	4.97	94
Bovine serum albumin	0.11	8.82	67
Ovalbumin	0.19	4.63	43
Lactosidase	0.33	4.48	30
Chymotrypsin	0.47	4.30	20.1
Ribonuclease	0.67	4.16	14.1
Protein 1	0.45		15
Protein 2	0.31		22
Protein 3	0.51		12

3.4 DISCUSSION

In preliminary investigations described in chapter 2, fibrinogenolytic activity was identified in the SGE of the tick, *O. savignyi*. The activity was assayed using SDS-PAGE analysis of fibrinogen degradation products. During the present study, partial purification of fibrinogenolytic activities has been achieved by the combination of various HPLC steps. In the first attempt, separation of the SGE with SE-HPLC indicated that the fibrinogenolytic activity eluted in one peak. This activity was found to separate into two regions on the anion exchange column, a basic pI region (unadsorbed proteins) and an acidic pI region (adsorbed proteins). Application of the latter region to RP-HPLC indicated that several proteins were associated with this region. However, RP-HPLC resulted in loss of activity.

Separation of the SGE with the cation exchange HPLC resulted in three regions (A, B, and C) exhibiting fibrinogenolytic activity. From this experiment it could be deduced that the proteins in region A, have an acidic pI, while the proteins associated with regions B and C are basic in nature. Region C (basic pI) was re-chromatographed on the cation exchange column and the active fractions were applied to the size-exclusion column. Tricine SDS-PAGE analysis of the active peak indicated that at least five different proteins of molecular masses greater than 25 kDa were present. It was at this stage that the fibrinogen affinity column was introduced as an additional step. Jennings *et al.* (1999) were able to successfully purify a fibrinogenase from the venom of the African puff adder (*Bitis arietans*) by means of fibrinogen affinity chromatography. Region C from several cation exchange HPLC runs were combined and applied to a size-exclusion column. The active SE-HPLC fraction was then subjected to the fibrinogen affinity column. Reducing SDS-PAGE analysis of the active fraction showed two bands of relative molecular masses of 31 and 39 kDa, respectively. Even though the fibrinogenolytic activity was not purified to homogeneity, it can be concluded from these results that the molecular mass of one of the enzymes responsible for fibrinogen degradation is either 31 or 39 kDa and is basic in nature.

Throughout the various isolation methods the fibrinogenolytic activity was found to be Aα-specific. However, during one experiment the activity was directed at Aα- Bβ- and γ–chains. Dilution of the fractions showed that the activity was Aα–specific at low levels, but that all three chains are susceptible to degradation at higher enzyme levels. Brown *et al.* (1995) observed that the fibrinogenolytic activity of the hookworm, *Necator americanus* ES (excretory secretory) products degrade human fibrinogen in a time-dependent manner. After incubation for 1 h, degradation of fibrinogen was Aα-specific. Prolonged incubation times caused increasing amounts of degradation, and at 24 h all fibrinogen chains had been degraded.

In an attempt to isolate the fibrinogenase activity associated with the acidic pI region, region A of the cation exchange column was further purified on the anion exchange column followed by the HIC. During HIC, the activity eluted in several peaks. It proved to be essential to desalt the HIC fractions with SE-HPLC, since the high salt concentrations used for the HIC buffers caused inactivation. The fact that activity was spread over eight peaks after HIC can possibly be due to the aggregation of the enzyme with other proteins or the presence of multiple structural isoforms. HIC separates the proteins on the basis of surface hydrophobicity, however the disadvantage of this column is that the resolution is not good since the surface hydrophobicity is not that discriminating a factor. There is a relatively slow association-dissociation process, meaning that columns are not run in equilibrium mode. The main obstacle to sharp separations is protein-protein interactions: similar proteins will interact with each other as well as with the adsorbent (whereas in ion exchange chromatography, similar proteins will repel each other because of like charges) resulting in a large degree of overlap between the eluting components (Scopes, 1994). Each HIC-peak was individually applied to a SE-HPLC-column and all activity containing peaks from each SE-HPLC run were pooled as they all had similar retention times on the anion exchange column. The combined samples were analyzed for purity using C5-reversed phase chromatography and reducing tricine SDS-PAGE revealed three bands of relative molecular masses of 15, 22 and 12 kDa, respectively. However, as expected the activity of the fibrinogenase was also lost on this column. It is known that RP-HPLC can lead to protein denaturation. This is

because of the harsh mobile-phase conditions, the use of the organic modifiers for elution and the low pH used in this method (Aquilar and Hearn, 1996).

Even though the fibrinogenase activity in both the acidic (region A) and basic (region C) CE-HPLC regions were not purified to homogeneity it would be useful to obtain the N-terminal amino acid sequences for these proteins. These sequences will enable a homology search in protein sequence databases for other proteins with similar sequences. The N-terminal amino acid sequence can also be employed to design a degenerate primer which together with a poli-T primer will enable the amplification of the corresponding cDNA by reverse transcription PCR. The 31 and 39 kDa proteins obtained after the fibrinogen affinity column were transferred to a PVDF membrane for possible sequencing. However, at that time sequencing of protein bands (other projects in the Department) with similar intensities to the 31 and 39 kDa protein bands were unsuccessful. It was therefore decided not to sequence the two proteins and to repeat the isolation procedure in order to obtain sufficient amounts for sequencing. Due to a shortage of ticks this was not possible.

The laborious and time consuming efforts of enzyme purification from natural sources, which are normally scares can be circumvented by cloning, sequencing, and expressing the gene encoding the enzyme of interest (Sakanari et al., 1989 and Eakin et al., 1990). In my opinion future attempts to characterize the tick fibrinogenase should maybe focus more on molecular biology techniques using the sequence of known fibrinogenases in designing degenerate primers for PCR-based amplification of the fibrinogenase cDNA. Conserved structural motifs, identified by alignment of several members of the protease family, can be used to design generic molecular probes for amplification of protease gene fragments from the DNA of parasitic organisms using PCR (Mulenga et al., 2001, Renard et al., 2000). An alternative strategy would be using a SE-HPLC column of fractionation range 5-100 kDa. This is expected to result in better separation of the low molecular mass proteins present in the SGE. SDS-PAGE analysis of the crude SGE indicated that most proteins present in the salivary glands are smaller than 94 kDa and the majority even smaller than 40 kDa (Gaspar, 1995). Considering these results, the exclusion range of 10-500 kDa of the size exclusion column used in the isolation

procedure was not ideal for separation of the SGE proteins. Because of the fact that proteins are denatured during the reversed phase isolation, copper staining and elution of the proteins from non-reducing SDS-PAGE gels instead of the RP-HPLC step could be attempted. The method fixes proteins within the gel in a reversible manner. The polypeptides can be quantitatively eluted from slices at any time following staining (Lee *et al.*, 1987).

Sufficient amounts of the pure proteins will enable studies on the protein structure and their role in tick physiology. Kinetics studies will give parameters such as Ki, Km and Vmax, which could indicate the suitability of the anticoagulant for therapeutic use. It will be interesting to establish whether the mechanism of action of the tick fibrinogenase is similar that of the snake fibrinogenolytic activities and whether the mechanism is unique or shared by other arthropod fibrinogenolytic enzymes. Ultimately further analysis of the blood feeding strategies employed by hematophagous arthropods may provide an important insight into the host-parasite relationships, as well as identify novel targets for future vaccine development.

The next chapter investigates the effect of the partially purified metalloproteinase (region C) on platelet disaggregation.

CHAPTER 4

DISAGGREGATION OF AGGREGATED PLATELETS BY THE PARTIALLY PURIFIED FIBRINOGENOLYTIC ACTIVITY

4.1 INTRODUCTION

Platelets play pivotal roles in the hemostatic process including detection of vascular lesions, adherence at sites of injury, recruitment of additional platelets, and consolidation into a hemostatic plug (McNicol and Israel, 1999). Aggregation of platelets into a mass is responsible for their main physiological function, that is, the formation of a hemostatic plug and sealing off the wound in an injured blood vessel. Whereas formation of platelet aggregates (thrombi) *in vivo* is difficult to measure, the process of platelet aggregation *in vitro* has been qualitatively analyzed and employed in many studies of platelet function (Zucker 1989).

4.1.1 PLATELET AGGREGATION

Damage to blood vessels triggers a prompt response of hemostatic reactions to prevent hemorrhage. These reactions include contraction of the vessel wall itself due to the action of released vasoactive agents, adhesion and aggregation of circulating platelets to form a hemostatic plug and activation of fibrin clots. In order to allow full tissue healing, the clots are subsequently removed by the fibrinolytic enzyme, plasmin (Bithell, 1993).

In situations where any component of these mechanisms is altered, hemostasis is compromised and the result could be either thrombosis or hemorrhage (bleeding due to platelet and/or clotting factor deficiencies). In small blood vessels, platelets alone can arrest bleeding (Bithell, 1993).

Platelet agonists are very diverse, consisting of low molecular-weight compounds, such as ADP, serotonin, and epinephrine; enzymes such as thrombin and trypsin; particulate material such as collagen and antigen-antibody complexes; lipids, such as platelet-activating factor and ionophores, such as A23187. These agonists induce changes in the membrane of platelets, evoking their aggregation under appropriate conditions (Holmes *et al.*, 1999). Each agonist stimulates platelets via a specific receptor. The receptor for collagen belongs to the super-family of $\alpha\beta$-dimeric proteins or integrins (Hynes, 1992). The first reaction of platelet to vessel damage is their adhesion via their GPIb/IX and GPIa/IIa receptors to the adhesive proteins, von Willebrand factor (vWF) and collagen, respectively, on the exposed sub-endothelium.

Fibrinogen (340 kDa) is an essential cofactor for platelet aggregation. Binding of fibrinogen to the platelet receptor, GPIIb/IIIa, anchors platelets to each other (Zwaal and Hemker, 1986). The interaction of fibrinogen with platelets is necessary for aggregation. Fig 4.1 adapted from Clarke *et al.* (1994) illustrates the role of fibrinogen in platelet aggregation.
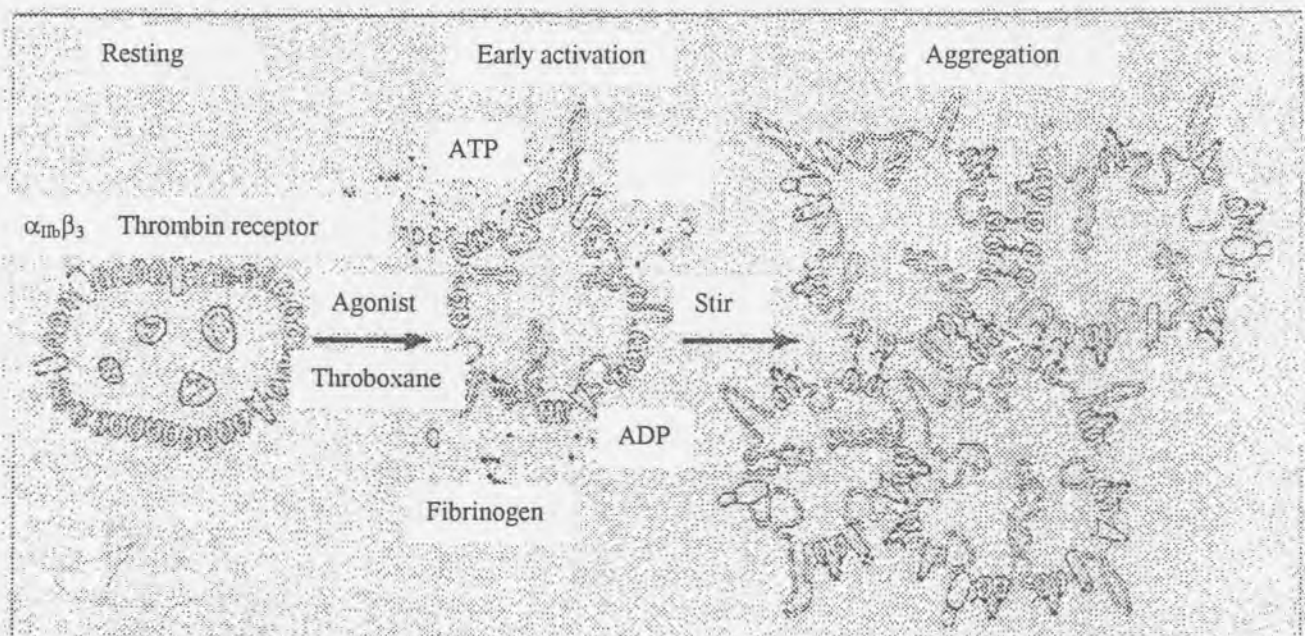
Fig 4.1: The role of fibrinogen in platelet aggregation. Treatment with agonist activates multiple intracellular signal transduction pathways that are responsible for the physiological responses of platelets. Induction of arachidonate metabolism leads to formation of thromboxane.

Platelet stickiness develops when the platelet membrane acquires the ability to bind fibrinogen. This dimeric molecule acts as a molecular glue via the fibrinogen receptor GPIIb/IIIa, bridging the gap between platelets. With agonists that can induce secretion without prior aggregation e.g., thrombin or collagen, fibrinogen can be secreted from the platelet α-granules, whereas with agonists that require aggregation before secretion occurs, e.g. ADP and epinephrine, fluid-phase fibrinogen must be present. Fibrinogen binding to platelets, and hence aggregation, also requires divalent cations. It is important to note that certain responses, e.g. secretion induced by ADP, and the association of GPIIb/IIIa with the cytoskeleton only occurs when platelets have actually aggregated, not when they have been simply stimulated or even when they have bound fibrinogen (Zucker, 1989).

Platelet activation is known to be involved in the development of atherosclerosis and retenosis after angioplasty. After exocytosis, ADP acts as an endogenous platelet agonist, potentially via multiple P2 receptors, and is important to the activation of additional circulating platelets and, hence to their recruitment to the site of injury. The significance of this effect is seen in the compromised hemostatic capacity of patients with dense granule deficiencies. Dense granules contain a non-metabolic pool of ATP, which is released during exocytosis. ATP is rapidly removed from the plasma by conversion to AMP and adenosine. An ATP receptor is present in platelets, and a role in activation has been proposed (McNicol and Israels, 1999). Stimulation of platelets with ADP has been shown to mediate platelet shape change, aggregation, and further release of ADP and ATP from activated platelets. ADP activates fibrinogen receptors, leading to platelet aggregation (Kunapuli and Daniel, 1998). Thromboxane A_2 produced by the action of ADP on platelets converts reversible aggregation into irreversible aggregation, which is also referred to as second wave of aggregation (Kunapuli and Daniel, 1998). The ADP receptor in platelets is reported to possess a unique, pharmacologically characteristic P2T-type purinoreceptor. Activation of the ADP receptor causes immediate activation of the nonselective cation channel that mediates calcium influx and mobilization of calcium via activation of phospholipase C, and inhibition of adenylyl cyclase by activation of Gi-protein (Kunapuli and Daniel, 1998). Thrombin, thrombin receptor-activating peptide (TRAP), and thromboxane analogues each activate platelets by

the well characterized phospholipase C-mediated hydrolysis of phosphatidylinositol 4,5-bisphosphate (PIP$_2$). This results in the bifurcating signal transduction pathways mediated by elevated cytosolic calcium levels and protein kinase C activity (McNicol and Israels, 1999). Thrombin, a serine protease, is a potent agonist for platelets eliciting shape change, secretion of granular contents, and aggregation (Vu *et al.*, 1991). Thrombin-induced platelet aggregation is critical for hemostasis and thrombosis (Hansen and Harker, 1988). Thrombin also evokes the release of arachidonic acid from platelet membrane phospholipids and induces the generation of prostaglandin endoperoxides, thromboxane, and other metabolites of arachidonic acid (Kramer *et al.*, 1995). These thrombin-induced platelet responses are mediated by cell surface receptors that belong to the family of seven-transmembrane domain receptors coupling to G proteins. They are activated by a novel mechanism in which thrombin mediated proteolytic cleavage of the receptor in effect unmasks a ligand within the thrombin receptor's amino-terminal extension, thereby affecting receptor activation (Vu *et al.*, 1991). SFLLRN peptides, containing the first six residues of the new amino terminus, have been shown to mimic the effect of thrombin, causing platelets to secrete and aggregate (Vu *et al.*, 1991 and Scarborough *et al.*, 1992). Like thrombin, they activate phospholipase C, phosphatidylinositol 3-kinase, and protein kinase C (Huang *et al*, 1991; Hung *et al.*, 1992; Rasmussen *et al.*, 1993), inhibit adenylate cyclase (Hung *et al.*, 1992), and induce phosphorylation on tyrosine residues of multiple proteins (Rasmussen *et al.*, 1993; Vassalo *et al.*, 1992). TRAP mimics (at least partly) the effects of thrombin with thrombin receptors on a cell membrane without exerting clotting action (Matsumoto *et al.*, 1999; Vu, *et al.*, 1991; Hung *et al.*, 1992).

Collagen aggregates platelets secondarily through the release of ADP by activated platelets (Zucker 1989). There are several proposed platelet collagen receptors including $\alpha_2\beta_1$ and $\alpha_{IIb}\beta_2$ integrin, GPIV and GPVI. It is proposed that collagen first binds to the I domain of the $\alpha_2\beta_1$ integrin; this binding generates signals for the activation and expression of the binding site on $\alpha_{IIb}\beta_2$ integrin (Shattil, 1995) resulting in collagen binding, presumably via the collagen RGD sequence. These reactions result in a full platelet aggregation response (Bithell, 1993).

4.1.2 INHIBITORS OF PLATELET AGGREGATION

Three groups of platelet aggregation inhibitors are recognized: The α-fibrinogenases, venoms containing 5'-nucleotidase or ADPase activities and fibrinogen receptor antagonists (Marsh 1994).

i. α-Fibrinogenases, such as the basic protease from the venom of the snake, *Tremesurus mucrosquamatous*, inhibit platelet aggregation by proteolysis of the Aα-chain of fibrinogen so that it cannot bind to platelet membranes.

ii. 5'-nucleotidase and ATP/ADPase both remove circulating ADP, which is necessary for platelet aggregation, and convert the nucleotide to adenosine, an inhibitor of aggregation. Apyrase, present in the salivary glands of ticks, hydrolyses the ADP released from injured cells and activated platelets. Both soft ticks (*O. moubata*, *O. savignyi*) and hard ticks (*I. dammini*) have been shown to contain apyrase (Ribeiro *et al.*, 1991; Mans *et al.*, 1998a). Apart from inhibiting platelet aggregation, *O. savignyi* apyrase has also been shown to disaggregate platelets (Mans *et al.*, 1998a).

iii. Several fibrinogen receptor antagonists have been purified from snake venoms. These compounds, known as disintegrins, are a class of highly homologous proteins mostly containing the RGD (Arg-Gly-Asp) sequence which act as antagonists of the integrin GPIIb/IIIa complex on the platelet membranes. They are comparatively small proteins in the molecular weight range 6800-7500 Da, lacking any recognizable enzyme activity. A disintegrin, barbourin, isolated from the venom of *Sistrurus m. barbouri* is a KGD (Lys-Gly-Asp) protein (Scarborough *et al.*, 1991). Other receptor antagonists have been isolated from the snake venom of *Agkistrodon halys*, *Bitis arietans*, *Calloselasma rhodostoma*, *Echis carinatus* and *Tremesurus gramineus* (Ouyang *et al.*, 1992). Moubatin which inhibits collagen-induced aggregation (Waxman and Connolly, 1993) and disagregin which inhibits ADP- induced aggregation (Karczewski *et al.*, 1994) have been identified in the salivary glands of *O. moubata*. Another disintegrin, named variabilin, was isolated from the salivary glands of the hard tick *Dermacentor variabilis*. It inhibits ADP-induced aggregation via binding to the platelet membrane receptor GPIIb/IIIa and therefore also inhibits the binding of

fibrinogen to the platelets (Wang *et al.*, 1996). Disagregin does not contain the Arg-Gly-Asp (RGD) recognition sequence characteristic of disintegrins, whereas variablilin does. Savignygrin, the platelet aggregation inhibitor isolated from the salivary glands of *O. savignyi*, was found to possess the RGD sequence (Mans *et al.*, 2002a).

4.1.3 DETERMINATION OF PLATELET AGGREGATION

Aggregation in a suspension of platelets is detected on a macroscopic level by the development of visible clumps and clearing of the suspension. More sensitive measurements can be made in a platelet aggregometer, which is simply a photometer that records the clearing of a stirred suspension of platelets. Aggregation of platelets constitutes a multistep process, which can be analyzed by recording the tracing of changes in light transmission. Following the addition of certain agonists there is a decrease in light transmission due to a change in the shape of platelets from discoid to spherical. This is followed by a gradual increase in light transmission as the platelets aggregate and clear the way for the light to be transmitted (Zucker, 1989).

The initial phase of platelet aggregation is reversible unless it is followed by secretion of pro-aggregatory factors from the dense granules (e.g. ADP and serotonin) and α-granules (adhesive proteins such as fibrinogen, von Willebrand factor, thrombospondin, and fibronectin). The irreversible phase reaches a plateau, which reflects the maximal level of light transmission (Holmes *et al.*, 1999 and Zucker, 1989).

The *in vitro* study of platelets is done with platelet rich plasma (PRP) using an aggregometer to monitor the increase in light transmission at a wavelength of approximately 600 nm. In this method PRP is prepared from citrated whole blood and is placed in a cuvette equipped with a magnetic stirrer operating at approximately 9000 rpm and 37 °C. The PRP is set at 0 % transmission while platelet poor plasma (PPP), which acts as a blank, is set at 100 % transmission. Aggregation is recorded on a chart recorder. Initially small oscillations are observed

on the chart tracing, which are due to the asymmetrical discoidal platelets. In this resting state before platelets are activated, they have the appearance of smooth biconvex discs, with few if any pseudopods. Addition of an agonist leads to a decrease in transmission due to the shape change from a discoid to a symmetrical spherical form, which causes the oscillations to disappear.

Severe lipemia decreases the apparent response to aggregating agents in the platelet-rich plasma because the plasma will remain turbid even if all of the platelets have aggregated. Hence a fatty meal should not be ingested within 6-8 hr before blood donation (Zucker, 1989). Ideally donors should not have taken any drugs for a week before blood donation. Non-steriodal anti-inflammatory drugs inhibit the enzyme cyclo-oxygenase and thus prevent secretion via the arachidonate pathway. The effect of most such drugs does not persist when the drug is cleared from the system, except for that of acetylsalicylic acid (aspirin). This drug irreversibly blocks the cyclo-oxygenase enzyme and hence affects platelets as long as they survive (about 10 days). Secretion appears to recover within 4-5 days, since there are enough young uninhibited platelets that can synthesize endoperoxides. An enormous number of drugs contain aspirin, including Alka-Seltzer and Dristan. Some antibiotics, tranquilizers and tricyclic antidepressants are known to inhibit platelet function as well (Matsumoto *et al.*, 1999; Zucker, 1989).

In chapter 2, it was found that the fibrinogenolytic activity in CE-HPLC region C was mainly due to the action of a metalloproteinase activity. In this chapter the effect of region C on platelet disaggregation was investigated, since disaggregation by the metalloproteinases may play a role in the maintenance of the fluidity of the blood pool during tick feeding.

4.2 MATERIALS AND METHODS

4.2.1 MATERIALS USED

ADP, epinephrine, TRAP and collagen were obtained from Diagnostica Stago, USA.

4.2.2 PLATELET AGGREGATION STUDIES

All experiments were performed with platelets from blood of healthy donors who were denied any drug consumption (alcohol, garlic and aspirin) in the two weeks preceding blood collection. Human blood from a forearm vein was collected in 3.8 % trisodium citrate dihydrate (9v/1v). PRP was prepared by centrifugation of whole blood (120 x g for 10 min) and plasma was collected in plastic tubes. PPP was prepared by further centrifugation of the remaining blood from the platelet rich plasma at 2700 x g for 30 min. PRP was diluted with autologous PPP to give a platelet count of 300 x 10^9 platelets/l using a Coulter counter. Aggregation was determined by the optical method as described originally by Born and Cross (1963), using a platelet aggregometer. Platelet poor plasma was set at 100 % transmission and platelet rich plasma at 0 % transmission.

4.2.3 THE EFEECT OF THE FIBRINOGENOLYTIC ACTIVITY ON AGGREGATED PLATELETS.

To test for disaggregation activity, platelets (400 µl of the diluted PRP) were incubated at room temperature for 2 min to monitor for spontaneous aggregation. Aggregation was then induced by adding 20 µl of the different agonists to the platelet rich plasma and platelets were allowed to aggregate for 4 min until a plateau was reached. Agonists used were ADP (5 µM), collagen (0.2 µM), TRAP (5 µM), and epinephrine (5 µM). Thereafter, SGE (the equivalent of 0.02 SG), 10 µl saline solution (0,15 M) or the partially purified fibrinogenolytic activity (prepared as in section 3.3.2 and concentrated by freeze drying and reconstituted in saline solution) were added and their effect on platelet disaggregation was observed over 4 min. Aggregation and disaggregation were monitored as the respective increase and decrease in transmission using an aggregometer (Chronolog Corporation).

4.2.3 SCANNING ELECTRON MICROSCOPY (SEM)

Platelet aggregation was induced by ADP and allowed to proceed for ~ 4 min until a plateau was observed on the recorder trace, before addition of SGE (2 salivary glands in 20 µl) or partially purified fibrinogenolytic activity (50 µl). Disaggregation was allowed until a baseline was observed (4 minutes). The disaggregated platelets, as well as the resting (non-aggregated) and aggregated (allowed to aggregate for 4 min) platelet controls were diluted with 0.15 % gluteraldehyde solution (0.15 M phosphate buffered saline, 0.015 M EDTA, pH 6.5) and centrifuged at 180 x g for 10 min. The supernatant was discarded and the pellet was resuspended in 2.5 % gluteraldehyde (0.15 M PBS, pH 7.4) and fixed for 1 hour before centrifugation (180 x g 10 min). After washing with 0.075 M phosphate pH 6.5 (twice for 15 min each time) the platelets were pelleted again (2700 g, 10 min). The pellet was resuspended in 1 % osmium tetra-oxide after which it was pelleted again once more, washed in 0.075 M phosphate buffer pH 6.5 (twice for 15 min each time) and dehydrated sequentially with 30 %, 50 %, 70 % and 100 % ethanol (three times for 15 min each time). The platelets were then critical point dried in CO_2 and sputter-coated with gold prior to SEM analysis using a JOEL 840 SEM. Scanning electrographs were obtained using an accelerating velocity of 5 kV and a magnification of x5000.

4.3 RESULTS

4.3.1 THE EFFECT OF SALIVARY GLAND EXTRACTS AND PARTIALLY PURIFIED REGION C ON PLATELET DISAGGREGATION

With aggregometry, disc to sphere transformation is observed in a stirred suspension of platelets as a decrease in the amplitude of the recorder pen oscillations. The change in amplitude is due to light- deflecting differences between discs and spheres. Because discoid platelets are asymmetrical, they are oriented by the shearing forces of the stir bar to produce a visible swirl, which is recorded as rapid oscillations. Immediately after addition of the aggregating agent, discoid platelets transform into spheres, which no longer show the swirl effect and the oscillations disappear. In Fig 4.1 it can be seen that the light transmission gradually increased in both cuvettes as aggregation took place after addition of ADP to the diluted PRP. In both cuvettes the aggregation reached a plateau 4 min after addition of ADP. The difference in aggregation response curves for the control and experiment cuvettes in Fig 4.1 was shown to be due to the difference in the measurement of the responses by the two channels of the aggregometer (Mans *et al.*, 1998a). In this experiment saline was added to both cuvettes after aggregation (indicated by thin arrows in Fig 4.2) and served as controls for the disaggregation experiments. As can be seen from Fig 4.1, addition of saline had no effect as no disaggregation effect was observed.
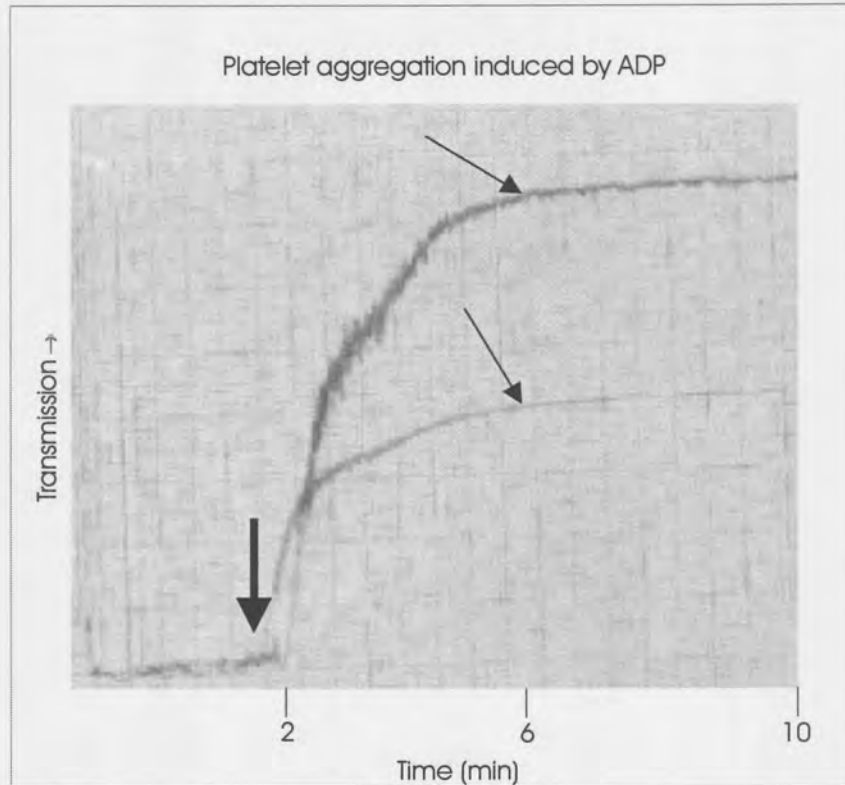
Fig 4.2: Aggregation of PRP induced by ADP. In both the control and experiment cuvettes, PRP was incubated alone for 2 min to monitor spontaneous aggregation, after which ADP was added to induce aggregation at the point of the thick arrow. Aggregation was then allowed for 4 min, before addition of 0.15 M saline solution (thin arrows) and monitored for a further 4 min.

Anti-platelet substances have previously been identified in the SGE of the tick, *O. savignyi* (Mans *et al.*, 1998a). These studies showed that extract prepared form 0.02 glands was able to disaggregate ADP-induced aggregated platelets. It is possible that the observed disaggregation by the SGE could also be due to the fibrinogenolytic activity since this activity has been identified in the tick SGE. Aggregation induced by any agonist should be affected, as proteolysis of fibrinogen results in physiological abrogation of the link between platelets. It was thus of interest to investigate disaggregation of platelets aggregated by different agonists: ADP, TRAP, collagen, and epinephrine.

The SGE (0.02 SG) showed a disaggregating effect on the platelets aggregated by different agonists: ADP (Fig 4.3B), epinephrine (Fig 4.4B), collagen (Fig 4.5B) and TRAP (Fig 4.6B).

Addition of the partially purified fibrinogenolytic activity (region C) to ADP aggregated platelets caused disaggregation of the platelets (Fig 4.3A). To determine whether the observed inhibition was specific for ADP-induced aggregation, the experiment was repeated with different agonists. The results with different agonists clearly show that the disaggregation effect is not specific to ADP-induced aggregation. The same disaggregation effect was observed when epinephrine (Fig 4.4A), collagen (Fig 4.5A) and TRAP (Fig 4.6A) were used as agonists.
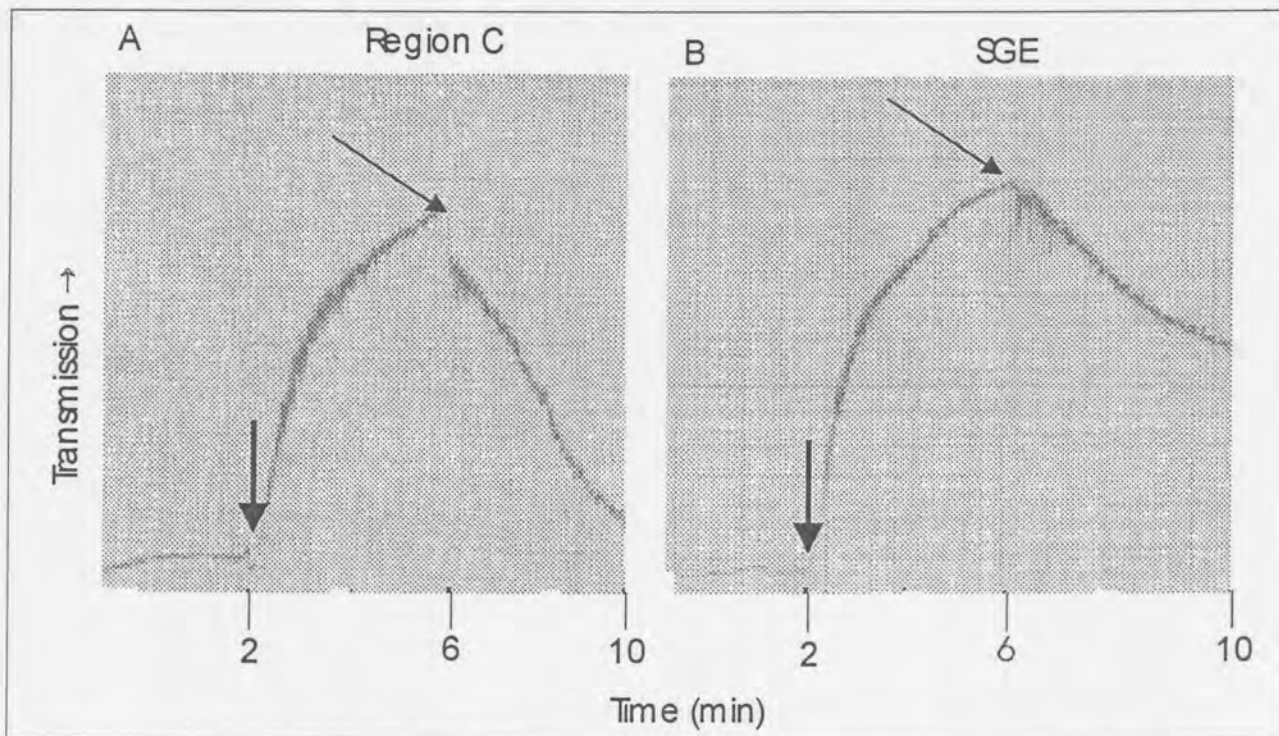
Fig 4.3: Effect of region C and SGE on platelets aggregated by ADP. PRP was incubated at room temperature for 2 min to monitor for spontaneous aggregation. Thereafter, platelet aggregation was induced by addition of ADP (thick arrows). After complete aggregation (4 min), in A) region C and in B) SGE was added (indicated by thin arrows) and disaggregation was monitored for 4 min.
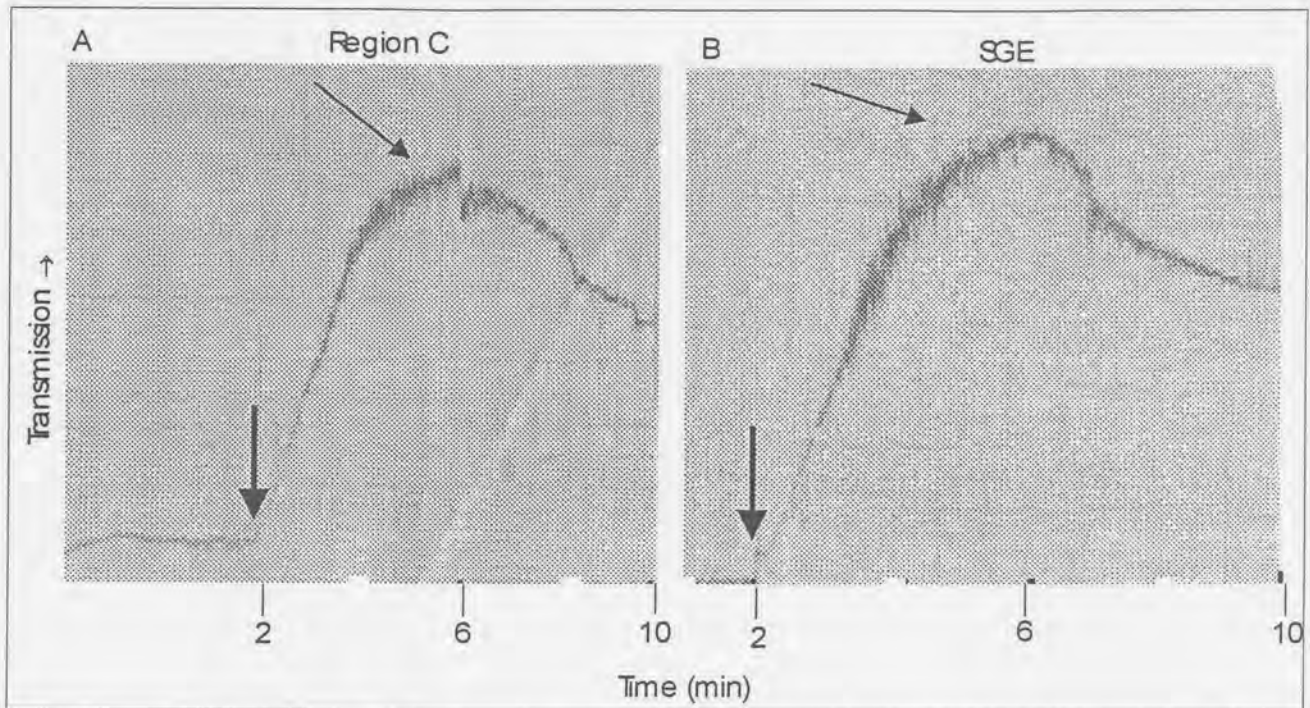
Fig 4.4: Effect of region C and SGE on platelets aggregated by epinephrine. PRP was incubated at room temperature for 2 min to monitor for spontaneous aggregation. Thereafter, platelet aggregation was induced by epinephrine (thick arrows). After complete aggregation (4 min), in A) region C and in B) SGE was added (indicated by thin arrows) and disaggregation was monitored for 4 min.
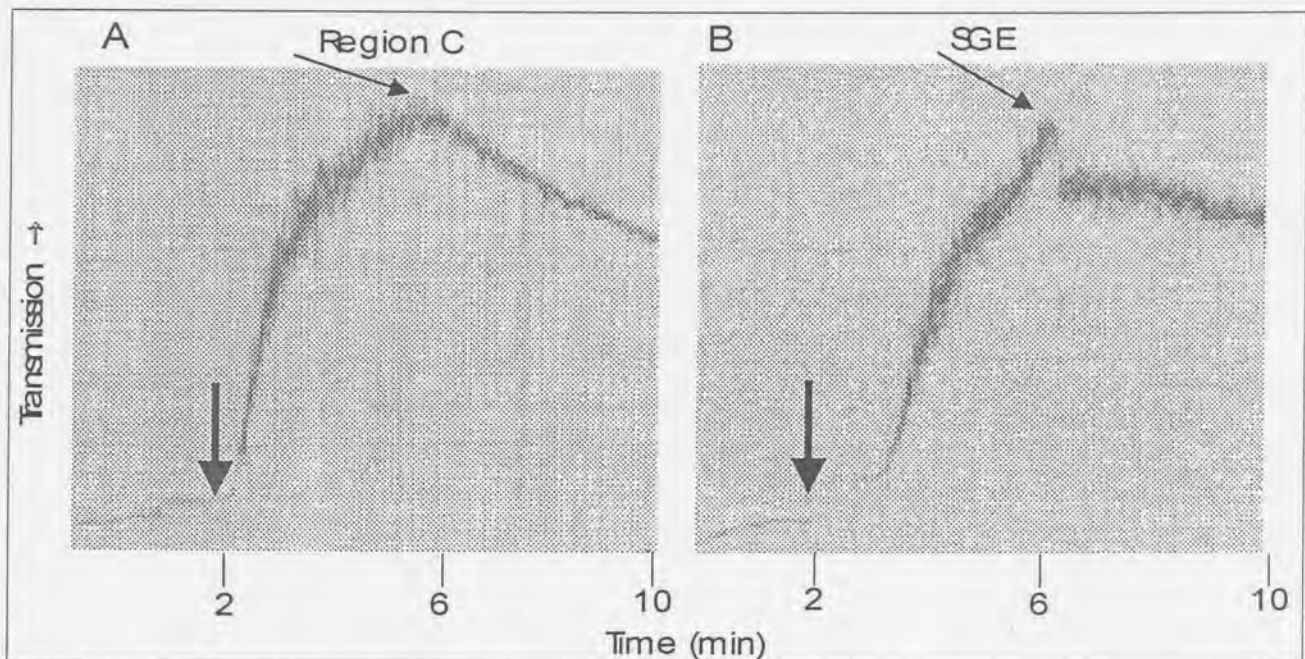
Fig 4.5: Effect of region C and SGE on platelets aggregated by collagen. PRP was incubated at room temperature for 2 min to monitor for spontaneous aggregation. Thereafter, platelet aggregation was induced by collagen (thick arrows). After complete aggregation (4 min), in A) region C and in B) SGE was added (indicated by thin arrows) and disaggregation was monitored for 4 min.
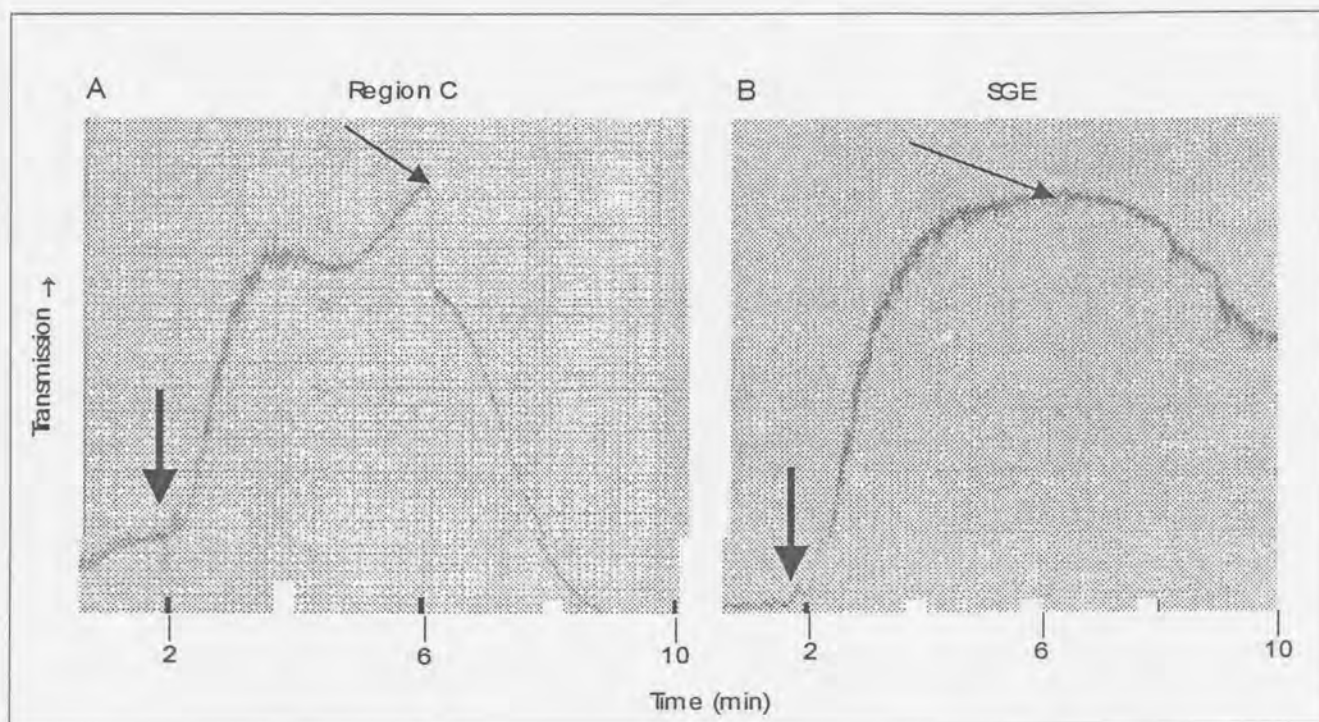
Fig 4.6: Effect of region C and SGE on platelets aggregated by TRAP. PRP was incubated at room temperature for 2 min to monitor for spontaneous aggregation. Thereafter, platelet aggregation was induced by TRAP (thick arrows). After complete aggregation (4 min), in A) region C and in B) SGE was added (indicated by thin arrows) and disaggregation was monitored for 4 min.

4.3.2 INVESTIGATION OF PLATELET DISAGGREGATION BY SEM

To investigate the mechanism of platelet disaggregation involved, platelet aggregation was induced with ADP, epinephrine, collagen and TRAP, respectively, and after 4 min partially purified fibrinogenase or the SGE was added and disaggregation was allowed for another 4 minutes. The resting (uninduced, non-aggregated) platelets, platelets aggregated by various agonists and disaggregated platelets by SGE/fibrinogenase (region C) were analyzed using SEM. The resting platelets show a dominating discoid form (Fig 4.7A). As can be seen in Fig 4.7B, ADP-induced aggregation resulted in change of the shape of the platelets from a discoid to a spherical form. Numerous extended pseudopods were also visible. disaggregation by the fibrinogenase or SGE showed that the disaggregated platelets retained a spherical form with extended pseudopods (Fig 4.8, 4.9, 4.10 and 4.11).

A

B
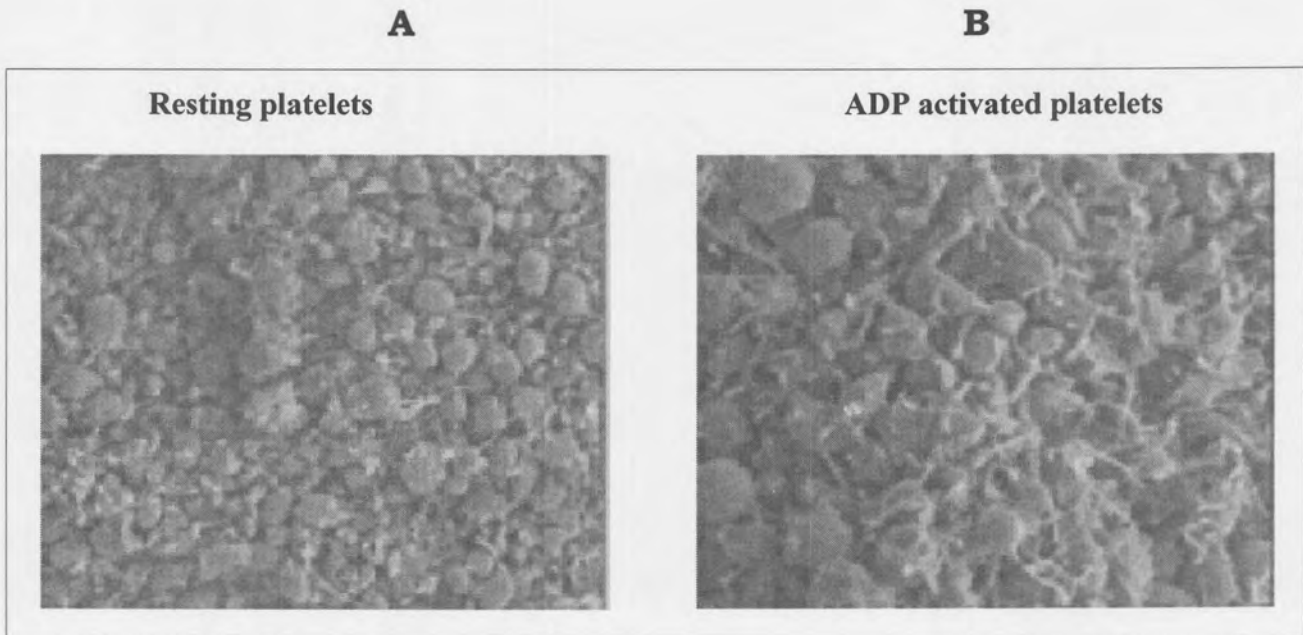
Fig 4.7: SEM analysis of A) resting platelets and B) ADP activated platelets. Scanning electrographs were obtained using an accelerating velocity of 5 kV and a magnification of x5000.

A

B
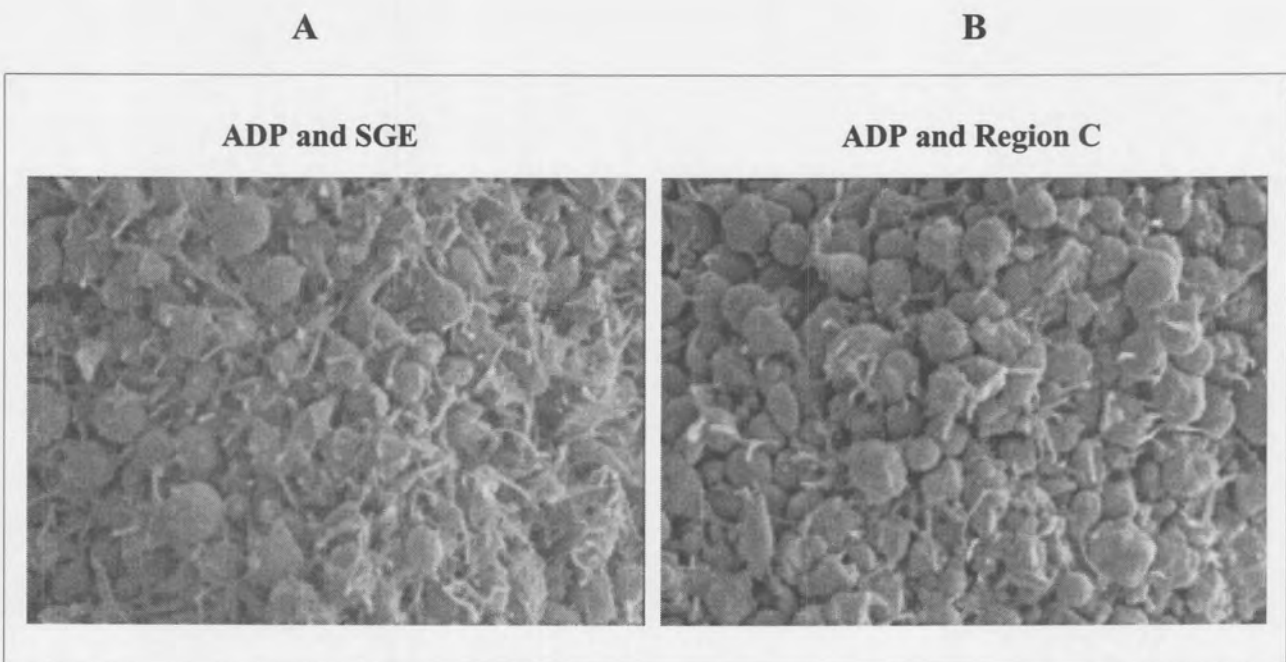
Fig 4.8: SEM analysis of the disaggregated platelets. Platelets were aggregated with ADP for 4 min and disaggregated with A) SGE and B) region C for 4 min. Scanning electrographs were obtained using an accelerating velocity of 5 kV and a magnification of x5000.

A   B

| Collagen and SGE | Collagen and Region C |
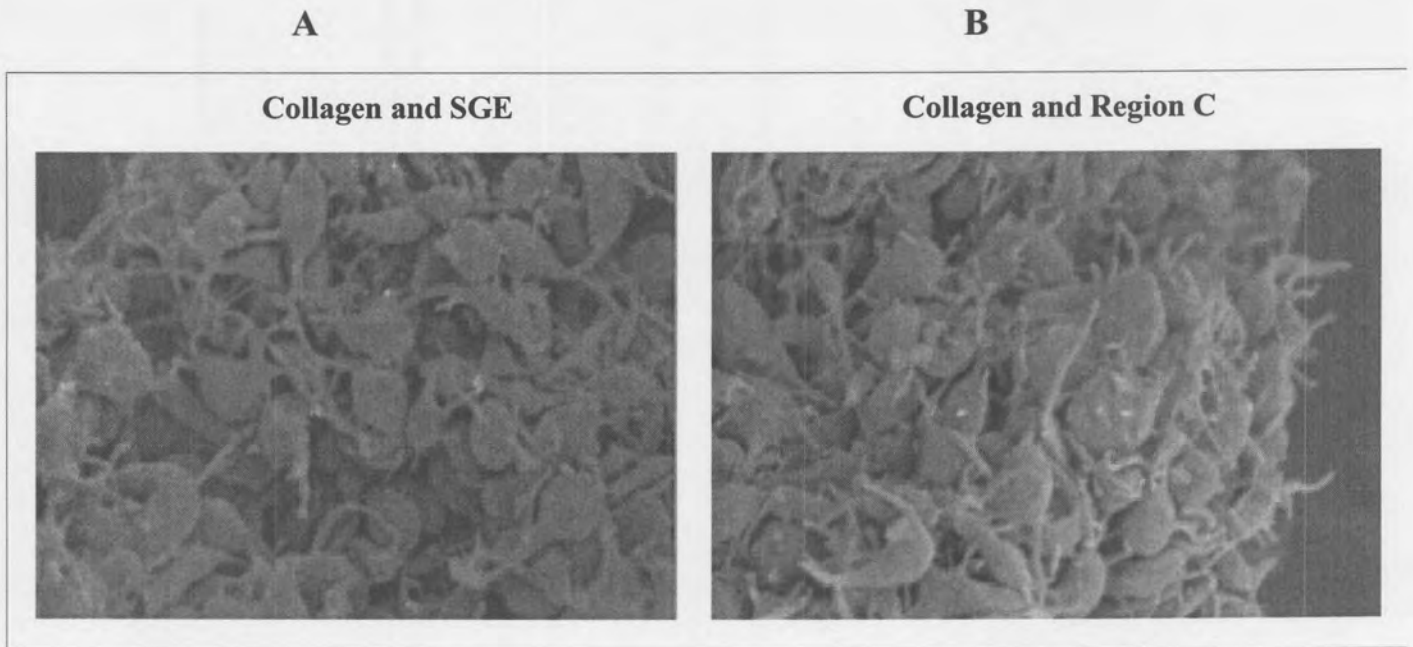
Fig 4.9: SEM analysis of the disaggregated platelets. A) Platelets aggregated with collagen for 4 min and disaggregated with SGE for 4 min. B) Platelets were aggregated with collagen for 4 min and disaggregated with region C for 4 min. Scanning electrographs were obtained using an accelerating velocity of 5 kV and a magnification of x5000.

A   B

| TRAP and SGE | TRAP and Region C |
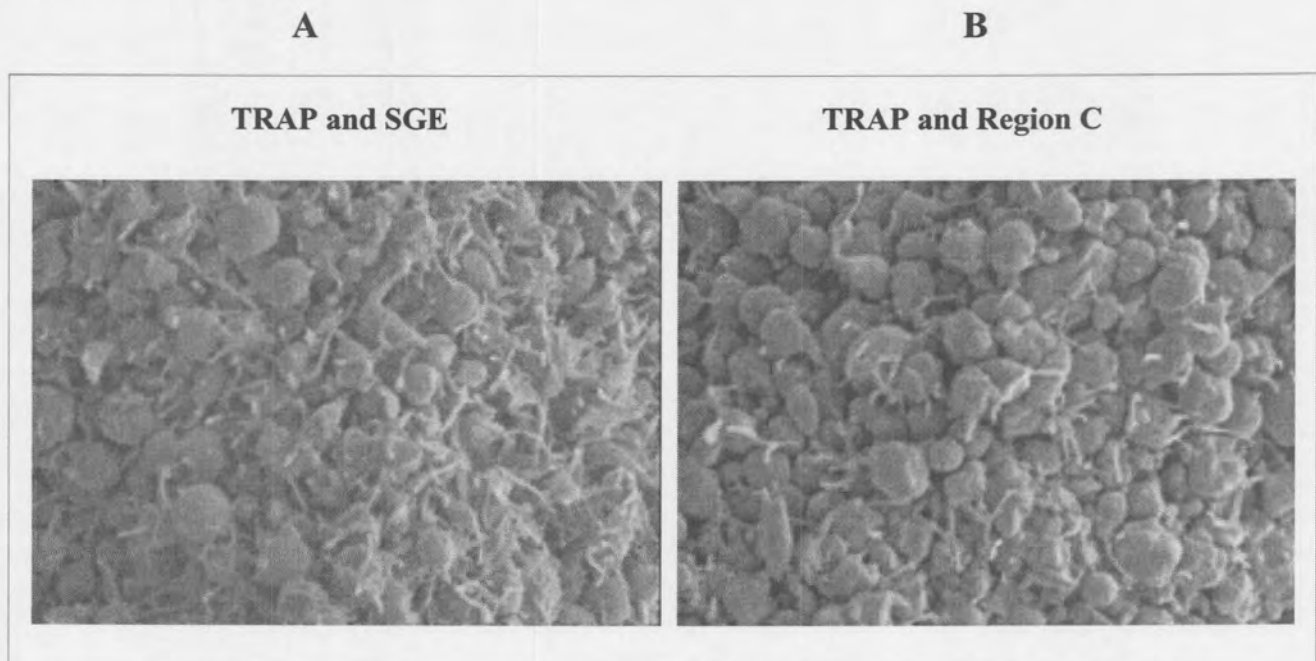
Fig 4.10: SEM analysis of the disaggregated platelets. Platelets were aggregated with TRAP for 4 min and disaggregated with A) SGE and B) region C for 4 min. Scanning electrographs were obtained using an accelerating velocity of 5 kV and a magnification of x5000.

A   **B**
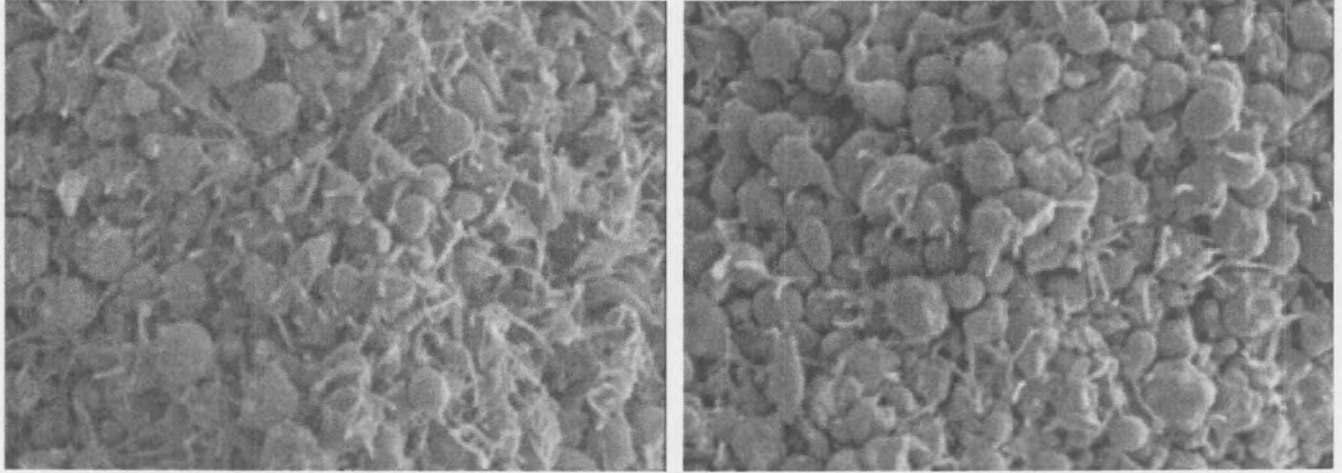
Fig 4.11: SEM analysis of the disaggregated platelets. Platelets were aggregated with epinephrine for 4 min and disaggregated with SGE A) and region C for 4 min. Scanning electrographs were obtained using an accelerating velocity of 5 kV and a magnification of x5000.

4.4 DISCUSSION

As mentioned previously investigations conducted by Mans *et al.*, (1998) showed that the SGE not only inhibited platelets aggregation induced by various agonists, but was also able to disaggregate platelets aggregated by ADP. In subsequent investigations apyrase was purified from the SGE and it was conclusively shown that this enzyme is responsible for both of the above observations (Mans *et al.*, 1998b, 2000). As fibrinogen cross-links activated platelets to form aggregates, it was hypothesized that the aggregation effect observed for the SGE could also be due to the fibrinogenolytic activity present in the extract. For this reason the effect of the partially purified fibrinogenase activity on aggregated platelets was investigated. It was found that SGE or partially purified fibrinogenase disaggregated the platelets and this was independent of the platelet agonist used (Fig 4.3-4.6). This is in agreement with the hypothesis by Peerschke (1985), that platelet aggregation, regardless of the agonist, is mediated by a final common pathway, namely, the exposure of fibrinogen receptors. During disaggregation, platelets revert from a spherical to a discoid form. By using SEM it would be possible to see whether fibrinogen induced disaggregation caused platelets to change back to the discoid form. It may distinguish between fibrinogenolytic activity induced disaggregation and an apyrase specific mechanism since when platelets are disaggregated by a fibrinogenolytic activity, a change in the shape of the platelet would be not be expected, because fibrinogen is cleaved, but the fibrinogen fragments will still be bound to its receptor. Platelets would thus retain their spherical form and pseudopods, while in the apyrase dependent platelet disaggregation (removal of ADP from the platelet surface), the platelets regain their discoid form as happens during reversible aggregation at low ADP concentrations (Mans *et al.*, 2000). Platelets disaggregated by fibrinogenase retained their spherical form and pseudopods (Fig 4.7-4.11). These results therefore clearly demonstrate that in addition to apyrase, the fibrinogenase activity is also involved in the disaggregation of aggregated platelets.

Platelet disaggregation has been observed in various hematophagous organisms. The enzyme, hementin in the salivary glands of the leech *Haementeria ghiliiani*, has

a fibrinogenolytic activity (Sawyer *et al.*, 1991). It showed a disaggregation effect on the aggregated platelets induced by ADP as well as collagen. The apyrase associated disaggregation was not observed with thrombin-aggregated platelets (Mans *et al.*, 1998), yet TRAP-activated platelets were disaggregated by the fibrinogenolytic activity.

Ouyang *et al.* (1992) proposed that α-fibrinogenases are platelet aggregation inhibitors assuming that the intact Aα-chain may be essential for the binding of fibrinogen to the platelet membrane. This suggestion was based on the reported inhibition of platelet aggregation by the products of fibrinogen degradation caused by some α-fibrinogenases (Markland *et al.*, 1991 and 1998). Although some previous work has demonstrated a limited role of the C-terminal parts of α-chains in platelet fibrinogen binding, cleavage of these parts by plasmin does not interfere with the interaction of fibrinogen with ADP-stimulated platelets (Kamiguti *et al.*, 1994). That the Aα 572-574 RGD sequence is not required for fibrinogen-platelet interaction was most convincingly demonstrated with recombinant fibrinogen altered by site directed mutagenesis (Farrel *et al.*, 1992). Jararhagin, an α-fibrinogenase from the snake venom of *Bothrops jararaca*, removes a 23 kDa fragment from the C-terminal of the Aα-chain and has no effect on ADP-platelet aggregation (Kamiguti *et al.*, 1994). This is because jararhagin and other venom α-fibrinogenases do not attack the fibrinogen γ-chain, which contains the more important platelet binding site (Kamiguti *et al.*, 1998). Jararhagin is known to inhibit collagen induced aggregation in both PRP and washed platelet suspensions (Kamiguti *et al.*, 1994). This provides further evidence that the Aα 572-574 RGD sequence is not required. The α-fibrinogenase of *Tremesurus mucrosquamatus* snake venom inhibits aggregation in the platelet rich plasma, but not in washed platelet suspensions (Ouyang *et al.*, 1979). Proteinase F1 of *Naja nigricollis* venom inhibits aggregation with collagen in PRP only (Kini *et al.*, 1991). In contrast the α-fibrinogenase from *Agkistrodon rhodostoma* venom inhibits platelet aggregation in washed platelet suspensions, but not in PRP (Ouyang *et al.*, 1985). The inhibition by both these enzymes is thought to be dependent on their catalytic activity and fibrinogen degradation. In this dissertation the effect of the partially purified

fibrinogenase on inhibition of platelet aggregation, was not tested on platelet aggregation induced by various agonists. It is logical to assume that the fibrinogenase will most probably also inhibit platelet aggregation, but this remains to be investigated in future experiments.

Inhibition of platelet aggregation was also observed by F1 proteinase, an α-fibrinogenase from *Naja nigricollis crawshawii* venom (Kini and Evans, 1991). This enzyme inhibits aggregation in whole blood, but not in washed platelet suspensions. Fibrinogen degradation products generated by F1 proteinase do not inhibit aggregation. The enzyme inhibits platelet aggregation in blood preparation reconstituted by defibrinogenated plasma. Thus, the inhibitor of platelet aggregation by this enzyme is dependent on fibrinogen degradation. Lebetase, a fibrinogenolytic enzyme from the snake venom, *Vipera lebetina*, inhibits ADP-induced and collagen induced platelet aggregation in PRP in a dose-dependent manner (Siigur *et al.*, 1998). Lebetase did not inhibit platelet aggregation on washed platelets (Siigur *et al.*, 1998). The enzyme may be inhibiting platelet aggregation by destroying the intact fibrinogen and the resulting fibrinogen degradation products concur to the inhibition process. Metalloproteinase L4 from the venom of Chinese mamushi (*Agkistrostron halys brevicaudus*) also inhibited ADP-induced platelet aggregation. Fujimura *et al.* (1995) suggest that protease L4 may release RGD-containing peptides which act as competitive inhibitors, as fibrinogen Aα-chain contains two RGD sequences that recognize and bind to glycoprotein IIb/IIIa. On the other hand, non-enzymatic inhibitors, disintegrins, interfere in the interaction between fibrinogen and GPIIb/IIIa complex by binding to the fibrinogen receptor (Gould *et al*, 1990).

The subsistence of ticks on a diet of fresh blood is dependent on their ability to interfere with the hemostatic system of the blood donor. The saliva of ticks contains a variety of factors, which presumably act in concert to satisfy two essential requirements: maintain prolonged bleeding from the wound and preserve blood fluidity following ingestion of a blood meal. Fibrinogen, which serves as a connecting protein during platelet aggregation is under attack during feeding. The salivary glands of *O. savignyi* produces an enzyme which cleaves fibrinogen and

disaggregates platelets that have aggregated. The fact that the enzyme can disaggregate platelets that have aggregated implies that if platelet aggregation does occur somewhere in the narrow lumen of the proboscis, the tick has a fibrinogenolytic activity mediated mechanism to dissolve such a platelet rich clot, thereby restoring blood flow through the proboscis.

Another point of attack for the fibrinogenolytic enzyme may be the blood coagulation pathway as fibrinogen is the precursor of fibrin. Degradation of fibrinogen will therefore also inhibit the process of blood coagulation. Future experiments should therefore include the testing of the effect of the fibrinogenase activity on blood coagulation. In these experiments, the fibrinogenase activity may either be tested using assays specific for the intrinsic or extrinsic coagulation pathways or directly on the thrombin-mediated conversion of fibrinogen to fibrin. It is expected that the coagulation of fibrinogen by thrombin will be significantly retarded when fibrinogen is incubated with the fibrinogenolytic activity before thrombin attack.

CHAPTER 5

CONCLUDING DISCUSSION

Successful feeding of hematophagous ectoparasites requires that the host's blood remain in a fluid state. Hence, the saliva of these ectoparasites contains a cocktail of vasodilators and anticoagulants in order to maintain blood in a liquid state at the site of the lesion as well as immunosupressors to evade their host's defense mechanisms.

The ubiquitous presence of anticoagulants in hematophagous arthropods illustrates that coagulation presents a major challenge to successful feeding. The numerous examples of anticoagulants that have gut origins in addition to secretion in the saliva indicate that these molecules are important in maintaining the flow of blood from the feeding site to the digestive tract (Stark and James 1996). The large number of anticoagulants produced in ticks and bugs suggests that a fluid bloodmeal is crucial for these organisms to facilitate their feeding strategies (Stark and James 1996). The potent pharmacological activities in vector saliva indicate that, in many cases, parasites may require the priming of the non-immune host's skin by its vector's saliva. It has been found that *Leishmania* transmission is enhanced by sandfly salivary homogenates, and Toghoto virus transmission is similarly enhanced by tick salivary homogenates. The development of an anti-tick vaccine could lead to a decrease in tick–borne diseases, including Lyme disease. Even if this approach fails, the spin off research may be worth it. Bloodsuckers are leading us to some novel potent pharmaceuticals. This is the time to mine drugs from bugs (Ribeiro, 1995b).

Because ticks have evolved to utilize mammals as a source of food, our understanding of the tick biochemistry will enhance the control of tick infestation as well as the possible exploitation of the tick's natural resources for beneficial purposes. Research in this field will undoubtedly remain a fertile field for innovative studies by scientists for many years to come (Sauer *et al*, 1995). Research on anti-

hemostatic substances is of considerable importance in terms of structure/function studies. The recombinant production of antihemostatic agents renders them ideal tools for evaluation as anti-tick vaccines and possibly therapeutic agents (Lehman, 1993).

The soft tick *O. savignyi* possesses numerous potent antihemostatics that could be used as potential protein vaccine candidates. This follows from the fact that these ticks pose a significant health risk to humans and domestic livestock due to the toxins they transmit. Because of the developed tick resistance to acaricides, we need alternative means to control them. The current study focused on the identification and characterization of proteolytic activities present in tick salivary gland extracts and more specifically on the fibrinogenolytic activity.

Previous experiments in our laboratory had shown that the SGE was not able to degrade fibrin, but degradation of fibrinogen was observed. In an attempt to investigate whether the fibrinogenolytic activity may be associated with antihemostatic activity, salivary glands were firstly tested for proteolytic activity against the nonspecific protease substrate, azocasein. Salivary gland extracts of *O. savignyi* hydrolyzed azocasein in a concentration-dependant manner. Proteolytic activity was found in the pH range of 3 to 11 with the highest activity at pH 9 followed by pH 7. Activity at the lower pH range, (pH 3 and 5) was found to be due to aspartic proteinases, while in the higher pH range, (pH 7 and 9), the activity was mainly due to the metallo- and serine proteinases. The activity observed at pH 11 was due to aspartic- and metalloproteinases. Aspartic and cysteine proteases normally function in an acidic environment. The observed broad activity range of the tick aspartic protease is uncertain and suggests that this enzyme is very stable. This stability may contribute to this enzyme still showing some activity (20 % relative to the highest activity at pH 9) in the basic range where they normally do not function. It would also be interesting to investigate the temperature stability of this enzyme in future experiments. The aspartic proteinases are most probably released from the lysosomes during the preparation of the salivary gland extract and are likely not to be directly involved in the feeding process. However, the serine- and metalloproteases are possibly involved in the feeding process as their pH

correspond with the pH of the tick saliva and the host blood. Therefore, the characterization of the proteolytic activity in saliva would give a more accurate indication of the enzymes involved in the feeding process.

The fibrinogenolytic activity present in the salivary gland extracts (SGE) of *O. savignyi* was confirmed by incubating human fibrinogen in the presence of SGE and monitoring the fibrinogen degradation products on Coomassie-blue stained polyacrylamide gels. The activity was found to be α-specific as the Aα-chain of fibrinogen was degraded within 2 hours of incubation with no apparent degradation of the Bβ- or γ-chains for fibrinogen even after 24 hours of incubation at 37 °C. The activity was found to be inhibited by the metal chelator, EDTA. Characterization of the fibrinogenolytic activity with protease inhibitors indicated that fibrinogenolytic activity was present at pH 3-9 and could be ascribed to the combined action of various classes of proteases. The fibrinogenolytic activity at pH 3 and 5 was mainly due to the presence of the cysteine and aspartic proteases, while the metalloprotease proved to be the major fibrinogenolytic activity present and active over the whole pH range of 3-9. The observed metalloprotease activity in the low pH range is uncertain and could indicate the stability of the enzyme and residual activity under extreme pH conditions. These experiments also showed that in the crude extract serine proteinases were not responsible for the fibrinogenolytic activity.

Attempts to characterize the activity with inhibitors on substrate gels were unsuccessful, as the activity was very sensitive to the conditions used in the zymography system. Even when SDS was eliminated in all steps, very little activity could be found at pH 3 and a smear of activity at pH 11 and only in the presence of 1 mM DTT in the incubation buffers. DTT is normally added to the reaction buffers so as to stabilize the SH-groups in proteins (Barrett, 1994). The activities at pH 3 and 11 were found not to be autohydrolysis at extreme pH values since no degradation was observed in the control gel (no SGE present) run under the same conditions.

Purification of proteins by conventional chromatography is usually achieved by combining methods including size exclusion-, ion-exchange-, hydrophobic- and reversed phase-HPLC. Separation of the SGE using cation-exchange chromatography resulted in three regions of proteolytic activity with azocasein as substrate. Characterization of the proteolytic activity associated with the various active fractions was done using protease inhibitors. From these results it was deduced that the tick SGE possesses a cocktail of different proteases. Region A was mainly composed of the cysteine and aspartate proteases; region B was mainly due to the activity of the metallo- and serine proteases, while region C was mainly due to the metalloproteases (Table 2.3). In this dissertation the metalloproteinase activity in the salivary gland extract was detected using azocaseine and fibrinogen as substrates. It would be interesting to test the substrate specificity of the purified metalloproteinase. Proteolytic degradation of the extracellular matrix proteins would imply that the enzymes may be in addition to its anti-hemostatic role be important in producing local bleeding by causing lesions in the walls of the small blood vessels as observed for various snake venoms (Ohsaka, 1979 and Ownby, 1990). It is believed that local bleeding is caused by proteolysis of components of the basal lamina of the microvasculature (Ohsaka *et al.*, 1973; Bjarnason and Tu, 1978, and Bjarnason *et al.*, 1988). These enzymes degrade all major proteins of the extracellular matrix (Baramova *et al.*, 1989; Baramova *et al.*, 1990 and Bjarnason and Fox 1989). This is also the case with cell-secreted soluble matrix metalloproteinases (MMP). It has been well documented that the venom metalloproteinases because of their broad substrate specificity cause digestion of the extracellular matrix proteins and damage the integrity of the blood vessels (Kamiguti *et al.*, 1998). The enzymes that degrade the components of the extracellular matrix have been reported to play an important role in tumor invasion and metastasis (Yamagata *et al.*, 1989). The extracellular matrix components are rich in various types of collagen (Fortunato *et al.*, 1997).

Past studies also shed little light on the possibility that metalloproteases can have effects on other components of the hemostatic mechanism and thereby also contribute to systemic bleeding (Kamiguti *et al.*, 1998). Serine proteinases have been linked to blood feeding activities in a number of blood hematophagous

arthropods. The trypsin-like enzymes from *A. aegypti* (Noriega *et al.*, 1996) and the black fly, *Simulium vittatum* (Xiong and Jacobs-Lorena, 1995).

The tick metalloproteinase may also be important in provoking rapid spreading of salivary gland antihemostatics from the injected area into the systemic circulation, as well as causing local tissue damage. It is likely that the components are easily diffused to the tissue and absorbed into the vessel by the degradation of extracellular matrix and vascular basement membranes by metalloproteinases. Tick metalloproteases may play key roles in the pathogenesis of both local and systemic complications resulting from tick bites and therefore may become target molecules for developing a novel therapy for tick bites.

Characterization of the fibrinogenolytic activity in the three CE-HPLC regions indicated that in region A all four enzyme classes were able to hydrolyze fibrinogen at pH 7 and 9. As explained before this was as a result of acidic proteins as well as unabsorbed basic proteins present in this region. Fibrinogenolytic activity in region B and C was due to the combined action of serine-, cysteine- and metalloproteinases. However, in region C the main fibrinogenolytic activity was due to the metalloproteinase. The presence of serine proteinase activity was unexpected, as such activity was not detected when the crude extract was incubated with fibrinogen. The serine proteinase activity in these regions is most likely as a result of the removal of a regulatory endogenous inhibitor from the proteinase during the purification step.

In contrast to the results found with the crude SGE, HPLC characterization of the fibrinogenolytic activity indicated the activity in the pH range of 7-9, while the crude indicated the activity in the pH 3-9 range. This could indicate that the activities associated with pH of 3 and 5 are less stable and lost their activity on the HPLC column. The other possibility could be the removal of a cofactor on the HPLC column and hence the loss of proteolytic activity. It must also be stressed that no protease inhibitors were added during the preparation of the crude extracts. During cell lysis many proteinases are released from intracellular organelles (e.g. lysosomes) and activate or inactivate many other released proteinases. It must also

be kept in mind that when SGE were prepared in buffer pH 3 or 5 many inactive forms may have been converted to active forms by the acidic conditions. SGE for the CE-HPLC on the other hand was prepared at pH 6.1 and would therefore not contain the proteinases resulting from autocatalytic activation.

Purification of the fibrinogenolytic activity was attempted by the combination of various HPLC steps. The problem encountered during the entire study was the lack of a more sensitive and faster bio-assay for the fibrinogenolytic activity in order to compile a purification table. SDS-PAGE analysis of the degradation products proved to be suitable for following the isolation procedure, but required more sample and was more labor-intensive. Separation with cation exchange HPLC resulted in three regions exhibiting fibrinogenolytic activity. From these experiments it could be deduced that proteins associated with region A possess an acidic pI, while proteins in regions B and C are basic in nature. Region A was further purified on the HIC-column and activity eluted in several peaks. Each HIC-peak was individually applied to a SE-HPLC-column and all activity containing peaks from each SE-HPLC run were pooled as they all had similar retention times. The fact that activity was spread over several peaks after HIC can possibly be due to the aggregation of the enzyme with itself or with other proteins. The combined sample was analyzed for purity using C5-reversed phase chromatography and reducing tricine SDS-PAGE. This revealed three bands of relative molecular masses of 15, 22 and 12 kDa, respectively. The proteins may be the aspartic and cysteine proteases but this remains to be confirmed.

In an attempt to purify the fibrinogenolytic activity associated with the basic pI, region C from several CE-HPLC runs was applied to a fibrinogen-affinity column. Analysis of the bound material with SDS-PAGE under reducing conditions showed two bands of relative molecular masses of 31 and 39 kDa, respectively. These proteins may be associated to the metallo- and serine proteases. Affinity chromatography was used recently in the isolation method of Ba100, a fibrinogenase from the venom of *Bitis arietans* (Jennings *et al.*, 1999). The enzyme was isolated in a two-step procedure employing SE-HPLC and affinity

chromatography. The authors also found two bands, which after sequencing were shown to share 66.7 % identity and also appear to be homologous to the N-termini of the C-type lectins from the crotalids and vipers. As mentioned previously in chapter 3, protein bands (31 and 39 kDa) were not sequenced due to insufficient amounts. The N-terminal sequence of these proteins would be useful as a primer could be designed and then used together with a poly-T primer to generate cDNA from the salivary gland mRNA in a reverse trancriptase- PCR reaction. Alternatively if a cDNA library is available the primer could be used as a probe to select fibrinogenolytic enzyme genes from the library. Once a full nucleotide sequence is known, it can be compared with that of known fibrinogenolytic enzymes.

CE-HPLC (region C), which was found to be mainly associated with the metalloproteinase activity, was found to disaggregate platelets aggregated by ADP, epinephrine, collagen as well as TRAP. The disaggregation is most probably due to the hydrolysis of the fibrinogen cross-liking platelets by the proteolytic activity present in region C. The platelet disaggregation by the fibrinogenolytic activity of ADP-induced platelets was steeper and complete and resembles the one observed with plasmin (Mans *et al.*, 2000). The fibrinogenolytic activity disaggregated platelets irrespective of the mechanism of aggregation, because it degrades fibrinogen, which serves as a molecular anchor between platelets. This together with the fact that there was no recognizable shape change (platelets retained a spherical form with numerous pseudopods) suggests that the mechanism of platelet disaggregation by the fibrinogenolytic activity resembles that of plasmin (Mans, *et al.*, 2000). The fact that the enzyme can disaggregate platelets that have aggregated implies that if platelet aggregation occurs somewhere in the narrow lumen of the proboscis, the tick employs a fibrinogenolytic activity mediated mechanism to dissolve such a platelet rich clot, thereby restoring blood flow thorough the proboscis.

Most venomous α-fibrinogenases, which degrade the Aα-chain are zinc metalloproteases. These enzymes also act on the β-chain of fibrinogen, but at a slower rate (Terada *et al.*, 1999). Some of these enzymes seem to be functionally similar to the fibrinogenolytic activity in the SGE of *O. savignyi* as they are also

metalloproteinases inhibited by EDTA, requiring a metal ion for activity and cleave fibrinogen. Studies on venom metalloproteinases showed that they can inhibit platelet aggregation *in vitro* (Ouyang *et al.*, 1979). As the majority of these enzymes are also known to hydrolyze the Aα-chain of fibrinogen (Markland, 1991) and since fibrinogen is an important cofactor in platelet aggregation (Hawiger *et al.*, 1982 and Plow *et al.*, 1984), it has been proposed that the inhibition of the platelet aggregation caused by these enzymes is due to the degradation of fibrinogen (Ouyang *et al.*, 1992 and Teng and Huang, 1991). However, Kamiguti *et al.* (1998) have shown that proteolysis of the C-terminal of the fibrinogen Aα-chain (which contains an RGD sequence) by jararhagin, does not affect fibrinogen-dependent platelet aggregation. This is mainly because jararhagin and other venom α-fibrinogenases do not attack the fibrinogen γ-chain, which contains the more important platelet-binding site (Kamiguti *et al.*, 1998).

Reviews by Markland (1991 and 1998) are indicative of the growing interest in this field owing to the potential therapeutic value of the fibrinogenolytic agents in clinical thrombotic disorders. Of all the different types of fibrinogenolytic enzymes that have been studied, more work has been carried out on snake venoms. To date no fibrinogenolytic activity has been isolated and characterized in any tick species. One approach to improving treatment of thromboembolic disorders involves the design and testing of inhibitors that block specific stages of the coagulation cascades. Anticoagulants isolated from ticks, earthworms, leeches, vampire bats and many more hematophagous organisms could all be evaluated as anti-thrombotic drugs. The drugs that have been used in the past, like heparin, all have certain drawbacks. These include a short half-life in circulation, systemic bleeding and a low affinity for the fibrin clot. There is a definite need for stable, potent and substrate specific anticoagulants since thrombosis is a major cause of death, resulting from the occlusion of diseased arteries and veins that leads to myocardial infraction, stroke and pulmonary embolism (Johnston, 1994). Additionally, these potent, naturally derived antihemostatics found in insect saliva may provide useful therapeutic agents and reagents for enhancing treatment and understanding of normal and abnormal homeostasis in human and animals.

It has been postulated that pool-feeding arthropods should rely more on anti-clotting factors than those feeding directly from the blood vessel, where vasoconstriction and platelet aggregation pose more serious challenges (Ribeiro, 1995a). Thus, the fibrinogenolytic activity, together with other antihemostatics known from the salivary glands of this tick may act in concert to disrupt the host hemostatic clotting mechanism thereby ensuring successful imbibement of blood by ticks. Identification and characterization of salivary components will not only provide important insights into vertebrate mechanisms for controlling parasites (including tick ectoparasites and tick-borne pathogens), but offer opportunities for new sustainable strategies of disease control (Nuttall, 1998). The results in this dissertation also suggest that the anticoagulant effect of fibrinogenase could be attributed mainly to its proteolytic action on fibrinogen, rendering possible the use of this protein to reduce plasma fibrinogen levels and blood viscosity, and to increase blood flow. It also disaggregates platelets that have aggregated. The studies on thrombolytic and pharmacological activities of fibrinogenases may be of value in selecting a tick enzyme enhancing the efficacy of thrombolytic therapy and research purposes.

Future aims should include the optimization of the isolation procedure, N-terminal amino acid sequencing, followed by cloning and sequencing of the fibrinogenolytic activity. Expression of the recombinant protein will allow the exploration of the enzyme *in vitro* and *in vivo* as well as evaluation of its therapeutic potential and application as an anti-tick feeding vaccine. Cloning and sequencing of the genes encoding the different proteases may aid in the elucidation of their function by screening the gene sequence against those in gene databases. Structure-function studies employing site directed mutagenesis may be employed to identify the various binding sites on the fibrinogenase. Sufficient amounts of pure proteins are also required for kinetic studies. Parameters such as Ki, Km and Vmax could indicate the suitability of the anticoagulant for therapeutic use. It will be important to establish whether its anticoagulant strategy is similar to that of the snake fibrinogenases or whether the mechanism is unique or shared by other arthropod fibrinogenolytic enzymes. It should also be established whether the fibrinogenase inhibits platelet aggregation and if so whether the effect results from proteolysis of

SUMMARY

The saliva of hematophagous ectoparasites contains a cocktail of vasodilators, anticoagulants and immunosuppressors that maintain blood in a liquid state at the site of the lesion and evade the host's defense mechanisms in suppressing the immune response. Since ticks have evolved to utilize mammals as a source of food, our understanding of the tick material, especially the salivary glands will enhance the control of tick infestation and allow the exploitation of the tick's natural resources.

SGE protease activity was determined by measuring the degradation of azocasein. Proteolytic activity was found in the pH range of 3 to 11 with the highest activity at pH 9 followed by pH 7. At pH 3-5 the activity was mainly due to aspartic proteases, whereas at pH 7-9 the activity was due to the action of metallo- and serine proteases. At pH 11, the activity was mainly ascribed to metallo- and aspartic proteinase activity

The fibrinogenolytic activity was determined by incubating human fibrinogen in the presence of SGE and monitoring the fibrinogen degradation by SDS-PAGE. SGE degraded the Aα-chain of fibrinogen within 2 hours of incubation and even after 24 hours incubation there was no hydrolysis of the Bβ and γ-chains of fibrinogen. Characterization of the fibrinogenolytic activity revealed that metalloprotease activity was present over pH range of 3-9 and at pH 3-5, the cysteine proteases were active. No serine protease activity was found under similar experimental conditions.

CE-HPLC separation of the SGE revealed three regions of proteolytic activity. Further characterization of the activity containing fractions using protease inhibitors at various pH values showed that the activity associated with region A is mainly due to the presence of aspartic and cysteine proteases in the lower pH range (< 5). Region B was mainly due to the activity of the metallo- and serine proteases,

while the activity in region C was mainly due to the metalloproteinases which were more active in the higher pH range (> 9).

CE-HPLC separation of SGE resulted in three regions exhibiting fibrinogenolytic activity at pH 7-9. In region A all four enzyme classes were found while in regions B and C, serine, cysteine and metalloproteinases were found to be responsible for the activity. Region A was further purified on the HIC-column and activity eluted in several peaks which after individual application on SE-HPLC column had similar retention times. The pooled samples were analyzed for purity using C5 RP-HPLC and reducing tricine SDS-PAGE and three bands of relative molecular masses 15, 22 and 12 kDa, respectively were found. In an attempt to purify the proteins in region C, four individual CE-HPLC runs were combined and applied to a fibrinogen affinity column. Reducing SDS-PAGE analysis of bound material showed two bands of relative molecular masses of 31 and 39 kDa, respectively.

CE-HPLC region C as well as the SGE control was found to disaggregate platelets aggregated by ADP, epinephrine, collagen as well as TRAP. No disaggregation was observed for the saline negative control. The disaggregation is most probably due to the hydrolysis of the fibrinogen cross-linking platelets by the metalloproteinase activity in region C.

Understanding of the proteolytic activities present in the salivary gland and therefore identifying molecules crucial for tick feeding could aid in the development of experimental vaccines. Even though the fibrinogenolytic activity was not purified to homogeneity, this study has laid the groundwork for further experiments in this field.

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